

A ‘biorelevant’ system to investigate in vitro drug released from a naltrexone implant

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Received 1 September 2006; received in revised form 31 January 2007; accepted 26 March 2007

Available online 30 March 2007

Abstract

This research is based on the recognized need for an in vitro release method for drug implants that better simulate physiological conditions at the site of implantation (‘biorelevance’). In this paper, we describe the evaluation of a ‘biorelevant’ approach for in vitro drug release testing of a biodegradable implant of naltrexone in a pre-clinical stage of development.

A miniature, capillary cell culture device was modified and tested as a biorelevant alternative for a standard commercially available flow-through cell. The real-time data generated through 90 days indicated a 48% lower rate of release for the capillary system. The profiles using both systems followed zero-order kinetics after an initial period of burst release. In vitro release data from the capillary device resulted in a 1-to-1 correlation with dog plasma pharmacokinetic data, and furthermore, the capillary device potentially simulated the lag-time in absorption more effectively than the flow-through cell. Scanning electron micrographs revealed that the sheath was continuous with no signs of cracks at the end of in vitro and in vivo studies. However, at the interface of the sheath and the core, intercalating, “finger-like” projections were observed consistent with penetration of the medium. No macroscopic or clinical toxicity signs were observed during the in vivo implantation study.

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Keywords: Naltrexone implant; Modified Hanks’ Balanced Salts Solution; Capillary and flow-through devices; In vitro release testing

1. Introduction

The development of subcutaneous pellets and implants as drug delivery devices had been necessitated over the years primarily to circumvent the disadvantages of oral administration (Ballard, 1961; Davis, 1974; Mohl et al., 2002). Implants are dosage forms that are subcutaneously placed with the aid of surgery or a hypodermic needle and are designed to release drugs over a prolonged period of time. From the perspective of patient acceptability, a longer duration of action through use of biodegradable polymers avoids the need for frequent invasive procedures.

Naltrexone, an opiate receptor antagonist (Resnick et al., 1974), is a good candidate for formulation as an implant. The drug has been marketed as an oral tablet for the clinical man-

agement of opiate addiction and as adjunctive treatment for individuals with alcohol dependence (Verebey and Mule, 1975). However, medication non-compliance is a difficult obstacle to treatment during naltrexone therapy. Two factors, the complete abstinence of opioid-induced reinforcing effect and the absence of adverse withdrawal effect (unlike methadone), are primarily responsible for patient non-compliance (Comer et al., 2002; Volpicelli et al., 1997). There is therefore, a need to develop sustained-release forms of naltrexone that could increase compliance and ultimately improve treatment effectiveness (Brewer, 2002; Bartus et al., 2003; Hulse et al., 2004). Prolongation of drug release from implants is achieved either through the use of polymers as controlled release matrices, or by the use of devices based on osmotic pump technology (Eckenhoff et al., 1987; Stevenson et al., 2000; Langer and Peppas, 2003). The majority of research studies have been focused on incorporation of the drug into biodegradable polymers, such as polylactic acid, polyglycolic acid and polycaprolactone. During formulation of these drug delivery systems, manipulation of

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polymer degradation rates has been achieved by blending polycaprolactone and poly(glycolic acid-co-lactic acid) to control chain scission (Pitt, 1990).

The need for improved methodologies for characterizing in vitro drug release that correlate better with in vivo drug release and absorption has been recognized by regulatory agencies, as well as industrial and academic groups Sirisuth and Eddington (2001). Systems for in vitro drug release testing of implants include, the unstirred vial method, the constant rotation method, the modified compendial flow-through method and the gel method (Kalkwarf et al., 1972; Chien, 1978; Shah et al., 1992; Miclau et al., 1993; Allababidi and Shah, 1998). Although a method modification for the compendial flow-through apparatus is recommended by the Fédération Internationale Pharmaceutique and the American Association of Pharmaceutical Scientists (FIP/AAPS), little published information is available. Possibly, this could be because of the high proprietary interests involved. Iyer et al. (2006) reviewed methodologies currently employed for in vitro drug release testing of subcutaneous implants, and acknowledged the need for studies aimed at improving interpretation of release data of these dosage forms. None of the methods referenced above simulate physiological condition (termed 'biorelevance') at the site of implantation entirely, and therefore, it was necessary to evaluate other approaches to set more meaningful specifications.

The priority of investigators, most often, is to develop methods that correlate in vitro dissolution rate to in vivo input rate, a prerequisite for point-to-point (Type A) correlations defined by the US Food and Drug Administration (FDA Guideline, 1997). Whereas a linear in vitro–in vivo correlation (IVIVC) with zero intercept, between the fraction of drug released in vitro and the fraction absorbed in vivo is ideal, predictive non-linear relationships can also be established, and are thus considered acceptable (Polli et al., 1996; Dunne et al., 1997).

Imaging techniques have been employed to understand drug diffusion and to visualize degradation changes in implant delivery systems (Narasimhan et al., 1999; Gao et al., 2001; Mikac et al., 2001; Weir et al., 2004). Scanning electron microscopy has been extremely useful in investigations of the degradation of biodegradable polymers in aqueous media (Göpferich, 1996; Rothen-Weinhold et al., 1999), which prove that degradation proceeds via a random, time-dependent, bulk hydrolysis of ester bonds in the polymer chain.

Miniaturized bioreactors for cell culture were first fabricated by the National Aeronautics and Space Administration (NASA Facts, 1995). These were employed to study effects on microgravity on cell adhesion and growth on board the Mir space station (Freed et al., 1997), with the aim of investigating the effects of space flight on the function and growth of musculoskeletal tissue. In order to explore the device for potential biomedical applications, hollow fibers were incorporated into the bioreactor to simulate capillaries in vivo (Hollingshead et al., 1995; Redmond et al., 1995, 1997, 2001).

This paper describes, for the first time, a 'biorelevant' approach based on the capillary bioreactor device, for investigation of in vitro drug release from a biodegradable, subcutaneous implant of naltrexone. The approach is expected to provide a

general methodology, with subtle modification as required on a case-by-case basis, for setting specifications on release testing of subcutaneous implants.

2. Materials and methods

2.1. Materials

Biodegradable implants for the study were obtained from Durect Corporation, Cupertino, CA, USA. Naltrexone hydrochloride (USP Grade, working standard) for the assay was obtained from Sigma (St. Louis MO, USA). The capillary device (Cellmax™) was obtained from Spectrum Labs., CA, USA. The sagittal saw for the study was generously provided by Stryker Corporation, MI, USA. Analytical grade triethylamine, ammonium hydroxide and trifluoroacetic acid, and Hanks' Balanced Salts (1 × 101) and HEPES buffer (10 mM) were purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide was procured from Fisher Scientific (Fairlawn, NJ, USA). HPLC grade acetonitrile was purchased from Burdick & Jackson (Honeywell International, Inc. MI, USA). Water was obtained in-house using the Nanopure Diamond water system (Barnstead International, IO, USA).

2.2. Description of the dosage form

The monolithic implant consisted of a biodegradable core of naltrexone: polycaprolactone enclosed in a sheath of poly-[DL-lactide: caprolactone]. The implant itself had been fabricated by melt-extrusion and ends of the cylinder were sealed with the same polymer that had been used for the membrane sheath. The mean length, outer diameter, ratio of the outer to inner diameters (R_o/R_i), and weight of the implants ($n = 15$) was 3.94 ± 0.07 cm, 3.57 ± 0.16 mm, 1.14 ± 0.04 , and 471.8 ± 44.8 mg, respectively. Each implant contained approximately 280 mg of naltrexone. Fig. 1 is a photograph of the implant having a homogenous matrix core covered by a smooth outer coating.

2.3. Theoretical considerations

Important variables that need consideration for incorporating 'biorelevance' into in vitro release tests include use of a medium having a physiologically relevant composition, and flow characteristics as determined by the type of apparatus and flow rate.

2.3.1. 'Biorelevant' medium and flow rate

A Modified Hanks' Balanced Salts Solution for use as a 'biorelevant' medium in implant release studies has been previously characterized (Iyer et al., 2007). The selection of a media volume between 900 and 1000 ml for compendial dissolution Apparatus 1 and 2 is based on the volume required to ensure adequate 'sink' conditions for most drugs. The same medium volume has been used, conventionally, for the flow-through apparatus also. This is not a correct representation of physiological flow characteristics in the subcutaneous region however.

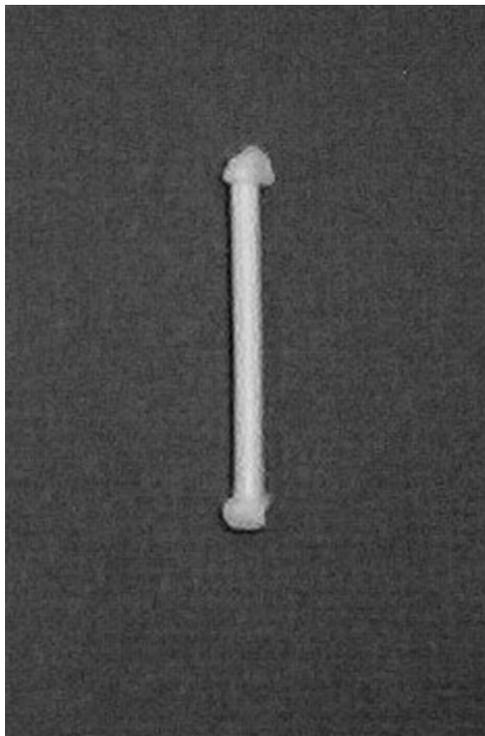


Fig. 1. The biodegradable naltrexone implant.

Post-implantation, the drug from the dosage form would be released into the extravascular interstitial tissue fluid, following which diffusion processes would predominate till the drug molecules reach the vascular barrier. To simulate this environment in vitro, the following equation was used for calculation of flow rate (Redmond et al., 1995):

$$Q = \frac{SS \pi r^3}{4\mu} \quad (1)$$

where Q is the flow rate (ml/s), SS the shear stress (dyn/cm²), r the radius of the capillary (cm), and μ is the viscosity of the medium (poise). The viscosity of the medium was taken to be 10 centipoise (Stinchcomb et al., 2002). The shear stress range that the endothelial wall of subcutaneous capillaries is exposed to is 0.07–20 dyn/cm² (Davies, 1989). This translated to a flow rate range from 2.47×10^{-5} to 7.06×10^{-3} ml/s/capillary. For this study, a shear stress of 1 dyn/cm² was used with the objectives of retaining the flow rate within the biorelevant range, and which could be reproducibly achieved with the pump system employed. Thus, the flow rate was calculated to be 3.53×10^{-4} ml/s/capillary, or 1.06 ml/min for the cartridge with 50 capillaries.

2.3.2. Calculation of 'sink' conditions

The solubility of naltrexone freebase in Hanks' Balanced Salts Solution at 32 °C is 5.42 mM (Stinchcomb et al., 2002). This reported value was used for calculation of 'sink' conditions. Based upon a conservative factor of 3 times the solubility of the drug, the threshold concentration warning of a departure from sink condition at 32 °C would be 1.63×10^{-2} M or 5.54 mg/ml.

Since release rate from the implants was expected to be much slower, this indicated a fair flexibility provided for accumulation of drug in the release medium prior to replacement, if at all concentrations reached that level.

2.3.3. Modeling the capillary diffusion process

In their seminal papers on the pore theory of capillary permeability, Renkin (1954, 1964, 1977) and Pappenheimer et al. (1951) had related the diffusional radii of molecules to predict their passage through pores of the endothelial wall. The assumptions made had been: (a) the molecules were perfect spheres, such that their diameters could be estimated using the Stokes–Einstein equation, and (b) the pores of the capillary wall were symmetrical.

The equation was:

$$\frac{A_x}{A_o} = \left[2 \left(1 - \frac{a}{r} \right)^2 - \left(1 - \frac{a}{r} \right)^4 \right] \times \left[1 - 2.104 \left(\frac{a}{r} \right) + 2.09 \left(\frac{a}{r} \right)^3 - 0.95 \left(\frac{a}{r} \right)^5 \right] \quad (2)$$

where A_x/A_o is the ratio of effective area of the opening to the total cross-sectional area of pore and a/r is the ratio of radii of the diffusing molecule to that of the pore.

The average diameter for a naltrexone molecule was 9.154 Å (R.S.D. = 10%), as determined using a molecular modeling approach described earlier (Iyer et al., in review). Since the molecule had a rigid conformation, this diameter was used for calculation of the radius, a , in the above equation. The average radii of pores, defined as r^{vitro} , for the in vitro capillary material was 0.5 μm. The average radii of pores in vivo (r^{vivo}) was determined by Pappenheimer et al. (1951), and found to be 24 Å. Based on these values, the ratio, A_x/A_o , was calculated separately for in vitro [defined as $(A_x/A_o)^{\text{vitro}}$], and in vivo [defined as $(A_x/A_o)^{\text{vivo}}$] scenarios.

Therefore, the factor, defined as R_{diff} , which accounted for the additional restriction to diffusion of molecules in vivo, was determined by the following ratio:

$$R_{\text{diff}} = \frac{(A_x/A_o)^{\text{vivo}}}{(A_x/A_o)^{\text{vitro}}} \quad (3)$$

The value of R_{diff} was calculated as 0.54, which implied that the molecule would be subjected to a greater barrier to diffusion in vivo than what was simulated by the in vitro capillaries.

Also, a pharmacokinetic model is proposed for subcutaneous absorption as shown in Fig. 2. The drug molecule, once released, diffuses as a function of its diffusion coefficient, D , through the displacement, R , from the outer surface of the implant to vascular barrier. The distance traversed by the molecule is variable because it depends one or more of the dotted paths that the molecule is likely to follow. An additional barrier to diffusion is provided by cells, simulated in the in vitro system by the glass beads. Furthermore, depending on the lipophilic character of the drug, it may be partitioned into the adipose cells, and be released back into interstitial fluid as a function of $-k_D$, the re-distribution rate. This could lead to formation of a localized depot, represented by the drug concentration, C_a , in adipose.

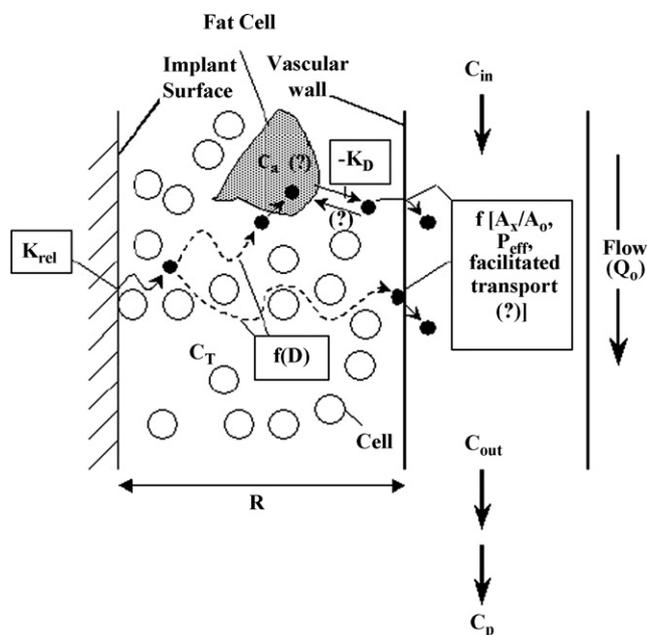


Fig. 2. A proposed pharmacokinetic model for drug absorption from the subcutaneous site of implantation. (The dotted lines indicate probable pathways the drug molecule might follow. The partitioning of the drug into subcutaneous fat would be a function of its lipophilicity.)

Passage of the drug molecule from interstitial fluid into vascular lumen would be a function of its permeability coefficient (P_{eff}), the resistance to diffusion (A_x/A_o), or possibly, facilitated transport. Assuming that naltrexone undergoes a fast intrinsic clearance from the implantation site, the rate-limiting factor to absorption would then be Q_o , the blood flow to the tissue. Normal blood flow to subcutaneous tissue is 1.5–2.5 ml/100 g/min (Benet, 1990; Enevoldsen et al., 2001). However, tissue blood flow is subject to high variability such as exercise, pathology,

diurnal changes, body position, etc. Estimation of the various rate constants would require an in vivo input rate, obtained through intravenous administration. In addition, an estimation of drug concentrations at the interstitial site, possibly involving microdialysis studies would also provide useful information regarding disposition of the drug.

2.4. System components and assembly

A schematic diagram for the in vitro system is given in Fig. 3. Details of each component are provided below. The set of flow-through cells (2 each mounted on 3 stands) and capillary devices ($n=6$) were maintained in the same water bath (Labline, PA, USA).

2.4.1. Glass beads and filters

Borosilicate glass beads (Chemglass, Inc., NJ, USA) having a mean diameter of 1 mm (R.S.D. = 10%) were employed for this study. The purpose of using beads in a flow-through system conventionally has been to establish laminar flow conditions within the cell. In a capillary in vitro system however, the beads were used to simulate the barrier formed by cells of the subcutaneous tissue. An approximation for the weight of beads required to fill the extracapillary space as uniformly as possible was calculated based on the porosity of the beads. The porosity was calculated to be 40%, based on the true weight of a known volume of beads and weight of water displaced in a graduated cylinder. Approximately 1.25 g of beads was required to fill the extracapillary space.

Glass fiber filters (1 cm diameter, Type GF/D) from Whatman International Ltd., Maidstone, England, with a low non-specific binding were employed at both ends of the flow-through apparatus. For the capillary device, no filters were required since the dosage form was placed in extracapillary space with no possibil-

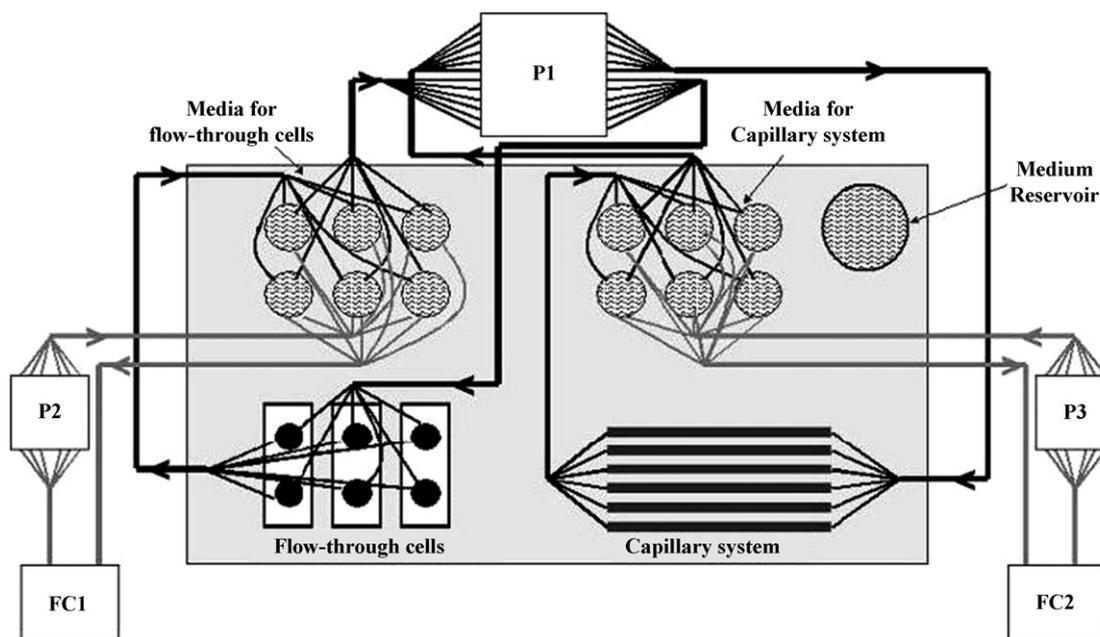


Fig. 3. Schematic of the system. P1 is a 12-channel peristaltic pump; FC1 and FC2 are fraction collectors for the flow-through and capillary devices, respectively, and for controlling 6-channel peristaltic pumps, P2 and P3.

ity of fragmentation that might penetrate through the membrane wall and reach the media reservoir.

2.4.2. Pump and tubing

Polytetrafluoroethylene (PTFE) tubing was employed throughout. A peristaltic pump (Masterflex Modular L/S drive; Cole-Parmer Instrument Company, Inc., PA, USA) with a 12-channel, 8-roller pumphead with a linear flow rate range linear between 0.0006 and 41 ml/min was employed. The larger number of rollers was employed to minimize pressure pulses in flow. Platinized peristaltic tubing was used that was resistant to mechanical abrasion and was chemically inert. Six channels each were used for the flow-through and capillary device.

A minimum possible length of tubing was employed for each leg of the closed loop: 45 cm for the distance between the pump and the drug release device, 30 cm between the device and the reservoir and another 45 cm between the reservoir back to the pump. When the set up was completed, the pump was calibrated for a flow rate of 1 ml/min. All connectors, Teflon unions, and flanges purchased from Upchurch Scientific, Oak Harbor, WA, USA were employed.

2.4.3. Flow-through cell

The flow cell used for this study was made of transparent polycarbonate. It had a cylinder with an inner diameter of 5.9 mm and a length of 37 cm. Two such cells were loaded with O-rings at both ends and mounted on a brass stand with the help of Teflon fittings. The stand was made of brass to prevent rusting upon immersion in a water bath at elevated temperature. Fig. 4 represents the flow-through cell and each of its components.

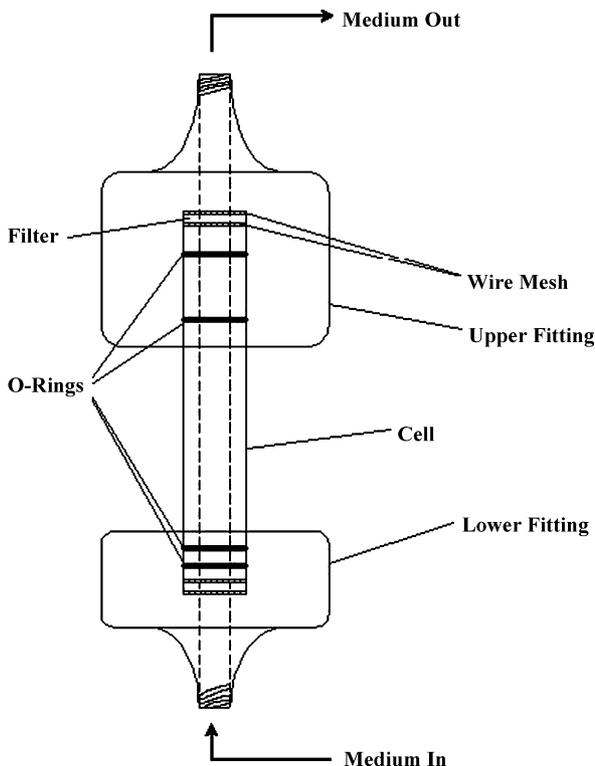


Fig. 4. The modified USP flow-through cell.

2.4.4. Capillary device

The miniature bioreactor can be described as a cylindrical tube with enclosed fibers. There are two end ports and two side ports as shown in Fig. 5a. The fibers of the capillary device are made of chemically inert polyether sulfone. Each fiber is 0.5 μm in diameter, and both ends of the bundle are provided sealed by the manufacturer with an inert resin. Since it was not possible to open the sealed end of the cartridge without significantly disturbing the arrangement of fibers, a slot was made through the body of the cartridge for placement of the implant. Using a sagittal saw with a blade size of 0.5 mm, a slot measuring 2 cm \times 4.2 cm was carefully made. The slot cover was retained. A layer of glass beads was placed into the opening

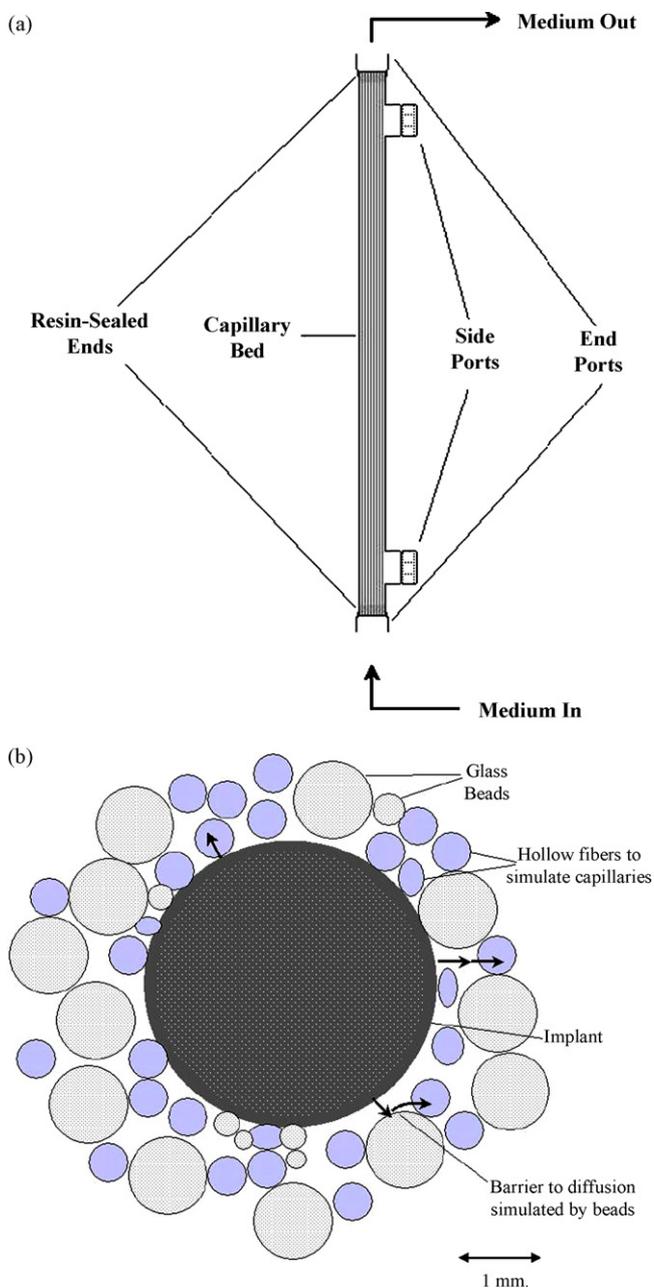


Fig. 5. (a) The new capillary device identified for 'biorelevance'. (b) Diagram of a transverse section of capillary device with the implant positioned ready for testing.

and the implant was placed in the capillary bed. The space was then filled with beads to provide a tight fitting. The slot cover was replaced back, and the gap was sealed thoroughly with an epoxy resin adhesive. Fig. 5b is a schematic representation of the implant placed inside the extracapillary space of the device. Twelve hours was allowed following placement of the implant and the filled cartridge was positioned vertically with clamps prior to use.

2.4.5. Fraction collector

Collection of samples was performed using a semi-automated method. Two fraction collectors (Intelligent Fraction Collector VK3000, Vankel Instruments, NJ, USA) were used independently for the flow-through cells and capillary devices. Each controlled six channels of each device type via a peristaltic pump (Taylor-Wharton, UK). These were calibrated for sample withdrawal of 4 ml for each sample into borosilicate glass tubes, with an additional 1 ml used for rinsing. Immediately after the sample was withdrawn, 5 ml of medium maintained in the same water bath as the devices was used to replenish to the reservoir. The samples collected were stored below -20°C prior to analysis.

2.4.6. Medium reservoir

Cylindrical borosilicate glass reservoirs (250 ml) were used with tight fitting silicone stoppers (50 mm \times 43 mm \times 25 mm) from Fisher Scientific, GA, USA. Perforations of 1 mm were drilled for ports as depicted in Fig. 6. Deflected non-coring septum-penetrating stainless steel needles (15.2 and 10.2 cm long for medium inlet and outlet, respectively) purchased from VWR International, Inc., NJ, USA, were employed in the ports.

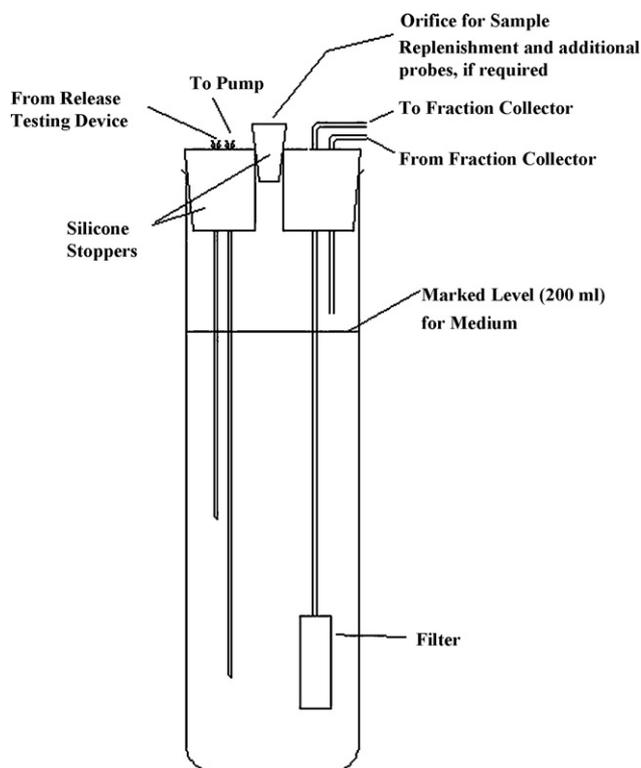


Fig. 6. Schematic of the reservoir.

An additional orifice 7 mm in diameter was drilled for insertion of temperature and sample probes, as and when required, and for media replenishments. The system was kept closed with another silicone stopper (10 mm \times 6 mm \times 20 mm). At the end of needles intended for sample withdrawal, filters were fixed to prevent any particulate matter from entering the tubing of the fraction collector.

2.5. Real-time *in vitro* drug release experiments

A final check of the flow rate was conducted before reservoirs containing fresh media were placed in the bath, and the flow path closed. For real-time study, six flow-through and six capillary devices each containing an implant were employed. When the system was ready, the pump was switched on. The side ports of the capillary device were kept open initially to allow air in the extracapillary space to escape. As soon as the medium made its way up the chamber, the ports were closed to enable the medium to exit via the end port into the reservoir. The system was operated continuously, except during replacement of buffers. The samples were stored for about 1 week and analyzed by a HPLC method with detection at 204 nm, as described earlier (Iyer et al., 2007).

Temperature measurements inside the vessel were made periodically. Samples were collected every 12 h for the first 30 days, and every second day thereafter up to Day-90. The flow rate and pH of the media were checked every seventh day, along with the media replacement. For medium replacement, the outlet from the reservoir was removed. After the medium in the loop drained back in the reservoir, the pump was stopped. The stopper was removed, and the needles and filter were rinsed thoroughly with water to prevent any carryover of naltrexone. A reservoir containing fresh medium pre-heated to 38°C was placed in the reservoir, and the stopper was replaced. The pump was switched back on and as described previously, side ports of the capillary device were manipulated to allow entrapped air to escape. The complete procedure required less than 10 min of time in which the pumps were stopped. No fluctuations in flow rate were observed, possibly because no clogging of filters occurred.

2.6. Scanning electron microscopy (SEM)

Following *in vitro* and *in vivo* release investigations, scanning electron micrographs of transverse and longitudinal sections of the implants cut with a surgical blade were compared to those obtained initially. The sections were coated using a Polaron platinum sputter coating system (Quorum Technologies, Newhaven, East Sussex, UK) at 10^{-2} psi. The energy setting on the JEOL Scanning Electron Microscope (Jeol Instruments, USA) was optimized to 25 keV, and sections were examined under low ($35\times$) and high ($2000\times$) magnification.

2.7. *In vivo* study

Following characterization of real-time *in vitro* drug release using both devices, the question remained which of the two

systems was more representative of an in vivo system. It was therefore necessary to conduct an in vivo study. General scientific opinion favors the use of larger animals for implant dosage form studies to minimize interspecies differences compared to small laboratory animals (Burgess et al., 2002); hence the dog was selected as the animal model.

2.7.1. Protocol development

Since the objective was to compare the two in vitro techniques, only one animal was implanted. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Virginia Commonwealth University. A mature, mixed hound dog weighing 31.8 kg was used. The dog was purchased from Covance Laboratories, PA, USA, and acclimatized to the test facility at the Division of Animal Research, Virginia Commonwealth University. A certified canine diet was provided along with water ad libitum.

2.7.2. Test material

Athanasίου et al. (1996) have compared standard sterilization techniques for their applicability to polylactide and polyglycolide polymers. They concluded that the mechanical and physical properties of the matrix could be altered significantly upon exposure to different techniques. Heat sterilization was ruled out due to a possibility of deformation and melting of the polymer. Prior knowledge regarding reaction of this implant matrix to gas (ethylene oxide) and gamma radiation was limited. So, it was decided to adopt a mild sterilization with ultraviolet (UV) radiation. One day prior to implantation, the test implant was laid aseptically on a horizontal laminar flow bench. The body of a sterile 3 ml syringe was cut slightly diagonally to resemble a trocar and placed in the laminar flow bench with 2 pistons. A polyethylene pouch was swabbed thoroughly with alcohol, and placed alongside the implant overnight with the UV lamp switched on. Care was taken to paste clear warning signs to prevent any accidental hazardous exposure of personnel to radiation. On the day of study, the ultraviolet radiation was switched off. The implant was gently sandwiched into the body of the syringe with one piston on either end. Following this, the intact syringe containing the implant was placed into the pouch that was sealed effectively with cellophane tape, and carried to the surgical suite ready for implantation.

2.7.3. Surgical procedure for implantation

The dog was pre-medicated with acepromazine (subcutaneous dose of 0.1 mg/kg) and moved to the surgical suite. Heavy anesthesia was maintained with xylazine (4 mg/kg administered as an intravenous bolus). Following induction, hair on the dorsal area between the forelimbs was shaved and cleaned aseptically. A 1-cm incision was made and the sterile implant was aseptically placed in the dorsal subcutaneous space. Two sutures held the skin in place until the area healed completely.

2.7.4. Clinical observations

Following recovery of the animal, it was transferred back to its cage. The dog was kept under observation for normal activity

and any clinical sign of toxicity. Body weights were recorded before treatment, and once every 2 weeks thereafter.

2.7.5. Blood collection and plasma storage

The VCU Division of Animal Resources Standard Operating Procedure on Blood Sampling Amounts & Techniques for Various Laboratory Animal Species was followed. Blood (5 ml) was collected from the cephalic or saphenous vein at pre-dose and at 0.5, 1, 2, 4, 8, 15, 30, 45, 60, 75 and 100 days post-implantation. No anesthesia was required. Blood was collected in tubes with sodium heparin as the anticoagulant and the tubes were centrifuged to obtain plasma that was separated and stored below -50°C , until analysis. Based on preliminary analytical results, the protocol was amended to collect blood samples at 1, 2, 3, 6, 8, 10 and 15 days after removal of the implant.

2.7.6. Sample analysis

The plasma samples were analyzed using a validated Liquid Chromatography–Tandem Mass spectrometry (LC–MS/MS) method described elsewhere (Iyer et al., in review). The peak height ratio of naltrexone to $[15,15,16\text{-}^2\text{H}]$ naltrexone (internal standard) was used for quantification. The calibration curves were linear in the range of 10–5014 pg/ml ($r^2 > 0.98$) using a weighting factor of 1/concentration, and precision and accuracy of quality control samples processed and analyzed along with the samples were within 2%. The results were expressed as picograms per milliliter of plasma.

2.7.7. Surgical removal of implant, and macroscopic and histological evaluations

Although the implant was biodegradable, its surgical removal was necessary because, biodegradation could not be expected to be complete at the end of the study period. Following collection of the blood sample on Day-100, the implant was surgically removed from its site (following the same pre-medication schedule as described in Section 2.7.3). Following the incision, the area around the implant was exposed for evaluation as described by Cukierski et al. (2001). The local tissue response was graded using the macroscopic scale that consisted of three parameters: capsule, vascularity and fluid accumulation, in the region of implantation. Each parameter was assigned a score between 0 and 4, representative of a minimal to a severe response. For example, a score of 0 for the capsule would indicate no visible proliferation of connective tissue; whereas a score of 4 would indicate a translucent to opaque capsule at least 3 mm. thick. Following macroscopic evaluation, the implant was removed along with 2–3 mm of surrounding tissue. The affected area was sutured aseptically and dressed. The dog was kept under observation until complete recovery.

For histological evaluation, the tissue was separated from the implant and immediately fixed in isotonic buffered formalin (10%) for histological evaluation (Zhao et al., 2000; Hulse et al., 2005). The specimen was embedded in paraffin. The paraffin sections were stained with haematoxylin-eosin and evaluated under a light microscope. The separated implant was stored in a sealed container at room temperature for 18 days until a constant

dry weight was attained on three consecutive days. The implant was then evaluated using the SEM technique as described earlier, for a comparison of in vivo changes to those that had occurred in vitro.

3. Results and discussion

3.1. In vitro release study

A description of the real-time in vitro release data is provided in the following subsections.

3.1.1. System performance

The system worked efficiently throughout the study period. At the end of Day-49, the peristaltic pump tubing was replaced to avoid possible leakages arising due to wear-and-tear. No visible contamination of microorganisms was observed confirming the efficiency of the antimicrobial agent, Primocin. The pump performance was rugged in terms of a constant maintenance of flow rate during the entire study period. No flow rate fluctuations were observed that might arise due to clogging of filters in the flow-through device. This indicated that, possibly, the dosage form had retained its integrity over the study period. This was further verified at the end of the study by removal of the dosage form from the respective devices.

Media replacement proceeded smoothly every seventh day with a 10-min pump stoppage time. The pH was observed to vary up to only ± 0.04 units as measured for the medium immediately after each replacement; thereby indicating that the Modified Hanks' Balanced Salts Solution had adequate buffer capacity for the study of this dosage form. A visible examination of media was also performed to ensure a lack of microbial contamination.

3.1.2. Sample analysis

The samples were analyzed by a validated High Performance Liquid Chromatography (HPLC) method described elsewhere (Iyer et al., 2007). The peak area at 204 nm for naltrexone was used for quantification. No chromatographic interference was observed from any degradation product. The calibration curves were linear in the range of 0.16–20 $\mu\text{g/ml}$ ($r^2 > 0.99$) using a weighting factor of 1/concentration, and precision and accuracy of quality control samples processed and analyzed along with the samples were within 5% of the nominal concentration. The results were expressed as micrograms per milliliter of medium, that were used for calculation of cumulative drug released by taking into account the volume of medium in the reservoir and a correction factor for the amount of drug lost at each sampling point.

Furthermore, based on the assumption of zero order release, a R.S.D. of 22.51% ($n = 6$) was obtained for mean concentrations of naltrexone in samples withdrawn 12 h after each consecutive replacement of medium, consistent with the facts that: (a) solid-state degradation of drug in the implant, if any, was negligible, and (b) no carryover problem in terms of non-specific binding of naltrexone to the flow path existed.

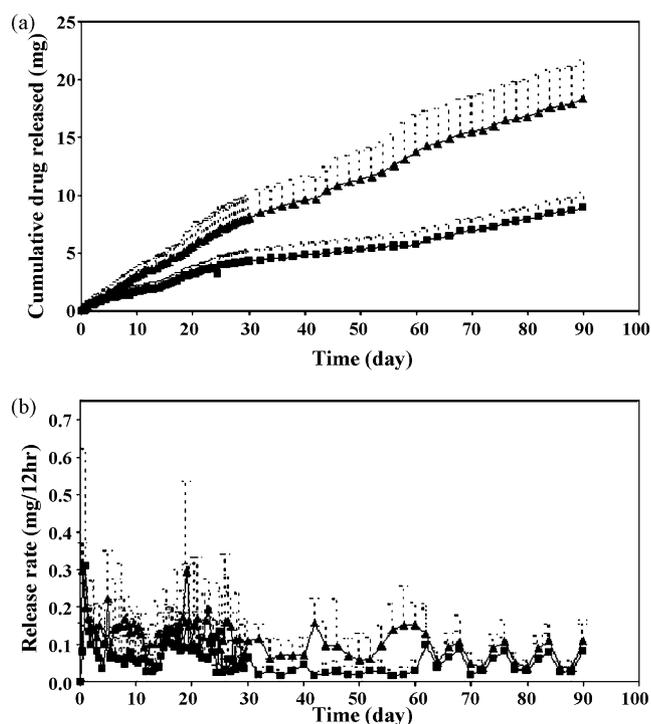


Fig. 7. (a) Comparison of real-time in vitro release profiles and (b) release rates of naltrexone using both devices ($n = 6$). Key: \blacktriangle , flow-through cell; \blacksquare , capillary device. (Error bars only representing positive standard deviations are shown.)

3.1.3. In vitro release data analysis

Fig. 7 compares the real-time release data obtained for 90 days using the modified flow-through and capillary devices ($n = 6$). Overall, the rates of release of naltrexone were 0.11 mg/12 h (95% CI: 0.105, 0.124), and 0.06 mg/12 h (95% CI: 0.050, 0.064), respectively, for flow-through and capillary devices between 3 and 90 days. The rate of release was significantly less ($p < 0.0001$) for the capillary device than the flow-through cell. This can be explained by a lower rate of flow of the medium in extracapillary space that the implant is exposed to. Thus, the capillary model effectively simulates a barrier to diffusion that would exist in vivo. Although the release rates fluctuated (0.04–0.29 mg/12 h and 0.01–0.16 mg/12 h for flow-through and capillary devices, respectively) as a function of time, the pattern was consistent for both devices. Usually, such a variation can be attributed to a variable pumping rate and the behavior of dosage form itself. Since the study had not been initiated simultaneously using both the types of devices, the former cause could be excluded.

The cumulative drug released was 18.31 ± 3.25 mg for the flow-through cell, and 8.97 ± 1.20 mg for the capillary device. Considering that each implant contained approximately 283 mg of naltrexone initially, these represented 6.5% and 3.2% of total drug. In conventional dissolution studies, it is expected that the cumulative drug released should be at least 80% of the total amount contained in the dosage form. This rule however, is not practical when applied to the study of dosage forms whose release is controlled over a period of months.

An initial period of burst release up to approximately 3 days with both devices was observed. However, the time for attaining

the peak rate of release (0.3 mg/12 h) is 12 h for the flow-through cell versus 24 h for the capillary system. Since other known variables (Huang and Brazel, 2001) related to the dosage form were similar in both devices and since the polymeric matrix was non-swelling, the reason for the longer time required for the capillary device could be speculated based on the percolation-limited diffusion theory (Tzafirri, 2000). The theory suggests that an initial loading of the drug occurs from two separate pools: a pool of mobile drug molecules, and a pool of immobilized drug that can diffuse only after pore sizes increase due to hydrolytic degradation of the matrix. Since the pool of mobile entities in both devices can be expected to be similar, a slower penetration of medium due to reduced flow in the capillary device as compared to the flow-through cell might offer a possible explanation. Without in vivo data, it could be speculated that a better simulation of the in vivo condition will be achieved with the capillary device.

3.2. In vivo study

3.2.1. Animal health

No toxic clinical symptoms were recorded during the entire study. Only once upon administration of xylazine prior to surgery, the animal had showed signs of convulsions unrelated to the dosage form. However, the vital signs regained normalcy fast and the surgery was continued. A constant body weight, responsiveness and normal bowel habits were observed. The area of implantation did not show any sign of infection and the sutures were removed within a week of surgery. In addition, at the end of 90 days, no perceptible shift was noticed from the original position of the implant.

3.2.2. Plasma data analysis

The plasma concentration–time profile of naltrexone in the dog is shown in Fig. 8. The pre-dose plasma sample showed no interference at the retention time of naltrexone and the internal standard. There was a lag-time in absorption as indicated by the BLQ (Below the Lower Limit of Quantification) value for the 12-h sample. Following this, a peak plasma concentration (C_{max}) of 391.5 pg/ml was observed at Day-2 post-implantation, indicating a burst release of naltrexone, as expected from the in vitro data. A mean steady state level of 198.0 pg/ml (R.S.D.: 16.2%;

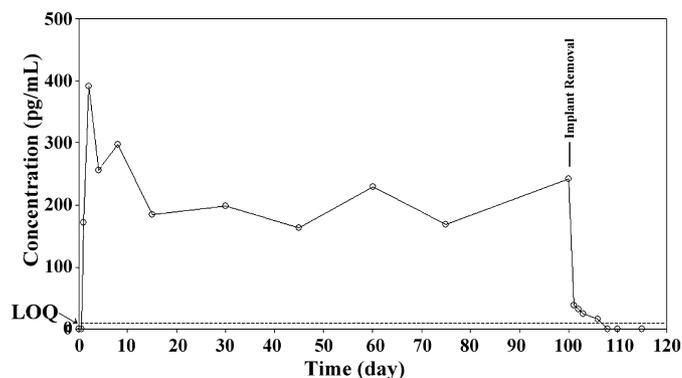


Fig. 8. Plasma concentration–time profile of naltrexone following subcutaneous implantation in dog. The dotted line represents the limit of quantification, 10 pg/ml, of the LC–MS–MS analytical method.

$n = 6$) was attained between days 15 and 100. This was far less than the desired therapeutic concentration (at least 1–2 ng/ml) in humans, but had been anticipated based on the low rate of release observed in vitro. Following removal of the implant on Day-100, the plasma concentration of naltrexone dropped quickly to BLQ after 6 days. However, a terminal phase having a half-life ($t_{1/2}$) of 3.84 days for naltrexone was determined. This value is consistent with the results of Lee et al. (1988), who reported that naltrexone had a significantly longer half-life due to receptor occupancy as compared to its plasma half-life.

3.2.3. Macroscopic evaluation

The scores of the three parameters as described in Section 2.7.7 were summed at the end of the evaluation to categorize tissue response. There was no visible proliferation of connective tissue, no visible increase in vascularity or redness, and none to a barely perceptible amount of fluid was present around the area of implantation. This indicated a “minimal” tissue reaction to the implant.

3.2.4. Histological evaluation

As shown in Fig. 9, the histological evaluation showed limited signs of abnormalities. There were localized regions indicating the formation of lymphoid follicles. The follicles and macrophages had engulfed bluish material covered by a loosely organized membrane. This is similar to the outcome reported by Bergsma et al. (1994), who identified the material within macrophages as birefringent polylactide particles that disappeared after 80 weeks. Also, regions of loose connective tissue possibly composed of keloidal collagen had formed, providing indications of body’s natural healing processes. Formation of connective tissue is consistent with the findings of Zhao et al. (2000); and demonstrates good biocompatibility feature of the implant (Anderson, 1994). During normal tissue healing, the replacement of giant cells and macrophages by fibro-vascular tissue indicated a progressive decrease in immune response (Goiss et al., 2003).

Another prominent characteristic observed in the slides was the presence of “clear pools” suggestive of foreign body material that may have been removed or destroyed during the slide fixing process. This area of “washed out” implant material had been observed in a number of studies (Li et al., 1990; Therin et al., 1992; Hulse et al., 2005) indicating the presence of disintegrated foreign material with slow metabolic properties.

The control tissue that was sampled at least 5 mm away from the wall of the implant showed the presence of normal adipose cells. In a few regions, a localized tissue reaction characterized by mild inflammation, fibrosis indicated by scarring, and foreign body reaction related to the implant was observed. A proliferation of eosinophils was observed in areas marked by inflammation. This condition has been attributed to the irritant effect of a local reduction in pH, because of the acidic polymeric degradation products that are released (Agrawal and Athanasiou, 1997). An effort to significantly reduce foreign body giant cell response in rodents by coating a subcutaneously implanted polymer with calcium phosphate has been reported (Lickorish et al., 2004).

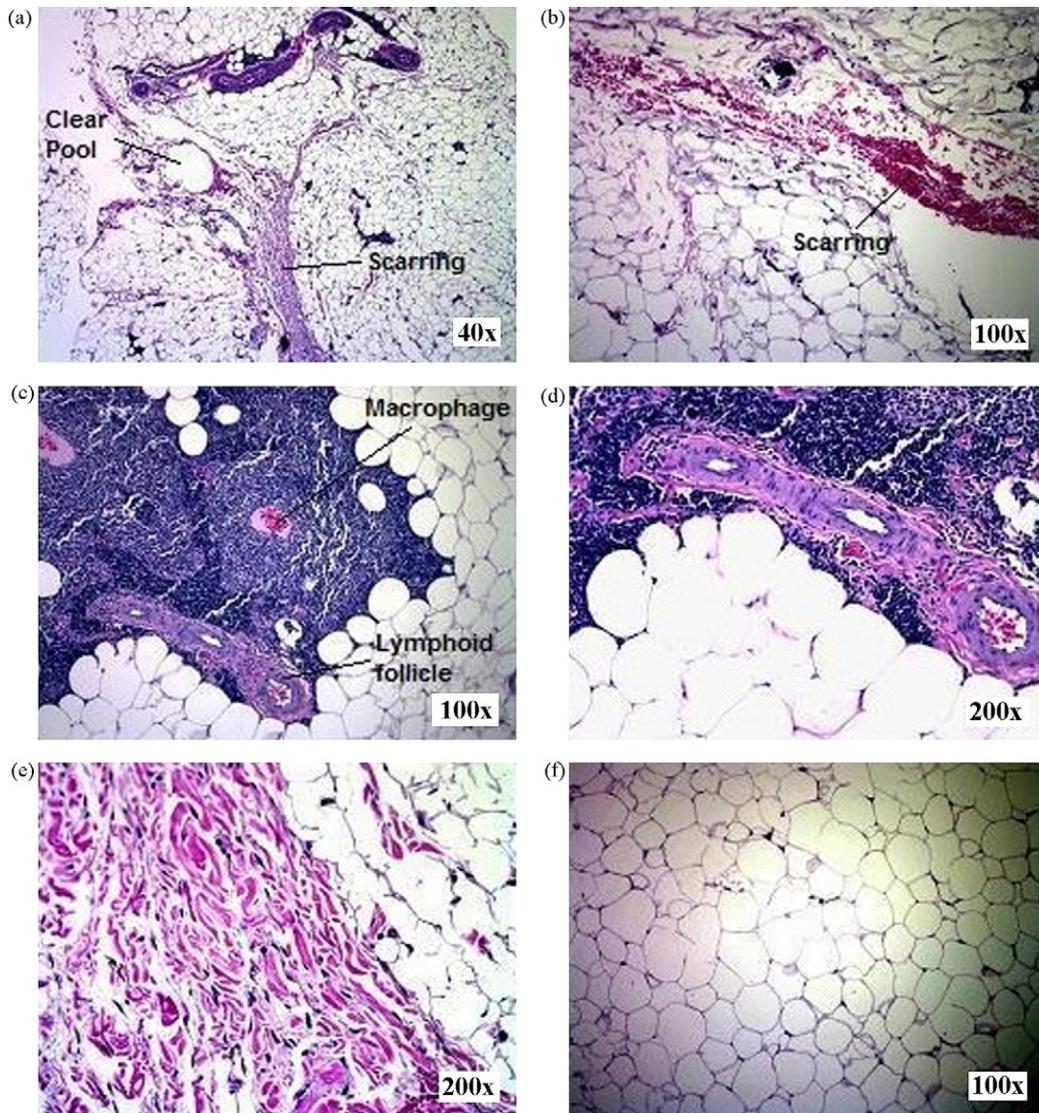


Fig. 9. Tissue slides depicting changes after 3 months of implantation. (a) Clear pool indicating presence of 'foreign material', (b) scarring caused due to washed out implant material, (c) chronic inflammation with lymphoid follicle, (d) view of lymphoid follicle showing engulfed material with a thin, crudely organized membrane, (e) loose connective tissue, and (f) control slide showing normal fat at regions further away from the implantation zone.

3.3. Scanning electron microscopy

In Fig. 10, the electron micrographs of the implant taken initially are compared those taken after 90 days of *in vitro* and *in vivo* release investigations. The initial condition showed a homogenous matrix core encapsulated by a polymeric sheath. The sheath was continuous with no signs of cracks. After 90 days of both *in vitro* and *in vivo* release (Fig. 10b and d), there is a distinct increase in thickness of the sheath. A careful examination of both sections under high magnification however, reveals the presence of channels in the sheath formed due to penetration of the medium. These channels, depicted in Fig. 10c and e, were formed at random but perpendicular to the longitudinal axis. Also evident was an increase in the roughness of the outer surface. These findings are consistent with the observations of Rothen-Weinhold et al. (1999), although the objective in that study was to compare properties of biodegradable matrices fabricated by melt-extrusion

and injection molding techniques. There was no distinction based on porosity from the initial conditions indicating that the integrity of the sheath had been maintained. At the interface of the sheath and the core, intercalating, "finger-like" projections were observed that had uniformly formed throughout the circumference of the core. This further indicated that the medium had penetrated through the sheath into the core. The core itself retained its homogeneity however, throughout the study period, as observed in both *in vitro* and *in vivo* experiments (Fig. 10f).

3.4. *In vitro*–*in vivo* correlation

Since *in vitro* drug release was evaluated for 90 days, the data point for Day-100 was calculated based upon an extrapolation of data from days 30 to 90. This is justified since the points were demonstrated an excellent linear relationship ($r^2 > 0.97$) for both devices.

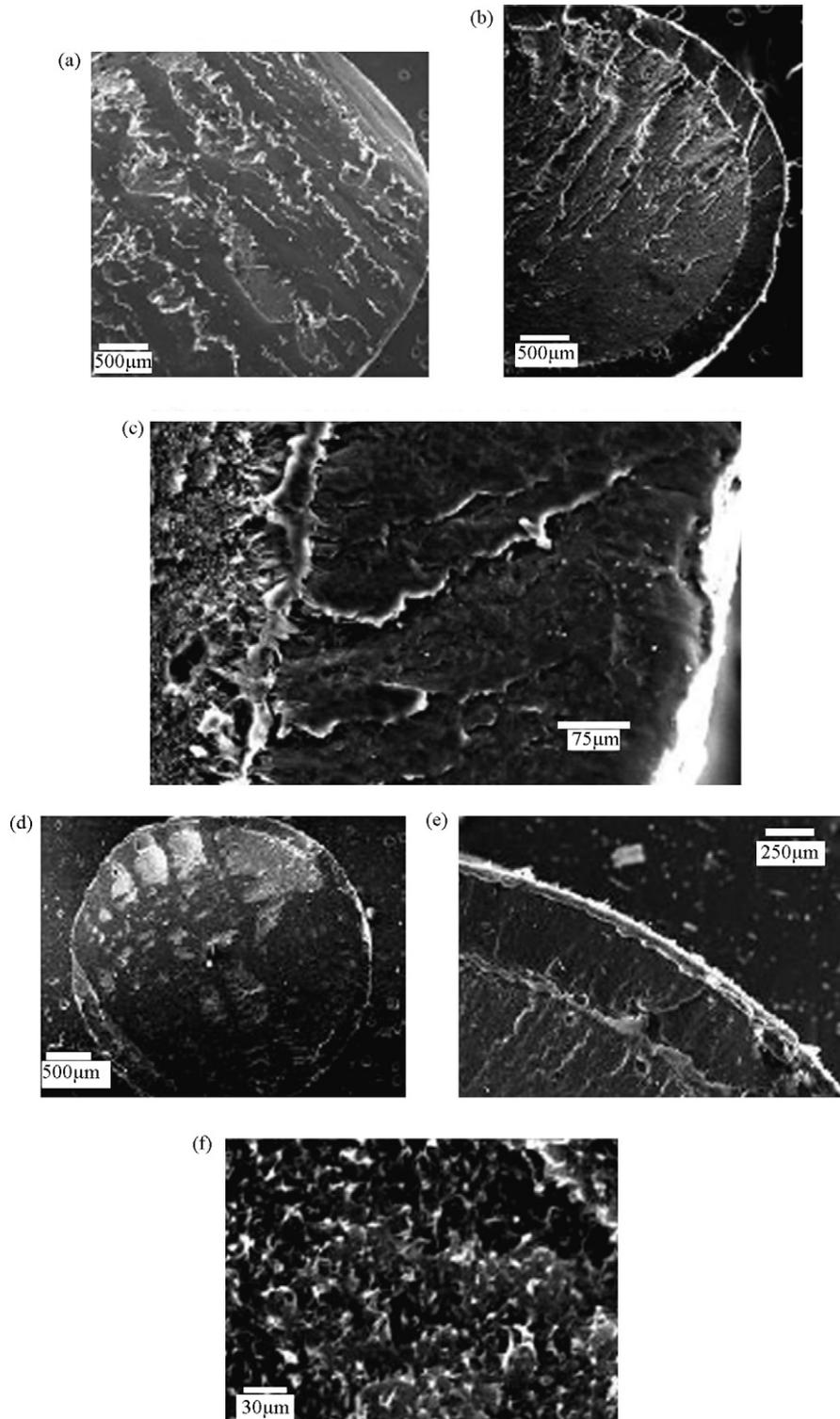


Fig. 10. Scanning electron micrographs of transverse sections of implant. (a) Initial condition (35 \times), (b) post Day-90 in vitro release (35 \times), (c) channels in membrane sheath after 90 days of in vitro release (500 \times), (d) post Day-90 in vivo study (35 \times), (e) channels in sheath after in vivo study of 90 days similar to the in vitro observation (350 \times), and (f) intact homogenous core after in vivo study (2000 \times).

The Wagner–Nelson approach, with an appropriate modification, was used to estimate the input function for data treatment (Wagner and Nelson, 1964). The trapezoidal rule was used for calculation of the area under curve (AUC_{0-100}). The value of β was estimated from the terminal slope of semi-log plot of the

plasma concentration–time profile (attributed earlier to receptor binding of naltrexone), and found to be 0.00751 h^{-1} . This was used for estimation of $AUC_{0-\infty}$, the area under curve to time infinity. Following this, the percentage absorbed in vivo was calculated and normalized to loss in weight of the implant.

This normalization was based on the following mass-balance equation:

$$\text{Total Drug}_{\text{initial}} = \text{Drug}_{\text{final}} + \text{Drug}_{\text{released in vivo}} \quad (4)$$

Therefore, if the initial drug loaded in the implant and final drug remaining at the end of implantation study are estimated, the amount of drug released in vivo can be determined. The implant was retrieved intact after the in vivo study, and loss in weight of the implant can be assumed to be equivalent to the amount of drug released in vivo. Assay of residual drug content was not done because the implant was needed for SEM evaluation comprising a destructive sample preparation step. A constant dry weight of the implant resulted, and microscopic evaluations showed minimal invasion of the implant material by connective tissue. We therefore consider this assumption of weight loss representing drug released to be a valid assumption. Furthermore, naltrexone has been reported to have excellent bioavailability following subcutaneous administration (Perez-Reyes and Wall, 1981) which suggests that the drug released into the subcutaneous tissue space would be in equilibrium with drug levels in the plasma.

It is important to note that the data manipulation in this work differs from the practice followed conventionally for oral modified-release dosage forms. In most cases where release close to 100% of total drug content is obtained, the input function calculated using the Wagner–Nelson approach is used directly. The potential importance of this data handling approach in development of IVIVCs for parenteral dosage forms (especially stents and other types of implants) that are designed for localized drug delivery is significant because these dosage forms most often, yield negligible systemic drug concentrations. This procedure however, may not be suitable for studies in which retrieval of the intact dosage form at the end of the study is difficult.

The normalized in vivo absorption data was then used for quantitative assessment of an in vitro–in vivo relationship (Fig. 11). In vitro release data using the capillary device were superimposable on the in vivo data, indicating a better correlation than that obtained using the flow-through cell.

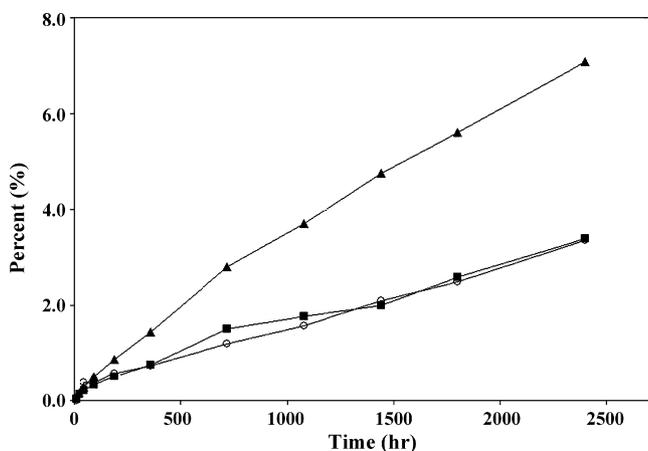


Fig. 11. Qualitative visualization of an in vitro–in vivo relationship. Key: \blacktriangle , flow-through cell; \blacksquare , capillary device; \circ , in vivo.

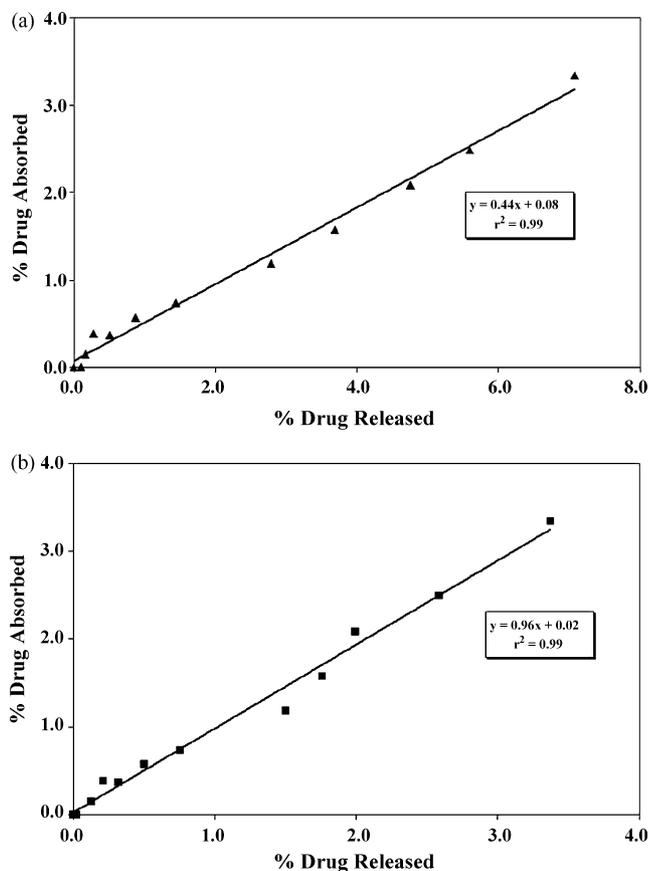


Fig. 12. Plots to show an in vitro–in vivo correlation using (a) the flow-through cell and (b) the capillary device. Key: \blacktriangle , flow-through cell; \blacksquare , capillary device.

Finally, the percent absorbed in vivo data was plotted versus the percent released in vitro to yield the correlations represented in Fig. 12. Overall, the data obtained with both devices could be well described by a linear relationship with an r^2 of 0.99. The slopes of the lines were significantly different from each other (0.44 versus 0.96 for the flow-through cell and capillary device, respectively). Both intercepts demonstrated low positive values that were not significantly different from zero ($p=0.65$ for the capillary data and $p=0.12$ for the flow through device). Also, the value of the intercept for the capillary device (0.02) was lower than that for the flow-through cell (0.08). To evaluate this observation further, the data up to 48 h were examined (refer Fig. 13). The difference in slopes and intercept during this period of burst release was clearly demonstrated. The capillary device simulated the lag-time in absorption more effectively than the flow-through cell, as indicated by its intercept (−0.07). It can be speculated that after attainment of peak burst release, the rates of release using both devices dropped before settling to an almost constant value. The magnitude of the dip in release rate with the capillary device, however, was larger compared to the flow-through cell. This may possibly, have accounted for the lower intercept value for the capillary device.

A further substantiation of the difference in capability of the two devices to simulate the overall lag of absorption relative to in vitro drug was obtained from the Levy's plot of

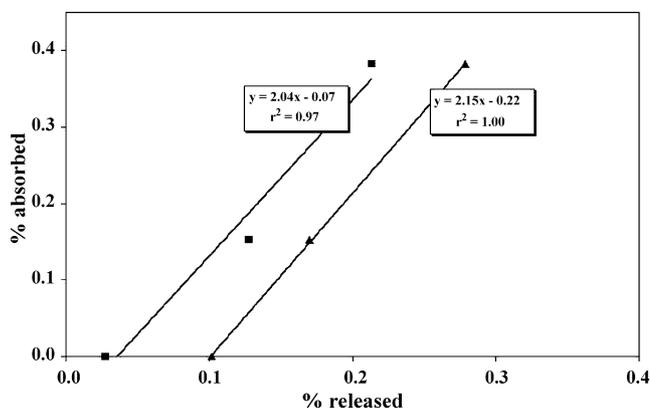


Fig. 13. Comparison of IVIVC using the flow-through cell and the capillary device during the first 48 h of release to show difference in slopes and lag-times. Key: ▲, flow-through cell; ■, capillary device.

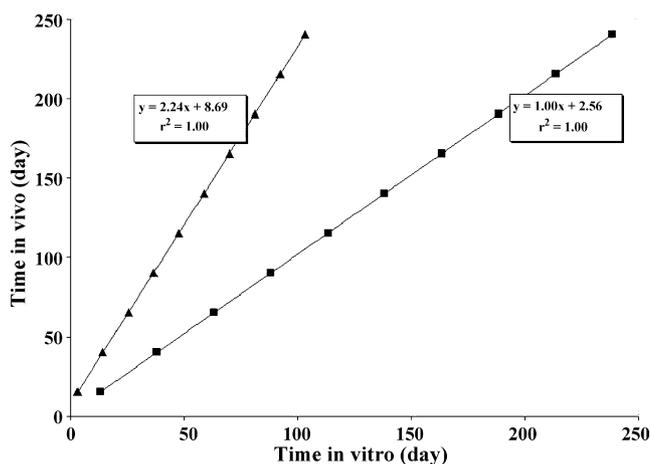


Fig. 14. Levy's plot to demonstrate a closer simulation of in vivo lag-time using the capillary device. Key: ▲, flow-through cell; ■, capillary device.

the data (Fig. 14). The time required for in vivo absorption of 0–0.75%, 0.75–1.50%, 1.50–2.25%, 2.25–3.00%, 3.00–3.75%, 3.75–4.50%, 4.50–5.25%, 5.25–6.00%, 6.00–6.75%, and 6.75–7.50%, was plotted versus the time for in vitro release of the same amounts of naltrexone using both devices. The slope for flow-through cell data was twice that of the capillary device, indicating that the time required for drug release from the flow-through cell was half the time taken for implants placed in the capillary device. The relationship also showed that the lines for the two devices had significantly different intercepts from zero ($p < 0.0001$). As expected, however, the value of intercept for the capillary device was closer to zero, indicating that the capillary device was able to simulate the barrier to diffusion more effectively than the flow-through cell. Also, it is important to understand that margin of error involved for in vivo data has not been considered in this study ($n = 1$).

4. Conclusion

A new 'biorelevant' approach to in vitro release testing of a naltrexone implant has been described this paper. The Modi-

fied Hanks' Balanced Salts Solution was found to be suitable for the real-time release study of the implant. A modified capillary bioreactor device was tested as the 'biorelevant' alternative to the commercially available flow-through cell. Sample analyses using a validated stability-indicating HPLC method resulted in zero-order in vitro release profiles with both devices after an initial period of burst release. No macroscopic and clinical toxicity signs were observed during the implantation study in dog. Regions of loose connective tissue possibly composed of keloidal collagen had formed, providing indications of body's natural healing processes. The dog plasma analysis yielded steady-state concentrations of naltrexone in the range of 200 pg/ml. An excellent 1-to-1 linear correlation was obtained with the capillary device. As expected from the flow characteristics and barriers to diffusion, the capillary device was able to simulate the lag-time in absorption more effectively than the flow-through cell. Therefore, the capillary device could also be useful for quick investigations of the initial period of burst release of implants in development. Further barriers to diffusion in the form of commercially available collagen gel may be created around the glass beads to simulate the healing process revealed by the histological evaluation. As proposed by the pharmacokinetic model of the site of implantation, it is speculated that blood flow to site of implantation may be the rate-limiting factor to absorption of drugs with good permeability that are administered subcutaneously. An investigation of the in vivo input rate would require intravenous administration of the drug. The additional modification in terms of decreasing the average capillary pore diameter (based on R_{diff} calculation described in Section 2.3.3) to mimic 'biorelevance' may be contemplated. This may be subject to manufacturing limitations of the capillary material however. Even with the existing pore size however, it can be speculated that the capillary device may provide sufficient rate-limiting barriers to large molecules, and could potentially be very useful for in vitro release studies of a few currently marketed implants and depots that contain peptides or hormones. Although further real-time studies on subcutaneous implants or depots with different release rate profiles are essential for validation, this research successfully demonstrates potential of the 'biorelevant' approach for investigation of in vitro drug release from other dosage forms.

Acknowledgements

The authors acknowledge Durect Corporation, CA, USA for providing implants for the study. The financial assistance and staff of the Biopharmaceutical Analysis Core Laboratory and Center for Drug Studies at School of Pharmacy are gratefully acknowledged. The Division of Animal Research, College of Humanities and Sciences, and the Departments of Anatomy and Pathology at Virginia Commonwealth University are thanked for assistance in specific portions of the study. Thanks are also due to Mark Schneider at Stryker Instruments for providing the sagittal saw. Sunil Iyer acknowledges financial support in the form of Dissertation Fellowship from School of Graduate Studies, Virginia Commonwealth University.

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