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Research Article

Reverse Engineering the 1-Month Lupron Depot®

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Abstract. The 1-month Lupron Depot® (LD) encapsulating water-soluble leuprolide in poly(lactic-co-glycolic acid) (PLGA) microspheres is a benchmark product upon which modern long-acting release products are often compared. Despite expiration of patent coverage, no generic product for the LD has been approved in the USA, likely due to the complexity of components and manufacturing processes involved in the product. Here, we describe the reverse engineering of the LD composition and important product attributes. Specific attributes analyzed for microspheres were as follows: leuprolide content by three methods; gelatin content, type, and molecular weight distribution; PLGA content, lactic acid/glycolic acid ratio, and molecular weight distribution; mannitol content; *in vitro* drug release; residual solvent and moisture content; particle size distribution and morphology; and glass transition temperature. For the diluent, composition, viscosity, and specific gravity were analyzed. Analyzed contents of the formulation and the determined PLGA characteristics matched well with the official numbers stated in the package insert and those found in literature, respectively. The gelatin was identified as type B consistent with ~ 300 bloom. The 11-μm volume-median microspheres in the LD slowly released the drug *in vitro* in a zero-order manner after ~ 23% initial burst release. Very low content of residual moisture (< 0.5%) and methylene chloride (< 1 ppm) in the product indicates in-water drying is capable of removing solvents to extremely low levels during manufacturing. The rigorous approach of reverse engineering described here may be useful for development of generic leuprolide-PLGA microspheres as well as other new and generic PLGA microsphere formulations.

KEY WORDS: generic drugs; leuprolide; Lupron Depot®; PLGA microspheres; reverse engineering.

INTRODUCTION

The 1-month Lupron Depot® (LD) is a poly(lactic-co-glycolic acid) (PLGA) microsphere product, which encapsulates and slowly releases leuprolide acetate, to reduce injection frequency relative to daily injections of soluble peptide for treatment of hormone-sensitive prostate cancer, breast cancer, endometriosis, and uterine fibroids (1,2). Since its launch in the USA in 1989, the LD has become a benchmark product with which modern long-acting release (LAR) PLGA products are often compared. Annual market sales of LD in the USA was $580 million in 2014 (3), making it an attractive candidate for generic competition. Despite expiration of patent coverage, no generic product for the LD has been approved in the USA. Three- and six-month LD formulations are also commercialized, which are also of interest for generic development.

For injectable PLA/PLGA-based drug products, the proposed generic product should be qualitatively (Q1) and quantitatively (Q2) the same as the reference listed drug (RLD) to be considered for approval in an Abbreviated New Drug Application (ANDA) according to the 505(j) pathway (4). The extensive list of ingredients of LD is expected to pose challenges to generic product development (5). Referring to publications of LD and the package insert (1,6), the 7.5 mg LD for 1-month administration formulation is prefilled in a dual-chamber syringe for better usability. The powder filled in the front chamber (chamber 1) contains microspheres loaded with leuprolide and gelatin (7.5 mg leuprolide acetate, 1.3 mg gelatin, and 66.2 mg PLGA) and 13.2 mg D-mannitol.

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The injection diluent filled in the second chamber (chamber 2) is composed of 5 mg carboxymethylcellulose sodium (Na-CMC), 50 mg mannitol, 1 mg polysorbate 80, water for injection (USP), and glacial acetic acid to control pH (USP) (6). Before administration, the microspheres will be mixed with the diluent thoroughly until a homogeneous suspension forms (6).

As an initial step in the generic drug development, the relevant analytical methods need to be established to determine the composition of the RLD. The characteristic properties of active and inactive ingredients are also of interest for the potential use in the selection of manufacturing materials for generic drug product development. For example, comprehensive characterization of PLGA is required in the generic application of polymer-based products (5). The key properties of PLGA, including lactic acid/glycolic acid (LA/GA) ratio, molecular weight distribution, and polymer end-group identity, all could affect the release mechanism and release rate of the drug from the microspheres. In addition, the PLGA synthesis method and presence or absence of specific catalyst could also potentially affect product performance. During the manufacturing process of microspheres, the PLGA polymer could potentially degrade resulting in changes in the formulated product, which may cause failure in an equivalence test. Another complex ingredient in the case of LD is gelatin. Gelatin was originally added to the leuprolide solution to increase encapsulation efficiency in the manufacturing of microspheres (7). Later, it was found that increasing the viscosity of the primary emulsion by cooling was the key step to achieve high encapsulation efficiency of leuprolide in the microspheres (1,8). Gelatin is a mixture of proteins and peptides derived from collagen in animal tissues and bones. Gelatins are derived most commonly from bovine and porcine sources as type A or B, according to acid or base hydrolysis, and possess a gel strength indicated by bloom number (9). However, the specific gelatin product used in the LD formulation is not disclosed to the best of our knowledge.

We describe the reverse engineering of the 1-month LD injection system to (a) determine the identity and quantity of specific components of this formulation, (b) characterize key aspects of the formulation critical to performance of the product, and (c) establish chemical assays that are useful to accomplish the above. By improving our understanding of the LD, the barrier to increasing the number of PLGA products can be reduced, especially to those pursuing generic PLGA products for leuprolide.

MATERIALS AND METHODS

Chemicals and Reagents

The 7.5 mg leuprolide dose for 1-month administration Lupron Depot® (LD, AbbVie Inc., North Chicago, IL, USA) was employed for reverse engineering the product composition. The LD products were purchased from the pharmacy department at the University of Michigan Health System. Leuprolide acetate with purity more than 98% by high-performance liquid chromatography (HPLC) analysis was purchased from Soho-Yiming Pharmaceuticals Co. Ltd. (Shanghai, China). This leuprolide acetate was detected by UV absorbance at 280 nm of wavelength on ultra-performance liquid chromatography (UPLC) and confirmed to be within 100.55 ± 2.16% (mean ± SEM, $n = 3$) of the USP standard (USP 36 NF 31; catalog number: 1358503; lot: I0M442) in the range of 0–600 μg/mL. Gelatin products used in this paper include: type B gelatin derived from porcine skin with bloom number 300 (beMatrix™ Low Endotoxin Gelatin LS-W) and type B gelatin derived from bovine bone with bloom number 250 were purchased from Nitta Gelatin Inc. (Osaka, Japan); type A gelatin derived from porcine skin with bloom number 300 and type B gelatin derived from bovine skin with bloom numbers 75 and 225 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hereafter, the gelatins are designated by "company name; type; bloom number." The AccQ•Tag Chemistry kit was purchased from Waters (Waters Corporation, Milford, MA, USA). All solvents used were HPLC grade and were purchased from Fisher Scientific. Wako 7515 PLGA polymer (catalog no. 823-11966) was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Determination of Leuprolide Acetate Loading

Two extraction methods were employed to determine the leuprolide content in the LD formulations. A single extraction method (method 1) was published by LD originator, Takeda Pharmaceutical Company Ltd. (7,10) where methylene chloride (DCM) was used to dissolve PLGA microspheres and 1/30 M sodium phosphate buffer at pH 6.0 was used to extract leuprolide acetate into the aqueous phase. Approximately 5 mg of formulation was weighed accurately and 10 mL of DCM and 20 mL of phosphate buffer (pH 6.0) were added. The supernatant of the aqueous phase was obtained after mixing the solution vigorously for 5 min and subsequent centrifugation (2000*g*, 5 min) at room temperature.

In method 2, 5 mg of the LD formulation was dissolved in 750 μL DCM and then leuprolide acetate was extracted with 750 μL of 50 mM sodium acetate buffer at pH 4.0 (11,12). In order to extract leuprolide from the organic phase, this extraction process was repeated five times (11) followed by additional six extractions with 50 mM sodium acetate buffer pH 4.0 containing 1 M sodium chloride (11 total extractions) (12). Between each extraction, the supernatant was collected by centrifugation at 6000*g* for 4 min at room temperature.

In both methods, the content of leuprolide acetate in the aqueous phase was determined by UPLC. The UPLC system consisted of an Acquity Quaternary Solvent Manager, Sample Manager-FTN, Column Manager, and TUV Detector (Waters, Milford, MA, USA). The separation of leuprolide was carried out with an Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 100 mm, Waters, Milford, MA, USA) and a gradient elution of acetonitrile with 0.1% TFA (solvent A) and water with 0.1% TFA (solvent B) at a flow rate of 0.5 mL/min as follows: 0 min (25% A), 2 min (35% A), and 2.5 min (25% A), followed by 1-min recovery with initial conditions. The concentration of leuprolide was detected by UV absorbance at 280 nm of wavelength and its peak appeared around a retention time of 2.4 min. Three batches of LD with different lot numbers were used and the experiment was performed in triplicate.

Amino acid analysis was used as the third method (method 3) to determine the content of leuprolide acetate in LD. Leuprolide contains nine amino acids, and tyrosine (Tyr) and histidine (His) are the specific amino acids that do not exist in the gelatin (1,9). Histidine was used to determine the content of leuprolide. About 25 mg of LD formulations or 5 mg of leuprolide acetate was weighed into hydrolysis tubes and 1.0 mL of 6 N HCl (Fisher Chemical, Fair Lawn, NJ, USA) was added. The tubes were purged under nitrogen, sealed under light vacuum, and incubated at 110°C for 24 h. After incubation, the solution was frozen with liquid nitrogen and lyophilized under vacuum at room temperature. Then, 400 μL of 20 mM HCl was added into each tube to reconstitute the samples. Standard solutions of leuprolide acetate were prepared by dilution of the hydrolyzed leuprolide samples. Derivatization and analysis were performed by using Waters AccQ•Tag Chemistry kit. Briefly, hydrolyzed amino acids were derivatized using the borate buffer (< 5% sodium tetraborate in water) with the Waters AccQ•Fluor reagent (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate). Norleucine was added to the samples during the derivatization and used as the internal standard. The derivatized samples were separated by reverse phase UPLC using a C18 column (AccQ•Tag Ultra C18, 1.7 μm (Millipore Corporation, Milford, MA, USA)) and a gradient elution of solvent A (5% solution of Waters AccQ•Tag eluent A concentrate (19 wt% sodium acetate, 6–7 wt% phosphoric acid, and 1–2% wt% triethylamine)) and solvent B (2% formic acid in acetonitrile solution) at a flow rate of 0.5 mL/min as follows: 0 min (99.9% A), 1 min (98.5% A), 11.5 min (78% A), 13.5 min (40% A), and 15 min (99.9% A), followed by a 2-min recovery with initial conditions. The urea derivatives yielded during the derivatization were detected by fluorescence (excitation emission, 250–395 nm). Three batches of LD with different lot numbers were used and the experiment was performed in triplicate.

Characterization of the Gelatin in the LD Formulation

Determination of Gelatin Type

Ion exchange chromatography was employed to differentiate the pI difference between type A and B gelatin in order to identify the gelatin type in the LD formulation. To extract gelatin from the LD formulation, the formulation powder was first suspended in ice-cold water to dissolve and remove the D-mannitol from the sample. Ice-cold water was used to inhibit degradation of PLGA. The suspended microspheres were collected using a nylon membrane filter with 0.20-μm pores under vacuum and then washed with another 5 mL of ddH_2O to rinse off mannitol bound to the microspheres. Then, the microspheres were transferred into a pre-weighed 2-mL tube and dried at room temperature under vacuum until the weight of the sample remained constant. The dried mannitol-free microspheres (i.e., microspheres without mannitol) were dissolved in 5 mL of DCM and 10 mL of ddH_2O was added. The mixture was heated to 60°C and mixed well to extract gelatin and leuprolide into the aqueous phase. After centrifugation at 2000*g* for 5 min at 40°C with slow brake, 8 mL of the aqueous phase was collected and replaced with the same volume of ddH_2O. The extraction was repeated one more time and then the extract was collected after lyophilization of the aqueous solution.

Since the extract contained leuprolide as well as gelatin, leuprolide was removed by using a centrifugal filter unit (Amicon Ultra-15, 10-KDa cut-off, EMD Millipore Corp., Darmstadt, Germany) to avoid the interference in the ion exchange chromatography. Briefly, the extract was reconstituted with 15 mL of 6 M acetic acid and transferred into the molecular cut-off filter device, followed by centrifugation at 5000*g* for 40 min at 30°C. Then, 12 mL of 6 M acetic acid was added to the concentrated extract and the separation was repeated one more time. To remove the acetic acid in the purified samples, 11 mL of 10 mM sodium chloride solution pre-warmed at 50°C was added to the tube. The excessive solution was removed by centrifugation at 5000*g* for 30 min at 40°C. This replacement process was performed twice. The purified gelatin extracts remaining on the upper layer of the filter tubes were collected after lyophilization. The dried extracts were reconstituted with ddH_2O to make the final concentration of gelatin around 2 mg/mL and heated to 60°C for 15–20 min with several times of vortexing, and immediately applied to ion exchange HPLC. Three batches of LD with different lot numbers were used. Type A and type B gelatins were dissolved in 6 M acetic acid, applied to a molecular cut-off filter device, and processed in the same manner and used as reference samples. Concentrations of all gelatin samples were 2 mg/mL.

The type of gelatin was analyzed by cation ion exchange HPLC installed with a TSKgel SP-NPR column (Tosoh Bioscience LLC, King of Prussia, PA) and a gradient elution of solvent A (10 mM citric acid buffer at pH 3), solvent B (20 mM sodium phosphate buffer at pH 11.5), and solvent C (1 M NaCl) at a flow rate of 1 mL/min as follows: 0 min (74:26, A:B), 2 min (53:47, A:B), 5.5 min (24:76, A:B), 12 min (100% B), and 14.5 min (100% C) followed by recovery with initial conditions for 3 min; the column temperature was 50°C. The wavelength of UV detection was 220 nm. After each run, acetic acid was used to wash the needle and ddH_2O was used to clean the residues on the column. A blank control was injected between samples to confirm there was no cross over contamination.

Molecular Weight of Gelatin

The gel strength of gelatin is typically determined by a texture analyzer and described by bloom number. Briefly, 6.67% gelatin water solution is prepared in a specified 150-mL standard bloom jar. After chilling, the rigidity of the gel is measured as the force required to depress a standard probe with a diameter of 0.5 in. to a depth of 4 mm into the gel (9). However, due to the limited quantity (1.3 mg) of gelatin in each syringe, preparing such a gelatin test solution is not a reasonable cost. As bloom number is related to molecular weight of gelatin (9), the distribution of gelatin molecular weight was studied instead of the bloom test, which requires extensive amount of sample to perform the assay. To determine the molecular weight (Mw), gelatin was extracted from the LD and purified as described in the gelatin typing section. Three batches of LD with different lot numbers were used. Three commercial gelatins with different bloom numbers (Nitta B 300, Nitta B 250, and Sigma B 75) were loaded

in the microspheres as described below and extracted and purified in the same way. Extracted and purified gelatin was reconstituted with ddH_2O at 2 mg/mL and 10 μL of the samples was injected to UPLC installed with a TSKgel UP-SW3000 column (Tosoh Bioscience LLC, King of Prussia, PA, USA). The mobile phase was composed of 0.1 M potassium dihydrogen phosphate buffer and 0.1 M disodium monohydrogen phosphate buffer (1:1, *v*:*v*) and the flow rate was set to 0.2 mL/min. The column temperature was 30°C and the sample temperature was 40°C. The wavelength of UV detection was 230 nm. Protein standards (Gel Filtration Markers Kit, Sigma-Aldrich, St. Louis, MO, USA) were used as molecular weight markers. The standard mixture contained carbonic anhydrase, albumin, alcohol dehydrogenase, β-amylase, apoferritin, and thyroglobulin. The molecular weight of the standard mixture ranged from 29,000 to 700,000 Da.

Preparation of PLGA Microspheres for Gelatin Analysis

Gelatin and leuprolide acetate were loaded into PLGA microspheres by solvent evaporation method. PLGA (600 mg) was dissolved in 1 mL DCM. Gelatin (10 mg) and leuprolide acetate (68 mg) were dissolved in 150 μL ddH_2O at 60°C. The water phase and the oil phase were mixed and then emulsified using a VirTis Tempest IQ2 homogenizer (SP Scientific 184 Inc., Warminster, PA, USA) at speed 15,000 rpm for 4 min to form a W1/O emulsion. The obtained W1/O emulsion was cooled to 18°C to increase the viscosity of the emulsion. Then, 4 mL aqueous 0.25% polyvinyl alcohol (PVA) (EG-40P) (Soarus L.L.C., Arlington Heights, IL, USA) solution was added to the W1/O emulsion and the mixture was homogenized at 12,000 rpm for 4 min. After homogenization, a W1/O/W2 emulsion was obtained. The W1/O/W2 emulsion was transferred into 200 mL 0.25% PVA solution and stirred with an overhead stir-tester (Glas-Col G.K.H. stir-Tester and Model HST20 stirrer, Terre Haute, IN, USA) at 700 rpm for 3 h to evaporate the methylene chloride and solidify the oil phase. The suspensions were rinsed with at least 1 L of water to wash off the unencapsulated drug and PVA. The microspheres were passed through a 90-μm-opening sieve to remove the large microspheres and collected by centrifugation at 4000 rpm for 5 min. The microspheres were freeze-dried under vacuum for 48 h.

Determination of Content of Gelatin by Amino Acid Analysis

Amino acid analysis was performed in the same way as described in the Determination of Leuprolide Acetate Loading section. Standard solutions of gelatin were prepared by dilution of the hydrolyzed Nitta B 300 gelatin samples. Gelatin has several specific amino acids such as alanine (Ala), asparagine and aspartic acid (Asx), hydroxylproline (OH-Pro), and valine (Val), which do not exist in the nonapeptide sequence of leuprolide (1,9). The second abundant amino acid in the gelatin, alanine, was used to determine the gelatin content in the LD formulation. Poor reproducibility was found when using glycine, the most abundant amino acid in the gelatin, likely because of poor peak separation. Three batches of LD with different lot numbers were used and the experiment was performed in triplicate.

Characterization of the Polymer in the LD Formulation

Determination of the PLGA Weight Average Molecular Weight, Number Average Molecular Weight, and Polydispersity Index

As the cryoprotectant in the LD, D-mannitol is insoluble in tetrahydrofuran (THF) and it was removed as described in the gelatin typing section. Then, mannitol-free LD microspheres were dissolved in dehydrated THF at 4 mg/mL. As the presence of moisture/water can induce degradation of the polymer, THF was dehydrated by 3-Å molecular sieves (Sigma-Aldrich, St. Louis, MO, USA). The samples were subjected to gel-permeation chromatography (GPC) installed with two styragel columns (HR 1 and HR 5E columns, Waters, Milford, MA, USA) and a refractive index detector (2414 refractive index detector, Waters, Milford, MA, USA). Polystyrene standards with Mw ranging from 1000 to 50,000 Da were dissolved in the dehydrated THF. Mw, number average molecular weight (Mn), and polydispersity index (PDI) of PLGA were calculated by Breeze software (Waters, Milford, MA, USA).

Quantitative NMR Analysis to Determine PLGA Content and Lactic Acid to Glycolic Acid Ratio

Quantitative ^{1}H NMR (qNMR) (Varian, Inc., Palo Alto, CA, USA) was used to determine the ratio of lactic acid and glycolic acid as well as the content of PLGA by using dimethyl terephthalate (DMT) as an internal standard (13). The mannitol in the LD formulations was removed as described in the gelatin typing section. The mannitol-free LD microspheres were dissolved in CDCl3 at 15–20 mg/mL with DMT at 1.0–2.0 mg/mL and subjected to NMR analysis. From the area of the peaks, the masses of LA and GA in PLGA are determined using the following equation (13):

$$M_s = M_{IS} \cdot \frac{Mw_s}{Mw_{IS}} \cdot \frac{nH_{IS}}{nH_s} \cdot \frac{P_{IS}}{P_s} \cdot \frac{A_s}{A_{IS}} \quad (1)$$

where "*s*" designates LA or GA in polymer and "IS" represents the internal standard; *Ms* and M_{IS} are the masses, *Mws* and Mw_{IS} are the molecular weights in g/mol; *Ps* and P_{IS} are the purities; *nHs* and nH_{IS} are the numbers of protons that contribute to the peak signals used for integration; and *As* and A_{IS} are the peak areas for the selected peaks (13). It is noted that *Ps* was set at 100% because the purity of PLGA to manufacture LD was undisclosed.

Determination of Acid Number of PLGA

The number of free carboxylic acid end group in PLGA was determined by organic phase titration (14). Approximately 10 mg of LD was dissolved in 5 mL of dehydrated acetone/tetrahydrofuran (1:1, *v*:*v*) mixture. Phenolphthalein methanol solution (0.1 wt%) was added as an indicator. The solution was immediately titrated with 0.01 M methanolic

potassium hydroxide to a stable pink end point. The acid number of PLGA was calculated using the following equation:

$$\text{Acid number[mgKOH/gPLGA]} = \frac{(\text{Volume of sample[mL]}) \times (N_{KOH}) \times (\text{Mw}_{KOH})}{(\text{Weight of PLGA[g]})} \quad (2)$$

Characterization of the Diluent

Determination of pH level of Diluent in the LD Formulation

The pH level of diluent was determined by a pH meter (430 pH Meter, Corning, Inc. Corning, NY, USA) equipped with a microelectrode (MI-410, Microelectrodes, Inc., Bedford, NH, USA). The pH meter was calibrated using standard solutions at pH levels 4 and 7 at room temperature.

Determination of Water Content of Diluent

The diluent is supposed to contain 5 mg (0.5%) Na-CMC, 50 mg (5%) D-mannitol, 1 mg (0.1%) polysorbate 80 in water for injection (1-mL injection diluent for a 7.5-mg dose of the drug), and glacial acetic acid (USP) to control pH level (1,6). The water content was estimated by the weight difference before and after drying of diluent. Approximately 300 μL of the diluent was added to pre-weighed vials and the weight of diluent was recorded. After the diluent was dried at reduced pressure at 60°C for 48 h, the weight of sample was recorded. In order to confirm the weights of the samples remained constant and the water has been completely removed, the samples were further dried under the same conditions for an additional 2 h and the weight was measured again. This step was repeated for one more time to determine the final weight of the samples.

Determination of D-Mannitol Content in the LD Formulation and in Diluent

The content of D-mannitol was determined using a D-mannitol colorimetric assay kit (Sigma-Aldrich, St. Louis, MO, USA). D-Mannitol was converted to D-fructose by mannitol dehydrogenase in the presence of NAD. This reaction produces NADH and the concentration of NADH could be determined by UV absorbance at 450 nm of wavelength. Approximately 1 mg of LD formulation was added to 2-mL tubes. A 1.5 mL aliquot of ddH_2O was added and the suspension was centrifuged at 8000*g* for 5 min. Then, 10 μL of the supernatant was added to the 96-well plate using a pipette pre-calibrated by a balance. To determine the content of mannitol in diluent, approximately 20 mg of the diluent was diluted 500 times with ddH_2O and 10 μL of the samples was added to the 96-well plate by a pre-calibrated pipette. The assay buffer and reaction mixture solution were added according to the instructions in the assay kit. After the incubation at 37°C, the plate stood for another 30 min until the air bubbles disappeared. The concentration of mannitol was determined by UV absorbance at 450 nm of wavelength (SpectraMax M3, Molecular Devices, Sunnyvale, CA, USA).

Determination of Viscosity and Specific Gravity of Diluent

The viscosity of the diluent in LD was determined by an Anton-Paar rolling-ball viscometer Lovis 2000 M/ME, which measures the rolling time of a ball through liquid according to Hoeppler's falling ball principle (15). The mimic diluent was prepared by adding Tween 80, Na-CMC (high viscosity or low viscosity, Sigma-Aldrich, St. Louis, MO, USA), and mannitol at the same ratio as the composition in diluent. Specific gravity was measured using a 1-mL pycnometer. The pycnometer was pre weighed and filled with the diluent in LD. Then, the pycnometer was placed in a thermostatic bath with temperature controlled at 25°C. After the temperature of the solution was equilibrated, excess volume of the solution that expelled from the top of the pycnometer was absorbed with Kimwipes (Kimberly-Clark Professional, Roswell, GA, USA). The weight of the filled solution was recorded to determine the specific gravity using the density of water at 4°C (density = 1 g/mL).

Determination of Tween 80 Content in Diluent

To determine the content of Tween 80 in the diluent, *bis*-ANS (4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid, dipotassium salt) was used as a fluorescent probe. This fluorescent probe is almost non-fluorescent initially, and the fluorescence increases when it reacts with the hydrophobic group in Tween 80 (16). Briefly, 50 μL of diluent was mixed with 950 μL water and then, 55 μL of 1 mM *bis*-ANS solution was added. Then, the mixture was vortexed for 5 s and shaken at 220 rpm for 5 min, followed by no agitation to equilibrate for 25 min. The stock standard solution of Tween 80 (Thermo Fisher Scientific Inc., Waltham, MA, USA) was prepared in ddH_2O with the presence of Na-CMC and mannitol at the same ratio as the composition in LD diluent and it was further diluted to make serial standard solutions that fell within the range of 30–100 ppm. Two hundred microliters of the mixture was loaded to Costar 96-well plates (black bottom polystyrene) and the concentration of Tween 80 was determined by fluorescence (excitation emission, 380–500 nm) (SpectraMax M3, Molecular Devices, Sunnyvale, CA, USA).

Characterization of Product Attributes

Particle Size Distribution

The median diameter of the microspheres was determined using a Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK). About 30–40 mg of LD formulation was suspended in 1 mL of diluent and vortexed vigorously before added to the instrument sample dispersion unit. Three measurements were performed per sample at a stirring speed of 2500 rpm and sampling time of 15 s.

Surface Morphology

The surface morphology of microspheres was examined using a Hitachi S3200N scanning electron microscope (SEM) (Hitachi, Tokyo, Japan). The LD microspheres were fixed on a brass stub using double-sided carbon adhesive tape and the

samples were prepared electrically conductive by coating with a thin layer of gold for 120 s at 40 W under vacuum (17). Images were taken at an excitation voltage of 10.0 kV.

Glass Transition Temperature

The glass transition temperature (Tg) of LD was determined with a modulated differential scanning calorimeter (mDSC) (TA Instruments, New Castle, DE, USA). LD microspheres (3–5 mg) were crimped in DSC aluminum pans. Temperatures were ramped between – 20 and 90°C at 3°C/min. All samples were subjected to a heat/cool/heat cycle. The results were analyzed by using TA TRIOS software and Tg was taken as the midpoint of the reversing heat event.

Residual Moisture

Residual water content in microspheres from the LD was determined by Karl Fischer (KF) titration. Eighty milligrams of LD was weighed into a vial and sealed with a septum cap. Anhydrous dimethyl sulfoxide (DMSO) was added to make the final concentration at 10 mg/mL and the sample was sonicated for 10 min before injected into the KF for titration. The moisture in the blank DMSO was also determined.

Residual Solvent

Residual solvent (methylene chloride) in Lupron Depot® was determined by a Trace 1310 gas chromatograph (GC) (Thermo Fisher Scientific Inc., Waltham, MA, USA). The LD microspheres were added into a glass vial containing anhydrous DMSO to make the final concentration at ~ 10 mg/mL and the vial was sealed. The samples were applied to the GC by two different methods: headspace and liquid injections. For headspace injection, the GC conditions were as follows: nitrogen gas was used as the carrier solvent at a flow of 25 mL/min; air flow was 350 mL/min and hydrogen flow was 35 mL/min; and the front detector temperature was 240°C and the front inlet pressure was a constant flow at 2 mL/min. Each sample was agitated for 20 min at 80°C and 1 mL of the headspace sample was injected into the front inlet with the temperature of 140°C, a split flow of 40.0 mL/min, and a split ratio of 20. The GC column temperature was initially set at 40°C for 15 min, then increased at 10°C/min to 240°C and held at 240°C for 2 min. For liquid injection, the GC conditions were as follows: nitrogen gas was used as the carrier solvent at a flow of 33 mL/min; air flow was 450 mL/min and hydrogen flow was 34 mL/min; and the front detector temperature was 220°C and the front inlet pressure was a constant flow at 12 mL/min. The injection volume was 1 μL and the inlet operation was in splitless mode with temperature at 200°C. The GC column temperature was initially set at 40°C for 1 min, increased at 5°C/min to 65°C, and then increased at 100°C/min to 190°C. A standard curve was prepared by adding methylene chloride to DMSO at 1, 10, 50, 100, 250, and 500 ppm.

Release Kinetics

Drug release of microspheres was carried out using a sample-and-separate method in release medium PBST (phosphate-buffered saline (PBS) + 0.02% Tween 80 + 0.02% NaN_3, pH 7.4). Microspheres (~ 10 mg) were suspended in 1 mL of medium and shaken mildly at 37°C. At each time point (1, 3, 7 days and weekly thereafter), the medium was completely collected after centrifugation at 8000 rpm for 5 min and replaced with fresh PBST buffer. The concentration of leuprolide in the supernatant was determined by UPLC as described in the "Determination of Leuprolide Acetate Loading" section.

Statistical Analysis

Statistical analyses were carried out using GraphPad Prism 7.04 software. One-sample *t* test was used to compare the measured values to the officially labeled numbers. The level of significance was established at the 95% confidence interval ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Characterization of LD Microspheres

Leuprolide Acetate Content

Leuprolide acetate content in the 1-month LD was determined by three different methods, as shown in Fig. 1. Method 1, as performed by the originator of the LD, indicated 8.31 ± 0.05 wt% (mean ± SEM, $n = 3$) of leuprolide acetate in the LD. However, cationic leuprolide is capable of binding to negatively charged terminal chains of PLGA even in the DCM phase (11). Note that the acetate counterion of leuprolide is less acidic than the end group of PLGA (18) and is therefore expected to deprotonate the polymer end group to some extent. Hence, method 2 with multiple extractions of leuprolide acetate was performed, giving 8.95 ± 0.31 wt% (mean ± SEM, $n = 3$) as the leuprolide acetate content. As expected, method 2 increased the recovery of leuprolide by 0.6 wt% more than method 1. In method 3, the leuprolide content was determined by the concentration of amino acid in the samples, which should not be affected by the interaction between the peptide and polymer that exists in the extraction method. The peak area ratios of histidine (retention time = 8.15 min) to the internal standard, norleucine (retention time = 12.7 min), were used to determine the concentration of leuprolide based on the standard curve. The measured value, 8.89 ± 0.13 wt% (mean ± SEM, $n = 3$), was slightly higher than the result in method 1 and comparable to that of method 2. All three methods provided reasonable measured values which were not significantly different (*t* test, $p > 0.05$) from the officially reported value 8.5 wt% in the package insert of LD (6). The leuprolide acetate standard solutions used in this study were compared to the USP leuprolide acetate standard solutions on three different days and were confirmed to be within 100.5 ± 2.2% (mean ± SEM, $n = 3$) of the USP standard by UPLC in the concentration range of 0–600 μg/mL.

Gelatin Type

Figure 2 displays representative ionic exchange chromatographs of gelatin samples. Pure type A and type B gelatins were differentiated based on their major peaks, which appeared at

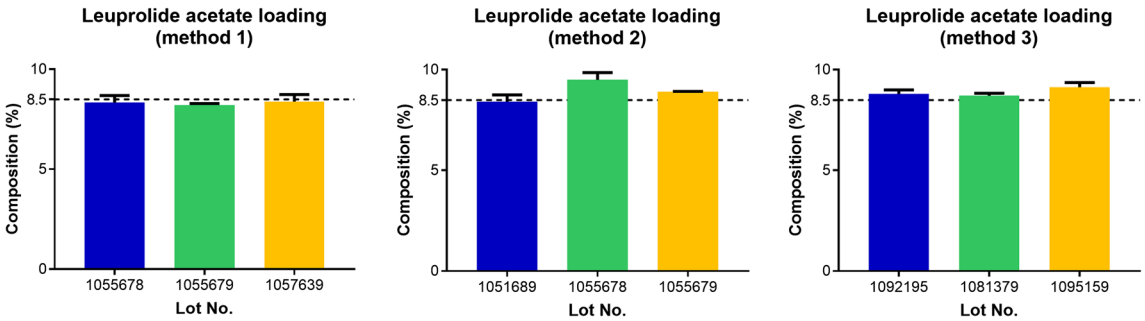


Fig. 1. Leuprolide content in LD formulations determined by two different extraction methods. All values present as mean ± SEM ($n = 3$). The dash lines indicate the official LD loading (6)

retention times around 13.5 min (Fig. 2h) and 4.2 min (Fig. 2f–g), respectively. The extracted gelatin from three different batches of LD shown in Fig. 2b–d exhibited major peaks at roughly the same retention time as that of type B gelatin, which were far from that of type A gelatin. Therefore, the gelatin loaded in the LD was identified as type B.

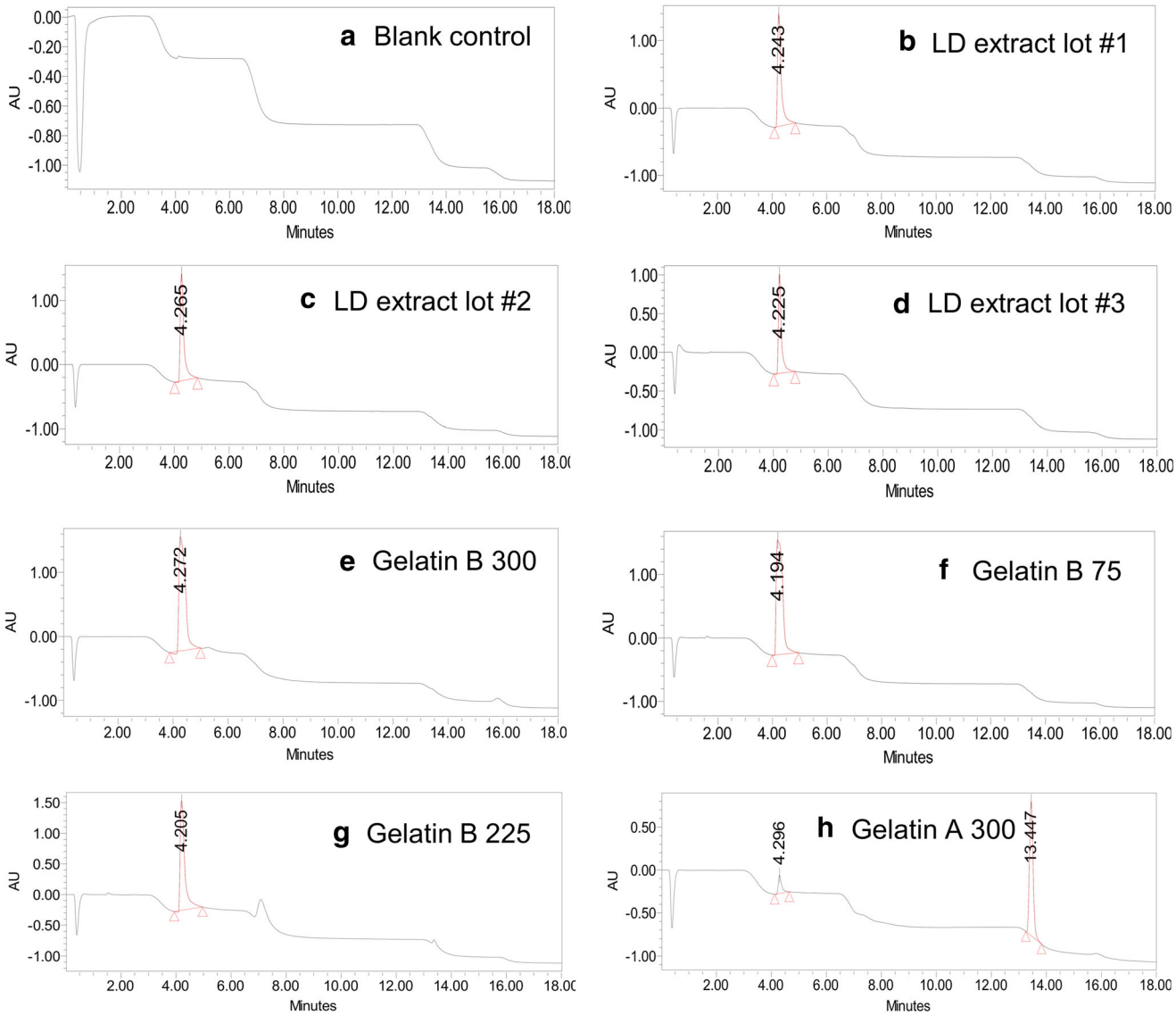


Fig. 2. Ion exchange chromatograms of blank control (**a**), LD extract lot no. 1 (**b**), LD extract lot no. 2 (**c**), LD extract lot no. 3 (**d**), type B gelatin with bloom number 300 from Nitta gelatin Inc. (**e**), type B gelatin from Sigma-Aldrich with bloom number 75 (**f**), type B gelatin from Sigma-Aldrich with bloom number 225 (**g**), and type A gelatin from Sigma-Aldrich with bloom number 300 (**h**). Note that negligible peaks are present in type A gelatin sample potentially due to the impurity in the product

Molecular Weight of Gelatin

The representative chromatography of extracted gelatin from LD is shown in Fig. 3a. As the retention time of peaks is related to the molecular weight, the peaks were fractioned into eight sections (Fig. 3a, b) based on the molecular weight standards. The percentages of the area of peak were obtained to plot the Mw distributions of gelatin samples (Fig. 3c). In Fig. 3c, Nitta type B gelatins of bloom numbers 300 and 250, Sigma type B gelatins of bloom numbers 75 and 225, and Sigma type A gelatin 300 bloom were dissolved in acetic acid and collected after being applied to molecular cut-off filter device before UPLC analysis in the same way as the purification process of extracted gelatin from LD. Considering the potential degradation of gelatin during dissolving, encapsulation, and extraction, we dissolved and loaded various gelatins into the PLGA microspheres using polymer produced by Wako and leuprolide acetate according to the W/O/W method. Then, we performed the extraction and purification process in the same way as described above for the LD. Three gelatins with relatively high, medium, and low bloom numbers were studied in this experiment and the Mw distributions are designated as *extracted Nitta B 300*, *extracted Nitta B 250*, and *extracted Sigma B 75* in Fig. 3c. Compared to the gelatin samples without the encapsulation process, all of the extracted gelatins showed higher levels of lower Mw fractions indicating of gelatin hydrolysis during microsphere preparation. The extracted Nitta B 300 showed very similar Mw distributions to the extract from the LD (Fig. 3c). To further confirm Nitta B 300 is comparable to the gelatin in LD in terms of their Mw distribution, we prepared three batches of microspheres loaded with Nitta B 300 gelatin and performed the extraction, purification, and Mw analysis as described above. The Mw distributions of extracted Nitta B 300 gelatin were compared to the extracts from three different batches of LD in Fig. 3d. From the results regarding peak shape and Mw fractions, it is reasonable to conclude that the LD was encapsulated with high Mw gelatin (i.e., bloom 300). Combined with the result from the gelatin typing section, the properties of Nitta B 300 gelatin matched the gelatin used in the LD. Furthermore, Nitta B 300 is manufactured with low endotoxin, is suitable for injection, and was used in publications (7) from the LD inventor. Therefore, Nitta B 300 gelatin was used as the reference gelatin in the measurement of gelatin content and identified as the probable source of gelatin in the LD.

Gelatin Content

The peak area ratios of alanine (retention time = 10.05 min) to the internal standard (retention time = 12.7 min) were used to determine the concentration of gelatin based on the standard curve. The average content of gelatin in the LD samples was determined as 1.55 ± 0.08 wt% (mean ± SEM, $n = 3$) (Fig. 4), which was not significantly different (t test, $p > 0.05$) from the labeled content of 1.5% gelatin.

Molecular Weight of PLGA

PLGA is a biodegradable polymer that degrades by hydrolysis of ester bonds and the Mw of the PLGA is an important attribute to control the duration and kinetics of drug release (10). Weight-averaged molecular weight (Mw) and number-averaged molecular weight (Mn) of LD were determined as approx. 13.0 and 8.7 kDa, respectively with a PDI of 1.5 (Fig. 5). The LD 7.5 mg is reported to be composed of PLGA with an LA/GA ratio, 75:25; Mw, 12.1 to 14 kDa (10,19); and a ratio of Mw to Mn (PDI) of 1.81 (10). However, these characteristic numbers are related to the raw polymer before encapsulating leuprolide with gelatin by double emulsion solvent evaporation technique and Mw, Mn, and PDI could potentially be affected during the formulation process. Therefore, the results reflect the numbers of the PLGA in the finished product and were in reasonable ranges for Mw and Mn (10). The PDI obtained in this study was slightly lower than the published one.

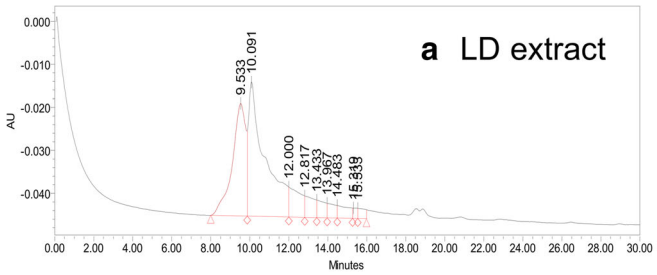
LA/GA Ratio and Content of PLGA

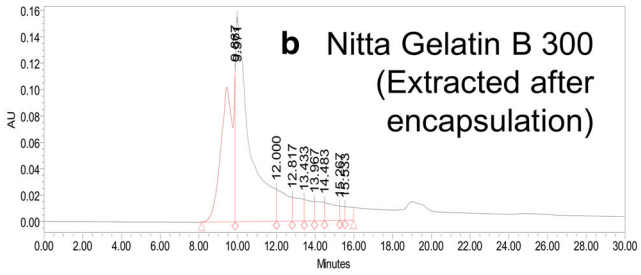
LA/GA ratio is another attribute of PLGA to control the duration of release. The PLGA ester bonds (pairs of GA-GA, LA-LA, and GA-LA or LA-GA) containing GA are less stable than the bonds with LA, and thus a higher content of glycolic acid facilitates the water uptake and increases the rate of degradation of the polymer (20). As the release progress depends on the degradation of PLGA ester bonds, the composition of monomer changes over time of release, typically resulting in an increase in LA/GA ratio (18).

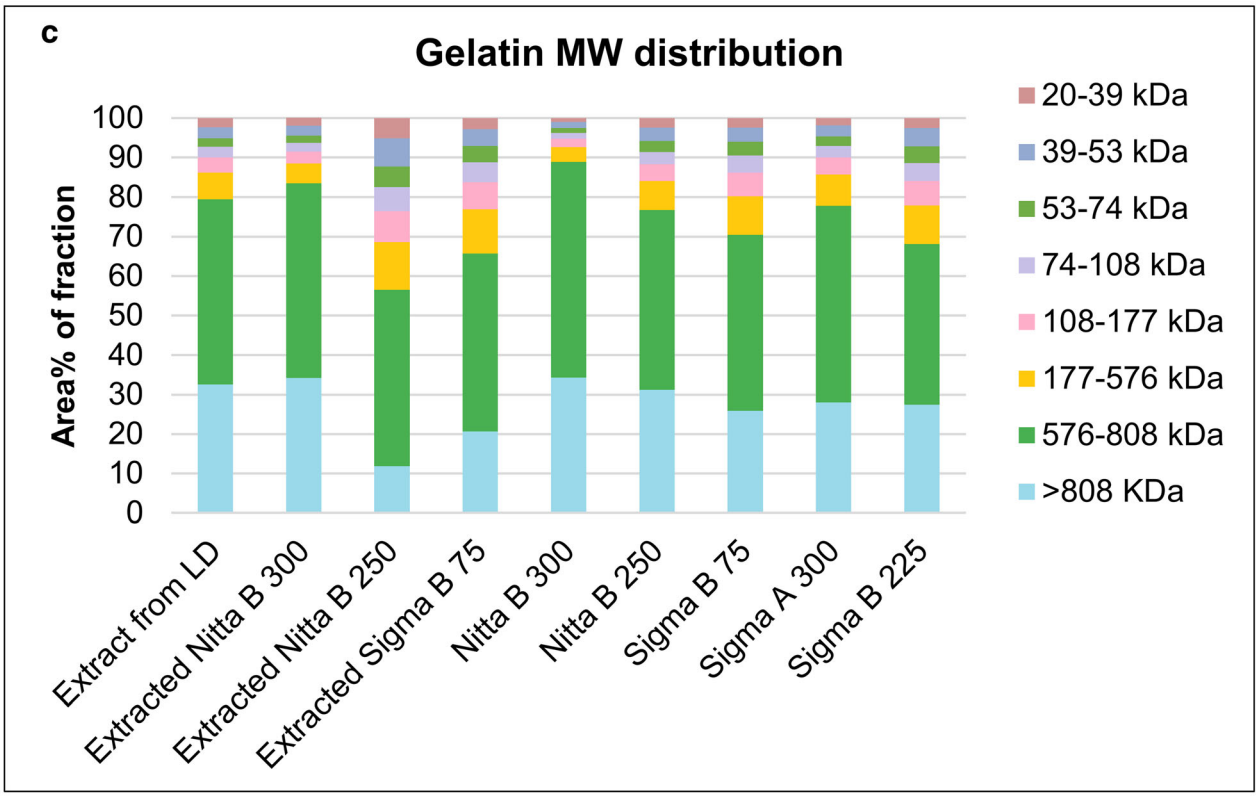
Figure 6 displays a representative NMR. The LA/GA ratio was determined from the proton signals generated by methyl ($–CH_3$) and CH groups of GA and methylene ($–CH_2$) groups of LA. The initial LA/GA ratio was found to be 74.3/25.7, which closely corresponds to the expected values of 75/25 (1,19). Additionally, the content of PLGA was determined by the sum of the masses of LA and GA calculated by Eq. (1). As summarized in Fig. 5, it was found that the content of PLGA was 87.0 ± 0.3% (mean ± SEM, $n = 3$), which is quite close to the officially reported PLGA mass 88.3% (6).

Acid Number of PLGA

The acid number represents the number of free carboxylic acid functionalities in the PLGA at the terminal of the polymer chain and is essential to evaluate whether the end group is a carboxylic acid or an aliphatic ester. PLGA is insoluble in the aqueous phase so titration was performed in acetone/tetrahydrofuran solution using methanolic KOH. The acid number of PLGA in a single lot of the LD 3.75-mg dose formulation was determined as 12.9 mg KOH/g PLGA. In the polymers with similar molecular weight, the polymer with a carboxylic acid end group always has higher acid number compared to the polymer with an ester-capped group (21). Schrier and DeLuca (21) studied the acid numbers of different Resomer® polymer products (manufactured by Boehringer-Ingleheim (Ingleheim, Germany)) with and without ester end-capping, and showed that for the polymers with free acid end and with molecular weight in the range of 8–12.5 kDa (RG 501H, 502H, and 752H), the acid numbers were above 14 mg KOH/g PLGA while the ester end-capping forms had acid numbers below 2 mg KOH/g PLGA. The polymer Resomer® RG 752H has comparable molecular weight (Mw 13 kDa) to the polymer used in LD and the acid number was reported as 14.3 mg KOH/g PLGA (21). The high value of the acid number







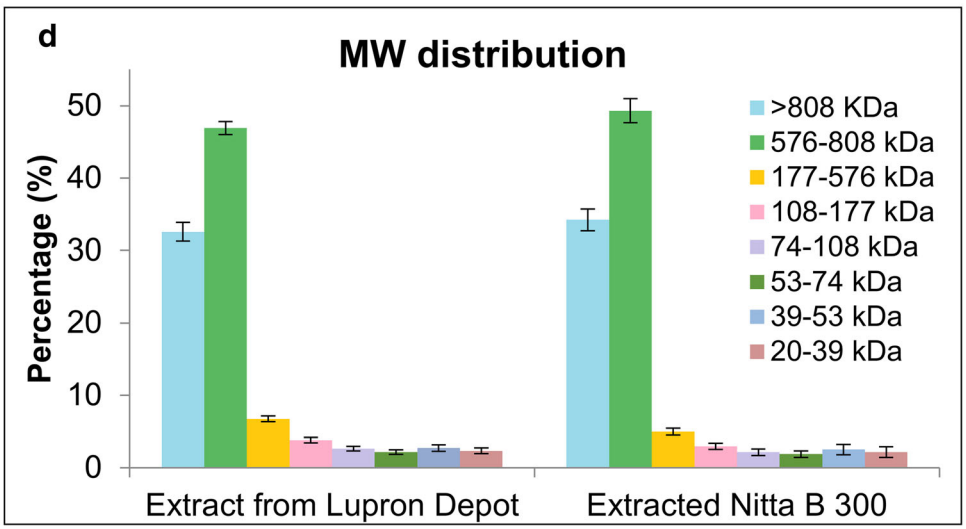


Fig. 3. GPC chromatograms of gelatin extract from the LD (**a**) and extracted Nitta gelatin B after PLGA encapsulation (**b**); Mw distributions of different gelatin products (**c**); and comparison of the Mw distributions between gelatin extract from the LD and extracted Nitta B 300 (**d**) (the bars indicate mean ± SEM, $n = 3$). Extracted gelatin samples were taken after PLGA encapsulation

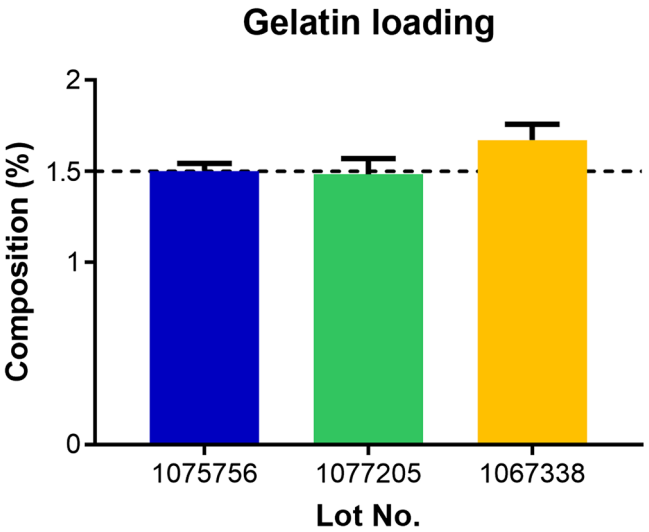


Fig. 4. Gelatin content in the LD. All values represent mean ± SEM ($n = 3$). The dash line indicates the official LD loading (6)

obtained in this study indicates the PLGA is the acid end-group polymer instead of ester end-group polymer, consistent with the innovators' publications and patents.

D-Mannitol Content in LD Formulation

After encapsulation, D-mannitol is added to LD microspheres to prevent aggregation during freeze-drying process and to help resuspension of the microspheres before administration (1). As shown in Fig. 7, the measured content of D-mannitol mixed with microspheres was 15.63 ± 0.43 wt% (mean ± SEM, $n = 3$), which was not significantly different (t test, $p > 0.05$) from the expected number, 15 wt% (6).

Characterization of the Diluent

pH Level, Water Content, and D-Mannitol of Diluent

The diluent of a LD kit displayed a pH level of 6.0–7.0. The content of water in the diluent was estimated as 94.55 ± 0.01 wt% (mean ± SEM, $n = 3$) (Fig. 7) and the results showed close values relative to the official content 94.4 wt%. The content of D-mannitol in the LD diluent was determined as 4.42 ± 0.07 wt% (mean ± SEM, $n = 3$) (Fig. 7). The value is close to but slightly lower than the official content of 5 wt%.

Characterization of Viscosity and Specific Gravity

It is considered that Na-CMC should be added to increase the viscosity of the diluent for maintaining the suspension of PLGA microspheres and for accurate injection. As characteristics of Na-CMC vary depending on Mw and viscosity, the diluent was initially subjected to microviscometry to identify the relative viscosity of Na-CMC. As a result, the viscosity of diluent was determined as 2.99 ± 0.06 cP (mean ± SEM, $n = 3$) and the simulated diluent containing low viscosity Na-CMC and all the other ingredients at the same quantity as the commercial diluent showed a similar value of 3.31 ± 0.03 cP (mean ± SEM, $n = 3$). The specific gravity of the LD diluent was determined to be 1.02.

Tween 80 Content

The critical micellar concentration (c.m.c.) of Tween 80 is 13–15 ppm (22,23) and the formation of Tween 80 micelles may affect the interaction between the hydrophobic group in Tween 80 and the fluorescent probe used in the assay. The presence of Na-CMC and mannitol may also affect the

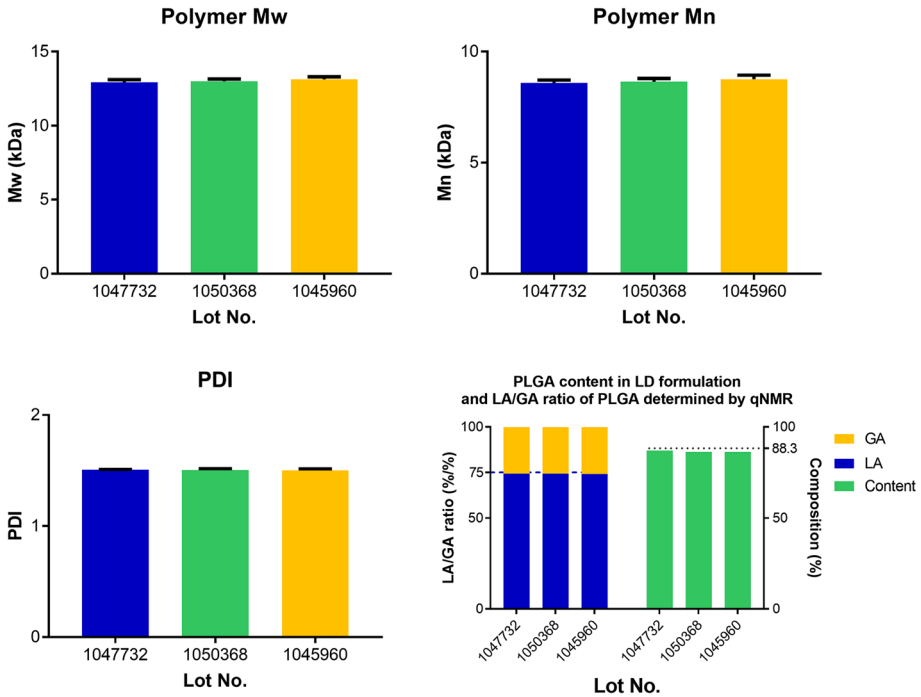


Fig. 5. Characterization of PLGA in the LD formulations. The values of Mw, Mn, and PDI represent mean ± SEM ($n = 3$). The dash lines indicate the official values

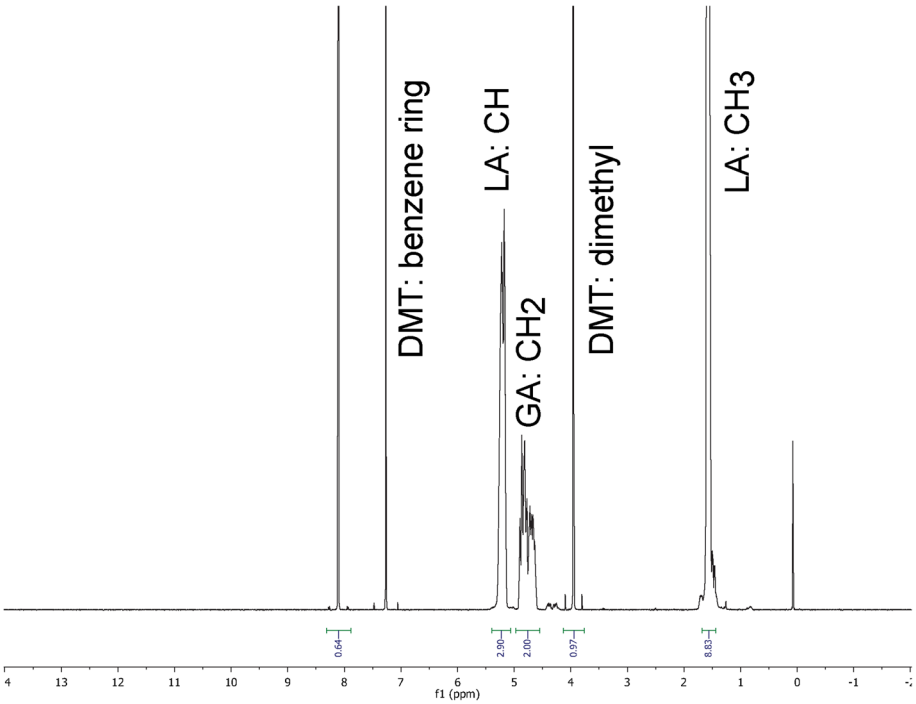


Fig. 6. ^{1}H NMR spectrum of PLGA from the LD with internal standard dimethyl terephthalate (DMT)

formation of Tween 80 micelles and the generated fluorescence. Several control studies were performed to avoid those influences in the measurement of Tween 80 content in LD. The results are not shown in this paper, but some key conclusions are summarized as follows: (1) Serial solutions of Na-CMC and mannitol were prepared in the absence of Tween 80 and showed negligible fluorescence; and (2) the standard Tween 80 solutions with the presence of Na-CMC and mannitol need to be prepared in the high concentration range (30–100 ppm) to achieve desirable linearity ($R^2 = 0.99$).

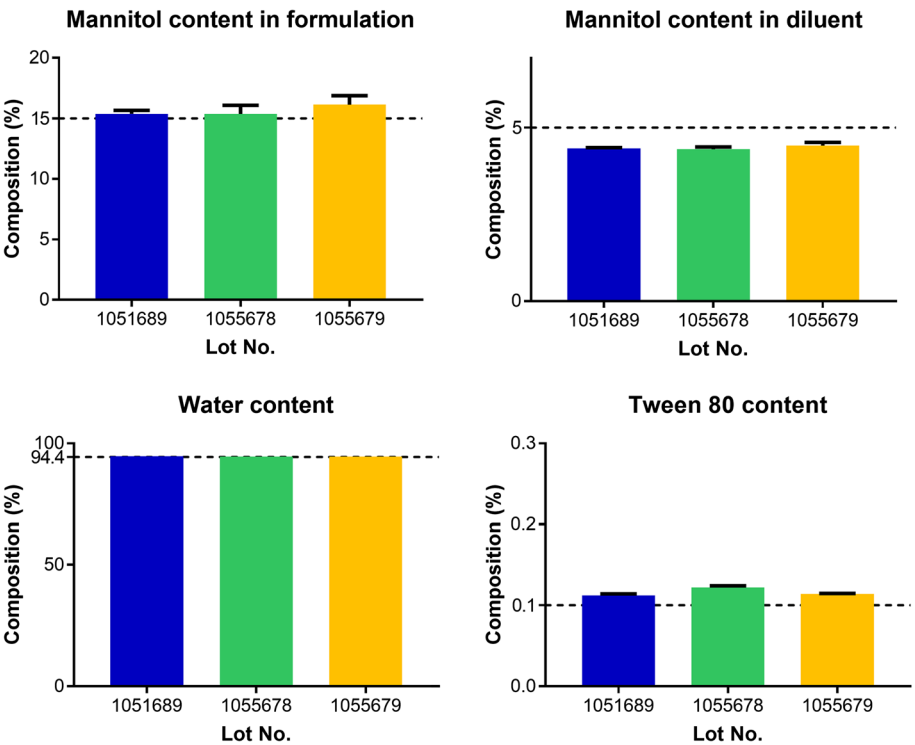


Fig. 7. Contents of D-mannitol in the LD formulation and diluent, and water and Tween 80 in the diluent. All values represent mean ± SEM ($n = 3$ for D-mannitol content in the formulation, water content, and tween 80 content; $n = 4$ for D-mannitol content in the diluent). The dash lines indicate the official LD compositions (6)

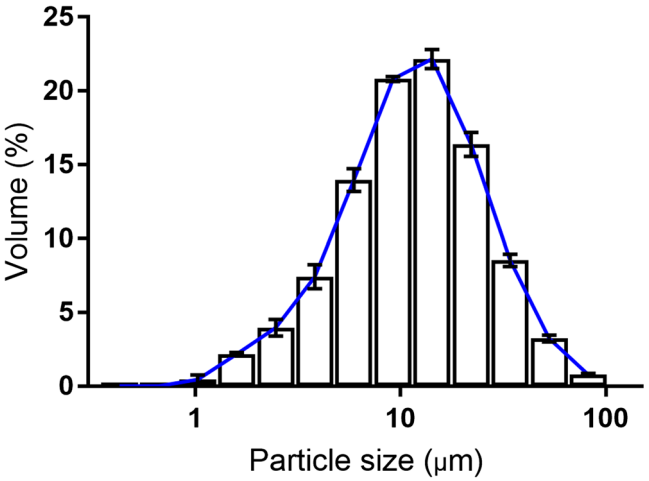


Fig. 8. Particle size distribution of the LD microspheres. The columns indicate mean ± SEM ($n = 3$)

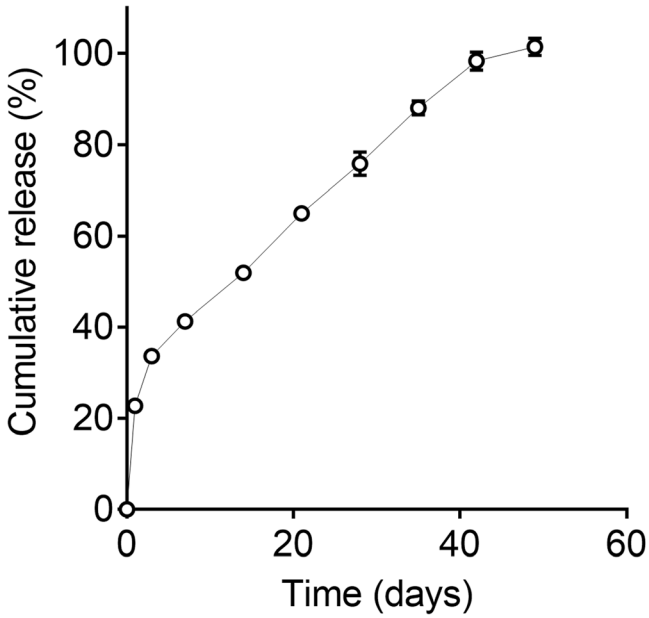


Fig. 10. *In vitro* release of LD formulation. Data represent mean ± SEM ($n = 5$). Error bars not plotted when smaller than symbols

The injection diluent from the LD was diluted to the concentration that fell within this range to generate reliable results. The content of Tween 80 in the diluent was determined as 0.116 ± 0.003 wt% (mean ± SEM, $n = 3$) which was close to the official content of 0.1 wt% (Fig. 7).

Characterization of Product Attributes

The particle size distribution of LD (Fig. 8) was narrow with a volume-median diameter of 11.4 ± 0.5 μm (mean ± SEM, $n = 3$) ($d(v, 0.5)$). Ten percent of the volume distribution was below 3.8 ± 0.2 μm (mean ± SEM, $n = 3$) ($d(v, 0.1)$) and 90% of the volume distribution was below 30.0 ± 0.6 μm (mean ± SEM, $n = 3$) ($d(v, 0.9)$). These results were supported by the SEM micrographs. As seen in Fig. 9, the LD formulations were spherical microspheres mixed with mannitol and the majority of the microspheres were < 20 μm. The Tg of LD was measured as 48.6 ± 0.1°C (mean ± SEM, $n = 3$). Note that the presence of leuprolide has been reported to increase Tg of the microspheres as a result of the peptide-polymer interaction (1,11). The water content of the LD was determined by Karl Fischer titration as 0.44 ± 0.10% (mean ± SEM, $n = 3$), indicating careful drying of the product. Very surprisingly, the residual content of methylene chloride determined by two different GC methods was < 1 ppm. Clearly, the in-water drying protocol is capable of achieving low levels of organic solvent in the final microspheres manufactured on a large scale. Lastly, as seen in Fig. 10, the cumulative release of LD in PBST lasted for 7 weeks with a 22.8 ± 0.4% initial burst on day 1 followed by a zero-order release after day 3. The release curves after day 1 were fit using linear regression and the time to 50% release ($t50$) was calculated to be 12.3 ± 0.2 days. Overall, these data are consistent with the existing literature (1,24) on the LD and a carefully formulated and manufactured product.

Comparing and contrasting our entire dataset with that previously reported, mostly from the LD manufacturer, Takeda Chemical Industries, Ltd. (Osaka, Japan), we find excellent agreement and some new insights. The comparison between the published values and measured values are summarized in Table S1. The determination of composition of chamber 1 provided reasonable measured values, which were not significantly different from the labeled values. The measured values of the composition of diluent were also close to the labeled values and the accuracy might be affected by the complexity of the diluent. The specific gelatin used in the formulation was identified in this report. The Mw of the polymer in the product was close to the reported Mw of raw polymer (1,10,19) indicating no significant degradation occurred during the manufacturing. The viscosity of the diluent was determined and was similar to the simulated diluent. The particle size distribution and SEM micrographs indicated the LD

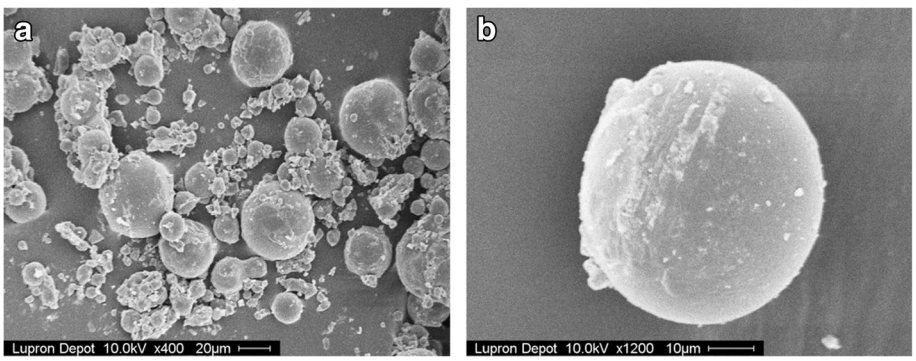


Fig. 9. SEM micrographs of the LD formulation

microspheres were fine and small particles, which matched the brief descriptions in the literature (1). The Tg of the LD formulation showed a higher value compared to the raw polymer due to the interaction between peptide and polymer chains and relatively high drug loading (10%) (1,24). The inventors stated the residual DCM in the formulation was below 100 ppm (1) and our observation indicated this value was below 1 ppm. Ogawa *et al.* (25) studied the release of leuprolide from PLGA by using rotating bottle method and phosphate buffer (pH 7) containing 0.05% Tween 80, and concluded the release kinetics followed zero-order release over 4 weeks by measuring peptide remaining in the microspheres. In this study, we used a sample-and-separate method and microspheres were incubated in PBST and shaken mildly. We observed a slightly faster initial release and a zero-order release after day 3. The release was more than 80% after day 35 and complete after day 49.

CONCLUSIONS

Analytical methods for analyzing the specific components of the 1-month Lupron Depot®, including its diluent, have been developed, and the ingredients have been identified and quantified. The results are consistent with the values reported in the drug label and literature, although we found the LD content by rigorous amino acid analysis and multiple extraction protocols slightly higher than listed in the drug label but not statistically significant. The most complex aspect of the analysis is the evaluation of gelatin in the LD, which may undergo hydrolysis during preparation of microspheres and extraction from drug product. The gelatin appears to be type B with bloom 300. Attributes including particle size distribution, residual water and solvent levels, Tg, and *in vitro* release demonstrate the unique features of this product. The analysis described here will be useful for further development of generic leuprolide microspheres and also could be applied for reverse engineering analysis of other PLGA-based long-acting release products.

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