An in-situ forming implant formulation of naltrexone with minimum initial burst release using mixture of PLGA copolymers and ethyl heptanoate as an additive: In-vitro, ex-vivo, and in-vivo release evaluation

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**ABSTRACT**

An in-situ forming implant formulation of naltrexone (NTX) was achieved based on a minimum initial burst release of NTX in the in-vitro release medium using a Box-Behnken design. Variables such as percent of copolymer by weight, copolymer composition (PLGA 756s: PLGA 504H), and percent ethyl heptanoate by weight as an additive in formulation were investigated. The in-vitro, ex-vivo, and in-vivo release of the optimized formulation was investigated. The rabbit-blood concentrations of the optimized formulation and Vivitrol\textsuperscript{®} were compared to ensure their equivalency. The initial burst release of the optimized formulation in the in-vitro release over the first 24 h, 6.18 ± 0.91%, was significantly (p < 0.05) lower than that of the formulation containing 100% of PLGA 504H (17.45 ± 1.07%) and 100% of PLGA 756s (11.82 ± 1.03%). The \( C_{\text{max}} \) of NTX (21.06 ± 2.9 ng/mL) from the optimized formulation was close to that of Vivitrol\textsuperscript{®} (21.11 ± 2.89 ng/mL). Also, the absolute bioavailability (F) and the range of serum concentration of NTX (C) of the ISFI formulation (F = 18.29, C = 6.18–22.84) were similar to Vivitrol\textsuperscript{®} (F = 16.83, C = 6.83–23.09). These results indicate that the optimized formulation can reach an effective therapeutic concentration for treating opioid and alcohol dependence.

1. Introduction

Naltrexone is a competitive opioid antagonist of the \( \mu \), \( \kappa \), and \( \delta \) opioid receptors that has been used as a maintenance therapy after withdrawal in detoxified opioid-dependent patients [1]. It has also been proven effective in treating alcohol dependence by the US Food and Drug Administration (FDA) [2]. One alternative to an oral naltrexone formula is a sustained release formulation, which can improve compliance in patients being treated for opioid or alcohol addiction.

Naltrexone implants, a long-acting form that is subcutaneously placed with the aid of a hypodermic needle or surgery, are composed of poly (D,L-lactide) with doses of 1.1, 2.2, 3.3 g NTX [3,4] and poly-[D,L lactide:caprolactone] containing 280 mg of NTX [5]. Application of implants is limited due to side-effects of inflammation, foreign-body reaction and fibrosis in the injection site [3,6].

Naltrel\textsuperscript{®} is a major commercial form consisting of 150 mg of naltrexone incorporated within microspheres of poly-(\( \alpha \)-lactide) polymer [7,8]. In Vivitrex\textsuperscript{®}/Vivitrol\textsuperscript{®} formulation, naltrexone 34% w/w (190 and 380 mg of NTX) is encapsulated into microspheres (size \( \sim 100 \) \( \mu \)m) made of a biodegradable polymer (poly-(\( \alpha \)-lactide-co-glycolide)) [9,10]. This formulation is supplied as a dry powder along with an aqueous diluent, and an injectable suspension is prepared just before use via intramuscular injection [11,12].

In-vitro release studies have been conducted on the form of injectable ISFI containing NTX HCl [13,14]. ISFI includes a water-insoluble biodegradable polymer, poly-(\( \alpha \)-lactide-co-glycolide) (PLGA), dissolved in a water-miscible and physiologically compatible organic solvent, NMP. Upon injection into an aqueous environment, the organic
solvent diffuses into the surrounding environment, while the water diffuses into the polymer matrix (phase inversion mechanism). Then, the polymer precipitates in contact with water and results in a solid polymeric implant [15]. PLGA is a biodegradable polymer that hydrolyzes into lactic and glycolic acids under physiological conditions and is subsequently eliminated from the body via the Krebs cycle [16]. NMP is an FDA-approved solvent that is inert, bioabsorbable and safe. This solvent is used in manufacturing some medical products such as Eligard® (leuprolin acetate using PLGA), RBP-6000® (buprenorphine using PLGA) and Atridox® (doxycycline hyclate using PLA). Upon absorption through body tissues, NMP is metabolized and excreted primarily in the urine [16–18]. Many in-vitro and in-vivo studies have been conducted on various drugs loaded on ISFI as leuprolide acetate [19–21], diltiazem hydrochloride, buserelin acetate [22], bupivacaine hydrochloride [23], fluorescein [24], bupernorphine [25], betamethasone [26], thymosin alpha 1 [27], haloperidol [28], montelukast [29], risperidone [30], cucurbitacin [31], asenapine maleate [32], doxycycline hyclate [33,34] and aldosterone [35]. Unfortunately, ISFI has a considerable capacity for a bursting effect, especially in the first few hours after injection into the body (15–80% of the total drug loaded) [36]. The high initial burst-release rates are directly related to the diffusion of NMP over short periods of time into water to form the solid implant, because the NMP carries the drug with itself to the release medium during the formation of the implants. The plasma concentration may significantly increase due to the initial burst release, which may lead to systemic toxicity. Because of this unwanted phenomenon, the use of ISFI has been limited only to drugs with a very high therapeutic index [20].

The objective of this study was to develop naltrexone in ISFI formulations that would release drugs over 1 month or longer, and particularly to reduce the initial burst release using a combination of poly (lactic-co-glycolic acid) 75:25 copolymer (RG 756s) and 50:50 copolymer (RG 504H) along with additives. The Box-Behnken design was used to obtain the optimized ISFI formulation results based on initial minimum burst release during 24 h. Variables such as percent of copolymer by weight, copolymer composition (PLGA 756s:PLGA 504H), and percent ethyl heptanoate by weight as an additive in formulation were investigated. Scanning electron microscopy (SEM) was applied to visualize the surface and cross-section morphologies. The in-vitro, ex-vivo, and in-vivo evaluation of the optimum formulation was investigated. The pharmacokinetic profiles of the NTX and its main metabolite 6β-naltrexol in rabbits were determined using blood samples after subcutaneous injection of the optimum formulations. The formulation’s in-vitro and in-vivo compatibility were evaluated. Finally, the rabbit-blood concentrations of optimized ISFI and Vivitril® were compared to ensure their equivalency.

2. Materials and methods

2.1. Materials

Poly (lactic-co-glycolic acid) 75:25 copolymer Resomer® RG 756s (719927 ALDRICH, ester terminated, MW = 76–115 kDa) and 50:50 copolymer RG 504H (719900 ALDRICH, acid terminated, MW = 38–54 kDa) were obtained from Sigma-Aldrich (US). N-methyl-2-pyrrolidone (NMP), ethyl heptanoate, potassium dihydrogen phosphate (K2HPO4), sodium hydroxide (NaOH) and hydrogen chloride (HCl) were purchased from Merck (Germany). Acetonitrile and deionized water were all HPLC grade and used after filtration and degassing. Penicillin-streptomycin, fetal bovine sera (FBS), Roswell Park Memorial Institute (RPMI) 1640 culture medium and trypsin were purchased from Gibco (Germany). MTT [3-(4,5-dimethylthiazol-2-yl)-2, diphenyltetrazolium bromide] was obtained from Promega (USA). Mouse fibroblast 1929 cell lines were obtained from the Pasteur Institute of Iran.

2.2. Solubility determination of drug in the phosphate buffer saline (PBS) and NMP

The solubility of NTX-HCl and NTX in PBS with pH 7.4 as an in-vitro release medium and NMP at 37 °C was investigated [21]. Excess amounts of NTX-HCl and NTX were dispersed and sonicated for 15 min. The suspension was then centrifuged for 10 min at 8000 rpm. The supernatant liquid was analyzed using high-performance liquid chromatography (HPLC) [32]. The HPLC conditions were an LC-6AD pump connected to a diode array detector (SPDM) at 205 nm, an analytical column (C18, Brisa LC2, 4.6 × 250 mm, 5 µm), a flow rate of 1.2 mL/min, and an injection volume of 20 µL, eluted with an isocratic mixture of 80% (v/v) HPLC-grade water (pH = 3.2 via phosphoric acid) and 20% (v/v) methanol at ambient temperature [37]. The experiments were conducted independently in triplicate.

2.3. Stability of NTX in the PBS at temperature of 37 °C

To verify the stability of NTX, at specified time intervals (1, 2, 4, 8 and 12 h and 1, 2, 4, 7, 14, 21 and 28 days), 1 mL of the solution of NTX with a concentration of 50 µg/mL in PBS at a temperature of 37 °C was sampled and analyzed using HPLC according to the method described in Section 2.2. The experiments were conducted independently in triplicate.

2.4. Experimental design for in-vitro release evaluation

A Box- Behnken design (BBD) was planned [38–40] for optimizing the formulation based on minimizing the initial burst release. The independent variables were percent of copolymer by weight, copolymer composition (PLGA 756s:PLGA 504H), and percent ethyl heptanoate by weight as an additive in formulation were investigated. Scanning electron microscopy (SEM) was applied to visualize the surface and cross-section morphologies. The in-vitro, ex-vivo, and in-vivo evaluation of the optimum formulation was investigated. The pharmacokinetic profiles of the NTX and its main metabolite 6β-naltrexol in rabbits were determined using blood samples after subcutaneous injection of the optimum formulations. The formulation’s in-vitro and in-vivo compatibility were evaluated. Finally, the rabbit-blood concentrations of optimized ISFI and Vivitril® were compared to ensure their equivalency.

<table>
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<tr>
<td>Copolymer composition (PLGA 756s: PLGA 504H)</td>
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<tr>
<td>Concentration of ethyl heptanoate (w/w %)</td>
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* Copolymer (Combination of PLGA 756s and 504H).
investigate the molecular-weight variations [42]. An Agilent GPC-Addon apparatus with Plgel™ columns was used to determine the molecular weights of implants. Tetrahydrofuran as an eluent with a flow rate of 1 mL/min and polystyrene standards were used as a calibration [43]. The experiments were done independently in triplicate.

2.6.3. Morphological studies
After 3 days, during which the NMP almost completely migrated from the formulations into the release medium, scanning electron microscopy (SEM) was applied to visualize the surface, and a cross-section morphological study of the implant was carried out using a JSM 35C F (Jeol, Japan) at 25 kV. Samples were freeze-dried, mounted on metal stubs with double-sided tape and coated with gold (30 mA for 5 min) [44].

2.6.4. Cytotoxicity studies
The Mouse 1929 fibroblast cell lines were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 100 IU/mL penicillin and 100 mg/mL streptomycin. The cells were incubated at 37 °C in a humidified incubator under 5% CO2 atmosphere. The cells were seeded into 12-well plates at a density of 5 × 104 cells/well and incubated overnight for 24 h. Then, the cells were treated with sterilized formulations (10 μL) and 10 mg each of PLGA 504H, PLGA 756s, NMP and ethyl heptanoate. The samples were incubated for 24 h. A well containing cells and growth medium with no sample was set up as a control. To determine the relative viability of the mouse fibroblasts, the culture medium was discarded and 500 μL of MTT solution (0.5 mg/mL in PBS) was added to each well. After 4 h, the absorbance of the wells was read at 570 nm (reference wavelength of 630 nm) using a microplate reader (Tecan Group Ltd., Switzerland) [21]. The reported values are the means of three trials.

2.7. Ex-vivo release evaluation
The formulations were administered subcutaneously using a 20G marked needle into hen drummets. The study was carried out in triplicate in which one set containing three drummets was used to visually observe the formed gel [32]. Each drummet was immersed in the vessel of an organ bath with 25 mL of phosphate buffer saline (pH 7.4) as a dissolution medium containing sodium azide (0.1% w/v) at 37 °C. The shape of the implant was observed by cutting into the injection site on the drummet after 24 h. At specified time intervals (2, 6, 10, 12, 18, 24, 48 and 72 h), 3 mL of the release medium was replaced with fresh

<table>
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<tr>
<th>Run</th>
<th>Concentration of copolymers (w/w %)</th>
<th>Copolymer composition (PLGA 756s: PLGA 504H)</th>
<th>Concentration of ethyl heptanoate (w/w %)</th>
<th>Observed initial burst release of NTX (%)</th>
<th>Predicted initial burst release of NTX %</th>
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<td>1</td>
<td>30</td>
<td>50:50</td>
<td>4</td>
<td>11.68 ± 1.02</td>
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<td>2</td>
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<td>4</td>
<td>73.29 ± 6.54</td>
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<tr>
<td>3</td>
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<td>50:50</td>
<td>8</td>
<td>58.03 ± 3.96</td>
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<tr>
<td>4</td>
<td>40</td>
<td>0:100</td>
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<td>45.78 ± 3.78</td>
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<tr>
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<td>50:50</td>
<td>0</td>
<td>73.65 ± 7.41</td>
<td>76.54</td>
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<td>6</td>
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<tr>
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<td>11.25 ± 2.07</td>
<td>12.45</td>
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<tr>
<td>9</td>
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<td>8</td>
<td>28.79 ± 4.48</td>
<td>29.25</td>
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<tr>
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<td>23.75</td>
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<td>59.41</td>
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<tr>
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<td>50:50</td>
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<td>18.93 ± 2.45</td>
<td>20.49</td>
</tr>
<tr>
<td>13</td>
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<td>100:0</td>
<td>4</td>
<td>13.18 ± 1.69</td>
<td>12.45</td>
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<td>14</td>
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<td>0</td>
<td>53.39 ± 4.98</td>
<td>52.96</td>
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<tr>
<td>15</td>
<td>40</td>
<td>50:50</td>
<td>8</td>
<td>45.21 ± 3.72</td>
<td>47.07</td>
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<tr>
<td>16</td>
<td>20</td>
<td>100:0</td>
<td>4</td>
<td>77.49 ± 8.06</td>
<td>76.15</td>
</tr>
<tr>
<td>17</td>
<td>30</td>
<td>50:50</td>
<td>4</td>
<td>13.01 ± 2.18</td>
<td>12.45</td>
</tr>
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</table>
medium to maintain a sink condition. The amount of NTX released during a sampling period was measured using HPLC as described in Section 2.2.

2.8. In-vivo evaluation

2.8.1. Pharmacokinetic studies

New Zealand male rabbits (2 ± 0.1 kg) were used for the in-vivo study. They were maintained at 22 ± 1°C in a 12:12 light-dark cycle with free access to food and water in an animal room. All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). The animal studies were approved by the Institutional Animal Care and Use Committee of Mashhad University of Medical Sciences (930788).

The animals were divided into six groups (n = 3 per group): control (Group I); NMP (670 mg, subcutaneous) (Group II); NTX solution in NMP (subcutaneous) (Group III); optimized formulation (subcutaneous) (Group IV); Vivitrol® (intramuscular) (Group V); and 111 mg of NTX hydrochloride (equivalent to 100 mg NTX free base) in 1.1 mL WFI solvent intravenously (Group VI) (Note: The subcutaneous groups was injected in the back of the rabbit). Naltrexone is extensively metabolized in humans. Production of the primary metabolite, 6β-naltrexol, is mediated by dihydriodiol dehydrogenase, a cytosolic family of enzymes. Two other minor metabolites are 2-hydroxy-3-methoxy-6β-naltrexol and 2-hydroxy-3-methoxynaltrexone. Naltrexone and its metabolites are also conjugated to form glucuronide products. For analysis of the NTX and major metabolite (6β-naltrexol) in serum for pharmacokinetic evaluation, 1 mL of blood samples was collected at regular times (2, 6, 8, 10, 12, 18 and 24 h, then at 2, 3, 4, 5, 7, 10, 14, 16, 18, 21, 28 and 35 days) from the animals' hearts via 2-mL syringe. The serum was obtained by centrifugation and stored frozen. NTX and 6β-naltrexol concentration vs. time data were analyzed by non-compartmental pharmacokinetic analysis. The elimination profiles for NTX and 6β-naltrexol were fitted to one- and two-compartment (two-compartment model was assumed when the two-compartment model was assumed for the pharmacokinetics of other substances) pharmacokinetic models, and the pharmacokinetic parameters were estimated using the WinNonlin software. The absolute bioavailability (F) of NTX and 6β-naltrexol was calculated by dividing their AUC values from the intravenous injection by those from the oral administration [47].

2.8.2. Histopathologic study

For the histopathologic study, the injection sites of all the rabbits were shaved using a sharp blade; the animals were sacrificed by suffocation with carbon dioxide after the end of study. The injection site was removed and the skin tissues of the in-vivo groups (Group I-IV) were fixed in 10% neutrally buffered formalin solution (Accustain®). The excess fixative was removed by washing with water. Then, the samples were dehydrated using alcohol and cleared by passing them through toluene. Finally, the samples were infiltrated and embedded in molten paraffin (AccumateTM) and cooled to room temperature, and paraffin blocks were prepared. Sections 10 μm thick were cut using a rotatory microtome, mounted on a glass slide with the help of glycerogelatin, stained with chrysoideine and hematoxylin and counter-stained with eosin. The slides were examined under a light microscope (Olympus) and photographed using an attached digital camera for any signs of chronic inflammation, granulation tissue, foreign-body giant cells and fibrous capsule formation [48,49].

2.9. Statistical analysis

Data are expressed as mean ± SD (standard deviation). The data were assessed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post-test. The value of p < 0.05 was considered statistically significant.

3. Results and discussion

3.1. In-vitro evaluation

3.1.1. In-vitro release evaluation

The solubility of NTX-HCl and NTX in the NMP were 23 ± 2.09 mg/mL and 100 ± 3.14 mg/mL, respectively. Hence, to increase drug loading in the formulation, NTX-HCl was converted to NTX. The results of stability studies of NTX in the release medium at different times are shown in Fig. 1. The results showed no significant changes in the concentration of NTX in the PBS during 28 days of study (p > 0.05).

The diffusion of NMP into water causes over short periods of time the initial burst release, because the NMP carries the drug into the release medium during the formation of the implants [13]. To control the initial burst release of the drug, the idea of using a combination of PLGA 756s (ester terminated) and PLGA 504H (carboxylic acid terminate) instead of separately was suggested.

The retention times of NTX and NMP using HPLC analysis were observed at 3.97 ± 0.09 min and 6.25 ± 0.31 min, respectively. In this in-vitro release evaluation, the linear calibration curves of NTX and NMP in the release medium were fitted using a linear regression line with R² ≥ 0.98. The limit of detection (LOD) and limit of quantification (LOQ) of NTX were 2 ng/mL and 6.7 ng/mL, respectively [45] and the LOD and LOQ of NMP were 1.2 μg/mL and 4.0 μg/mL, respectively.

For minimum initial burst release (6.76 ± 0.38%), concentration of copolymers (w/w %), copolymer composition (PLGA 756c: PLGA 504H), and concentration of ethyl heptanoate (w/w %) were 31.6%, 37.64% and 6.18% respectively. A second-order polynomial equation is proposed for the prediction of initial burst release (Y₁) as a function of different independents variables as follows:

![Fig. 1. Stability of NTX in the PBS at 37°C.](Image)
regression coefficients, significant $p$-values for the model estimated using Minitab software.

<table>
<thead>
<tr>
<th>% Initial burst release of Naltrexone</th>
<th>Regression coefficients</th>
<th>$p$-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C^*$</td>
<td>+12.32</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>$X_1^{**}$</td>
<td>-13.08</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>$X_2$</td>
<td>+1.46</td>
<td>0.1298</td>
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<tr>
<td>$X_3$</td>
<td>-13.34</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>$X_1^2$</td>
<td>+32.28</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>$X_2^2$</td>
<td>+15.84</td>
<td>&lt; 0.0001</td>
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<tr>
<td>$X_3^2$</td>
<td>+10.02</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>$X_1 \times X_2$</td>
<td>-1.19</td>
<td>0.3561</td>
</tr>
<tr>
<td>$X_1 \times X_3$</td>
<td>-4.55</td>
<td>0.0070</td>
</tr>
<tr>
<td>$X_2 \times X_3$</td>
<td>+2.91</td>
<td>0.0469</td>
</tr>
</tbody>
</table>

*Significant factor.

$^a$ Concentration of copolymers (w/w %).

$^b$ Copolymer composition (PLGA 756s: PLGA 504H).

$^c$ Concentration of ethyl heptanoate (w/w %).

$\text{Y}_1 = + 12.32 - 13.08 \times X_1 + 1.46 \times X_2 - 13.34 \times X_3 + 32.28 \times X_1^2 + 15.84 \times X_2^2 + 10.02 \times X_3^2 - 4.55 \times X_1 \times X_2 + 2.91 \times X_2 \times X_3$ (2)

where $X_1$ is concentration of copolymers (w/w %), $X_2$ is copolymer composition (PLGA 756s:PLGA 504H), and $X_3$ is concentration of ethyl heptanoate (w/w %). The response surface model obtained from an experimental design was evaluated using ANOVA and analysis of residuals. The results of the statistical analyses, including the $p$-values of the initial burst release, are given in Table 3. The linear regression coefficient ($R^2$) for the initial burst release was 0.9947, which showed good performance of the model based on the observed and predicted initial burst release. Based on the statistical results (ANOVA) with a confidence level of 95%, the effect of each term in the models could be significant provided that its $p$-value were smaller than 0.05 ($p < 0.05$) [39]. It is imperative to realize that even though $p$-values are $> 0.05$ (Table 3) for the linear term of $X_3$ (concentration of ethyl heptanoate (w/w %)), due to the hierarchy rule, in which the $p$-values are $< 0.05$ for the higher order (quadratic) of this variable, the effect of the linear term must be considered in the model [50].

Fig. 2A shows the effect of different concentrations of copolymers (w/w %) and copolymer composition (PLGA 756s:PLGA 504H) on the initial burst release of NTX. Since a polymer concentration less than 20% (w/w) gives a high initial burst release due to the lower viscosity of the formulations, the lowest polymer concentration was chosen to be 20% in all experiments [28]. Increasing the copolymer concentration of PLGA 504H to 31.56 (w/w %) decreased the % initial burst release, after which the burst released slightly increased until the percent of polymer by weight reached the 40%. Higher concentrations of polymer in the formulation tend to give higher solution viscosities, lower the implant network porosity and increase the tortuosity, thus decreasing the diffusion rate into the medium; this results in a smaller burst [29,43]. Increasing the weight percent of polymer to more than 31.56 (w/w %) led to decreased solubility of NTX in NMP, such that it could be dispersed in NMP instead of dissolving; this resulted in the drug being deposited on the surface of the implant and a greater initial burst release.

PLGA 756s (ester terminated) contains 75% lactide and 25% glycolide with a molecular weight of 76–115KD, and PLGA 504H (carboxylic acid terminated) contains 50% lactide and 50% glycolide with a molecular weight of 38–54KD. When the copolymer composition (PLGA 756s: PLGA 504H) was increased to (37.64:62.36) (Fig. 2A and B), the % initial burst release decreased significantly; it then started to increase until the copolymer composition (PLGA 756s: PLGA 504H) reached (100:0). The molecular weight and proportion of lactide (LA) to glycolide (GA) increased as the copolymer composition increased. The addition of the higher-molecular-weight polymer (PLGA 756s) to the solution tends to give higher solution viscosities, thus decreasing the diffusion rate of NMP and NTX into the medium. The release rates as well as the extent of the initial bursts can be adjusted by manipulating the total LA/GA ratio in the copolymer. The molar ratio of LA/GA was found to be important for determining the crystallinity, solubility and water uptake of the final polymer, and, most important, the in-vivo degradation rate. Hence, the drug release rate and initial burst decreased with increases in the LA content of the implants until reaching the optimum formulation. By increasing the LA/GA molar ratio, the copolymer became less hydrophilic and absorbed less water, and subsequently NMP diffused more slowly, thus trapping more of the drug in the implants [28].

Increasing PLGA 756s to more than 37.64 (w/w %) led to a decrease in the proportion of PLGA 504H to PLGA 756s, and consequently an increase in the formulation's hydrophobicity. This phenomenon

Fig. 2. (A) Surface and contour plot of % initial burst release of NTX as a function of concentration of copolymers (w/w %) ($X_1$) and copolymer composition (PLGA 756s: PLGA 504H) ($X_2$); and (B) as a function of copolymer composition (PLGA 756s: PLGA 504H) ($X_2$) and % ethyl heptanoate (w/w %) ($X_3$).
decreases the formulation’s ability to dissolve NTX completely, again leading to a large initial burst release. In addition, with an increase in the ratio of PLGA 756s to more than 37.64%, the syringability of the formulation using a 20-gauge needle was reduced, and it could be not injected.

Ethyl heptanoate, due to its finite solubility in water, may have slowed NMP exchange with the buffer, potentially minimizing pore formation in the formed implant and slowing the rate of drug release [29]. The increase in ethyl heptanoate from 1 to 6.18% decreased the initial burst release, although subsequent increases did had no significant effect on initial burst release, as shown in Fig. 2B.

The implant of the optimized formulation in the release media after 24 h is shown in Fig. 3A. Fig. 4A shows the cumulative release profile of NTX from the optimized formulation (concentration of copolymers (31.56 w/w %), copolymer composition (PLGA 756s: PLGA 504H) (37.64:62.36), concentration of NMP (62.26 w/w %), concentration of ethyl heptanoate (6.18 w/w %)), pure PLGA 504H (concentration of PLGA 504H (33 w/w %), concentration of NMP (62 w/w %), concentration of ethyl heptanoate (5 w/w %)) and pure PLGA 756s concentration of PLGA 756s (33 w/w %), concentration of NMP (62 w/w %), concentration of ethyl heptanoate (5 w/w %)). Fig. 4B shows the in-vitro degradation profile of copolymers without NTX based on the decrease of molecular weight [42,51] in the release medium. The cumulative amount of drug released (Fig. 4A) for the optimized formulation (combination of PLGA 504H and PLGA 756s) over the first 24 h, 6.18 ± 0.91%, was significantly (p < 0.05) lower than that for the formulation containing PLGA 504H (17.45 ± 1.07%) and PLGA 756s (11.82 ± 1.03%). NMP release was shown to significantly influence the release rate of the studied formulations. The optimized formulation exhibited a lower initial NMP release (14.54 ± 1.73%) within the first day than the formulations containing PLGA 504H (36.17 ± 2.98) and PLGA 756s (28.18 ± 2.08) (Fig. 4C). These results confirmed that the combination of PLGA 756s and PLGA 504H was used correctly.

The SEM picture shows the surface and cross-section morphologies for implants of formulations containing PLGA 504H and PLGA 756s, and for the optimized formulation after 3 days in the release medium (Fig. 5). As the PLGA/NMP-based solutions injected into PBS solution, a fast diffusion of NMP toward PBS and subsequently a fast quenching of the solutions occurs. This results in an immediate formation of a porous polymeric implant [52]. Implants containing pure PLGA 756s had finger-like pores (Fig. 5A), and those containing pure PLGA 504H had a sponge-like structure with large pore size (Fig. 5B). Combining PLGA 756s and PLGA 504H reduced the rate of the exchange between water and the solvent. This caused a more compact structure with a smooth surface, as it reduced the porosity of the surface, preventing the formation of the finger-like structures and permitting the formation of sponge-like structures with small pore size (Fig. 5C) [53].

The time required for the NMP to escape almost completely from the formulation (80% of initial content) and for the matrix to form for PLGA 504H, PLGA 756s and the optimized formulation were 6, 8 and 10 days, respectively. During this time, the mechanism of release was by diffusion from a polymeric system, and the molecular weight of the copolymer was almost constant (Fig. 4B). After 10 days, polymer

Fig. 3. (A) Formation of implant inside PBS using optimized formulation after 24 h; (B) formation of implant inside hen drummets using optimized formulation after 24 h.

Fig. 4. (A) Cumulative in-vitro release of NTX from formulation; (B) cumulative in-vitro release of NMP; (C) in-vitro degradation of formulation without NTX; and (D) cumulative ex-vivo and in-vitro release of NTX.
degradation began and the release rate of the drug increased, with a much higher release rate (slope) than for shorter durations. The degradation rate of the formulation containing PLGA 504H was faster than those for the formulation containing PLGA 756s and the optimized formulation due to the presence of carboxylic acid terminated PLGA 504H, which caused more water to penetrate the matrix [54]. Furthermore, due to the more rapid degradation of PLGA 504H than other components, the release rate of NTX from formulation containing PLGA 504H was higher than for PLGA 756s and the optimized formulation. Also, the observed in-vitro drug release profiles are in good agreement with in-vitro degradation and NMP release.

3.1.2. In-vitro cytotoxic evaluation

The cytotoxic effects of formulations were studied against mouse L929 fibroblast cell lines. The cytotoxicity of samples after 24 h was determined via quantitative MTT assay based on the reduction of MTT dye by mitochondrial dehydrogenases in living cells to blue formazan; this method is used for the evaluation of cell viability. The MTT assay was carried out on fibroblast cells in contact with samples after 24 h (Fig. 6). The fibroblast cell viability values were in the range of 86.4–100%, demonstrating that the samples were not cytotoxic. The cell viability for the optimized formulation (96.2 ± 1.15%) was higher than that for the formulations containing PLGA 504H (86.4 ± 0.76%) and PLGA 756s (92.63 ± 2.03), as the burst release of NMP and NTX from the optimized formulation over 24 h was lower than for the formulations containing PLGA 504H and PLGA 756s. The MTT assay measurements were in good agreement with the evaluation of in-vitro release. thus, the optimized formulation was shown to be biocompatible, and can be used for subcutaneous administration [27].

Fig. 5. Surface (scale bar: 10 μm) and cross-section (scale bar: 100 μm) morphologies of implants after 3 days from release medium: (A) formulation containing PLGA 756s; (B) formulation containing PLGA 504H; and (C) optimized formulation.
3.2. Ex-vivo release evaluation

An ex-vivo study was carried out to confirm the shape and formation of the implant and to investigate the release of NTX from hen drummets. The formation of the deposit and implant was confirmed by removing a section of hen drummet after 24 h injecting the formulation. The star in Fig. 3B shows the subcutaneous formation of an implant in a drummet.

The ex-vivo drug release data was obtained for 3 days because after 3 days, the release medium was corrupted. The results of ex-vivo release evaluations for the optimized formulation (Fig. 4D) showed no significance difference from in-vitro release (p > 0.05). This confirmed that the implant behaved similarly in a subcutaneous environment.

3.3. In-vivo evaluation

3.3.1. Pharmacokinetic study

The retention times of NTX and 6β-naltrexol using HPLC were observed at 4.12 min and 4.83 min, respectively. The calibration curve of NTX and 6β-naltrexol in the release medium were fitted using a linear regression line with $R^2 \geq 0.98$. The LOD and LOQ for NTX and 6β-naltrexol were 2 ng/mL and 6.7 ng/mL, respectively, in rabbit serum [45]; these results were later applied in the drug-release and pharmacokinetic study.

An intravenous administration of NTX-HCl resulted in its high initial concentration followed by an immediate decrease concentration; the concentration then gradually decreased (Fig. 7A). Fig. 7B shows the blood profile of the subcutaneous solution, which displayed an initial increase in NTX and 6β-naltrexol concentrations and a gradual decrease over the 48 h for NTX and 72 h for 6β-naltrexol period [55]. In contrast, constant serum levels of NTX were detected for nearly 4 weeks for the optimized formulation (Group IV) and Vivitrol® (Group V), indicating a constant rate of NTX release in-vivo upon single injection after $T_{\text{max}}$ (Fig. 7C–D). The AUC and absolute bioavailability (F) of NTX were enhanced by using the optimized formulation and Vivitrol® in comparison with subcutaneous injection (Group III). The results of the in-vivo release studies for the optimized formulation were similar to those of the in-vitro release studies. Tables 4 and 5 list the pharmacokinetic data for this study. The $C_{\text{max}}$ of NTX (21.06 ± 2.9 ng/mL) for the optimized formulation was close to that for Vivitrol® (21.11 ± 2.89 ng/mL). The $C_{\text{max}}$ of 6β-naltrexol (32.63 ± 5.27 ng/mL) for the optimized formulation was also close to Vivitrol® (21.11 ± 2.89 ng/mL). Furthermore, the AUC, absolute bioavailability (F) and range of serum concentration of NTX for the ISFG formulation was similar to Vivitrol®. These results indicate that the optimized formulation can reach the effective therapeutic concentration for treatment of opioid and alcohol dependence.

The ISFI formulation composed of PLGA/NMP showed a considerable initial burst effect, especially in the first few hours after injection into the body. During this time the initial burst of the drug may increase the plasma concentration achieved above the required one. Furthermore, this initial burst of drug has led to severe inflammation at the injection site, and occasionally to systemic toxicity. Because the initial burst release for the optimized formulation in the in-vitro, ex-vivo and in-vivo studies was significantly lower than that for the formulations containing PLGA 504H or PLGA 756s, the optimized formulation was preferable. Thus, a combination of PLGA 504H with PLGA 756s is a
good formulation for developing a long-acting, controlled-release, injectable solution-delivery system for NTX with minimum initial burst release.

3.3.2. Histopathological study
The histological section from Group I (Control), Group II (NMP) (Fig. 8A) and Group III (NTX solution in NMP) (Fig. 8B) showed normal architecture. Subcutaneous injection of the optimized formulation (Group IV) (Fig. 8C) induced a marked granulomatous reaction characterized by the presence of epithelioid histiocytes and multinucleated foreign-body giant cells. In addition, macrophages and other mononuclear inflammatory cells such as lymphocytes were found surrounding the implant particles, having infiltrated the deep layer of dermis, representing chronic inflammation. These responses were considered as a component of the normal tissue or cellular host reaction to injury [48,56].

4. Conclusion
Naltrexone is a competitive opioid antagonist of the μ, κ and δ opioid receptors that has been used as a maintenance medication after withdrawal in detoxified opioid-dependent patients. Furthermore, naltrexone has been proven by the FDA to treat alcohol dependence. One alternative to an oral naltrexone formula is a sustained release formulation, which can increase compliance in opioid or alcohol patients. The aim of the present investigation was to develop a long-acting, injectable implant of NTX with improved therapeutic performance. This study provides evidence that the combination of PLGA 756s (ester terminated) and PLGA 504H (carboxylic acid terminated) can control the delivery of NTX both in-vitro and in-vivo for 28 days after a single subcutaneous injection. The delivery system was biocompatible and biodegradable. Thus, the optimum formulation was a good formulation for developing a long-acting, controlled-release, injectable solution-delivery system for NTX with minimum initial burst release.

Conflicts of interest
The authors declare no conflict of interest.

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