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Research paper

Drug release from and sterilization of *in situ* cubic phase forming monoglyceride drug delivery systems

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ABSTRACT

Since a monoglyceride-based cubic phase is too viscous to be injected parenterally, mixtures of monoglyceride, water and water-miscible cosolvents were investigated as low viscosity injectable *in situ* cubic phase-forming formulations. Upon contact with the release medium, a highly viscous cubic phase formed rapidly and served as an extended release matrix for the oligonucleotide drug. Extended drug release was obtained with all formulations. The drug release followed the square root of time relationship indicating a diffusion-controlled release mechanism. The release depended on the type of cosolvent and followed the order of ethanol > PEG 300 > 2-pyrrolidone > DMSO. Higher water or monoglycerides contents decreased the drug release because of an increased viscosity and increased swollen matrix thickness. The bioburden of different commercially available monoglycerides and of the prepared *in situ* cubic phase-forming formulations met USP XXIII requirements. Monoglycerides can be successfully sterilized by gamma irradiation or by autoclaving and the *in situ* cubic phase-forming formulations by autoclaving and aseptic filtration. The monoglycerides and *in situ* cubic phase-forming formulations retained their phase behaviour and release properties after sterilization.

1. Introduction

Macromolecular drugs (e.g. proteins, peptides and siRNA/oligonucleotides) are becoming a very important class of therapeutic agents as a result of gaining more understanding of their role in physiology and the rapid advances in the field of biotechnology and genetic engineering. However, naked delivery of these drugs suffered from short half-life, unfavourable pharmacokinetics and systemic toxicity [1]. Therefore, efficient drug delivery systems are necessary to deliver these drugs to achieve the desired therapeutic action [2–7]. Monoglyceride-based drug delivery systems are an interesting system for macromolecular drugs [8–9].

The monoglyceride (glycerol monooleate and glycerol monolinoleate) swell upon contact with an aqueous medium at 37 °C by taking up water. During this process, monoglycerides are transformed into various liquid crystalline phases (anisotropic hexagonal and lamellar) and finally into a very viscous (isotropic) cubic phase. The cubic phase has a thermodynamically stable structure [10] and due to its amphiphilic nature can be used as carrier for both hydrophilic and lipophilic drugs [11–17]. The release of incorporated drug occurs primarily from the swollen cubic phase since the swelling of monoglycerides into the cubic phase is a fast process [10]. The drug release from the cubic phase is usually diffusion-controlled and follows square root of time kinetics [13].

Monoglycerides are non-toxic, biocompatible and biodegradable and show good chemical and physical stability of incorporated drugs and especially macromolecular drugs (e.g. proteins) [8–9]. A wide variety of drugs with different physico-chemical properties have been incorporated into monoglycerides-based drug delivery systems, e.g. a lamellar liquid crystalline phase shows promising buccal drug carriers for peptide drugs, as well as acting as permeation enhancer [15–16]. *In vitro* studies and subcutaneous injections of insulin-loaded cubic phase in rats have shown that insulin is protected from agitation-induced aggregation and retains biological activity [18–19]. A number of different proteins in cubic phase appear to retain their native conformation and bioactivity and are protected from chemical and physical inactivation due to the reduced activity of water and biomembrane-like structure of the cubic phase [17]. Cubic phase monoglyceride systems exhibit strong bioadhesion [20]. Therefore, monoglyceride-based cubic phase is an interesting candidate for oral, gastrointestinal, lung, nasal, rectal and vaginal drug delivery. Camurus AB has been marketing glycerol monooleate-based formulation for Parodontitis use (e.g. Elyzol dental gel), and several products for parenteral use are in phase II studies.

However, the cubic phase is highly viscous and thus difficult to handle or to inject. As an alternative, low viscosity monoglyceride-based formulations were developed by the addition of either drugs

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(drug-induced) or organic solvents (solvent-induced) to the monoglyceride–water system, which transform into the cubic phase after contact with dissolution fluids [14]. Extended release of chlorpheniramine maleate and propranolol HCl was observed from these low viscosity formulations.

The objective of this study was to prepare a monoglyceride–oligonucleotide-based delivery system, which *in situ* form cubic phase after contact with aqueous fluid and to investigate suitable methods to sterilize these formulations.

2. Materials and methods

2.1. Materials

The following materials were used as received and were at least of reagent grade: phosphorothioate oligodeoxynucleotide (ISIS Pharmaceuticals Inc., Carlsbad, CA, USA), monoglyceride (GMO) (glycerol monooleate: GMOrphic®80; Eastman Chemical Company, Kingsport, TN, USA, Rylo™ MG 15; Danisco Ingredients Deutschland GmbH, Quickborn, Germany., glycerol monolinoleate: Rylo™ MG 13; Danisco Ingredients Deutschland GmbH, Quickborn, Germany), acetone, ethanol, 2-pyrrolidone, PEG 300, DMSO, potassium dihydrogen phosphate, sodium hydroxide, sodium chloride, crystal violet, methylated spirit, ammonium oxalate, iodine, potassium iodide, sodium azide (Merck KGaA, Darmstadt, Germany), dialysis bag (cellulose ester, MWCO:100,000, Spectra/Por-CE, Spectrum Medical Industries Inc., Houston, Texas, USA), microorganisms (*Bacillus pumilus* ATCC 27142; *Bacillus stearothermophilus* ATCC 7953; *Pseudomonas diminuta* ATCC 191469; Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany).

2.2. Preparation of in situ cubic phase-forming formulation

The low viscosity *in situ* cubic phase-forming formulations were obtained by mixing of molten monoglyceride (glycerol monooleate or glycerol monolinoleate) and cosolvents (ethanol, PEG 300, 2-pyrrolidone, DMSO) in glass vials, followed by the addition of the aqueous drug solution. The samples were vortex mixed for 5 min, centrifuged and stored tightly closed for 24 h for equilibration at room temperature. After 24 h, the clarity of the phases of the samples was confirmed by examination for their isotropic nature and any phase transition occurred under the polarized light microscope.

2.3. Optical microscopy

The liquid crystalline phases with different monoglyceride/water contents or *in situ* cubic phase-forming formulations were identified by polarized light microscopy (Axioskop, Carl Zeiss Jena GmbH, Jena, Germany). The lamellar phase could be identified by its anisotropic textures (birefringence). The cubic phase was isotropic (transparent).

2.4. Measurement of the viscosity

The viscosity of *in situ* cubic phase-forming formulations was measured using a rotational Rheometer in the controlled stress mode (Rheostress RS 100, Haake Meß-Technik GmbH, Karlsruhe, Germany). The samples were analysed using a plate/cone equipment (60 mm diameter, 1° angle) at 25 °C ± 0.5 °C ($n = 3$). The viscosity values were calculated using the software (RheoWin® Software Für HAAKE Viskosimeter und Rheometer (Thermo Haake, Karlsruhe, Germany).

2.5. In vitro drug and solvent release

The *in vitro* drug release was performed in 0.1 M phosphate buffer, pH 7.4, with 0.1% sodium azide as preservative in a horizontal shaker (37 °C, 75 rpm; GFL 3033, Gesellschaft für Labortechnik GmbH, Burgwedel, Germany) ($n = 3$). Then, 0.1–0.2 g of the *in situ* cubic phase-forming formulations was filled in dialysis bags. The dialysis bags were then immediately put into 25-ml pre-warmed release medium. At predetermined time intervals, 2 ml samples were withdrawn and replaced with fresh medium. The drug concentration was measured spectrophotometrically at $\lambda = 260$ nm Shimadzu UV 2101 PC UV–vis scanning spectrophotometer, Kyoto, Shimadzu Japan).

The solvent (2-pyrrolidone) concentration in the buffer medium was measured with a computer connected Shimadzu-HPLC system at 220 nm (UV-detector) at room temperature. A 40 μl sample volume was injected onto a LiChrospher-100 RP 18.5 μm vertex column (Knauer GmbH, Berlin, Germany) using an acetonitrile/pH 7.4 phosphate buffer mixture (10:90 v/v) as the mobile phase at a flow rate of 1.0 ml/min [21].

2.6. Bioburden determination

Bioburden in commercially available monoglycerides and that of the *in situ* cubic phase-forming formulations were determined according to the USP XXIII method for ointments and oils.

2.6.1. Total plate count

One gram monoglyceride or *in situ* cubic phase-forming formulation was dissolved in 100 ml pre-sterilized isopropyl myristate (aseptically filtered through 0.22-μm membrane filter). The resulting monoglyceride or *in situ* cubic phase-forming formulation solution in isopropyl myristate was aseptically filtered through a 0.22-μm membrane followed by twice rinsing with 200 ml of sterile 0.1% w/v peptone water containing polysorbate 80 and 100 ml 0.1% w/v peptone water without polysorbate 80. After rinsing, the membrane filter was aseptically transferred onto a pre-incubated tryptic soy agar (TSA) plate and incubated at 35–37 °C for 48 h. The whole procedure was repeated, but the membrane filter was placed on a sabourad dextrose agar (SDA) plate (for yeast/mould) and incubated at 25 °C for 7 days. After incubation, the microbial colonies were counted; each colony was the result of the growth of one bacterial or yeast/mould cell in the original samples (results expressed as cfu/g sample).

2.6.2. Coliforms test

Repeat the procedure as described earlier, the membrane filter was aseptically transferred into a pre-incubated lactose broth and incubated for 24 h at 35–37 °C. After 24 h, a small amount of culture was withdrawn from broth with the help of a sterile loop, streaked on MacConkey agar and on cetrimide agar. Both plates were incubated at 35–37 °C for 48 h. No growth on the MacConkey agar plate assumed the absence of coliforms and absence of any growth on cetrimide agar plate assumed absence of *Pseudomonas aeroginosa.*

2.7. Sterilization techniques

Commercial products of monoglycerides (GMOrphic®80, Rylo™ MG 15 and Rylo™ MG 13) and *in situ* cubic phase-forming formulations were sterilized by the following techniques: (1) autoclaving at 121 °C for 15 min at 2 bar pressure (MMM Münchener Medizin, Mechanik GmbH, Germany), (2) gamma irradiation at doses of 10, 15, 20 and 25 KGy, gamma irradiation was performed using a ^{60}Co source (Willi Rüsch, Waiblingen, Germany), and (3) aseptic filtration through a 0.22 μm pore size membrane.

All sterilization methods were challenged with standard strains of microorganisms. In a challenge test, the respective spore/bacterial (10^6) suspension was added to pre-sterilized (by autoclave) monoglycerides and *in situ* cubic phase-forming formulations prior to sterilization. Autoclaving and irradiation were challenged with spore suspension of *Bacillus stearothermophilus* and *Bacillus pumilus*, respectively. In case of the aseptic filtration, *Pseudomonas diminuta* was added to the pre-sterilized *in situ* cubic phase-forming formulation, which was then aseptically filtered through 0.22-μm membrane. After the sterilization, a sterility test was applied as described below (2.8) to confirm that the challenged samples were successfully sterilized.

2.8. Sterility test

The sterility test was performed according to the USP XXIII method. As described earlier, 1 g sample was dissolved in isopropyl myristate, filtered and rinsed with 0.1% w/v peptone water. After rinsing, the membrane filter was cut into two pieces with the aid of sterile forceps and scissors. One piece was aseptically transferred into pre-incubated tryptic soy broth (TSB) and the other piece into fluid thioglycolate broth (FTB) and incubated for 14 days at 25 °C and 37 °C, respectively. The sample bottles were visually inspected daily; turbidity indicated a non-sterile sample. After 14 days of incubation, staphylococcus aureus (<100 cfu) was added into the bottles without growth (turbidity) followed by incubation

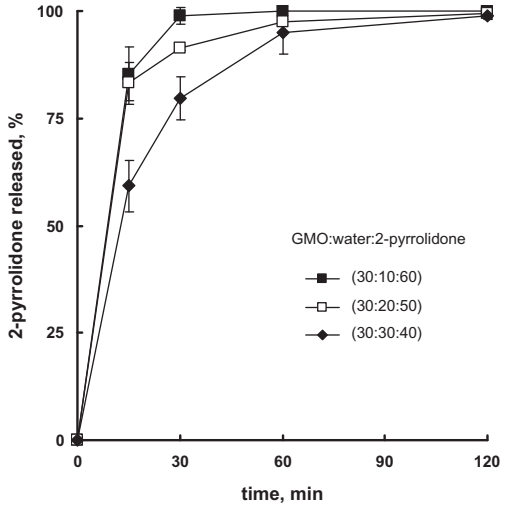

Fig. 1. 2-Pyrrolidone release from *in situ* cubic phase-forming formulations (Rylo™ MG 13, $n = 3$).

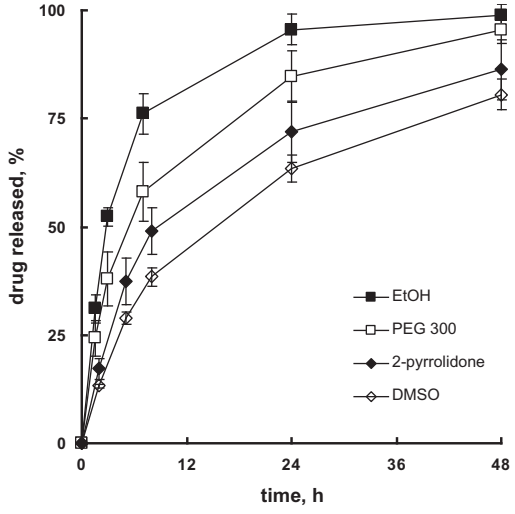

Fig. 2. Effect of cosolvents on drug release from *in situ* cubic phase-forming formulations (Rylo™ MG 13:water:cosolvent, 35:15:45; drug loading 5% w/w, $n = 3$).

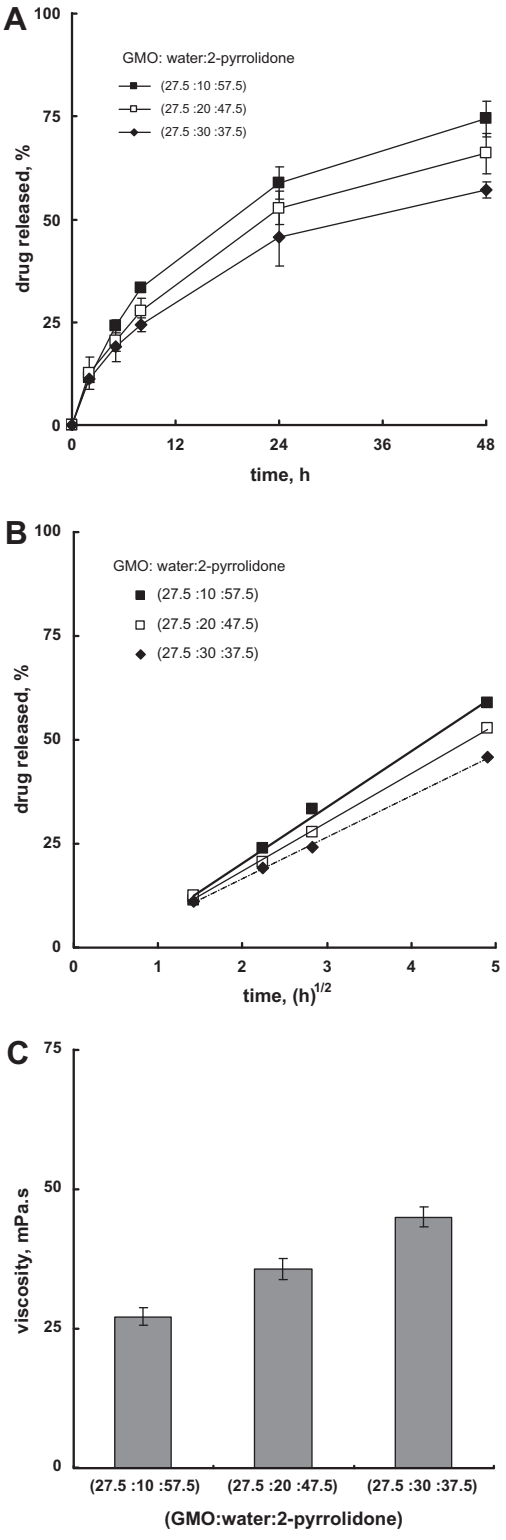

Fig. 3. Effect of different water/solvent ratios of *in situ* cubic phase-forming formulations on (A) drug release, (B) amount release vs. square root of time and (C) viscosity (Rylo™ MG 13, drug loading 5% w/w, $n = 3$).

for 3 more days. The presence of microbial growth (turbidity) in the medium after these 3 days confirmed that the previous absence of microbial growth was not due to any inhibitory factor, but that the samples were successfully sterilized by the respective sterilization technique.

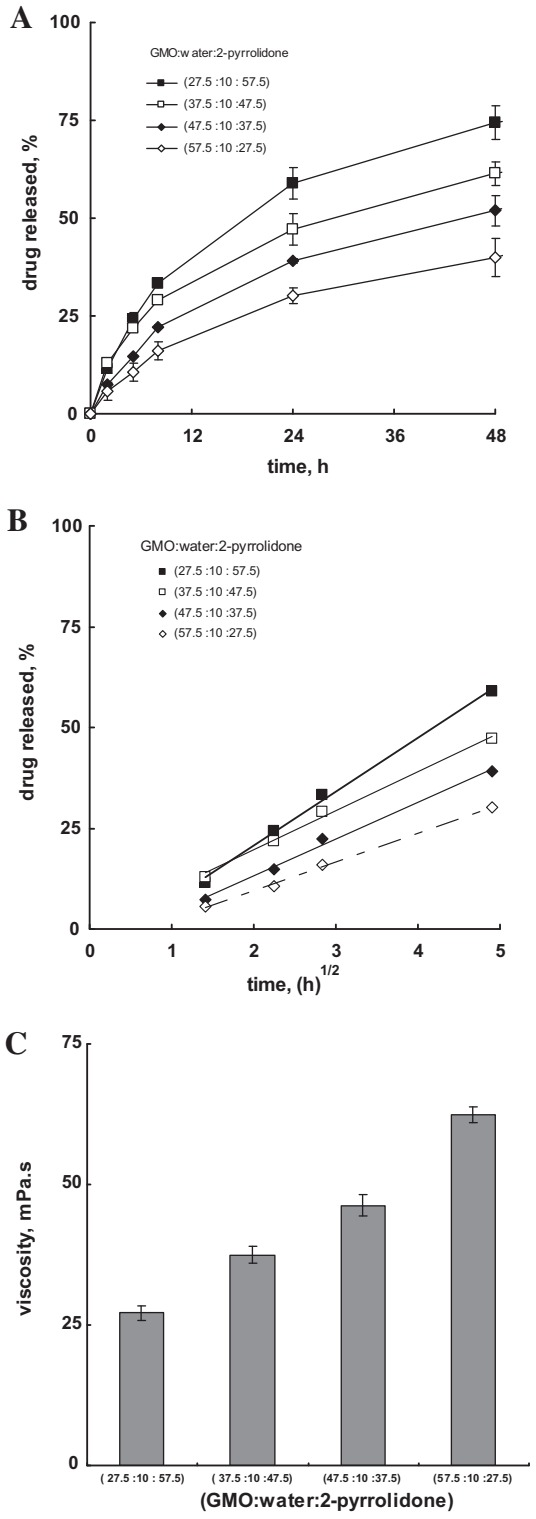

Fig. 4. Effect of different monoglyceride/solvent ratios of *in situ* cubic phase-forming formulations on (A) drug release, (B) amount release vs. square root of time and (C) viscosity (Rylo™ MG 13, drug loading 5% w/w, $n = 3$).

3. Results and discussion

In this study, low viscosity and injectable *in situ* cubic phase-forming formulations were prepared from three components: monoglyceride, water and water-miscible cosolvents like ethanol, polyethylene glycol 300 (PEG 300), 2-pyrrolidone and DMSO [4]. These formulations were of low viscosity and isotropic at room temperature (clear solution observed under a polarized light microscope). Upon contact with release medium at 37 °C, cosolvent rapidly leached out was exchanged by release medium within 60 min as exemplified by 2-pyrrolidone (Fig. 1) resulting in immediate transformation into the cubic phase. This fully swollen cubic phase served as an extended release matrix for the oligonucleotide.

All formulations with different cosolvents resulted in typical matrix-type drug release profiles. The release was in the order of ethanol > PEG 300 > 2-pyrrolidone > DMSO (Fig. 2). The drug was completely released after 24 h from ethanol-based formulations.

The drug release decreased with increasing water, increasing monoglyceride and decreasing cosolvent content (Figs. 3 and 4A) because of an increase in viscosity (Figs. 3 and 4C). This viscosity order might therefore be a possible explanation for the order of drug release. Additionally, increasing monoglyceride or decreasing cosolvent contents (at constant water content) also increased the matrix thickness of the fully swollen cubic phase thus also explaining the decreased drug release. The release of oligonucleotide from the fully swollen cubic phase matrix followed a diffusion-controlled release mechanism as shown by a linear relationship between the amount of drug released and the square root of time (initial 24 h) for all formulations (Figs. 3 and 4B).

Parenteral products have to be sterile. To achieve higher sterility assurance level, the initial product should have minimum bioburden before sterilization. Therefore, starting raw materials according to the USP XXIII should have total bacterial and yeast/mould count < 100 cfu/g and should be free from coliforms (gram negative) per 0.1 g sample. The obtained results met the USP requirements (Table 1). Gram staining showed that all the isolated colonies were from gram positive bacteria, which were mainly responsible for the initial bioburden (contaminations). The low initial bioburden support that the commercial product can be used to investigate different sterilization techniques.

In the present study, three techniques were applied to sterilize monoglycerides or *in situ* cubic phase-forming formulations: (1) autoclaving (2) gamma irradiation and (3) aseptic filtration. Monoglycerides samples were autoclaved and gamma irradiated at different doses (10, 15, 20 and 25 KGy). Monoglycerides samples were sterile when autoclaved or irradiated at doses at and above 15 KGy (Table 2). *In situ* cubic phase-forming formulations were successfully sterilized by autoclaving or aseptic filtration (Table 2).

Each sterilization technique was challenged by introducing known amounts of spores/bacterial suspension (10^6) into pre-sterilized samples of monoglyceride or *in situ* cubic phase-forming formulations followed by sterilization (Table 3). All samples were sterile except the challenge sample exposed to 10 KGy.

Table 1
Bioburden of monoglycerides and *in situ* cubic phase-forming formulations (monoglyceride:water:PEG 300, 35:25:40).

Monoglyceride	Monoglyceride			Formulations		
	Total plate count (cfu/g)	Yeast and mould (cfu/g)	Coliforms/0.1 g	Total plate count (cfu/g)	Yeast and mould (cfu/g)	Coliforms/0.1 g
GMOrphic®80	32.7	1.3	Absent	22.3	1.3	Absent
Rylo™ MG 15	41.0	0.0	Absent	16.3	0.0	Absent
Rylo™ MG 13	27.3	0.7	Absent	19.7	0.0	Absent

Table 2
Sterility test of pure monoglycerides and *in situ* cubic phase-forming formulations (monoglyceride:water:PEG 300, 35:25:40) sterilized by different techniques.

Monoglyceride	Sterilization technique	Samples			
		Monoglyceride		Formulations	
		TSB	FTB	TSB	FTB
GMOrphic®80	Autoclave	S	S	S	S
	Aseptic filtration	NA	NA	S	S
	Irradiation 10 KGy	NS	S	NA	NA
	Irradiation 15 KGy	S	S	NA	NA
	Irradiation 20 KGy	S	S	NA	NA
	Irradiation 25 KGy	S	S	NA	NA
Rylo™ MG 15	Autoclave	S	S	S	S
	Aseptic filtration	NA	NA	S	S
	Irradiation 25 KGy	S	S	NA	NA
Rylo™ MG 13	Autoclave	S	S	S	S
	Aseptic filtration	NA	NA	S	S
	Irradiation 25 KGy	S	S	NA	NA

S: sterile.
NS: non-sterile.
NA: not applicable.

Table 3
Challenge test of the sterilization techniques (monoglycerides: GMOrphic®80, Rylo™ MG 15 and Rylo™ MG 13; spore/bacterial suspension: 10^6).

Sterilization technique	Challenge organism	Sample	
		Monoglyceride	Formulations[a]
Autoclave	*Bacillus stearothermophilus*	S	S
Aseptic filtration	*Pseudomonas diminuta*	NA	S
Irradiation (10 KGy)	*Bacillus pumilus*	NS	NA
Irradiation (15 KGy)	*Bacillus pumilus*	S	NA
Irradiation (20 KGy)	*Bacillus pumilus*	S	NA
Irradiation (25 KGy)	*Bacillus pumilus*	S	NA

S: sterile.
NS: non-sterile.
NA: not applicable.
[a] Monoglyceride:water:PEG 300 (35:25:40).

After autoclaving or irradiation, the monoglycerides and *in situ* cubic phase-forming formulations were examined by visual inspection and by polarized light microscopy. The visual inspection revealed no changes upon sterilization. The monoglyceride was semisolid with white crystals, whereas the *in situ* cubic phase-forming formulations were isotropic (clear solution). The monoglycerides formed different mesophases upon contact with different amounts of water (Table 4). The *in situ* cubic phase-forming formulations after sterilization (autoclaving or aseptic filtration) also formed the cubic phase upon injection into the release medium (pH 7.4, 37 °C). Sterilization also did not affect the drug release (Fig. 5).

Table 4
Fluidity and optical properties of sterilized (autoclave/gamma irradiation) monoglycerides and water mixtures (monoglycerides: GMOrphic®80, Rylo™ MG 15 and Rylo™ MG 13).

Monoglyceride/water ratio	Fluidity	Optical properties	Phase
95:05	–	Isotropic + crystals	L_2 + crystals
90:10	++	Isotropic	L_2
85:15	+	Anisotropic	Lamellar
80:20	–	Isotropic	Cubic
75:25	–	Isotropic	Cubic
70:30	–	Isotropic	Cubic

– No flow.
+ Flows if force applied.
++ Flows freely.

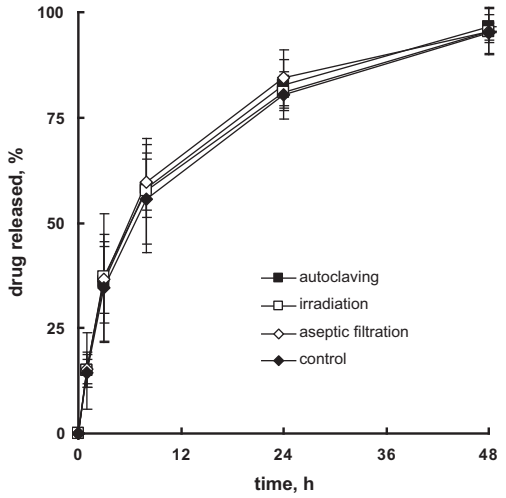

Fig. 5. Drug release from sterile *in situ* cubic phase-forming formulations (GMOrphic®80:water:PEG 300, 35:15:45; sterilization: GMOrphic®80, autoclave: 121 °C for 15 min; gamma irradiation 25 KGy; aseptic filtration: 0.22-μm membrane, drug loading: 5% w/w, $n = 3$).

4. Conclusions

Low viscosity *in situ* cubic phase-forming formulations based on monoglyceride/water/cosolvent were prepared. A viscous, extended release matrix cubic phase formed rapidly upon addition of the *in situ* formulations to the release medium. Different drug release profiles were obtained with different ratios of *in situ* cubic phase-forming formulations components (monoglycerides:water:cosolvent). Commercially available monoglyceride samples met USP bioburden requirements. The monoglycerides and *in situ* cubic phase-forming formulations can be sterilized by gamma irradiation, autoclaving or by aseptic filtration without significant changes in phase behaviour and drug release.

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