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# Long-acting intramuscular injections of ELQ-331, an antimalarial agent

Dipu Karunakaran [a], Shravan K. Mutyam [a], Melody Fu [a], Jiaming Chen [a], Kim Hue Nicky Pham [a], Sovitj Pou [b], Rolf W. Winter [b], Aaron Nilsen [b], Rozalia A. Dodean [b], Martin J. Smilkstein [b,c], Michael K. Riscoe [b,c], Gita Shankar [a,*]

[a] Pharmaceutical Sciences Group, Biosciences Division, SRI International, 333 Ravenswood Avenue, Menlo Park, CA, USA
[b] VA Medical Center, Experimental Chemotherapy Lab, 3710 SW, US Veterans Hospital Road, Portland, OR, USA
[c] Oregon Health & Science University, Department of Molecular Microbiology & Immunology, 3181 SW Sam Jackson Boulevard, Portland, OR, USA

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ABSTRACT

The overarching premise of this investigation is that injectable, long-acting antimalarial medication would encourage adherence to a dosage regimen for populations at risk of contracting the disease. To advance support for this goal, we have developed oil-based formulations of ELQ-331 (a prodrug of ELQ-300) that perform as long-acting, injectable chemoprophylactics with drug loading as high as 160 mg/ml of ELQ-331. In a pharmacokinetic study performed with rats, a single intramuscular injection of 12.14 mg/kg maintained higher plasma levels than the previously established minimum fully protective plasma concentration (33.25 ng/ml) of ELQ-300 for more than 4 weeks. The formulations were well tolerated by the rats and the tested dose produced no adverse reactions. We believe that by extending the length of time between subsequent injections, these injectable oil-based solutions of ELQ-331 can offer a more accessible, low-cost option for long-acting disease prevention and reduced transmission in malaria-endemic regions and may also be of use to travelers.

## 1. Introduction

Chemoprophylactic medicines can provide significant economic and health benefit to populations, but they are only effective when users follow the prescribed dosage regimen. Non-adherence defeats the purpose of chemoprophylactics because, after an interval, plasma levels of the drug can fall below the concentration necessary for disease prevention (Stirratt and Gordon, 2008; Trussell and Portman, 2013). Long-acting chemoprophylactic formulations have been shown to improve adherence by reducing the number of user-controlled dosing events, which lowers or eliminates the probability of missing a dose. These kinds of dosage forms have the longest history of use in spacing childbirth (Winner et al., 2012). More recently, they have been investigated for use in HIV pre-exposure prophylaxis (Krovi et al., 2021).

Malaria (Nosten et al., 2022) is another disease for which a prophylactic strategy could be useful. The disease continues to cause significant numbers of deaths in regions where it is endemic. In 2021, there were an estimated 247 million cases of malaria worldwide resulting in an estimated 619,000 deaths, mostly in malaria-endemic countries (World Health Organization, 2022). These malaria-endemic regions, generally financially resource-challenged countries in the tropics, face difficulties in raising funds that can be allocated to healthcare. These regions are also where the economic costs of malaria is mostly felt (Andrade et al., 2022). Even when medications are available, patient compliance during the treatment regimen of malaria is poor (Beer et al., 2009; Fogg et al., 2004; Khantikul et al., 2009). Adherence failure, which worsens disease and death rates, reduces the possibility of eliminating the malarial parasite and likely leads to the development of drug resistance (White and Pongtavornpinyo, 2003). Suitably used (*e.g.*, during times of increased mosquito breeding or in areas with increased prevalence of waterlogging), chemoprophylaxis appears to promise a reduction of the disease burden in malaria-endemic regions over a period of time by interfering with the reproductive cycle of the parasite (Macintyre et al., 2018).Travelers to these areas could also benefit from a conveniently dosed antimalarial chemoprophylactic (Marasinghe et al., 2020).

Developing successful pharmaceutical products involves the tailoring of the formulation to patient needs. One way this can be done is by meeting the needs stated in the target product profile (TPP). We used the TPP stated by Medicines for Malaria Venture (MMV) for an injectable prophylactic medicine for malaria, which states dosing to occur not more than once every three months, an acceptable injection volume,

* Corresponding author.
*E-mail address:* gita.shankar@sri.com (G. Shankar).

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suitable for intramuscular (IM) injection with minimal preparation, able to be administered with a 27-gauge needle, a cost of less than 5 USD per injection, and a shelf life of at least 2 years (Macintyre et al., 2018). It appears to be possible to meet these requirements with current formulation strategies and the drug formulations described in this report.

A few reports have described the use of small molecules as possible long acting injectable chemoprophylactic (LAI-C) using existing approved small molecule drugs (Bakshi et al., 2018; Fan et al., 2022; Pooda et al., 2023). However, commercially feasible LAI-C formulations have only become possible with the appearance of potent molecules that have the attributes necessary to support long-acting dosage forms. ELQ-331 (Smilkstein et al., 2019), a prodrug of ELQ-300 (Frueh et al., 2017), appears to fulfill the requirements for an antimalarial LAI-C. ELQ-300 is active against liver stage, blood stage, and sexual forms of *Plasmodium* parasites (Nilsen et al., 2013) and the ELQ-331 prodrug substantially enhances ELQ-300 exposure. As a result, ELQ 331 is a preclinical candidate in MMV's pipeline of malaria prophylactics (Pou et al., 2021). The minimum fully effective protective plasma concentration of ELQ-300 ($MEP_{100}$) to prevent sporozoite-induced infection is about 80 nM in mice. Allometric scaling indicates that a dose of 2.5 mg/kg (translated to 175 mg for a 70 kg person) is a reasonable estimate of the required injectable dose for long term protection in humans (Smilkstein et al., 2019).

The work reported in this article formulates ELQ-331 into an LAI-C against malaria. As for all prophylactic medications, since the target group is a pool of healthy users, the risk profile is particularly important. We have formulated the product with vehicles and excipients used in marketed products. An oil-based solution strategy was used to develop the formulation.

## 2. Materials and methods

### 2.1. Materials

The prodrug (ELQ-331) and the active form (ELQ-300) were synthesized at the Veterans Administration Medical Center, Oregon Health & Science University (VAMC/OHSU) in Portland, OR, using previously reported procedures (Frueh et al., 2017; Pou et al., 2021). Deionized (DI) water was obtained from a Millipore (Burlington, MA) water-purification system. Analytical reagents were analytical research grade or better. Inactive ingredients in the formulation were USP/NF grade. Phosphate buffered saline (10X) was purchased from Alfa Aesar (Ward Hill, MA) and diluted to 1X with DI water. Formic acid was purchased from EMD Millipore (Billerica, MA). Brij®35 was purchased from Acros Organics (Pittsburgh, PA). Glycerin was purchased from J. T. Baker (Pittsburgh, PA). Sesame oil, corn oil, cottonseed oil, peanut oil, castor oil, safflower oil, soybean oil, benzyl alcohol, benzyl benzoate, and polyethylene glycols were obtained from Spectrum Chemical Company (Gardena, CA). Sodium Lauryl sulfate (SLS), Tween 20, Acetonitrile, methanol, ethanol, disposable syringes, and needles were purchased from VWR International (Radnor, PA).

### 2.2. High performance liquid chromatography (HPLC) method

Chromatography was performed on an HP 1100 series HPLC system (Santa Clara, CA) controlled with ChemStation software (Agilent Technologies, Santa Clara, CA) as reported earlier (Potharaju et al., 2020). The HPLC gradient is shown in the supplemental section (Table S1). Standards of ELQ-331 and ELQ-300 were injected during each run to quantify the prodrug and the active drug.

### 2.3. Solubility of ELQ-331

The solubility of ELQ-331 in selected solvents, suitable for IM administration, was evaluated by a modified shake flask method. Excess quantity of ELQ-331 was added to each vehicle in glass vials followed by vortexing and overnight agitation at 25 °C on a VWR Model 3500 ADV orbital shaker (Radnor, PA) set to 200 rpm. The vortexing and agitation steps were repeated until saturated solutions were obtained. The vials were then agitated for an additional day. The saturated samples were centrifuged. The supernatant was collected, diluted with methanol, filtered through a 0.2 μm syringe filter, and analyzed by HPLC. The solubility of ELQ-331 in oil-based binary and ternary combinations was also determined using this procedure. Three replicates were performed for each solvent system. In addition, solubility of ELQ 331 in phosphate buffer saline (PBS, pH 7.4), and PBS containing surfactants (SLS, Tween 20 and Brij 35) was determined for the purpose of selecting an appropriate release media.

### 2.4. Preparation of oil-based solution formulations

The vehicle components were measured at the required volumes into a glass container. The container contents were blended with a magnetic stirrer until a homogenous solution was obtained. The active formulations were prepared by adding the required quantity of ELQ-331 to the oil or oil-solvent blend. The mixture was briefly sonicated. The vials were agitated, by vortexing and shaking, to dissolve the ELQ-331 completely and obtain the oil-based solution formulations.

### 2.5. Characterization and testing of oil-based formulations

The oil-based solution formulations were characterized for assay, viscosity, density, injectability, and *in vitro* release.

#### 2.5.1. Assay

A volume (75 μl) of the ELQ-331 oil formulations was transferred to 7 ml of methanol contained in a 10 ml volumetric flask. The flask was agitated on a vortexer for ~ 30 s and then transferred to an VWR Model 3500 ADV orbital shaker (Radnor, PA) set to 200 rpm. The flask was shaken for about 2 h. The volume was made up to 10 ml with methanol. The flasks were inverted three times end over end and then placed undisturbed on the benchtop for ~ 30 min. Samples were collected from the volumetric flask and filtered through a 0.45 μm syringe filter to HPLC vials. They were analyzed for ELQ-331 and ELQ-300 by the HPLC method.

#### 2.5.2. Viscosity

A Brookfield cone and plate viscometer assembly (Brookfield Instruments, Middleboro, MA) was used to measure the viscosity of the formulations. The viscometer (Model DV3TLV), assembled with a jacketed cup (CPA-44PSYZ) and spindle (CPA-40Z), was connected to a circulating refrigerated bath (TC-650). A speed of 5 rpm and 5 revolutions was used. The measurements were recorded at 25, 37, and 60 °C.

#### 2.5.3. Density

The density of each oil-based solution formulation was measured by weighing the formulation in a specific gravity bottle at room temperature (RT).

#### 2.5.4. Injectability parameters

The injectability of the formulations was determined with a Brookfield CT3-1500 texture analyzer (Middleboro, MA). The force was measured using an assembly to hold the syringe (Fig. 1, a). A close-up view of the syringe fixture is shown in Fig. 1b and c. The procedure used was similar to that reported by other authors (Burckbuchler et al., 2010; Zhang et al., 2018). The syringe (BD Luer-Lok™ Tip, 3 ml) was positioned in the syringe test fixture (TA-STJ), sourced from the texture analyzer manufacturer, and held in place on the base table. A needle (BD Precision Glide 21 G 1″ Needle) was attached to the syringe. Testing was carried out at a speed of 1 mm/s. The loading force (N) required to displace the plunger was measured as a function of plunger displacement (mm). The plunger was depressed for a distance of 17 mm while

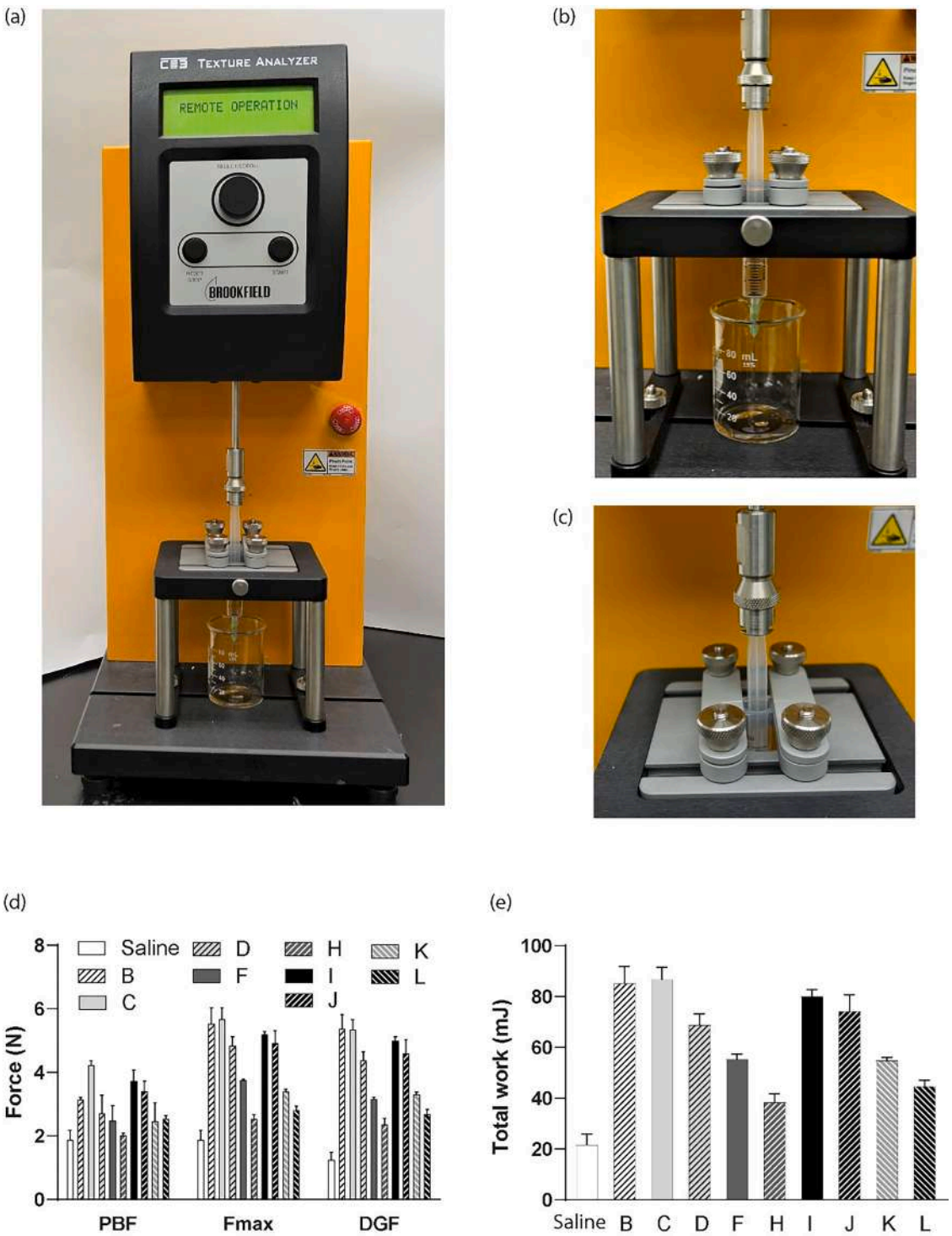


**Fig. 1.** (a) Texture analyzer equipped with a syringe test fixture. (b, c) Close up of the syringe test fixture. (d) Plunger-stopper break loose force (PBF), maximum force (Fmax), and dynamic glide force (DGF). (e) Total work. Refer to Table 3 for formulation composition. Values are reported as mean ± S.D.;$n = 3$ measurements.

the force (N) was measured. A force-distance plot was generated. The plunger-stopper break-loose force (PBF, the force required to initiate the movement of the plunger); maximum force ($F_{max,}$ the maximum force measured when the plunger completes displacing material from the front end of the syringe); dynamic glide force (DGF, the force required to maintain the movement of the plunger to extrude the content of the syringe); and the total work done (the work of injection between the time when the probe touches the plunger and the time when the plunger stops moving) were determined by the instrument's data processing software.

### 2.5.5. *In vitro release study*

The Oil Floating on Release Medium (OFRM) model was used for the *in vitro* drug-release study to screen the formulations. The method used was modified from that described in the literature (Larsen and Larsen, 2009). The release medium was 100 ml of 2% w/v Brij 35 in PBS, pH 7.4, which was added to a glass bottle (Qorpak 120 ml/4 oz Beaker Bottle). A transfer pipette was cut at both ends and converted to a tube with the

required length to be placed on the inside wall of the bottle (Fig. 2, a). The tube was affixed to the wall with either a hot glue gun or tape. The oil-based solution formulation was carefully placed on the surface of the aqueous release medium. The tube, affixed to the inside wall, was used to collect samples without disturbing the oil layer. An ELQ-331 concentration of 3 mg/ml was used for the study samples, and a volume of 1 ml of the sample was used. The release system was agitated by placing the bottle on a VWR 3500 ADV orbital shaker in an Imperial III Incubator (Lab-Line Instruments, Melrose Park, IL) maintained at 37 °C. The speed of the shaker was set to 100 rpm. The oil formulation and aqueous phase were shaken but not mixed with each other at this speed. Samples (1 ml) were collected through the tube from the aqueous phase at predetermined intervals. The collected samples were transferred to HPLC vials after filtration through a 0.45 μm syringe filter. The volume in the release container was replenished with 1 ml of fresh release medium (prewarmed to 37 °C) through the tube after each sample was collected. The collected samples were analyzed with the HPLC method described above. Sink condition was maintained during the study. Six replicates were performed for each formulation.

A study was performed to visualize possible oil micelles in the release studies. A placebo formulation was used. Nile red (0.5 mg) was added to a volume (1.5 ml) of vehicle composed of sesame oil, benzyl benzoate and benzyl alcohol (50:40:10 % v/v) and vortexed for 30 s. The dye containing placebo oil (1 ml) was added to the release media as described above. After 3 days shaking using the experimental conditions described earlier, a sample of the release media was collected through the sampling tube without filtration. The collected sample was gently mixed by inverting the tube several times and a 50 μL sample was loaded into the well of a 96-well plate. Automated digital microscopy (ADM) was performed using a CellCelector instrument (ALS Automated Lab Solution GmbH/ Sartorius, Jena, Germany) using the whole-well imaging and analysis function capabilities (Avital-Shmilovici et al., 2022). Images were captured using both bright-field and TRITC channel (excitation wavelength 555 nm) with a 20X objective lens. Oil droplets were identified and counted based on intensity contrast, size, and sphericity features observed in the bright-field images and confirmed through fluorescence.

### 2.5.6. Partition coefficient of oil formulations

Data from the *in vitro* release studies was used to determine the

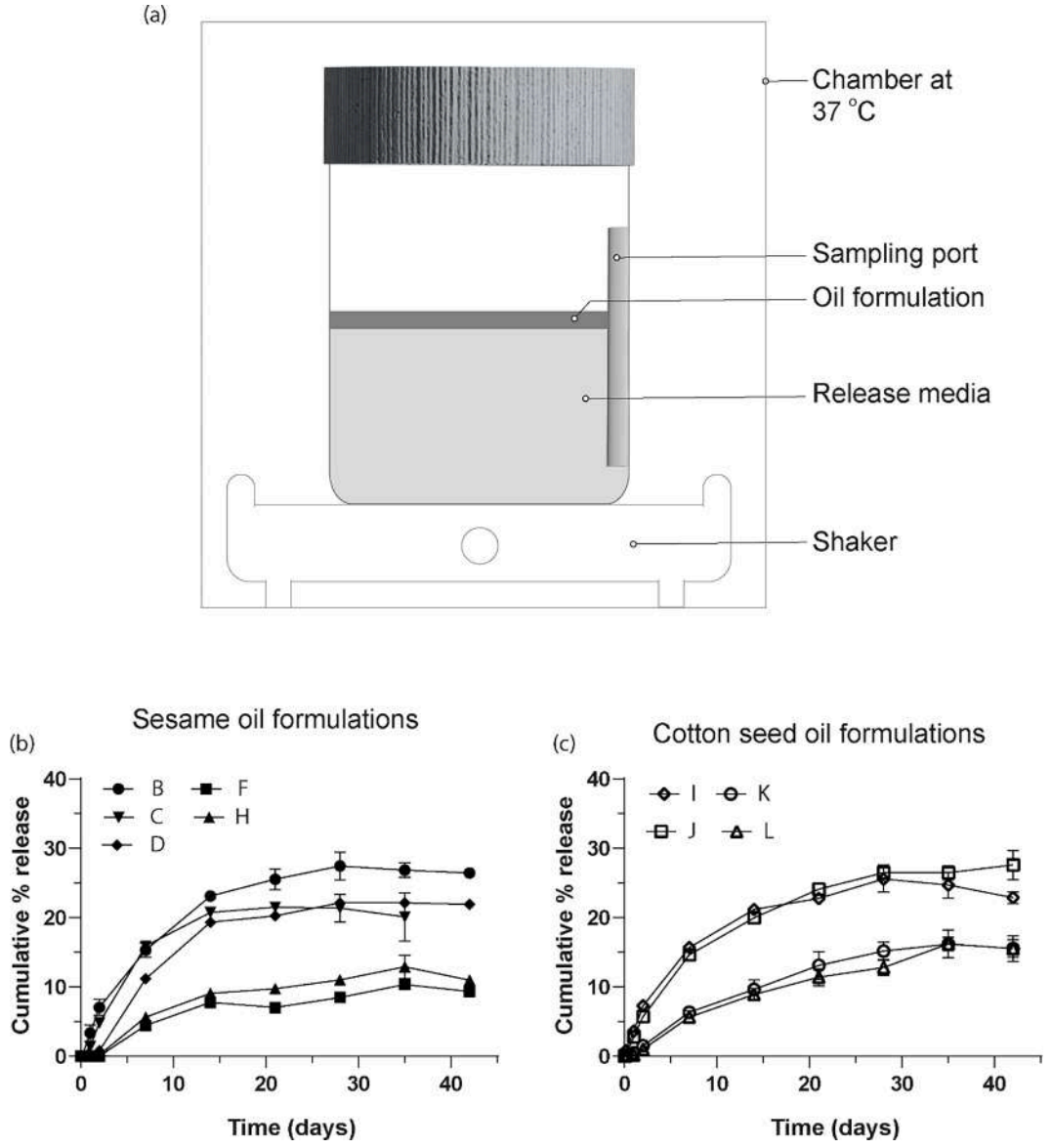


**Fig. 2.** (a) *In vitro* release testing arrangement. Cumulative percent release profiles of ELQ-331 oil-based solution formulations with the oil floating on release medium model. (b) Sesame oil formulations. (c) Cottonseed oil formulations. Refer to Table 3 for formulation composition. Values are reported as mean ± *S*.D., $n = 6$.

partition coefficient of ELQ 331 between the oil phase and the release medium. Equilibrium was assumed to have been established between the oil-based solution formulations and the aqueous buffer when the release curves plateaued (day 40). The partition coefficients were then calculated using Eq. (1) (Schultz et al., 1997)

$$P = \frac{\left(\frac{M}{Vaq}\right) - Caq}{Caq} \cdot \frac{Vo}{Vaq} \quad (1)$$

The total mass of drug used in the sample is M, Caq is the equilibrium drug concentration in the aqueous buffer, and Vaq, Vo are the volumes of aqueous and the oil phases, respectively.

### 2.6. Short term accelerated stability testing

Two formulations, selected for the animal studies, were placed on short-term accelerated stability testing at 40 °C/75 % Relative Humidity (RH) for three months. At the end of three months, the formulations were assayed and visually examined for any changes compared to the same solutions at RT.

### 2.7. Pharmacokinetic (PK) study

The general procedures for animal care and housing were in accordance with the recommendations of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), the Guide for the Care and Use of Laboratory Animals (National Research Council), and the U.S. Department of Agriculture through the Animal Welfare Act and Animal Welfare regulations. Every effort was made to minimize pain and suffering in all animals used in this study

Single jugular-vein catheterized (JVC) male Sprague Dawley (SD) rats sourced from Charles River Laboratories (Hollister, CA) were used. The age at first dose was 10–11 weeks. The rats were randomly assigned to treatment groups by computerized body weight stratification (Provantis® version 10.5.0.2), and each animal was identified by a unique ear punch. Water and food were provided *ad libitum*.

The concentration of ELQ-331 in the oil formulations used for the study was 12.14 mg/ml (equivalent of 10.00 mg/ml of ELQ-300). The IM injection was administered in the hind back quadriceps of unfasted animals at two sites with a 25 G 5/8″ needle. A dose of 12.14 mg (ELQ-331)/kg was administered. No more than 0.2 ml was injected per site on one leg, and both legs were used. The animals were checked at least once daily during the course of the study for mortality or morbidity. The animals were examined for any altered clinical signs, including gross motor and behavioral activity and observable changes in appearance. The injection site was also routinely examined during the study duration of four weeks.

### 2.8. Plasma collection

Blood samples were collected through the JVC port from each rat to $K_3$EDTA-containing tubes. Samples were collected for each dosed rat on day 1 (4 and 8 h post dose), day 2 (24 h post dose), day 3 (48 h post dose), day 7 (144 h post dose), day 14 (312 h post dose), day 21 (480 h post dose), and day 28 (648 h post dose). Sodium fluoride (NaF) was added promptly to the blood samples by transferring 300 µl of whole blood, from the $K_3$EDTA tube, into a microcentrifuge tube containing 34 µl of 500 mM NaF. These mixtures were placed on wet ice until processed to plasma by centrifugation at 2–8 °C. The plasma samples were placed on dry ice prior to being frozen below −60 °C for storage. Further breakdown, post blood collection, of the prodrug ELQ-331 during sample processing and storage was prevented by NaF. Blood was also collected using other Institutional Animal Care and Use Committee (IACUC) approved methods: tail vein (for one rat on days 14 and 21) and retro-orbital sinus (for two rats on day 28). Anesthesia was used when blood was collected via retro-orbital sinus.

### 2.9. Bioanalytical method

Drug levels of ELQ-300 and the prodrug ELQ-331 were determined in the collected plasma samples using a bioanalytical method reported earlier (Potharaju et al., 2020).

### 2.10. Pharmacokinetic data analysis

Plasma drug-level data of ELQ-300 and ELQ-331 were analyzed using Phoenix WinNonlin® (v 8.3) by noncompartmental approaches. The following PK parameters and constants were determined: observed maximum plasma concentration (Cmax), time to reach maximum plasma concentration after the IM dose (Tmax), area under the plasma concentration-time curve (AUC), and mean residence time (MRT).

## 3. Results and discussions

### 3.1. Physical attributes of the oil-based formulations

Oil vehicles for parenteral administration have to be inert, stable, syringeable, injectable, and safe (Spiegel and Noseworthy, 1963). Except for containing a drug (thus, not inert), the active oil formulation should have the same characteristics. The vegetable oils and vehicles tested in this study were selected based on published reports and from the US FDA inactive ingredient database. In addition to the vehicle, excipients are used in formulations to introduce or improve certain properties of the vehicle. These include preservatives (if intended for multi-dose administration), excipients to improve the ease of administration, and solvents to improve the solubility of the drug (Larsen et al., 2012; Rowe et al., 2009). Some excipients perform more than one function. For example, benzyl alcohol, in addition to being a preservative, has local anesthetic properties and serves to reduce the viscosity of the oily vehicle (Nair, 2001). For injectable products, where constraints of administration volume and limited selection (compared to oral or topical dosage forms) of excipients are possible, it appeared reasonable to limit our excipients and choose those that perform multiple roles.

The solubility of ELQ-331 (Figure S1) ranged from 11.2 to 17.7 mg/ml in the vegetable oils tested, while in non-oil solvents, solubility was in the range of 0.4 to 330.9 mg/ml (Table 1). Among the materials tested, the solubility of ELQ-331 was the highest in benzyl alcohol (330.9 mg/ml) and the lowest in glycerin (0.4 mg/ml). Castor oil has a high viscosity (Table S2) and is expected to have low injectability properties. Since the solubility of ELQ-331 was not very much increased in castor oil, it was eliminated from the choice of oils. The viscosity of the other oils was similar enough that all were possible choices as the primary vehicle if a short-acting injection was being formulated.

The safety profile during the intended long duration (possibly greater than one month) residence at the site of injection was an important

**Table 1**
Solubility of ELQ-331 in vegetable oils and solvents. Values, measured at 25 °C, are reported as mean ± S.D., $n = 3$.

| Vegetable Oil or Solvent | ELQ-331 solubility (mg/ml) |
|---|---|
| Benzyl Alcohol | 330.93 ± 7.66 |
| Benzyl Benzoate | 203.64 ± 2.04 |
| Polyethylene Glycol 300 | 57.50 ± 3.79 |
| Polyethylene Glycol 400 | 31.14 ± 0.07 |
| Castor Oil | 17.75 ± 0.22 |
| Ethanol | 16.43 ± 0.42 |
| Safflower Oil | 16.22 ± 0.36 |
| Soybean Oil | 13.97 ± 0.02 |
| Cottonseed Oil | 13.72 ± 0.22 |
| Corn Oil | 12.26 ± 0.29 |
| Sesame Oil | 11.90 ± 0.42 |
| Peanut Oil | 11.20 ± 0.38 |
| Propylene Glycol | 1.35 ± 0.03 |
| Glycerin | 0.38 ± 0.47 |

consideration in selecting suitable candidate oils and narrowing the choices (Wilkinson et al., 2022). Considerations of viscosity, safety during prolonged use, and prior precedent in approved products led to the choice of sesame oil and cottonseed oil for additional studies. These two oils were used to prepare binary and ternary combinations. The solvents in which the highest solubilities of ELQ-331 were obtained, benzyl alcohol and benzyl benzoate, were used as the additional components in the different combinations. The maximum concentrations of benzyl alcohol and benzyl benzoate were set at 10 and 40 % v/v, respectively, of the total volume in testing the different combinations.

The cosolvent formulations with benzyl alcohol and/or benzyl benzoate were colored yellow (Figure S2). The active formulations were similar in visual appearance to the corresponding placebo. The formulations of ELQ-331 and the placebo formulations were visually stable in color, physical appearance, and homogenous with no sign of phase separation at RT. The assay of the active formulations was within ±10 % of the nominal concentration (Table S3). ELQ-300 was not observed when the prepared solutions were assayed by HPLC.

The solubility of ELQ-331 in the cosolvent formulations was more than twice that with oil alone (Table 2). When 5 % of benzyl alcohol or benzyl benzoate was added to sesame oil, the solubility increased by a factor of 2.6 and 2.3. For cottonseed oil, the increase with the addition of these excipients was 2.4X. The increase in solubility with increasing quantities of the benzyl benzoate/benzyl alcohol appeared to be linear. Based on the solubility results, seven formulations (indicated with asterisks in Table 2) were selected for additional studies. Six were selected based on the increased solubility compared to the oils; one formulation (Formulation C containing 98.8 % sesame oil, 1.2 % benzyl alcohol) was prepared to enable comparison with a prior study (Smilkstein et al., 2019). Two formulations contained ELQ-331 in sesame oil or cottonseed oil without additional excipients. Thus, nine formulations (Table 3) were carried forward to additional studies. Formulations obtained by blending the two oils, and adding the excipients did not improve the solubilities compared to a single-oil formulation and were not tested further.

Viscous liquids can be difficult to inject, and high forces of injection are required to move the plunger forward in the syringe barrel to displace the solution out of the needle and into the injection site. This can cause patient distress and may also have other undesired effects (Roberts et al., 2022). As the oil was replaced with other excipients (Table 3), the viscosity of the ELQ-331 formulations decreased. The observed viscosity changes differed in sesame oil and cottonseed oil formulations with the addition of the excipients. Sesame oil-based formulations had a slightly higher viscosity compared to the corresponding cottonseed oil formulations. Testing was also performed at 25 and 60 °C in addition to 37 °C (Figure S3). The viscosity trend of the formulations was similar to that at 37 °C, with the sesame oil formulations having slightly higher viscosity values. The viscosity of formulations at different drug loadings in the formulation consisting of 60 % sesame oil, 40 % benzyl benzoate (placebo and drug loadings of 1, 3, and 30 mg/ml) were determined. Higher drug loading (up to 30 mg/ml) did not appear to significantly affect the viscosity of the sesame oil-based formulations. The increase in viscosity was less than 2 cP with the 30 mg/ml formulation compared to the placebo (Figure S4). Commercial parenteral solutions have viscosities in the range of 29–60 cP. (Allahham et al., 2004) and concentrated biologicals have viscosities of 10–200 cPs (Zhang et al., 2018). Since the viscosity of ELQ-331 formulations with 30 mg/ml is about 25 cP, we anticipate that these formulations can be injected without causing significant pain or distress. It appears likely that even if additional ELQ-331 is loaded in the formulation, within the solubility limits, the viscosity will be within the range for practical use. The density of the formulations ranged from 0.92 to 1.04 g/ml for the active formulations (Table 3). Replacement of oil with other cosolvents resulted in slightly increased density for both sesame oil and cottonseed oil. Density and viscosity are also important parameters for the filling of vials on production lines. The values affect the speed of filling and also are used for ensuring accurate fill of the product to meet content homogeneity requirements. They also affect the upper and lower rejection limits. The values we obtained for our formulations appear suitable for use on modern sterile liquid fill lines.

**Table 2**
Solubility of ELQ-331 in sesame and cottonseed oil-based cosolvent formulations. Values are reported as mean ± S.D., $n = 3$.

| Solvent (% v/v) | | | | | ELQ-331 Solubility (mg/ml) |
|---|---|---|---|---|---|
| Sesame oil | Cottonseed oil | Benzyl alcohol | Benzyl benzoate | | |
| 98.8 | – | 1.2 | – | * | 23.64 ± 0.06 |
| 95 | – | 5 | – | | 31.07 ± 0.32 |
| – | 95 | 5 | – | | 28.89 ± 0.27 |
| 95 | – | – | 5 | | 27.24 ± 0.93 |
| – | 95 | – | 5 | | 28.34 ± 0.93 |
| 90 | – | 10 | | * | 48.33 ± 2.24 |
| – | 90 | 10 | | * | 49.22 ± 0.52 |
| 90 | – | – | 10 | | 32.93 ± 1.15 |
| – | 90 | – | 10 | | 33.34 ± 1.69 |
| 80 | – | – | 20 | | 48.49 ± 0.78 |
| – | 80 | – | 20 | | 49.29 ± 0.96 |
| 60 | – | – | 40 | * | 97.33 ± 3.35 |
| – | 60 | – | 40 | * | 101.52 ± 2.21 |
| 47.5 | 47.5 | – | 5 | | 27.76 ± 0.58 |
| 45 | 45 | – | 10 | | 34.07 ± 0.43 |
| 40 | 40 | – | 20 | | 51.95 ± 1.59 |
| 50 | – | 10 | 40 | * | 160.74 ± 3.21 |
| – | 50 | 10 | 40 | * | 163.32 ± 9.18 |

* Used for detailed studies.

While administering injections, withdrawal of the dose into the syringe from the primary container (syringeability) and delivery to the administration site (injectability) are important parameters. This is especially true for formulations with high viscosity (Cilurzo et al., 2011; Zhang et al., 2018). While syringing and injecting are two different events, we use the term injectability to refer to the overall resistance of the liquid formulation to flow during either event. This includes the force required for injection, evenness of flow, and freedom from needle clogging. The measurement of injectability parameters of ELQ-331 oil-based solution formulations was performed by recording the force-distance plots (**Figure S5**) using the assembly shown in Fig. 1a. The mean PBF, $F_{max}$, DGF, and total work values were collected (Fig. 1d, e). Lower values indicate better injectability. All oil-based formulations had higher values of PBF, $F_{max}$, DGF, and total work when compared to saline. Combinations of the oils with benzyl benzoate or benzyl alcohol showed lower values for PBF, $F_{max}$, and DGF than the oils alone. This also resulted in the total work of the injection sequence being lower for the cosolvent formulations. The cottonseed oil formulations had slightly better injectability values compared to corresponding sesame oil formulations when the concentration of the oils was greater than 50 %. However, once the concentration was at 50 %, the major contribution appeared to be due to the property of the cosolvents. The addition of cosolvents resulted in better injectability values for both series of oil formulations.

The highest value of $F_{max}$ is about 6 N (Fig. 1a) when a 21 G 1″ needle is used for the test. The study allowed rank ordering of the different formulations. Since these values were obtained with a free-standing needle set up, the force when injecting into muscle is expected to be much higher. The results of the test indicate the likelihood of an uncomplicated and safe manual injection with these formulations in humans. Other authors have used the maximum force (when injected into air) of 30 N as a reasonable upper limit (Burckbuchler et al., 2010).

### 3.2. *In vitro release from oil formulations*

Sustained release parenteral products do not have a specified

**Table 3**
Density, viscosity, and partition coefficient of oil-based solution ELQ-331 formulations. ELQ-331 concentration is 3 mg/ml.

| ID | | Composition (% v/v) | | | | Density (g/ml) | Viscosity (cP) | Calculated Partition Coefficient |
|---|---|---|---|---|---|---|---|---|
| | | Sesame oil | Cotton seed oil | Benzyl alcohol | Benzyl benzoate | | | |
| B | | 100 | – | – | | 0.92 ± 0.00 | 34.64 ± 0.43 | 279.04 ± 5.79 |
| C | | 98.8 | – | 1.2 | – | 0.93 ± 0.00 | 34.89 ± 0.11 | 372.98 ± 46.07 |
| D | | 90 | – | 10 | – | 0.94 ± 0.01 | 23.89 ± 0.31 | 352.72 ± 14.36 |
| F | # | 60 | – | – | 40 | 1.02 ± 0.00 | 15.35 ± 0.19 | 992.23 ± 38.10 |
| H | # | 50 | – | 10 | 40 | 1.04 ± 0.05 | 11.55 ± 0.09 | 818.14 ± 56.43 |
| I | | – | 100 | – | – | 0.92 ± 0.00 | 35.95 ± 0.15 | 289.08 ± 32.29 |
| J | | – | 90 | 10 | – | 0.93 ± 0.00 | 18.72 ± 0.44 | 277.98 ± 15.75 |
| K | | – | 60 | – | 40 | 1.01 ± 0.00 | 8.79 ± 0.03 | 549.09 ± 48.34 |
| L | | – | 50 | 10 | 40 | 1.02 ± 0.02 | 9.01 ± 0.27 | 553.28 ± 70.27 |

Density measured at room temperature (25 °C), viscosity measured at 37 °C, values are reported as mean ± S.D., $n = 3$ measurements for density and viscosity, $n = 6$ for release data used for partition coefficient calculations. # Selected for PK study.

standard method for drug-release testing. Reports in the literature have used a variety of methods to measure *in vitro* release of extended-release products. We used the OFRM model for the *in vitro* release studies. A suitable release medium is an important aspect in the *in vitro* release-method development for a poorly soluble drug. Factors such as sufficient solubility of drug in the release media to ensure sink conditions under experimental set up and stability of the drug in the release media were considered important aspects of the development phase of the method. ELQ 331 does not dissolve in detectable quantities in 1X PBS. In 1X PBS containing 1% w/v of SLS, Tween 20 or Brij 35, ELQ 331 has a solubility of about 50, 22, and 45 μg/ml, respectively. In the SLS solutions we noticed a greater fraction of ELQ 300 (6 %) at the end of the solubility study compared to the Brij 35 solutions (3 %). Our target was to reach ELQ-331 solubility close to 100 μg/ ml in the dissolution media. This was driven by a desire to allow 10 mg to be solubilized in 100 ml, since we planned to use a 3 mg/ml oil formulation sample for our release studies. So, we chose to select Brij-35 over SLS for further studies. In a 2 % w/v Brij 35 in PBS, pH 7.4, the solubility of ELQ-331 is ~90 μg/ml. We also performed the solubility study with 0.5 % w/v Brij 35 in PBS, where we obtained ELQ 331 solubility of about 22 μg/ml. The solubility of ELQ 331 appears to be linear in the concentration ranges of 0.5- 2% w/v of Brij-35 in 1X PBS. Based on these studies, the 2% w/v Brij 35 in PBS, pH 7.4 was used for the *in vitro* release studies. A volume sufficient to ensure sink conditions (100 ml) was used.

Our attempts, using automated digital microscopy, to image possible oil micelles formed during these experiments indicate that about 6–8 globules (Figure S7), which could represent micelles, were present in 50 ul of unfiltered release media on day 3. The size of the brightly fluorescent globules ranged from 2 to 5 uM in size. Thus, these would be expected to be screened out by the 0.45 μm filter we used. We conclude that the fraction of the oil phase in the collected release media using our methods and after filtration through 0.45 μm syringe filter, is most likely very small.

The release profiles of ELQ-331 formulations are shown in Fig. 2. All the formulations resulted in sustained *in vitro* release, but to varying degrees. The cumulative release was in the range of 9 % to 27 % over 42 days. In release studies of oily formulations, the lipophilic drug will always prefer the oily phase, among the two liquids used; thus, it may be impossible to reach a higher cumulative release under most *in vitro* release conditions. Organic solvents are sometimes selected for *in vitro* release studies. However, for our formulations it is likely that such an organic phase would end up dissolving much of the oil itself. It is also likely that if the release studies are conducted at different conditions (for *e.g.* 25 °C or at 40 °C, or with a different surfactant or a different concentration of the surfactant in the release media, or with vessels having a different surface area) these cumulative data would be different. We believe that since our purpose is rank ordering and discrimination of the formulations, both of which appear to be possible with these experiments, the release studies have served their purpose in down selecting the formulations. Among the sesame oil formulations (Fig. 2b), drug release was higher when 100 % oil was used. Sustained release was greatest in formulations prepared in combination of co-solvents (benzyl alcohol, benzyl benzoate). Similar release trends were observed with cottonseed oil formulations in combination with co-solvents (Fig. 2c). The difference in release between the 100 % sesame and cottonseed oil formulation, using the AUC, was significant ($p < 0.05$). Sesame oil formulations in combination with co-solvents had similar or slightly more sustained release when compared with corresponding formulation prepared with cottonseed oil.

The conversion of ELQ-331 to ELQ-300 was monitored during the *in vitro* release studies (**Figure S6**). The amount of conversion to ELQ-300 was minimal with oil-based formulations. The partition coefficient values between the oil vehicle and release medium for the ELQ-331 oil-based formulations were calculated (Table 3). The formulations that combined sesame oil with co-solvents had higher partition coefficient values compared to corresponding cottonseed oil formulations ($p < 0.001$ for formulations F and K, $p < 0.01$ for formulations H and L).

The *in vivo* performance of oil-based solution formulations is affected by various processes that are difficult to simulate with *in vitro* tests. These include physical factors (spreading and disappearance of the oil-based solution from the injection site) or physiological factors (blood flow at the site and depth of injection, shape, and surface area of oil depot at site of injection). Nevertheless, *in vitro* release tests allow the rank ordering of these different formulations. It is known that drug partitioning between the oil vehicle and the aqueous tissue fluid play an important part in modifying the drug release rate from oil-based solutions (Larsen and Larsen, 2009; Larsen et al., 2012). Hence, it is possible to design oil-based formulations with customized release rate characteristics by modifying the partition coefficient. An increase in the partition coefficient can be achieved by increasing the drug partitioning to the oil phase of formulations by using mixtures of triglycerides/oils or by the addition of excipients such as nonaqueous vehicles. Oil-based solution formulations of ELQ-331 in combination with solvents (benzyl benzoate and benzyl alcohol) showed improved solubility, increased partition coefficient, and relatively sustained *in vitro* release. The observed release differences and the sustained release of ELQ-331 from the oil-based formulations appear to be primarily due to the change in oil–water partition coefficients by altering the oil vehicle composition. Over time, the cosolvents are also released from the oil, leading to a continous change in the formulation composition and the partition coefficient (Kalicharan, 2017). In summary, drug release and absorption from an oil depot is complex and may not be entirely described by simple models. Thus predicting the drug release and absorption from the injection site may prove difficult without *in vivo* studies.

In prior reported *in vivo* studies in mice (Smilkstein et al., 2019), a single IM injection of 30 mg (ELQ-331)/kg in sesame oil and 1.2 % benzyl alcohol (Table 3, Formulation C) maintained an ELQ-300 concentration above 100 nM and provided more than four months of causal prophylaxis against malaria. In our *in vitro* release studies (Fig. 2), other

formulations appeared to show a more sustained release, compared to Formulation C. These formulations with significantly enhanced solubility of ELQ-331 and an improvement in sustained release *in vitro* will allow higher ELQ-331 loading and were expected to achieve greater long-term release *in vivo* studies.

The objective going into the PK study was to down-select two oil-based formulations from among the various possible oil combinations. Formulation H had the best injectability (the lowest total work value during force testing) among the formulations tested and the second highest calculated partition coefficient value. This led to the choice of formulation H as one of the two candidates. While K, L, and F had good results in the injectability tests, formulation F had a higher calculated partition value than K or L. Formulations F and H also showed the greatest delay in the ELQ-331 being converted to the ELQ-300 during the *in vitro* release study. Since the objective was to have a stable prolonged release formulation and the intent was to narrow down the field of formulations to two candidates for the PK study, the sesame oil formulations (F and H) were selected. No significant advantage was thought to be obtained by carrying forward formulations of cottonseed oil. In an earlier section, we have mentioned the importance of viscosity and density to the product fill line. Looking at the values for the two formulations (Table 3), while the density of the two formulations is similar the viscosity of Formulation H (11.6 cP) is lower than that for Formulation F (15.4 cP). This makes a case for prioritising Formulation H (due to possibly higher speed of filling) over Formulation F.

The formulations stored at 40 °C for three months were visually like those stored at RT with no phase separation, precipitation, or visually discernible color changes. The assay values were similar to when initially tested at the time of manufacture.

### 3.3. Pharmacokinetic study

The two oil-based formulations (Formulations F and H, see Table 3 for composition) used for the study were selected based on solubility, *in vitro* release studies, and injectability parameters.

No animals were required to be euthanized prior to the study's scheduled end points due to adverse health status. The weight of the animals ranged from 331 to 351 g. Split dosing (~ 0.17 ml of the formulation at each of the two injection sites) was carried out without incident. The trained personnel performing the injections did not report any difference from other aqueous-based injections while administering these oil-based formulations. The formulations were well tolerated by the rats used in the study. The animals showed no adverse clinical observations during the entire study from the test article injection, and no animals died. Any clinical observations (**Table S5**) during the study did not appear to be test-article related, since they were located distal from the IM administration site. Significantly, the IM injection sites showed no changes during the study. The concentrations of ELQ-331 were below the lower limit of quantitation (LLOQ, 1.00 ng/ml) in all plasma samples, indicating that ELQ-331 was efficiently and completely converted to ELQ-300 *in vivo*. Earlier studies have indicated that it is most likely hepatic esterases that cleave the ELQ 331 to the active drug (Frueh et al., 2017).The plasma concentrations of ELQ-300 (Fig. 3) were above the LLOQ throughout the study. The PK parameters (mean values) are shown in Table 4. Individual values are in the supplementary section (**Table S6**).

The $T_{max}$ for ELQ-300 was from 8 to 48 h. The mean peak plasma concentration of ELQ-300 ($C_{max}$) was 268 ± 106 ng/ml (formulation F), and 216 ± 49 ng/ml (formulation H). The mean $AUC_{last}$ values were 88,800 ± 11,700 h•ng/ml and 88,700 ± 10,400 h•ng/ml for formulations F and H, respectively. The $AUC_{inf}$ was also calculated for each group, but as much as 26 % to 85 % of each value was extrapolated, which impacts the accuracy of the calculation. The terminal $t_{1/2}$ values were 509 ± 262 h (formulation F) and 394 ± 39.5 h (formulation H). The $MRT_{last}$ values were 262 ± 24.1 h (formulation F) and 272 ± 13.3 h (formulation H). $MRT_{inf}$ were 718 ± 383 h (formulation F) and 575 ± 48.9 h (formulation H). There was no significant difference ($p > 0.05$), using the $AUC_{last}$ values, between the two tested formulations.

The $MEP_{100}$ of ELQ-300 is 80 nM (Smilkstein et al., 2019). Plasma concentrations were well above this concentration for the duration of the study.

The oil-based injectable formulations provides the advantages of straightforward manufacturing, long-term stability, suitability for terminal sterilization, and the possibility for drug depots with tailored delivery characteristics (Fredholt et al., 2000; Larsen and Larsen, 2009; Larsen et al., 2012). LAI-C, formulated as solutions, may require the addition of a lead-in phase dosage regimen to rule out the possibility of adverse reactions since the injections cannot be retrieved once injected. In earlier work (Potharaju et al., 2020), we reported on the formulation of ELQ-331 as oral suspensions or spray-dried dispersion (SDD)

**Table 4**
Pharmacokinetic parameters for ELQ-300 in Sprague Dawley rats administered ELQ-331 by IM administration (mean ± S.D., $n = 3$).

| ID | $t_{1/2}$ (hr.) | $T_{max}$ (hr.) | $C_{max}$ (ng/ml) | $AUC_{last}$ (hr·ng/ml) | $MRT_{last}$ (hr.) | $MRT_{inf}$ (hr.) |
|---|---|---|---|---|---|---|
| F | 509 ± 262 | 27 ± 20 | 268 ± 106 | 88,800 ± 11,700 | 262 ± 24.1 | 718 ± 383 |
| H | 394 ± 39.5 | 48 ± 0 | 216 ± 49.2 | 88,700 ± 10,400 | 272 ± 13.3 | 574 ± 48.9 |

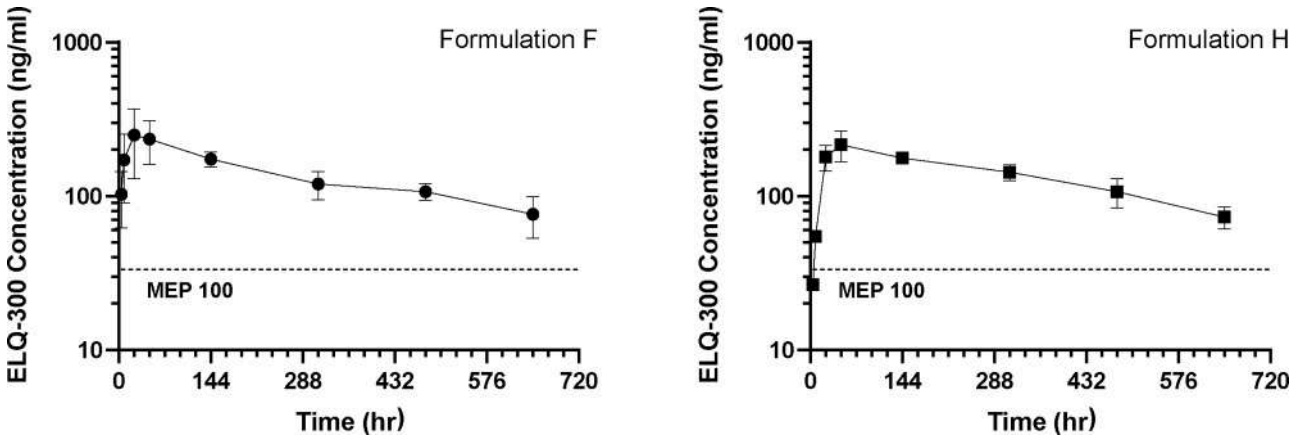


**Fig. 3.** Plasma concentrations of ELQ-300 in rats, administered ELQ-331 IM at a dose of 12.14 mg/kg, IM (the dose is the equivalent of 10.00 mg/ml ELQ-300). Each data point represents the mean and S.D. of $n = 3$ rats. Formulation F: 60 % sesame oil, 40 % benzyl benzoate. formulation H: 50 % sesame oil, 40 % benzyl benzoate, 10 % benzyl alcohol.The minimum fully effective protective plasma concentration ($MEP_{100}$) of ELQ-300 is shown as a dotted line.

preparations that could possibly be compressed as tablets. Oral formulations can be used as a lead in regimen prior to the administration of the long-acting injections to guard against the possibility of unanticipated adverse reactions to ELQ-331 in some individuals.

## 4. Conclusions

In this study, a series of oily solutions of ELQ 331, a new antimalarial drug, were tested for suitability as long-acting injection formulations. We performed physical tests, solubility studies, release studies and injectability parameter testing to shortlist two formulations for further development. The selected formulations have acceptable viscosity and injectability properties, demonstrated increased drug solubility, and displayed sustained release under *in vitro* testing. The formulations underwent a four-week pharmacokinetic study in rats, where they were able to maintain a systemic plasma concentration higher than that necessary to act as a prophylactic against malaria. The formulations show promise as sustained-release IM injections.

## CRediT authorship contribution statement

**Dipu Karunakaran:** Writing – original draft, Validation, Formal analysis, Data curation. **Shravan K. Mutyam:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Melody Fu:** Validation, Investigation. **Jiaming Chen:** Validation, Investigation. **Kim Hue Nicky Pham:** Validation, Investigation. **Sovitj Pou:** Writing – review & editing, Resources, Conceptualization. **Rolf W. Winter:** Resources. **Aaron Nilsen:** Resources. **Rozalia A. Dodean:** Formal analysis. **Martin J. Smilkstein:** . **Michael K. Riscoe:** . **Gita Shankar:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

## Data availability

Data will be made available on request.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2024.106795.

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