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In Vitro and In Vivo Release of Naltrexone from Biodegradable Depot Systems

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ABSTRACT The aim of this study was to prepare poly(d, l-lactide) (PLA) microspheres containing naltrexone (NTX) by a solvent evaporation method, and to evaluate both in vitro and in vivo release characteristics and histopathological findings of tissue surrounding an implant formulation in rats.

This method enabled the preparation of microspheres of regular shape and relatively narrow particle size distribution. The in vitro release profiles of NTX from PLA microspheres showed the release of NTX did not follow zero-order kinetics. An initial burst release was observed, subsequently followed by a nearly constant rate of 0.4% per day after ten days. The cumulative amount of NTX released at the end of 60 days was 80%. Compressed microspheres showed near zero-order sustained release of NTX for 360 days. The plasma NTX levels in rats showed that for compressed microspheres NTX concentrations were constant and exceeded 2 ng/mL for 28 days. Throughout the 28 days of study, the implantations cause a minor inflammatory response, which can be regarded as a normal defence mechanism. The sustained release performance of NTX from the biodegradable depot systems may provide a reliable, convenient, and safe mechanism for the administration of NTX for the long-term treatment of opioid dependence.

KEYWORDS Naltrexone, Microspheres, Tablet, Biodegradable system, HPLC Poly(D,L-lactide), Release in vitro, Release in vivo, Histopathology

INTRODUCTION

Heroin dependence is described as a "chronic relapsing disease," which requires long-term psychotherapy and pharmacological treatment (Leshner, 1997; McLellan, 2002; Millery et al., 2002). There is a large number of pharmacological and psychosocial interventions available for the treatment of heroin dependence, such as methadone maintenance (Woody et al., 1993) and buprenorphine treatment (Johnson et al., 1992). Naltrexone (NTX) is another promising option for maintenance therapy (San et al., 1991). As a pure narcotic antagonist, NTX effects occur by competitive displacement of opioid molecules at receptors, as well as the blocking of opioid access to the receptor sites (Emmerson et al., 1994). The recommended daily oral dose of NTX for maintenance therapy is 50–100 mg (Stine

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et al., 1998). As a result of first-pass metabolism, orally administered NTX has a low bioavailability of approximately 40% (Wall et al., 1981). Clinical data has indicated that this method is less successful owing to low compliance rates (Bell et al., 1999; Hulse & Basse, 2000; Brever, 2001). Better NTX compliance has been observed when a responsible adult is available to supervise daily NTX dosing (Hulse & Basso, 2000). Previous studies have reported that the method of daily oral NTX maintenance is not appropriate (Brewer, 2000; Brewer et al., 1998). On the other hand, adverse effects of NTX administered orally also contribute to non-compliance (Gongalez & Brogden, 1988). In order to improve the therapeutic efficiency of NTX in the treatment of opioid drug addiction, it has been proposed that long-term sustained delivery of NTX would be more appropriate. In a previous study (Wise, 1984), a NTX (70%)-poly(D, L-lactide-co-glycolide) copolymer (30%), which was the first biodegradable drug delivery system approved by the Food and Drug Administration (FDA) for clinical testing, was limited by "burst release" in human trials. Previous findings also suggested that polymeric NTX sustained release systems were biocompatible formulations (Anderson & Wise 1975; Yamaguchi & Anderson, 1993). Several other dosage forms have also been proposed, for example, a NTX-poly(lactic acid) composite (Yolles et al., 1975) which had an effective blocking action to morphine in rats for 24 days; a NTX-copolymer (90% L-lactic acid and 10% glycolic acid) beads (Chiang et al., 1984) which provided constant NTX levels for one month; and a NTX pamoate linear poly(ortho esters) disk (Maa & Heller, 1990) which released NTX pamoate for 21 days. Recently a new formulation, namely an injectable implant system, has been investigated (Shively et al., 1995). Clinical studies have shown that a depot formulation of NTX (Depotrex®) provided stable plasma NTX levels for approximately 3 and 4 weeks after administration of 192 mg and 382 mg NTX (Comer et al., 2002). In addition, transdermal NTX prodrugs have also been investigated in an animal model (Valiveti et al., 2005). The development of a controlled release system for NTX was first described in the 1970's (Anderson & Wise, 1975; Yolles et al., 1975), however, thus far a product providing sustained release over several months has not been achieved. In contrast to the above formulations, the present study involves the development and evaluation of new formulations involving compressed NTX-poly (D, L-lactide) (PLA) loaded microspheres. The aims of this project were: 1) to prepare NTX microspheres by a solvent evaporation method; 2) to evaluate both in vitro and in vivo the release characteristics; 3) to observe the tissue response at the implant site.

MATERIALS AND METHODS

Materials

Poly (D,L-lactide) (PLA) (inherent viscosity 0.53 dL/g) was purchased from PURAC Biochem (Gorinchem, The Netherlands). Naltrexone (NTX) hydrochloride was obtained from Inpharzam Trading Company (Lugano, Switzerland), and was converted to the free base form as described previously (Negishi et al., 1987). Poly(vinyl alcohol) (PVA; degree of hydrolysis 87 to 89%, viscosity of 4% aqueous solution at 20°C, 40–46 cP) was obtained from BDH (Poole, England). All other chemicals were purchased commercially as analytical grade reagents.

Methods

Preparation of Microspheres and Implants

The microencapsulation technique was based on the formation of a water-in-oil-in-water double emulsion under controlled stirring, modified according to the method of Uchida et al. (1997). A solution of 5 mL PLA (0.5 g) in dichloromethane, in which NTX (0.5 g) was dissolved, formed the dispersed phase. The solution was emulsified with 0.2 mL water using a vortex mixer. The water-in-oil emulsion was poured into 500 ml of 0.5% PVA solution containing 5% of sodium chloride. A stable multiple-emulsion was obtained by mechanical stirring (1,000 rpm) at room temperature until the organic solvent was evaporated. Microspheres were collected by filtration (10 μm) after multiple washings with deionized water at room temperature and were dried by freeze-drying. After mixing with 1% (w/w) magnesium stearate, some dried microspheres were directly compressed with a manual single-punch tablet machine (F3, Manesty, Liverpool, England). The diameter and thickness of the resulting implants were approximately 3 and 2 mm, respectively.

Evaluation of NTX Loading Microspheres

The NTX-loaded microspheres of known weight (50 mg) were dissolved in 2% methanolic KOH (100 mL) and stirred overnight at room temperature. The

amount of drug in solution was quantified by a UV spectrophotometer (HP 8452A Diode Array Spectrophotometer, Hewlett Packard, Waldron, Germany) at the maximum absorbance at 291 nm (aqueous alkali) (Negishi et al., 1987).

Evaluation of Release In Vitro

For in vitro release, 50 mg NTX-loaded microspheres was placed in a Sigma dialysis tubing (15 mm i.d. × 100 mm length) containing 2 mL of 0.02 M phosphate buffer solution at pH 7.4. This bag was immersed in 100 mL of the same buffer and maintained at 37°C. Samples were collected daily from the release medium after shaking the contents which were replaced daily with fresh buffer. The amount of NTX released in the buffer solution was quantified by a UV spectrophotometer at the maximum absorbance at 281 nm. Five replicate tablets were also evaluated by using a similar process. Linear regression analysis of the calibration plots resulted in the following equations (n = 6): $y = 0.0077x - 0.0017$ ($r = 0.9998$) for 291 nm, and $y = 0.0049x - 0.0008$ ($r = 0.9999$) for 281 nm, y representing the absorbance and x the concentration of NTX in the samples.

In Vitro Polymer Degradation

The microspheres (50 mg) were placed in microcentrifuge tubes and 1 mL phosphate buffer (pH 7.4) was added and maintained at 37°C as described previously (Pays et al., 2002). At each time point, microsphere samples (n = 5) were collected, dried, and then dissolved in tetrahydrofuran (THF;1 mg/mL). The solution was analyzed by size exclusion chromatography (SEC) using a styragel column (4.6 × 300 mm; HR 4E THF, Waters Co. Ltd, Milford, MA) with a differential refractometric detector at 45°C (RID-10A, Shimadzu, Kyoto, Japan). Tetrahydrofuran (THF) was used as mobile phase at a flow rate of 0.3 mL/min. The weight average molecular weight (Mw) and number average molecular weight (Mn) of each sample was calculated using polystyrene standards (Mw 1,260 to 189,000; Showa Denko, Tokyo, Japan).

Evaluation of Release In Vivo

Six-week-old Sprague Dawley rats, weighing 200–220 g were used in this study according to the procedure modified from a previous report (Yamaguchi & Anderson, 1993). The rats were randomly divided into three groups each of ten rats, and were then ear-punched with unique codes. The rats were housed in standard cages and were given free access to feed and water at all times. In brief, rats were lightly anesthetized (using ketamine) and a single incision (5 mm) was made in the midline (3 cm from the tail). Two NTX tablets were placed in one side, and one placebo tablet was implanted on the opposite side per rat subcutaneously. In another experimental group, NTX microspheres [15 mg (approximately equal to 2 tablets) dispersed in 0.3 mL polyethylene glycol 400] were injected into the subcutaneous area of rat through a trochar; and microspheres without NTX were separately injected into the other side.

Blood samples (0.5 mL) were collected at 1 (24 hours after implantation), 7, 14, 21, and 28 days from each animal by nicking the base vein in the rat's tail. The blood samples were centrifuged to separate the plasma, and were later stored at −20°C until further analysis. At each of the time intervals above, one animal in each group was sacrificed; at the end of 28 days all animals were sacrificed. Samples of tissues in the region of the implant were examined for adverse reactions arising due to the different formulations. Ethics approval was received for this study (Animal Experimentation Ethics Committee No. 46/99).

HPLC Analysis

The HPLC system for plasma samples consisted of a Waters Alliance M2690 chromatograph (Waters Co. Ltd., Milford, MA) equipped with a gradient pump, an autosampler, a Waters Symmetry C_{18} column (5 µm, 150 × 4.6 mm), Waters 996 photodiode array detector, and Millenium 32 software was used. The mobile phase was acetonitrile: 10 mM sodium dihydrogenphosphate (10:90, v/v, pH 6.6) and the flow rate was 1.2 mL/min at ambient temperature (Lai et al., 1997). The peaks were recorded at 210 nm, and the limit of quantitation was approximately 1 ng/mL. The calibration curve for the concentrations 1–100 ng/mL (six-point calibration) was linear [$y = 0.2038x - 0.02065$ ($r = 0.9973$), y representing the peak height ratio of NTX concentration to the internal standard (nalorphine) and x the concentration of the samples].

Microsphere Morphology

Microsphere morphology was observed using a scanning electron microscope (SEM) (Philips XL-30,

The Netherlands). Samples were coated with gold prior to examination.

Particle Size Distribution

Particle size was determined with a Malvern Mastersizer MS 2000 laser diffraction system (Malvern Instruments Ltd, Malvern, England). The microspheres were dispersed in approximately 75 mL of 1000 ppm sodium hexametaphosphate using a Cole-Plamer 8851 ultrasonic bath for 20 min. The sample was analyzed in the diffraction system using a single optical lens with two laser sources. The weighted average of the volume distribution $D[4,3]$ was used to describe the particle size.

Evaluation of Histopathology

After the animals were sacrificed, tissue samples within the implantation sites were removed and fixed in 10% buffered formalin. The samples were processed through a histological routine with a Shandon Pathcentre Tissue Processor. Paraffin sections were cut at 5 μm thickness. Sections were stained with (1) hematoxylin and eosin to study tissue morphology, and (2) Giemsa stain to examine the monocytes and plasma cells.

RESULTS AND DISCUSSION

The double emulsion technique is the most frequently used method for the encapsulation of water-soluble drugs into biodegradable microspheres, and this technique may lead to acceptable encapsulation efficiency values (Uchida et al., 1997; Pays et al., 2002; Benichou et al., 2004; Determan et al., 2004; Pekarek et al., 1996). The solubility of NTX free base in water is 4 mg/mL (Wise, 1984). To prevent the diffusion of NTX to the aqueous phase during solvent evaporation, the double emulsion technique was employed. The SEM photographs of the microspheres are shown in Fig. 1.

From analysis of different SEM photographs, the blank PLA microspheres (Fig. 1A) and drug-loaded microspheres (Fig. 1B) were spherical with a smooth surface. This observation suggested that NTX is homogeneously distributed in the PLA matrix and does not diffuse to the external aqueous phase, and thus, form a roughened surface. The microspheres were all less than 200 μm in diameter (Fig. 2) with 60% within the size range 20–150 μm, considered suitable for subcutaneous injection. On the surface of drug-loaded microspheres, a number of small drug-like crystals were observed.

The distribution of particle size of the microspheres was measured by laser diffraction. The particle size distribution is presented in Fig. 2.

Weighted average particle size $D[4,3]$ of the different microspheres was estimated to be 63.6 μm, and the surface weight mean diameter $D[3,2]$ was approximately 27.24 μm.

The target drug loading was optimized in preliminary studies, and microspheres with a loading amount of 50% (theoretical loading) of NTX were used for further investigation. The encapsulation efficiency was 82.73 ± 2.38% for six batches.

The amount of NTX in PLA microspheres was found to be approximately 41.4% (w/w). NTX encapsulation efficiency was 82.7% based upon the initial formulation objective of 50%. The results showed that high encapsulation efficiencies were achieved using the solvent evaporation technique selected.

NTX as the base is slightly soluble in water and it is possible diffusion occurred to the external aqueous

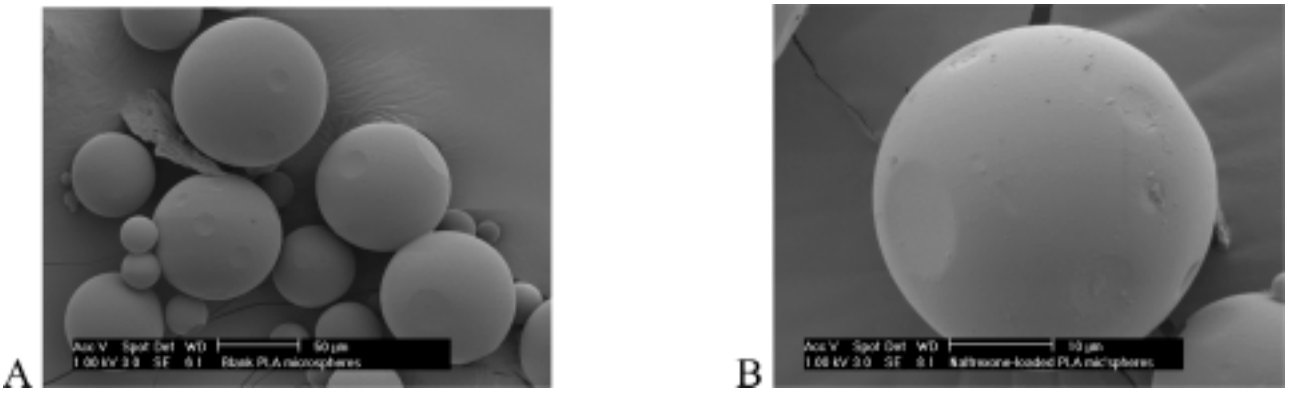

FIGURE 1 **SEM Photographs of Blank (A) and NTX-loaded PLA (B) Microspheres.**

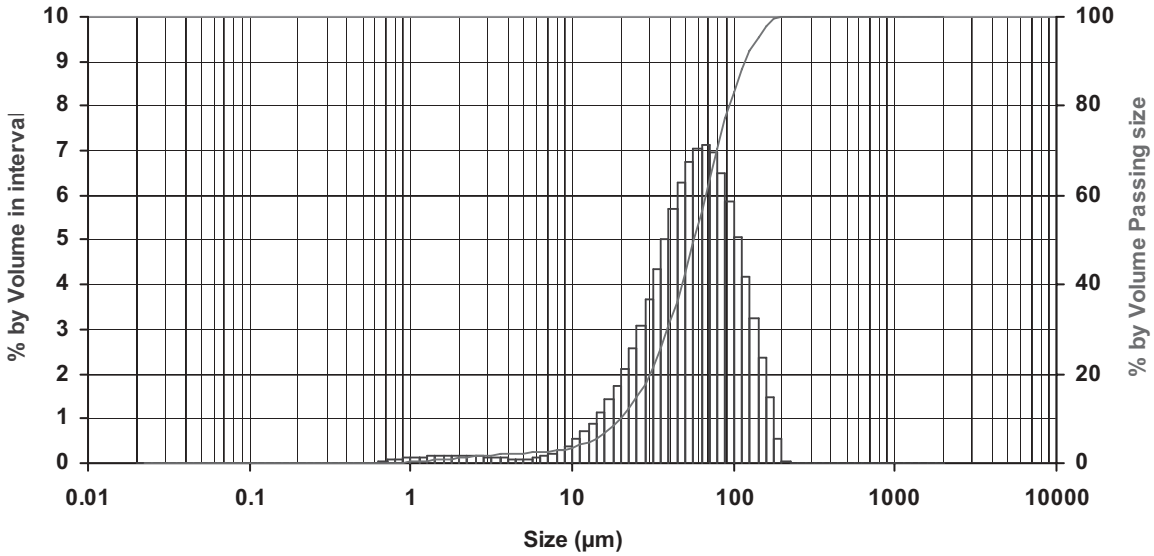

FIGURE 2 **Particle Size Distribution of NTX-loaded PLA Microspheres.**

phase during the microspheres formation process. In order to improve the loading efficiency, the addition of sodium chloride into the continuous aqueous phase was applied. Previous reports have shown that the addition of sodium chloride into the external aqueous phase significantly improved the drug loading efficiency (Uchida et al., 1997; Ogewa et al., 1988).

The in vitro release profiles of NTX from PLA microspheres are shown in Fig. 3.

These data indicate that the release of NTX initially did not follow zero-order kinetics. An initial burst release of 20% of NTX was observed on each of day one and day two, followed by a second phase of release, achieving a nearly constant rate after ten days. As mentioned above, NTX is slightly soluble in water, therefore it is difficult to avoid the initial burst of 41.4% of NTX loaded PLA microspheres. In fact, the burst effect might be especially related to dissolution and diffusion of NTX from near the surface of the microspheres due to the high drug to polymer ratio. It is likely that the relatively broad distribution range of the particle size could be another factor influencing the drug release patterns (Siepmann et al., 2004). The cumulative amount of NTX released at the end of 60 days was approximately 80%. These data support the concept that the microspheres are capable of producing a sustained release of NTX for more than one month if the burst release could be overcome.

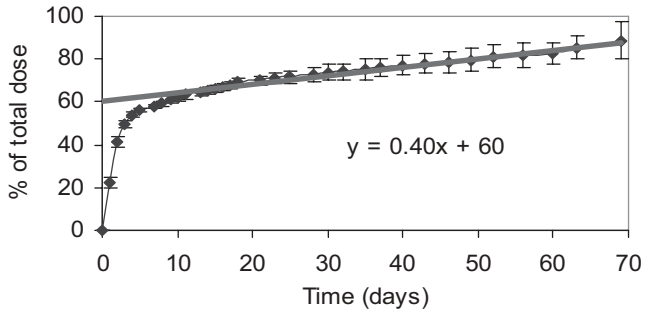

FIGURE 3 **Release Studies of NTX Microspheres (*n* = 3).**

The tablets were prepared by direct compression of drug-loaded microspheres in order to control the release rate. Different release patterns were achieved from microspheres when compressed as tablets. In contrast to the microspheres, an almost zero-order release pattern of NTX was obtained from tablets of approximately 0.4% per day for 12 months (Fig. 4).

There is an absence of burst release from this formulation which overcomes the main disadvantage of the previous microspheres. The release profile of NTX from microspheres was biphasic, however, it showed a monophasic profile from the tablet.

In vitro polymer degradation was characterized by SEC. The initial Mw of PLA was found to be 55,325 g/mol (Mw/Mn = 1.0313) which depolymerized to 51,512 g/mol (Mw/Mn = 1.0315) at day seven, and after 14 days 48,746 g/mol (Mw/Mn = 1.0316), and 36397 g/mol (Mw/Mn = 1.0324) by day 188.

In vitro degradation of microspheres showed an initial rapid phase over the first 14 days followed by a subsequent slower approximately zero-order rate over the remaining period. There was a 5.1% initial mass

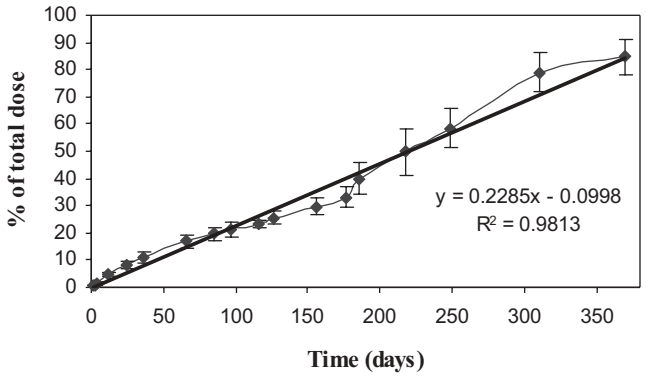

FIGURE 4 **Release Studies of NTX Tablets (*n* = 5).**

loss by seven days. By contrast, there was 11.98% of mass loss at day 14 and 34.11% by day 188 as shown in Fig. 5. The lag time in polymer degradation may be due to slow penetration of water into the polymer matrix. It may suggest that after an initial burst release, the zero-order release of NTX from the microspheres was controlled by the erosion rate of PLA matrix.

The SEC assay of the in vitro degradation of polymer partially correlated with NTX release profiles from the tablets as shown in Fig. 6.

The release kinetics of drug-loaded polymer microspheres appear to involve chemical and physical processes (Siepmann et al., 2002, 2004). Previous studies have reported that drug diffusion from the polymer and the degradation of the polymeric matrix play important roles in the drug release mechanism (Iwata & Mebinity, 1993; Wang et al., 1997). In addition, the molecular weight of polymer and the particle size of the microspheres also are the major factors influencing the drug release kinetics (Siepmann et al., 2004). The results of the present study suggest that the release of NTX from PLA microspheres could be diffusion controlled (e.g., NTX diffusion from the microspheres matrix) and/or chemically controlled (e.g., erosion of PLA) mechanisms. These data are in keeping with a previous finding that polymer degradation was governed by a "reaction/diffusion phenomenon" (Therin et al., 1992). The most likely model in the present study is erosion from a non-disintegrating matrix which followed the Higuchi relationship for the first 180 days (Higuchi, 1961, 1963).

The NTX-loaded microspheres showed an initial burst release followed by a stepwise decrease then achieved a constant release rate which is similar to most drug release profiles from polymer microspheres (Benichou et al., 2004; Determan et al., 2004; Ogawa et al., 1988; Siepmann et al., 2002, 2004; Iwata & McGinity, 1993; Wang et al., 1997). In contrast, the drug release patterns from implantable tablets have been reported as a biphasic process. Firstly, the initial release phase was based on the swelling properties of the tablets; secondly, the end phase was dependent on erosion of the tablets due to matrix degradation (Murakami et al., 2000). As a hydrophobic drug, NTX diffusion in the PLA matrix may be slower than the PLA degradation. Therefore, the release of NTX from the tablets was presumed to be controlled mainly by the degradation of PLA. Our results support the finding that polymer degradation was governed by a "reaction/diffusion phenomenon" (Therin et al., 1992).

The mean plasma concentrations versus time profiles obtained after implantation of NTX-loaded microspheres and tablets in rats are illustrated in Fig. 7. It should be noted that the sample size at each time point was different as indicated in Fig. 7. However, the sample size at each time point met the criteria for statistical analysis.

The data suggests that controlled NTX release in vivo was occurring from both the microspheres and tablet formulations. In the microspheres implantation group, plasma NTX levels increased to a peak of 14.2 ng/mL after seven days, and then showed a diminishing plasma NTX profile. Over the 28 days the plasma NTX concentrations were at or above 2 ng/mL. The particle size of the microspheres showed some variability which may give rise to some of the variation reported.

Data for the implanted tablets showed a relatively constant plasma level of NTX from day one (6.38 ± 2.48 ng/mL) to day 28 (5.64 ± 1.93 ng/mL) which suggested a relative constant release rate supported by the in vitro NTX data. This formulation eliminated some potential variation arising from the microspheres. The levels of

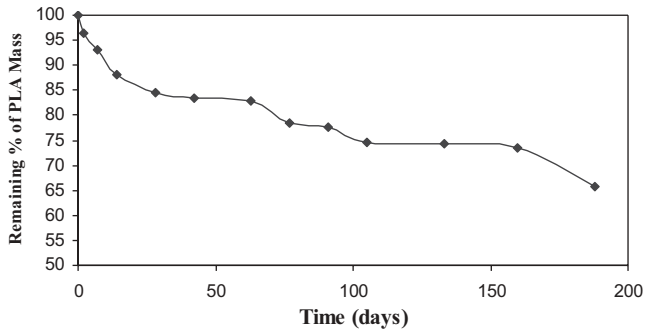

FIGURE 5 **Mass Loss of NTX Loaded PLA Microspheres in PBS at 37 °C (*n* = 5).**

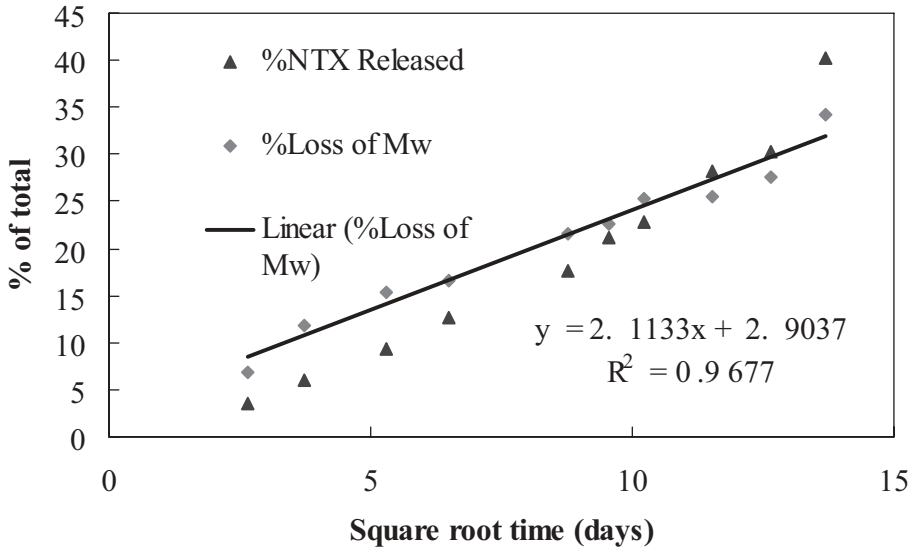

FIGURE 6 **NTX Tablet Dissolution and Polymer Mw Loss vs. Square Root Time (*n* = 5).**

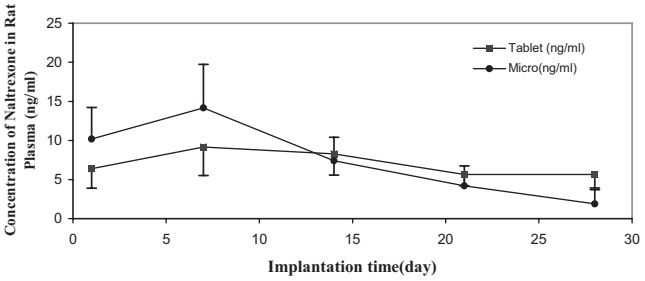

FIGURE 7 **In Vivo Release of NTX from Microspheres and Tablets (Day 1 *n* = 10; Day 7 *n* = 9; Day 14 *n* = 8; Day 21 *n* = 7; Day 28 *n* = 6).**

NTX in rat plasma samples were all above 2 ng/mL in both formulations throughout the 28 days study. It has been reported that the minimum effective plasma NTX concentration is approximately 2 ng/mL (Vereby et al., 1976), which was also confirmed in clinical studies of sustained-release systems in humans (Chiang et al., 1984; Comer et al., 2002). These studies provide further evidence indicating that the present tablet formulation has potential for clinical evaluation in humans.

Histological examinations of tissue surrounding the implants site are shown in Figs. 8 and 9.

The histopathologic investigation indicated that throughout the 28 day study, the inflammatory responses from both NTX tablets and microspheres implantation was not significant. It was observed that on day one local inflammation was found around the implanted NTX tablet site (surrounded by monocytes and plasma cells); only minor monocytes and plasma cells were observed for implanted NTX microspheres. It was found that on day seven a lower number of monocytes were evident; plasma cells were observed around the NTX tablet. The connective tissue had started to grow in, and small blood vessels were also evident; for NTX microspheres, scar tissue was observed and tended to grow as normal from this stage. In contrast, tissue surrounding the tablet appeared to grow as normal and the tablet was gradually covered by scar tissue from days 14 to 28.

In conclusion, the implantations caused a minor inflammatory response. This observation can be regarded only as a normal defense mechanism. Other tissue damage such as necrosis and degeneration of the connective tissues was not observed. Our observations are in agreement with previous studies that PLA is considered as very compatible with living tissues (Anderson & Wise, 1975; Yamaguchi & Anderson, 1993; Yolles et al., 1975). Therefore, the combination of NTX and the microspheres made of PLA were found to be biocompatible formulations.

CONCLUSIONS

It was found that controlled release formulations of NTX could be prepared at a high level of drug loading (more than 40%) based upon PLA. The microspheres showed a very significant "burst release" which was an unacceptable profile for a prolonged release implanted product. The tabletting of

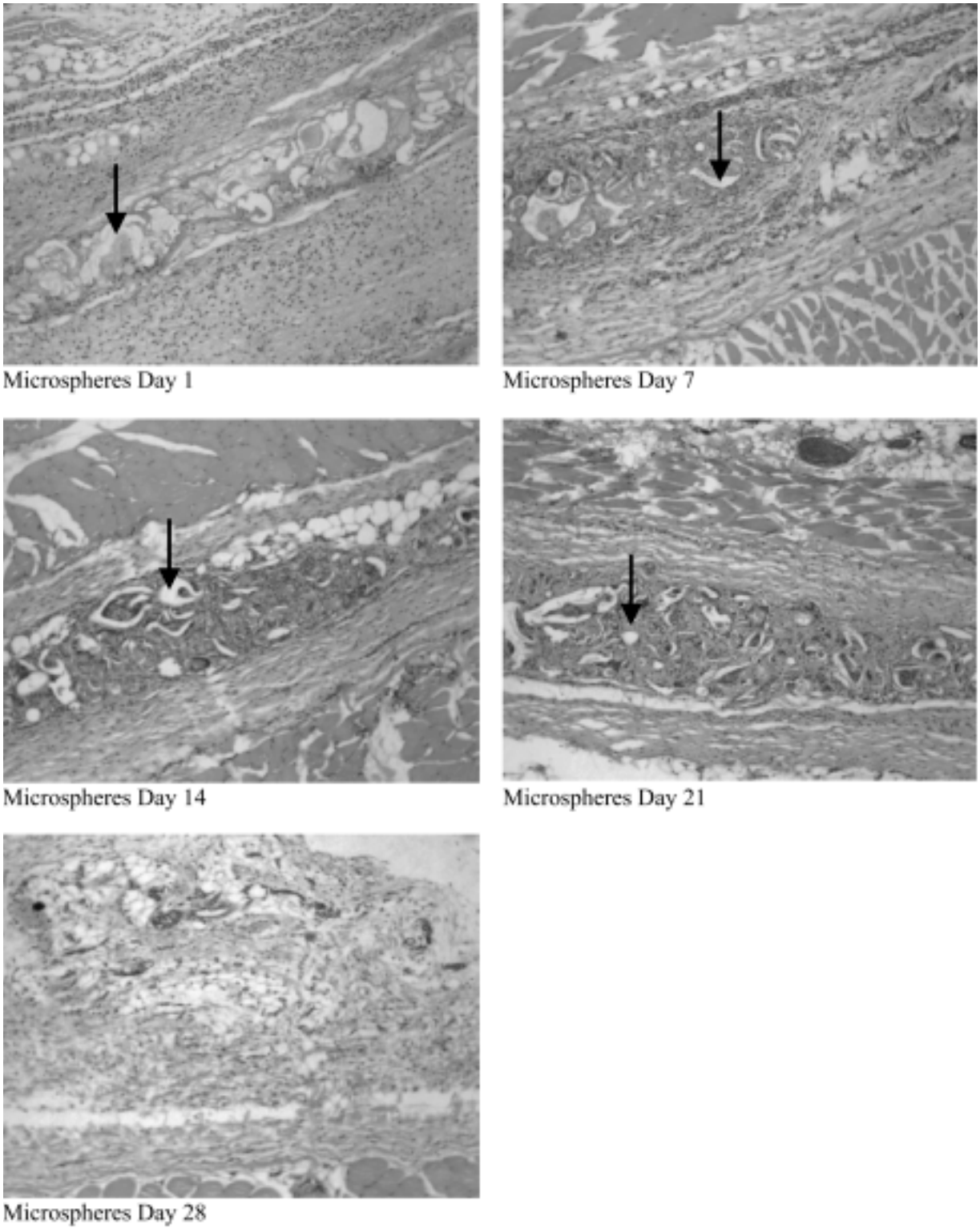

FIGURE 8 **The Optical Microscopic Examination of the Tissues Surrounding the Naltrexone Microsphere Implants. The Arrow Denotes Microspheres in the Tissue. Hematoxylin and Eosin Stained, Original Magnification 40×.**

these microspheres produced elimination of the burst release and a controlled release of NTX in vitro over a 12 month period. The release in vivo in rats indicated that adequate levels of NTX could be achieved for at least one month and the tablet formulation demonstrated a relatively constant NTX concentration. The loading levels and prolonged reliable release from the tabletted formulation produces

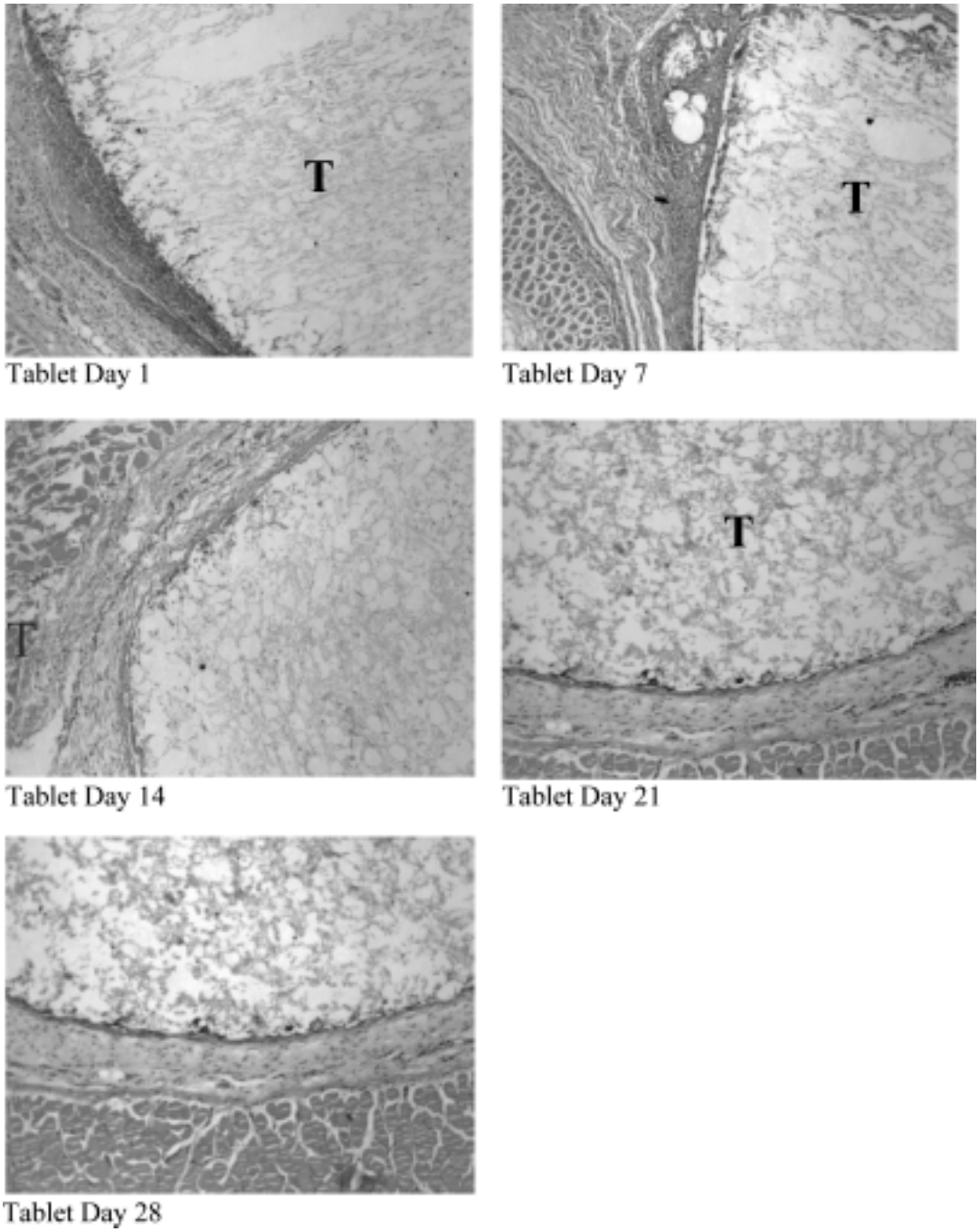

FIGURE 9 **The Optical Microscopic Examination of the Tissues Surrounding the Naltrexone Tablet Implant. "T" Denotes the Naltrexone Tablet in the Tissue. Hematoxylin and Eosin Stained, Original Magnification 40×.**

a pathway for further evaluation in humans where larger drug payloads are required (Hulse et al., 2003, 2004). The sustained release performance of NTX from the biodegradable implant system may provide a reliable, convenient, and safe mechanism for the administration of NTX for the long-term treatment of opioid dependence.

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