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# A new injectable liquid crystal system for one month delivery of leuprolide

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ABSTRACT

An injectable liquid crystal-forming system (LCFS) was prepared by using sorbitan monooleate (SMO) as a new liquid crystal-forming material for injections, and its potential use of clinically available sustained-release formulation was evaluated. LCFS was prepared using SMO mixed with phosphatidyl choline and tocopherol acetate, and contained 3.75 mg of leuprolide acetate as a monthly dose in 90 μl in liquid form. The semi-solid mesophase was formed from the liquid LCFS when it contacted water. The mesophase showed typical characteristics of the liquid crystalline phase, which was classified as the hexagonal phase. The safety of the LCFS was studied by an *in vitro* extraction colony assay and by examining the injection site in rats and white rabbits after an autopsy. Both *in vitro* release test and *in vivo* pharmacokinetic and pharmacodynamic studies showed a sustained release of leuprolide. When compared with a commercial depot formulation of leuprolide, the LCFS showed a similar $AUC_{last}$ value and significantly reduced initial burst with sufficient suppression of testosterone after subcutaneous injections in rats and dogs. The LCFS can serve as a new type of sustained-release injection formulation for its safety, ease of preparation, and sustained release properties.



## 1. Introduction

Sustained-release (SR) injections are designed to release a drug substance at a predetermined rate to maintain its effective plasma concentration for a specific period of time for months. Leuprolide (pGlu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt) is one of the most successful drugs in SR depot injection formulation of poly(lactic-co-glycolic acid) (PLGA) microspheres [1–4]. It is a potent luteinizing hormone-releasing hormone (LHRH) analog that stimulates the release of luteinizing hormones. Leuprolide has been used in treating prostate cancer by saturating and down-regulating pituitary receptors, thereby suppressing testosterone production. Though PLGA microspheres are available for clinical application, they are difficult to prepare and are known to decrease the stability of protein drugs [5–7]. Unfortunately, alternative polymers with proven safety for injectable excipients have not been reported yet in spite of many research efforts [8].

Recently, liquid crystal technology has emerged as a new, injectable SR formulation for its sustained drug release properties. Although no commercial product based on this technology has been developed, it has gained increasing interest for drug delivery [9,10]. Lyotropic liquid crystal systems composed of amphiphiles can be classified into lamellar (La), hexagonal and cubic phases based on their assembly shape. Among them, the reversed hexagonal phase ($H_2$) and the reversed cubic phase ($Q_2$) have been extensively investigated for their ability to control the release rate of numerous drug substances, from low-molecular-weight chemicals to macromolecular drugs (proteins, peptides and nucleic acids) [11]. Their structures consist of a linear arrangement of alternating lipid bilayers and of aqueous channels arranged in periodic minimal surface geometries. The reversed hexagonal phase consists of an infinite rod-type water channel arranged in a two-dimensional lattice and separated by lipid bilayers, and the reversed cubic phase comprises a curved water channel and a bicontinuous lipid bilayer that extends in three dimensions. The reversed hexagonal and cubic mesophases are spontaneously formed from the liquid crystal-forming system (LCFS) in an aqueous fluid. The formed tortuous networks of aqueous nano-channels in the mesophases or mesophase particles play important roles as passageways for the sustained release of drugs from liquid crystals [9–12].

Various amphiphilic liquid crystal-forming materials (LCFMs), such as glycerol monooleate (GMO), glycerol dioleate (GDO), glycerol oleyl ether, oleyl glycerate, phytanyl glycerate and phytantriol, have been reported [13–16]. The drug release patterns of different types of LCFMs have been evaluated for the development of lyotropic liquid crystals as

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controlled release dosage forms. The results show that the drug release rate was reduced as the diameter of the aqueous channel, located inside the liquid crystal, was decreased and the drug in the similar molecular structure was more hydrophobic [17,18]. Aside from LCFMs, additives that change the physical properties of liquid crystal have also been investigated, such as fat materials including phospholipid, tocopherol, tocopherol acetate and tri-acyl glycerol. A mixture of phospholipid and GDO (45/55 to 55/45%, w/w) that formed the liquid crystalline phase has been used for subcutaneous drug delivery [19]. Fat materials, such as tocopherol, tocopherol acetate and tricaprylin, that are added to LCFMs (up to 10 or 15% weight ratio), could help the fabrication of cubic or hexagonal phase by increasing the curvature of the bicontinuous layer inside the liquid crystal [20,21].

Although there were many results regarding *in vitro* drug release tests of LCFSs, their *in vivo* pharmacokinetic study over one month was not thoroughly investigated. Moreover, while many studies on SR injections using LCFMs, such as GMO, GDO, oleyl glycerate or phytantriol, have been conducted, no drug delivery systems based on LCFM have been approved by the FDA. In this study, sorbitan monooleate (SMO) (also known as Span 80) was used as a new LCFM because it has been used as a pharmaceutical excipient with acceptable daily intakes up to 25 mg/kg/day [22]. Unlike other LCFMs reported, SMO is regarded as safe for injection formulations. To the best of our knowledge, this study is the first case of applying SMO in SR (monthly) injections based on liquid crystal technology which could replace the PLGA depot systems. The non-polar tail structure of SMO is made up of oleic acid, the same as the previously used LCFMs. But its polar head group has a distinguished sorbitan structure, compared to the polyol structures in other LCFMs, such as glycerol or glycerate (Fig. 1) [13–16]. SMO has advantages in terms of safety and quality control when it is applied in SR injections, because it is an injectable emulsifier that has been used in various pharmaceutical formulations.

In this study, the LCFS composed of leuprolide acetate and SMO was assessed for its use as a SR injection formulation. The inner structure and the drug release profiles of liquid crystal phases from the LCFS were investigated. Also, the pharmacokinetic and pharmacodynamic properties of leuprolide acetate from the developed LCFS were compared to the commercial PLGA depot formulation.

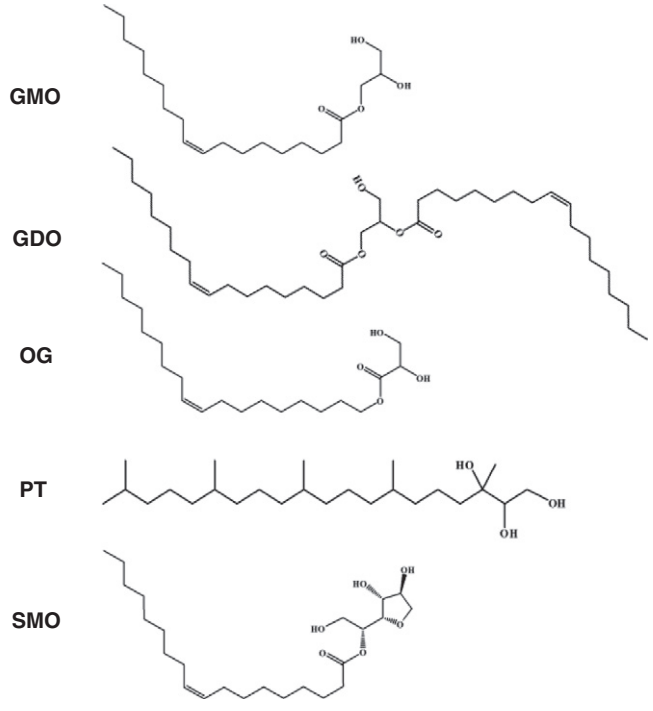


**Fig. 1.** Chemical structures of liquid crystal-forming materials, GMO: glyceryl monooleate; GDO: glyceryl dioleate; OG: oleyl glycerate; PT: phytantriol and SMO: sorbitan monooleate.

## 2. Materials and methods

### 2.1. Materials

Leuprolide acetate and leuprolide-$d_5$ acetate were obtained from Teva (Petah-Tiqva, Israel) and Toronto Research Chemicals (Ontario, Toronto, Canada), respectively. Testosterone and testosterone-$d_3$ were obtained from TLC PharmaChem (Ontario, Toronto, Canada) and BDG Synthesis (Wellington, New Zealand), respectively. SMO, phosphatidyl choline, tocopherol acetate, and Tween 80 were purchased from Seppic (Puteaux, France), Lipoid GmBH (Ludwigshafen, Germany), DSM Nutritional Products Limited (Sisseln, Switzerland), and Croda (Edison, NJ, USA), respectively. Leuprolide acetate conforms to 97–103% of the assay specification. SMO and phosphatidyl choline correspond to 65–88% as oleic acid and >94% of the assay specification, respectively. Na-heparin was obtained from JW Pharma(Seoul, Korea) and aprotinin solution (30 TIU/5 mL) was purchased from Phoenix Pharmaceuticals (Hanau, Germany). The mouse fibroblast (NIH 3T3) was obtained from ATCC (Manassas, VA, USA). Giemsa stain solution 5% and fetal bovine serum (FBS) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and NOVA Biologics (Oceanside, CA, USA), respectively. Penicillin, streptomycin, Eagle's minimum essential medium (EMEM) and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (Grand Island, NY, USA). All the other chemicals were of analytical grade.

### 2.2. Preparation of LCFS and its formation test

The LCFS was prepared by adding and mixing a leuprolide solution into the LCFS vehicle solution. The leuprolide solution was prepared by dissolving 3.75 mg of leuprolide acetate in 5 μl of DMSO. The LCFS vehicle solution was prepared by mixing SMO, phosphatidyl choline, tocopherol acetate, Tween 80, and ethanol (33:45:10:2:10, w/w%). SMO, phosphatidyl choline, and tocopherol acetate were used as core components for the LCFS, and Tween 80 and ethanol were used to prevent the separation of components which could make a homogenized vehicle solution. The final LCFS was designed to contain 3.75 mg of leuprolide acetate for one month dose in 90 μl. The viscosity of the liquid form, being approximately 650 cPs, was suitable for injection. The commercialized Leuplin DPS Injection® 3.75 mg (Takeda Pharmaceutical Company Limited, Osaka, Japan) was used as a reference product.

To confirm the transition from the oil phase to the gel-like mesophase after the LCFS came in contact with water, 100 μl of the LCFS in the oil phase was added to 3 ml of phosphate buffered saline (PBS, pH 7.4) at room temperature. The apparent appearance and the half-dissected section of the mesophases were observed after water on the surface of the formed liquid crystalline mesophases was cautiously wiped off at 0.5, 6, 72, 168, 240 and 336 h after the loading. The water content of the mesophases was measured by Karl Fischer titration (758 KFD Titrino, Metrohm, Herisau, Switzerland).

### 2.3. Cryo-transmission electron microscopy (Cryo-TEM) and polarized optical microscopy

Cryo-TEM (Tecnai G2 F20 Cryo-TEM, FEI Company, Hillsboro, OR, USA) was used to observe the inner structure of the liquid crystal that was formed with exposure to water [23–25]. For convenient observation of the liquid crystalline phase, 15 μl of the LCFS in the oil phase was added to 3 ml of triple distilled water and dispersed using a probe sonicator (CL-334, Qsonica, LLC, Newtown, CT, USA) to form mesophase particles. The dispersed liquid crystalline phase was placed on the holey carbon-coated grid (Quantifoil Micro Tools GmbH, Jena, Germany) like a water film and quickly frozen at −170 °C. The frozen grid was fixed in