Beijing Institute of Pharmacology and Toxicology, Beijing, People's Republic of China

Preparation and *in vivo* evaluation of thienorphine-loaded PLGA microspheres

YANG YANG, YONGLIANG GAO

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Yang Yang, Yongliang Gao, Beijing Institute of Pharmacology and Toxicology, 27 Taiping Road, Haidian District, Beijing, 10085, People's Republic of China
jiamusi101@126.com; ylgao868@yahoo.com

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Thienorphine-loaded microspheres composed of poly(D,L-lactide-co-glycolide) were prepared by an O/W emulsion solvent evaporation method. HPLC was used to determine the drug loading and drug release, while a LC-MS-MS system was employed to analyze the plasma drug concentration. Results indicated that the PLGA particles obtained were spherical and of appropriate size. The formulation was stable during the test period. *In vitro* drug release from the microspheres was sustained for about 28 days mostly by the diffusion mechanism. The plasma drug concentration-time profiles were relatively smooth for about 28 days after subcutaneous injection of the drug-loaded microspheres to rats, compared with that for drug suspension. *In vitro* and *in vivo* correlation was established.

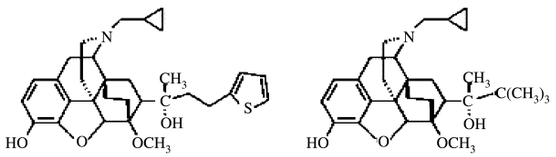
1. Introduction

The number of narcotic abusers in China is quite staggering. According to government statistics, there are estimated to be 0.791 million heroin abusers. Many relevant medical agencies have made continued efforts to reduce the number of narcotic abusers, but have seldom achieved the ideal goal. Statistics show that 98.2% of subjects who entered treatment programs had a dismal record of relapse, and only a small proportion remained clean for long after the program. The damage caused by narcotics abuse remains a scourge of society. It is becoming increasingly important for us to help addicted individuals to stop their dependency or at least decrease the level of dependency to make them a functional membesr of society.

Relapse of addiction is often caused by poor compliance and lack of retention in programs (Hulse et al. 2005). A repetitive treatment act, such as asking subjects to take a pill daily, is not easy, even they have no uncertainty about taking the pill. When the narcotics abuser has physiological and emotional needs for the abused substance, maintaining a therapeutic routine becomes more difficult. The lack of perseverance of the subject decreases the chance of success of the treatment. Therefore, it is of great importance to be able to reduce the level of involvement of the subject with medicinal treatments, particularly those treatments involving a specific regimen.

Many experts have adopted sustained-release methods to reduce the involvement of subjects in compliance. Biodegradable injectable microspheres have been studied widely over the last 30 years (Wu et al. 2006). They can prolong the duration of a drug effect significantly and improve patient compliance. The total dose and some adverse reactions may be reduced because a steady plasma concentration can be sustained. There is also no need for them to be implanted by a surgical operation and removed after the drug is completely released. Among various biodegradable polymers, poly(lactic-co-glycolic acid) (PLGA) is the one most widely studied and used (Fu et al. 2005). The properties of the microspheres are sensitive to many variables of preparation and conditions of the preparation and selection process.

Thienorphine [*N*-cyclopropylmethyl-7(-[(*R*)-1-hydroxy-1-methyl-3-(thien-2-yl)-propyl] -6,14-endo-ethano-tetrahydronororipavine] is a new compound, synthesized by the Beijing Institute of Pharmacology and Toxicology, China. As an analog of buprenorphine, thienorphine is a partial agonist of the μ-opioid receptor, as is buprenorphine (Gong et al. 2001), which has been widely used in the therapy of opioid addiction (Fudala et al. 1998; Umbricht et al. 2003). Like buprenorphine, it has been reported that thienorphine is bound potently and non-selectively to μ-, δ-, and κ-opioid receptors stably expressed in CHO (Chinese hamster ovary) cells and behaves as a partial agonist at the μ-opioid receptor. However, some differences have been observed between the pharmacological profiles of thienorphine and buprenorphine. *In vitro*, thienorphine was more potent than buprenorphine in inhibiting [^{3}H]diprenorphine and stimulating guanosine 5′-O-(3-[^{35}S]thio)triphosphate binding to rat μ-opioid receptor stably expressed in CHO cells. *In vivo*, thienorphine exhibited a more potent antimorphine effect compared with buprenorphine. Moreover, compared with buprenorphine, thienorphine showed a similar long-lasting antinociceptive effect but a much longer antagonism of morphine-induced lethality (Yu et al. 2006). In contrast to common opioid agonists, it was observed that thienorphine induced hypoactivity in mice and that this effect declined after repeated administration. In addition, coadministration of thienorphine dose-dependently suppressed the development, transfer, and expression of behavioral sensitization to morphine in mice (Zhao et al. 2004). These results indicate that thienorphine is a potent, long-acting partial opioid agonist and may have a possible application in treating addiction.



(A) (B)

Thienorphine (A) and buprenorphine (B)

Accordingly, in the present study, a newly developed controlled release system based on PLGA microspheres loaded with thienorphine was prepared and evaluated. Besides the evaluation of physicochemical characteristics, the *in vitro* release of the PLGA microspheres was also investigated. An analytical method based on the LC-MS technique was also developed for simultaneous determination of thienorphine in *in vivo* samples. Finally, the correlation between *in vitro* and *in vivo* release was established.

2. Investigations, results and discussion

2.1. Morphology, size, drug loading and in vitro release of thienorphine-loaded microspheres

The surface morphology of the microspheres was examined visually by scanning electron microscopy. Photomicrographs of PLGA microspheres loaded with thienorphine produced by the solvent evaporation method are shown in Figs. 1A and B. It was observed that the drug loaded microspheres were spherical in shape with a smooth surface. The microspheres are seen to be well formed and homogeneous, with no crystalline drug or fragments of polymer adhering.

As seen from Fig. 2, the PLGA microsphere particles were mainly distributed around 40 μm, with an average value of 34.52 ± 4.56 μm. Span was 1.04 ± 0.08 (n = 3). The results demonstrated that the particle size of the prepared system was uniform and appropriate for administration to rats via subcutaneous injection. The encapsulation efficiency and drug loading were 87.30 ± 0.59 and 4.57 ± 0.04 % (n = 3), respectively.

Fig. 3 shows the *in vitro* release profiles of three batches of thienorphine-loaded microspheres. The *in vitro* release behavior had a good reproducibility. In the initial 7 days, release was nearly linear with 10% released per day. Thereafter a gradual slow release was observed with about 90% released by day 21.

The *in vitro* release data were analyzed with Higuchi ($Q = 25.365t^{1/2} - 22.433$ ($R^2 = 0.9704$)), first-order ($\ln(100\text{-}Ft) = -0.1697t + 5.0483$ ($R^2 = 0.8759$)) or zero-order ($Ft = 4.3915t + 7.3355$ ($R^2 = 0.9972$)) equations, respectively. As a result, the best fit was given by the zero-order model as shown by the higher R^2 value (>0.99).

2.2. Pharmacokinetic studies of thienorphine solution and microspheres

Plasma concentration–time profiles of thienorphine after s.c. administration of thienorphine solution and thienorphine microspheres are shown in Fig. 4 and Fig. 5. As shown in Fig. 4, after subcutaneous injection of the drug suspensions, drug concentrations peaked within 2 min, then decreased, reaching the minimum at about 7 h, suggesting that the elimination of thienorphine was very fast. The peak value for thienorphine was about 1321.2 ng/ml. On the other hand, as shown in Fig. 4, the profiles for drug-loaded microspheres were rather smooth. Drug plasma concentration was maintained at a relatively high level (more than 3 ng/mL) in the initial 2 days after subcutaneous injection of thienorphine-loaded microspheres. The concentration reached its maximum values within the first 0.5 days, about 4.79 ng/ml. Drug concentration decreased markedly after 3 days. Relatively steady state concentrations for thienorphine were reached from day 7 to day 21.

The pharmacokinetic parameters are listed in the Table. Obviously, much higher values of AUC, $t_{1/2}$ and MRT, as well as much lower values of $C_{max,}$ were obtained for the drug-loaded PLGA microspheres, compared with those for the drug suspension.

2.3. In vitro and in vivo correlation (IVIVC)

The linear regression plots of drug released (%) vs. F_a for PLGA microspheres loaded with thienorphine are shown in Fig. 6 and the relative regression equations are also included. Good linear regression correlation was demonstrated between the percentage of drug released in PBS at 37 °C and the percentage of drug absorbed from the microspheres in rats. Based on this result, it seems reasonable to predict the drug absorption *in vivo* by an *in vitro* release test. The correlation of *in vitro* and *in vivo* behavior is always a crucial issue for dosage forms in which the drug needs to be absorbed into the systemic circulation, since it is important for both formulation development and quality

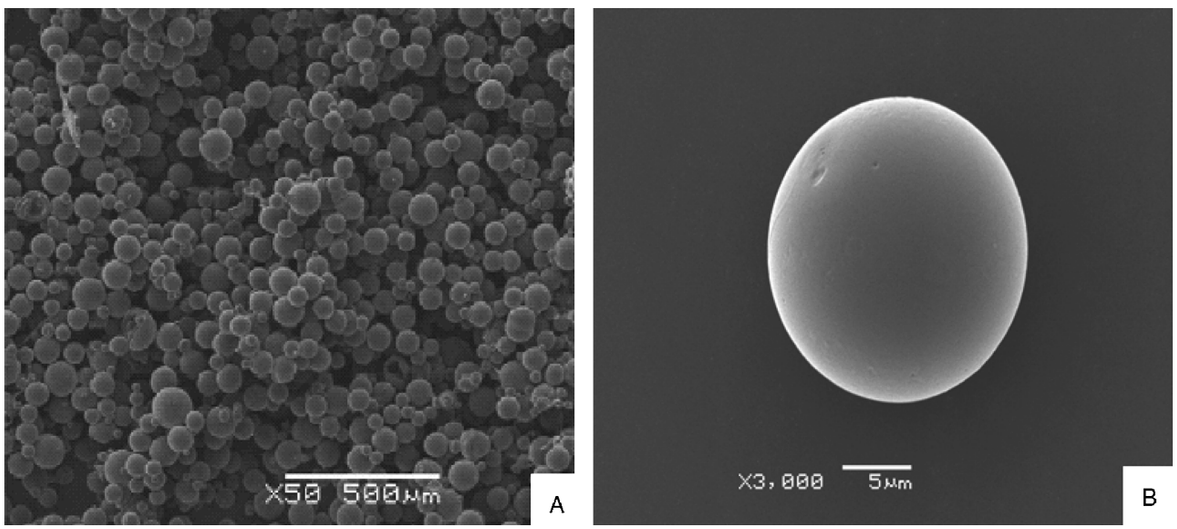


Fig. 1: Scanning electron micrographs of thienorphine loaded PLGA microspheres (magnification (A) 50 times, and (B) 3000 times)

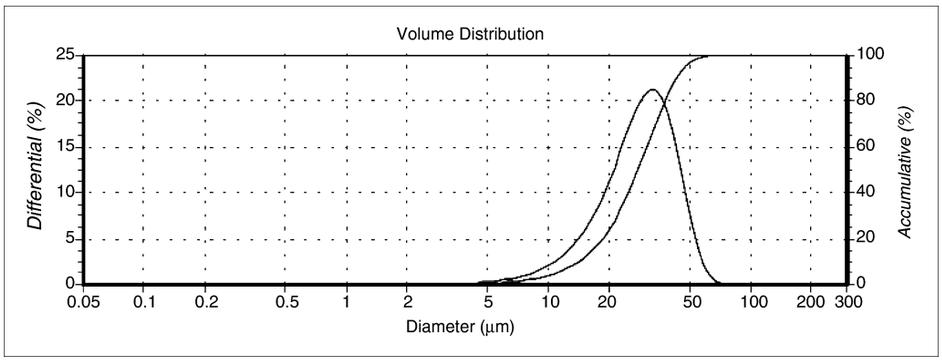


Fig. 2: Particle size distribution of thienorphine loaded PLGA microspheres

Table: Pharmacokinetic parameters following subcutaneous injection of thienorphine solution and microspheres to rats

Parameter	Thienorphine solution (3 mg/kg)	Thienorphine microspheres (9 mg eq thienorphine/kg)
T_{max}(h)	0.65 ± 0.09	12.0 ± 4.0
C_{max} (ng/ml)	1321.20 ± 296.73	4.79 ± 8.79
$t_{1/2}$(h)	4.34 ± 1.97	578.96 ± 1.83
$AUC_{(0\text{-}t)}$ (ng.h/ml)	1162.68 ± 144.35	1700.93 ± 178.67
$AUC_{(0\text{-}\infty)}$ (ng.h/ml)	1166.89 ± 145.50	3225.58 ± 418.64
$MRT_{(0\text{-}t)}$ (h)	2.85 ± 0.73	329.00 ± 23.56

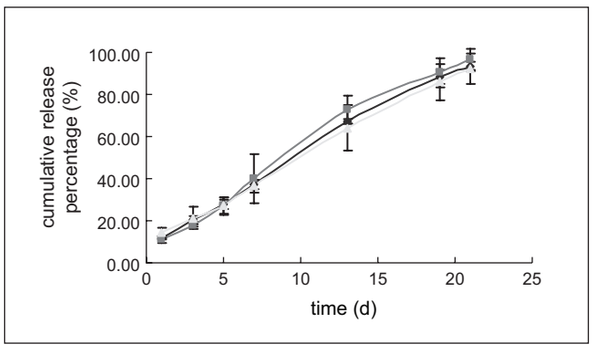


Fig. 3: *In vitro* cumulative drug release from thienorphine loaded PLGA microspheres in 0.01M PBS (pH 7.4) at 37 C (n = 3)

control (Freitas et al. 2005). Therefore, it will be beneficial to develop an *in vitro* release process that will enable the *in vivo* absorption of a subcutaneous injection to be predicted. The data presented above suggest that the method using a shaken flask was acceptable in IVIVC studies of subcutaneous microsphere formulation.

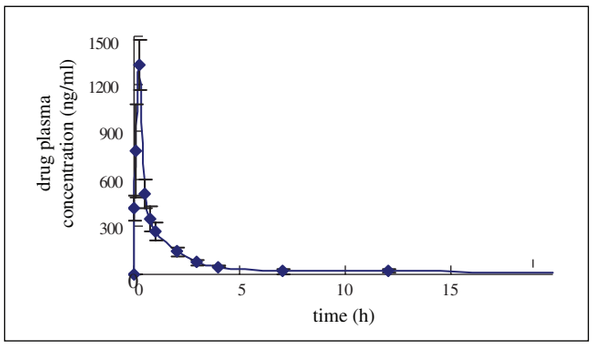


Fig. 4: Drug plasma concentration versus time profile after subcutaneous injection of thienorphine solution to rats (mean ± S.D., n = 6)

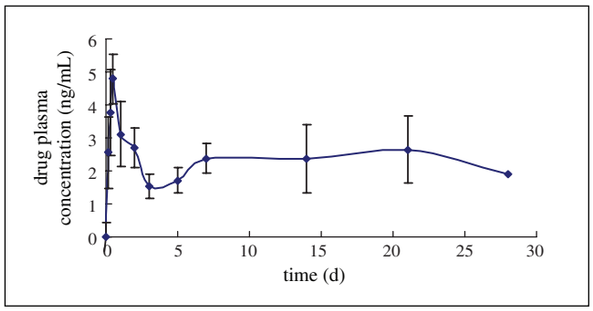


Fig. 5: Drug plasma concentration versus time profile after subcutaneous injection of thienorphine microspheres to rats (mean ± S.D., n = 6)

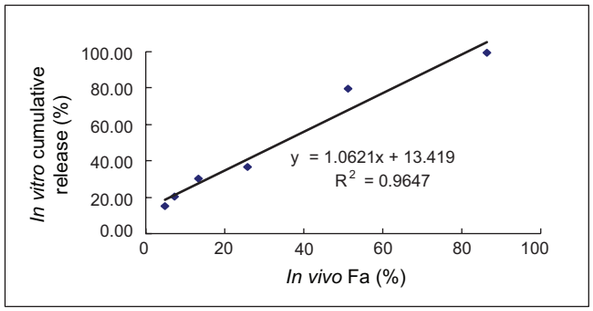


Fig. 6: Linear regression plots of cumulative absorption vs. percent dissolution of thienorphine from PLGA microspheres

3. Experimental

3.1. Materials

PLGA (Wh15000, lactide/glycolide ratio, 75/25) was generously donated by Prof. Shao-bing Zhou of Southwest Jiaotong University. Thienorphine (99% purity) was supplied by the Beijing Institute of Pharmacology and Toxicology. Buprenorphine [internal standard (I.S.)] (>99% purity) was obtained from Sigma (St. Louis, MO, USA). Polyvinyl alcohol (PVA-124), dichloromethane (DCM), and ethanol were obtained from Beijing Chemical Reagents Company. All other materials or solvents were of reagent or analytical grade.

3.2. Microsphere preparation

An O/W emulsion solvent evaporation method was used to prepare thienorphine-PLGA microspheres. About 1500 mg of PLGA and 82.85 mg of thienorphine were added to 10 mL of a mixture of DCM:ethanol (4:1). After being completely dissolved, it was poured into a 5.4 % aqueous solution of PVA-124 and then the mixture was emulsified by using a propeller stirrer (SXJQ-1, Zhengzhou, China) at 800 rpm for 10 min at 25 °C. Stirring at 300 rpm was then continued for 8 h to evaporate the organic solvent. The resulting microspheres were washed three times with distilled water and dried under vacuum.

3.3. Characteristics of microspheres

3.3.1. Determination of thienorphine content of microspheres

Thienorphine was extracted from the microspheres with 25 mL of a mixture of DCM and ethanol (1:1). The solution was directly measured using HPLC (LC-10AT VP, Shimadzu, Japan). HPLC conditions were as follows: C_{18} column (250 mm × 4.6 mm, 5 μm), a mixture of acetonitrile-methanol-0.02 mol·L^{-1} phosphate (40:15:45) buffer containing 0.2% triethylamine (pH = 3) as eluant, detection at 220 nm.The polymers did not interfere with absorbance of the drug at the specified wavelength. The encapsulation efficiency is expressed as the ratio of the amounts of drug detected and added. Each measurement was performed in triplicate.

3.3.2. Particle size analysis

A light-scattering particle size analyzer (BT-9300, BETTER, China) with a circulation disperser (BT-600, BETTER, China) was used to determine the size distribution of the prepared microspheres. The lyophilized particles were suspended in a large volume of distilled water and analyzed under continuous stirring. Particle size was expressed as volume mean diameter in micrometers (SEM, n = 3) of three batches.

3.3.3. Microscopic observations

The microspheres were mounted on metal stubs using double-sided adhesive tape. After vacuum coating with a layer of gold, the surface of the thienorphine microspheres was observed by scanning electron microscopy (Hitachi S-450, Japan).

3.3.4. In vitro release assays

About 25 mg of microspheres were weighed and added to a dialysis bag with molecular weight cut-off 1 kDa, and 1 mL phosphate buffered saline (PBS, 0.01 M, pH 7.4) was then added. The dialysis bag containing the microsphere suspension was placed in a flaskfilled with 30 mL phosphate buffered saline (PBS, 0.01M, pH 7.4) containing 0.02 % sodium azide. Incubation was conducted at 37 °C by shaking at a rate of 72 rpm. At predetermined intervals, 1 mL of medium was drawn out and replenished with the same volume of fresh medium. Thienorphine concentration was determined in triplicate at 220 nm by HPLC. Each measurement was performed in triplicate.

3.4. In vivo studies

3.4.1. Animals and experimental method

Wistar rats (adult male, 220 ± 20 g, Beijing Institute of Pharmacology and Toxicology) were used for the pharmacokinetic studies. All animal experiments complied with the requirements of the National Act on the use of experimental animals (PR China). All rats were fasted for 12 h before the experiments with free access to water. The rats were randomly divided into two groups, the test group and a control group. The animals in the test group were treated by subcutaneous injection of the suspension containing microspheres equivalent to 9 mg thienorphine/kg and 1 ml of dispersed solution (containing 0.5% CMC-Na and 0.05% Tween-80). The rats in the control group were administered subcutaneously a dose of 3 mg/kg, as well as the dispersed solution. All suspension used was vortex-mixed before administration. After administration, all the centrifuge tubes were collected and freeze-dried to determine the residual drug and calculate the exact amount of drug injected. At the designated time point, 0.8 ml blood samples were collected from the retroorbital plexus of the anesthetised rats, and centrifuged at 8000 rpm for 10 min within 2 h. Then 0.3–0.4 ml of plasma was obtained and stored at -20 °C until analysis.

3.4.2. Sample preparation

0.1 ml I.S. solution (buprenorphine, 40 ng/ml) was added to 100 μl plasma sample in a 1.5 ml test tube. The sample mixture was deproteinized with 0.8 ml of methanol and vortex-mixed for approximately 1 min, and the precipitate was removed by centrifugation at 12,000 rpm (revolutions per minute) for 10 min. Then 800 μl of supernatant was transferred to another clean test tube and evaporated to dryness at 37 °C with a CentriVap Concentrator. The dry residue was reconstituted in 100 μl of the mobile phase, vortex-mixed, and centrifuged at 12,000 rpm for another 10 min. Twenty microlitres of the clean supernatant were injected into the LC/MS/MS for analysis.

3.4.3. In vivo release assays

The plasma drug concentration in animals was very low according to the preliminary experimental results. HPLC/UV methods are not sensitive enough to detect thienorphine in rat plasma. The LC/MS/MS method has been described in a previous report (Kong et al. 2007). We repeated the LC/MS/MS (Agilent1200, Agilent, USA) method. LOQ of thienorphine was 0.25 ng/ml. The intra- and inter-batch precisions were less than 12% and recoveries were greater than 80%.

3.4.4. Data analysis

The plasma concentration-time data were analyzed with a noncompartmental model by DAS to obtain the maximum plasma drug concentration (C_{max}), terminal elimination rate constant (k_e), terminal half-life ($t_{1/2}$), area under the plasma concentration-time curve from time 0 to last point (AUC_{0-t}) or infinity ($AUC_{0-\infty}$) and mean residence time up to last point (MRT_{0-t}). The correlation between the drug released (%) *in vitro* in PBS at 37 °C and the drug absorbed *in vivo* (F_a) was examined. F_a was determined using the Wagner–Nelson method by the following equation: $F_a = (C_t/k_e + AUC_{0-t})/AUC_{0-\infty}$ (Wagner et al. 1963). The values of the correlation coefficient (R^2), slope and intercept were calculated, respectively.

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