2006

A 'Biorelevant' Approach for Accelerated In Vitro Release and In Vitro-In Vivo Relationship of a Biodegradable, Naltrexone Implant

Sunil S. Iyer
Virginia Commonwealth University

A 'BIORELEVANT' APPROACH FOR ACCELERATED *IN VITRO* RELEASE AND *IN VITRO-IN VIVO* RELATIONSHIP OF A BIODEGRADABLE, NALTREXONE IMPLANT

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

Sunil S. Iyer,
M.Pharm., India, Birla Institute of Technology, 1998
B.Pharm, India, K.L.E. Society's College of Pharmacy, 1996

Director: **H. Thomas Karnes**, Ph.D.,
Professor, Department of Pharmaceutics

Virginia Commonwealth University
Richmond, Virginia
August 2006

Dedication

". . . . . I had an extreme desire to acquire instruction. But so soon as I had achieved the entire course of study, at the close of which one is usually received into the ranks of the learned, I entirely changed my opinion. For I found myself embarrassed with so many doubts and errors that it seemed to me that the effort to instruct myself had no effect other than the increasing discovery of my own ignorance."

Rene Descartes, *Discourse on the Method*, ed. James Fieser (Internet Release, 1996)

Acknowledgements

I would like to take this opportunity to sincerely thank my major advisor, Dr. Thomas Karnes for his untiring efforts and support during the past four years. He has been my teacher, philosopher and guide; as well as a constant source of inspiration that I could approach whenever I needed any kind of opinion, whether academic or otherwise.

Special thanks are due to Dr. William Barr, who, in spite of his busy schedule always found time to sit down with me for discussion of data, and for designing experiments. I have learnt a great deal from both Dr. Karnes and Dr. Barr, and will be grateful to them for letting me attempt even the craziest of my ideas.

I would like to acknowledge Drs. Glen Kellogg, Robert Bell, and Leslie Edinboro for their valuable inputs to various stages of the project. Thanks to Drs. Mario Dance and Peter Coleman, without whom the *in vivo* part of the study would not have been possible.

I am thankful to Dr. Randy James for his help during my work at the Biopharmaceutical Analysis Core Laboratory. He had been generous enough to work around laboratory schedules of various instruments to suit my own. Thanks to Mr. Sadanand Ghatge for his assistance during initial bioanalytical method development on the mass spectrometer. Thanks also to Annmarie and Kim at the Center for Drug Studies for assistance provided during the *in vivo* study. I thank James Spivey, Dr. Scott Henderson, Deborah Reynolds and Jing Li at VCU College of Humanities & Sciences, Department of Anatomy and Immunology, Department of Anatomic Pathology, and the General Clinical Research Center, respectively, for assistance in microscopy, histology and osmometry experiments. Mr. Mark Schneider at Stryker Instruments is appreciated for the donation of and assistance in handling the sagittal saw during modification of the capillary device.

I thank the VCU Graduate School for the Dissertation Fellowship Award and the School of Pharmacy for financial assistance. Mia, Laura and Dee deserve mention for prompt assistance in office work, as and when required.

I appreciate the friendship and help of present and past members of the Bioanalytical Research Group, especially Songmei, Mike, Angela, Bhaskar, Dan and Kumar; as well as other SOP graduate students and alumni including Pankaj, Satjit, David, Pravin, Xihao, Soniya, Reshma, Amit, Alaa, Jessica and Bhaskar Rege, during my memorable stay. Special thanks to my apartment mate, Rajesh; and to Amol, Laxmikant, Naveen, Rajneesh, Ramesh and Nagesh, for making my stay at Richmond very pleasant.

Last but not the least, I take this opportunity to thank members of my family back in India: Parents, Brother and Grandparents, for their love, sacrifice and immense support, as always, all through the years.

Table of Contents

List of Tables

Page

List of Figures

Page

List of Abbreviations

AAPS	American Association of Pharmaceutical Scientists
ACDRA	Accelerated Dissolution Rate Analysis
BCS	Biopharmaceutical Classification System
COP1	Interstitial Colloid Osmotic Pressure
EUFEPS	European Federation of Pharmaceutical Sciences
FDA	Food and Drug Administration
FIP	Fédération Internationale Pharmaceutique
HBSS	Hanks' Balanced Salts Solution
HEPES	4-(2-hydroxy ethyl) piperazine-1-ethanesulfonic acid
HPLC	High-Performance Liquid Chromatography
IVIVC	*In vitro-In vivo* Correlation
IVIVR	*In vitro-In vivo* Relationship
LC-MS	Liquid Chromatography - Mass Spectrometry
LC-MS-MS	Liquid Chromatography - Tandem Mass Spectrometry
MRT	Mean Residence Time
NAL	Naltrexone
NAL-d_3	[15,15,16-^{2}H]-naltrexone
NIDA	National Institute on Drug Abuse

PLGA	Poly (lactic-co-glycolic) acid
SEM	Scanning Electron Microscopy
SUPAC	Scale-up and Post Approval Changes
USP	United States Pharmacopeia

Abstract

A 'BIORELEVANT' APPROACH FOR ACCELERATED *IN VITRO* RELEASE AND *IN VITRO-IN VIVO* RELATIONSHIP OF A BIODEGRADABLE, NALTREXONE IMPLANT

by Sunil S. Iyer, M. Pharm.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, Richmond VA, USA 23298.

Director: H. Thomas Karnes, Ph.D.,
Professor, Department of Pharmaceutics

Characterization of *in vitro* and *in vivo* drug release profiles constitutes an important step in developing and optimizing an effective, long acting delivery system for naltrexone. Accelerated *in vitro* methods are also important for quality assurance of manufactured dosage forms. For drug release testing of sustained release parenteral dosage forms, the modified USP Apparatus 4 (flow-through cell) has been recommended by the The Fédération Internationale Pharmaceutique/American Association of

Pharmaceutical Scientists (FIP/AAPS) Guidelines. Details on such studies however, are generally not found in the literature. To incorporate 'biorelevance' to implant drug release studies, this research investigated an approach to apparatus design and media selection that is significantly different from conventional dissolution studies involving oral dosage forms.

Biodegradable implants of naltrexone were obtained from Durect Corporation, USA. A modified Hanks' Balanced Salts Solution was characterized as a 'biorelevant' medium for *in vitro* drug release studies. Naltrexone was found to be sufficiently stable in the medium, as determined by a stability-indicating High Performance Liquid Chromatography (HPLC) assay. A miniature, cell-culture, capillary system was modified and tested as a 'biorelevant' alternative to the modified flow-through apparatus, to mimic significant barriers to drug release that would be expected *in vivo*. The *in vitro* release profiles generated up to 3 months using both devices indicated considerable (2-fold) variation in rates, as expected from the difference in media flow characteristics. An implantation study in a dog was conducted to determine which of the two devices could provide a better simulation of the *in vivo* conditions. Analysis of *in vivo* samples was carried out by a Liquid Chromatography-Tandem Mass Spectrometry (LC-MS-MS) method that also employed a molecular model approach to demonstrate the absence of Internal Standard Deuterium Isotope Effects. A good *In vitro-In vivo* Correlation (IVIVC) resulted from both devices; however, the capillary device provided a superior simulation for the lag-time in absorption. The accelerated study at 45°C and 55°C established a predictable increase in release rates (2-fold and 4-fold increases, respectively). The

approach described in this work could provide the basis for future method modification of *in vitro* drug release tests of subcutaneous implants.

CHAPTER 1. Background and Significances

1.1 Introduction

Dissolution testing is a critical quality attribute for formulation and process development (Gray, 2004a). Developments in these critical methodologies have come from regulatory agencies, as well as industrial and academic groups seeking *in vitro* drug release methods that better correlate with *in vivo* drug release and absorption; and provide improved precision, accuracy, predictability and ease of use (Sirisuth et. al., 2004).

Folkman and Long (1964) recognized the use of implants as sustained release drug delivery systems. Ballard gave an earlier review on the physicochemical characterization of subcutaneous pellets in 1961. Further development of these dosage forms over the years has become necessary due to the short half-life of many pharmaceutical agents after parenteral administration (Davis, 1974; Mohl et. al., 2002). A longer duration of action is also required for patient acceptability since it avoids the need for frequent invasive procedures. 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. A wide variety of drugs are good candidates for formulation as implants. Agents that are used in long-term therapeutics, such as goserelin, leuprolide, carmustine, recombinant nerve growth factor (rhNGF) and levonorgestrel are a few examples (Okumu et. al., 2003). Also, stents, which are small

cylindrical tubes placed in vessels, are an important new development for delivery of drugs that must be administered continuously for a long period of time. Prolongation of drug release from implants can be effected 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 et. al., 2003). The implants are fabricated from polymers in the molten state either by extrusion or by injection molding (Martin, 1995). Generally, the first step in the melt-extrusion process involves feeding the pharmaceutical active constituent with polymer and excipient, if required, into the feed section of the extruder for compression into pellets. These are conveyed to the transition section in which thorough mixing is performed along with the application of heat. This melts the polymer into a homogeneous, viscous liquid that is pumped accurately at a constant rate through a metering device, which determines the shape of the final product. In the injection molding process, the drug and polymer mix is melted and fed into a die cavity. The molten mix cools and solidifies under pressure. The mold is then opened to obtain the dosage form.

Naltrexone is a long-acting, opiate receptor, pure antagonist (Resnick et al., 1974) that blocks the subjective and objective responses produced by opioid challenge. The drug has been marketed as an oral tablet since the mid-1990s for the clinical management of opiate addiction, and as adjunctive treatment for individuals with alcohol dependence (Verebey and Mule, 1975; Food and Drug Administration NDA 18-932/S-010, 1994). The drug is known to be well absorbed from the gastro-intestinal tract. The drug undergoes extensive and variable first-pass metabolism however, that influences the

bioavaliability after oral administration in humans. This results in a bioavailability range between 5 and 60% (Gonzalez and Brogden, 1988). Dose linearity was observed following oral administration of 50, 100, and 200 mg doses. The drug is 20% plasma protein bound, and the apparent volumes of distribution at steady-state were 16.1 L/kg and 14.2 L/kg after oral administration of 100 mg single and long term doses, respectively. 6β-naltrexol is the major metabolite of naltrexone found in man, but not in dog. It is 1/50 to 1/12 times as potent as naltrexone (Cone et al., 1974). Initial studies reported mean elimination half-lives for orally administered naltrexone from 1.1 hours (Cone et al., 1974) to 10.3 hours (Verebey et al., 1976), and 2.7 hours after intravenous administration (Wall et al., 1981)

Pace et al. and Reuning et al. in 1979 studied the pharmacokinetics of naltrexone in dog after intravenous administration. The mean terminal half-lives was reported to be 85 minutes and 60 minutes, respectively. During data analysis, the latter group of workers had excluded a terminal point of the plasma concentration-time profile as an outlier. However, the potential influence of this point on prolonging the half-life of naltrexone was recognized. It was not until the late 1980s that the longer duration of action of naltrexone could be accounted for. In their study to determine duration of blockade of the mu-opiate receptors by naltrexone, Lee et al. (1988) demonstrated that the half-life of naltrexone on the specific receptor site ranged from 72 to 108 hours. This study also suggested that plasma clearance half-life of any drug may not be an accurate indicator of its duration of action. Also studies to improve bioavailability and for masking the bitter

taste using esters of naltrexone as prodrugs have been published (Hussain et al., 1987 and 1988).

Medication non-compliance is an additional obstacle to treatment during naltrexone therapy. Two factors, namely, the complete abstinence of opioid-induced reinforcing effects and the absence of adverse consequences (unlike methadone) upon discontinuation of medication are primarily responsible for patient non-compliance (Volpicelli et al., 1997; Comer et al., 2002). Therefore, sustained-release dosage forms of naltrexone, such as implants and microspheres, are under development with the aim to increase compliance and ultimately improve treatment effectiveness (Brewer et al., 2002; Bartus et al., 2003; Hulse et al., 2004). The majority of the research is focused on incorporation of the drug into biodegradable polymers, such as polylactic acid, poly glycolic acid and polycaprolactone. These polymers offer the advantage of a single surgical procedure. During formulation of drug delivery systems, manipulation of polymer degradation rates has been achieved by blending polycaprolactone and poly (glycolic acid-co-lactic acid) to control chain scission (Pitt, 1990).

A topic of current research interest is the selection of an appropriate dissolution medium that provides 'sink conditions' based on the solubility of the drug and the dose rate, as discussed in Pharmacopeial Previews, Chapter 1092 (2004). Another important topic is the development and validation of a standard apparatus for the quality control of implants that release drugs. A report (Burgess et. al., 2004a) of the workshop of the European Federation of Pharmaceutical Scientists (EUFEPS) held in February 2003 summarizes scientific support for the urgent development of a regulatory standard for

controlled-release products. Of particular concern was the necessity for standards on *in vitro* release methods and for science-based guidance in the areas of *in vitro* release testing and *in vitro–in vivo* correlations (IVIVC). An IVIVC imparts *in vivo* validation to the *in vitro* dissolution test, which can then be used as a surrogate for bioequivalence testing (Sunkara and Chilukuri, 2004). In addition, more meaningful dissolution specifications can be set using the concept of an IVIVC (Uppoor, 2001). A Guidance document on the role of development of *In vitro-In vivo* Correlations for Extended Release Oral Dosage Forms was issued in 1997 by the Food and Drug Administration in an effort to: (a) reduce the regulatory burden by decreasing the number of biostudies needed to get approval and maintain an extended release product on the market and (b) set dissolution specifications that are more meaningful clinically. The ultimate goal is that demonstration of valid IVIVCs would allow many of the biostudies that are generally required for major manufacturing changes to be replaced by relatively simpler *in vitro* dissolution tests. Four categories of correlations (A–D) have been described in the guidance. A Level A correlation represents a point-to-point relationship, generally linear, between *in vitro* dissolution rate and the *in vivo* input rate. It is usually considered the best type of correlation to claim biowaivers. A Level B correlation involves the principles of statistical moments. The mean *in vitro* dissolution time is compared either to the mean residence time or the mean *in vivo* dissolution time. This is not considered to be a point-to-point correlation and because a number of different *in vivo* curves will produce similar mean residence time (MRT) values, this cannot be considered discriminatory for different formulations. A Level C IVIVC represents a single point relationship between a

dissolution parameter (such as percent dissolved at a particular time) and a pharmacokinetic parameter of interest, e.g. the area under curve (AUC). However, it does not reflect the complete shape of the plasma concentration curve, which is the critical factor that defines the performance of extended release products. A Level C correlation, although useful to screen and rank-order formulations in animal models during drug development, cannot be used for biowaivers or bioequivalence (Shah, 2004). The fourth category, D, is a multiple Level C correlation and it represents a relationship between one or more pharmacokinetic parameters, and the amount of drug dissolved, at multiple points of time on the dissolution profile.

The FDA Guidance issued is applicable, directly, only to oral extended release drug products. Principles of the Guidance can be employed for peroral dosage forms, such as implants. Additional work is needed in this area, however. Several challenges exist in method development for implants and these need to be dealt with on a case-by-case basis. Thus, it is necessary to summarize the scientific issues related to drug release from subcutaneous implants, followed by a discussion of the current practices for these types of studies.

1.2 Apparatus Selection

A dissolution process involves two sets of variables relating to the apparatus and the medium. The apparatus variables include the type of apparatus and its hydrodynamics, including agitation and flow-rate. The medium variables include volume, composition, and concentration of the drug as function of time. When the drug

concentration continues to increase in the dissolution medium beyond one-third of the saturation value at a particular temperature, it is commonly referred to as a 'nonsink condition'. On the contrary, a 'sink condition' refers to the excess solubilizing capacity of the dissolution medium, a condition at which the concentration of the drug is maintained at a constant low level, well below saturation (Rohrs, 2001).

According to the flow characteristics and sink conditions, Banakar (1992) classified methods of dissolution devices into:

(a) Natural-convection non-sink methods (e.g. the static disk method of Levy, 1963), in which the dissolution medium surrounding the dosage form is continuously replaced by fresh medium based on density differences alone, and without the application of any agitation.

(b) Forced-convection non-sink methods, as exemplified by the Apparatus 1 and 2 of the United States Pharmacopeia (USP28/NF23, 2005) that have assemblies or rotors for agitation. These methods have a fixed volume of medium, and therefore offer restrictions in terms of application to compounds with low intrinsic solubilities.

(c) Forced-convection sink methods (e.g. the dialysis method of Barzilay and Hersey, 1968) that also have mechanisms for agitation, but in addition provide adequate simulation of 'sink' conditions.

(d) Continuous flow/ flow-through methods (e.g. Langenbucher method, 1969) described later in this section.

Most of the tablets and capsules administered orally are investigated either by Apparatus 1 or 2, specifications for which are provided in the compendium (USP28/NF23, 2005). The basic design for the USP 1 Apparatus comprises a stainless-steel mesh wire basket in which the dosage form is placed. The basket is fixed to a shaft that rotates at a constant speed, usually between 25-150 rpm, and is immersed in a flask having a fixed volume of dissolution medium. Samples are aliquoted at predetermined time intervals for analysis. Different mesh screens ranging from 10 to 40, and reservoir volumes between 200-2000 ml have been employed.

The USP Apparatus 2 has similar specifications as Apparatus 1, except that a precisely defined paddle is employed instead of the basket. The dosage form is placed directly in the reservoir, and a spring shaped device called the "sinker" is used at times for floating dosage forms. Problems such as clogging of the mesh, vibration due to the use of non-calibrated equipment, and "coning" (conical formation due to accumulation of cone shaped material) are dealt with on a case-by-case basis.

Wagner (1971) stated that the application of a universal dissolution or release rate test, in a compendial sense, is desirable but largely impractical. Each drug and the dosage form prepared from it have to be studied individually and usually retrospectively, after *in vivo* data are available. This is because achievement of the ideal correlation requires selection of the *in vitro* parameter that has the greatest relevance to the drug absorption characteristics. A dissolution rate apparatus suitable for both research and quality control purposes should meet certain criteria, summarized in Table 1.

Table 1. Criteria for Apparatus Selection

a) Simple design
b) Convenience in handling, operation and cleaning
c) Well-defined components for reproducible results
d) Provision for an easy introduction of the test product and sample withdrawal for analysis
e) 'Biorelevant' to the extent feasible i.e. should mimic physiological condition at the site of implantation
f) Allow effective and controlled agitation
g) Potential for use in accelerated drug release tests
h) Economical

The apparatus should have simplicity of design, convenience of operation and provision for an easy introduction of the dosage form under investigation. The inherent variability in the apparatus must be less than the inherent variability in the products being tested. This requires that the essential components be specifically definable and reproducible. It must be capable of reproducing a given intensity of agitation or flow, with fixed geometry for successive runs under a constant setting. It must be flexible in the effective degree of agitation; by altering the stirring rate or flow rate or some similar parameter. The apparatus should be economically practical. Ideally, apparatus variables should allow testing of formulations in such a way that facilitates elucidation of the mechanism(s) and critical variables for drug release e.g. disintegration, deaggregation, erosion, permeation, etc.

Current reports on testing drug release from implants involve subjecting the implants to unstirred conditions in vials containing media. In their study with buserelin implants, Schliecker et al. (2004) withdrew samples at different time intervals for the estimation of drug released, and the volume withdrawn at each time point had been replenished with fresh media. Other workers report complete media replacement at each time point (Malmsten, 2002; Mohl et. al., 2002). Neither of these approaches, however, employs physiologically relevant media and flow rates; and thus there is a need to devise new strategies for the investigation of drug release from implants.

Shah et al. (2002) describe *in vitro* release testing and dissolution of many special dosage forms. They state that the methodologies are well evolved for several special dosage forms and provide specific recommendations for drug release testing of

suppositories, transdermal patches and semi-solid topicals (creams, ointments and gels). However, for several other dosage forms, e.g. implants, chewing gums, powders, granules, solid dispersions and microparticles, more method development and refinement is needed before a final recommendation on standardized drug release methods can be made. The cell of the compendial flow-through apparatus (Apparatus 4), for which specifications are provided in Chapter 724 of the USP, has been suitably modified to accommodate implants (see Figure 1).

The inner diameter of these cells has been considerably reduced, compared to the tablet cell. This results in a low volume of the acceptor compartment. The implant is placed in a vertically mounted cell on a screen that permits fresh dissolution medium to enter from the bottom. The cell is closed by a second screen at a height h (defined by the length of the implant) that filters the liquid and prevents the removal of any undissolved particles. The dissolution medium is pumped through the cell from a reservoir after having passed the heat exchanger for temperature control. The medium leaving the cell is analyzed for drug content, either continuously or at fixed intervals. An important advantage is that the continuous flow-through type of arrangement, when used as an open system, ensures the existence of sink conditions since fresh medium flows past the implant throughout the period of study. Furthermore, the effects of pH on drug release can be easily investigated using this system, although alteration in pH would not be appropriate for 'biorelevance'. There remains, however, a need to establish suitable calibrators and standardization of this apparatus. Although the USP recommends that media flow be pulsed to avoid clogging of filters, other options such as a high pressure

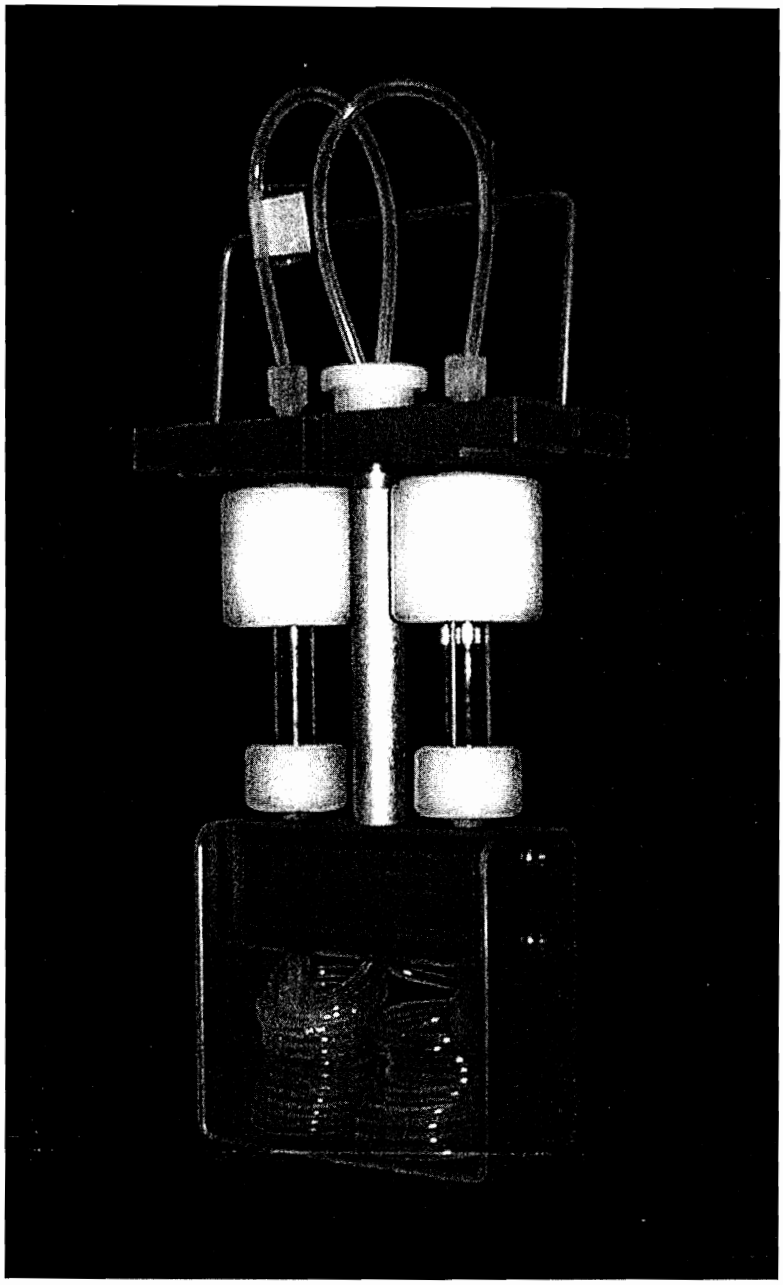


Figure 1. A flow-through cell apparatus

pump or intermittent flow should also be considered (Siewart et. al., 2003). The standardization process may also include temperature measurements in the cell, wherever feasible, to verify that readings of the instrument match the temperature inside the cell. Also, flow rate should be checked, and adjusted before the start of a run, and verified at the end of a run.

The flow-through apparatus has also been recommended in the Fédération Internationale Pharmaceutique/American Association of Pharmaceutical Scientists (FIP/AAPS) Guidelines on Dissolution/*In vitro* Release Testing of Novel/ Special Dosage Forms (Siewart et. al., 2003). The article presents the consensus of experts in a series of sponsored workshops. The authors advise against the 'unnecessary' proliferation of modified apparatuses for special products when the standard compendial equipment can produce equivalent results. In such cases, the compendial apparatus should be used. They also state, however, that for special non-oral dosage forms, it is difficult to find an appropriate balance between the general recommendation to avoid 'unnecessary' proliferation of dissolution apparatus and to acknowledge the formulation specific characteristics and requirements of a new product under development. For example, the development of USP apparatus 3 was based on recognition of the need for better *in vitro* and *in vivo* correlations that could not be achieved by the dissolution results obtained with USP apparatuses 1 and 2 (Borst et al., 1997; Yu et al., 2002). The USP apparatus 3 offered the advantages of mimicking changes in physiochemical conditions and the mechanical forces that products were subjected to in the gastrointestinal tract, and

avoided results to be affected by variables, such as shaft wobble, location, centering, and coning.

The flow-through cell represents conditions similar to those encountered *in vivo* because the entire content of the receptor compartment is replaced on a continuous basis (Langenbucher, 1969). However, a major limitation of the apparatus is that the implant is directly placed in the flow of the medium and this may not be a true representation of the *in vivo* environment. When placed subcutaneously, the implant is expected to release drug into its immediate vicinity (comprised of tissue fluid and cells), followed by passive diffusion or possibly facilitated transport into the cells and the vascular system before finally reaching the systemic circulation (Figure 2).

Nicolaides et al. (2000) reported discrepancies in the dissolution behavior even with tablets that could be attributable to inherent design problems in the *in vitro* system and to inadequate simulation of *in vivo* hydrodynamics (including slow flow rates, vertical positioning of the cell and possible sedimentation of the solids). The authors also speculated that the piston pump did not allow for simulation of bi-directional movement of chyme or segmental mixing, and had been operated at flow rates (between 2.5 and 12 ml/minute) that represented the average net flow in the aboral direction. The need for an alternative flow pattern has also been suggested as a means to mimic actual hydrodynamics *in vivo*. Fyfe et al. (2000) employed NMR imaging to better understand the physical changes that solid oral dosage forms undergo within a flow-through cell under dynamic media flow conditions. Understanding the flow-through cell therefore, presents an opportunity for future research. The quote by Salvador Dalí (1904–1989), the

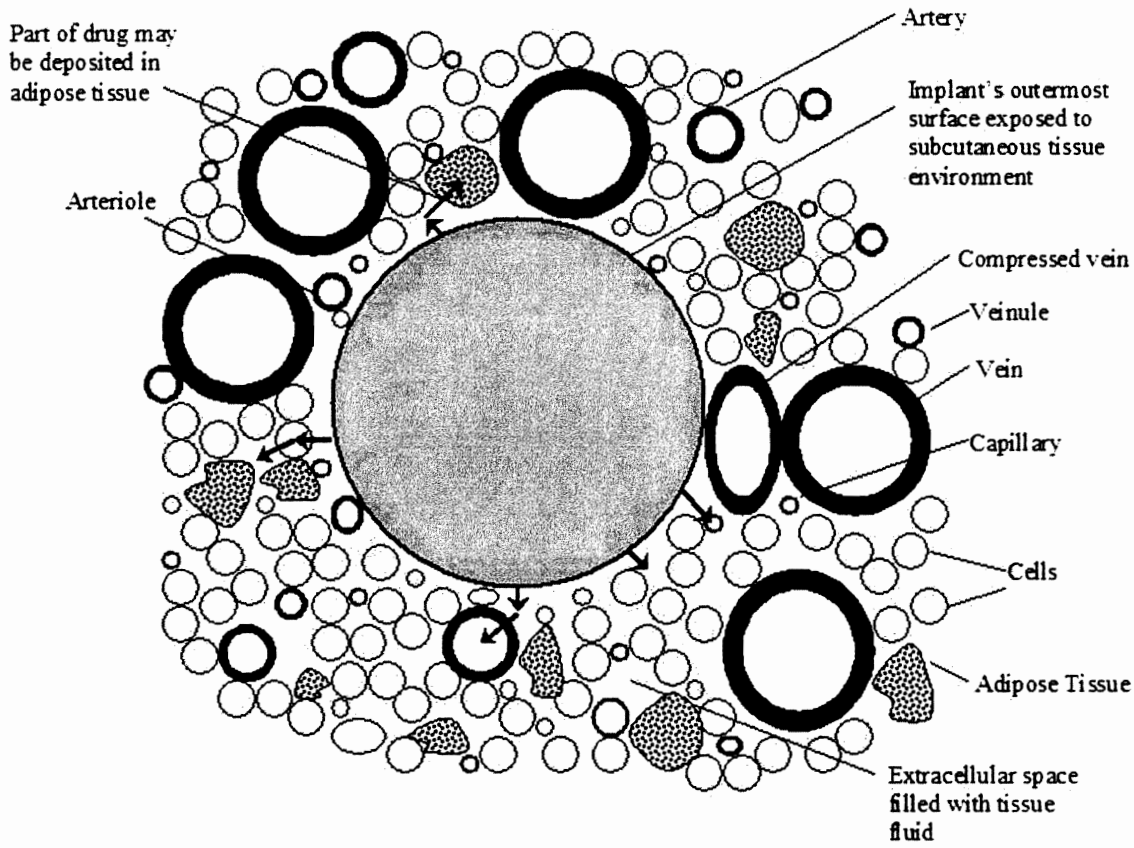


Figure 2.Visualization of subcutaneous tissue environment around an implant (Arrows indicate probable routes for movement of drug molecules)

Note: Sizes are not to scale

(Ref: Iyer et al., 2006).

famous Spanish artist, "Have no fear of perfection, you'll never reach it", would best describe this endeavor.

To design a 'biorelevant' apparatus for implant drug release studies, it is important to have an understanding of the conditions at the subcutaneous site.

1.3 Physiological Factors at the Subcutaneous Site of Implantation Influencing Drug Absorption

The structural characteristics of interstitial space are similar in all tissues, consisting of a fibrous collagen framework supporting a gel phase made up of glycosaminoglycans, salts and plasma derived proteins (Porter and Charman, 2000; Langevin et. al., 2004). The glycosaminoglycans are polyanionic polysaccharides that are charged at physiological pH (7.4) and are bound covalently to a protein backbone to form immobilized proteoglycans. Hyaluronan is an exception that may be removed from the interstitium via lymph vessels. The proteins present in the interstitial space are qualitatively the same as those present in plasma, although quantitatively, they are present in lower concentrations. This results in the interstitial colloid osmotic pressure (COP1) being less than that in plasma (Auckland and Reed, 1993). The interstitium however, displays a high degree of structural heterogeneity. It is believed that a network of endogenous macromolecules effectively reduces the distribution volume such that the interstitial space acts in a size exclusion manner, excluding very large molecules, and thereby affecting their interstitial occupancy (Watson and Grodrins, 1978; Bert et. al., 1982). The blood capillaries supplying the subcutaneous space are generally continuous

in structure and are characterized by tight endothelial junctions and an uninterrupted basement membrane. Whang et. al. (1982) found that the subcutaneous vessels are substantially larger than those in the dermal tissue (1500 mm for the veins and 600 mm for the arteries). These are relatively permeable to the exchange of small, lipophilic molecules. In addition, Porter and Charman (2000) found that aqueous 'pores' or channels are present which provide diffusivity to some hydrophilic molecules. A series of classic papers by Renkin (1954, 1964 and 1977) and Pappanheimer et. al. (1951) attempt to offer a functional understanding of the 'pore theory' of capillary permeability and its relation to restricted diffusion of solutes. The measurement of permeability revealed that the fractional area available for exchange decreased as the size of the diffusing molecule increased. The relationship is described by the equation 1 below:

$$A_x/A_o = \left[2\,(1 - a/r)^2 - (1 - a/r)^4\right].\left[1 - 2.104\,(a/r) + 2.09\,(a/r)^3 - 0.95\,(a/r)^5\right] \quad \ldots\ldots (1)$$

where A_x/A_o is the ratio of effective area of the opening to the total cross-sectional area of pore, a/r is the ratio of radii of the diffusing molecule to that of the pore. Furthermore, using a simple model of permeability pathways as uniform cylindrical pores penetrating an otherwise impermeable membrane, the authors have calculated that the pores occupy only 0.02–0.2% of the area of capillary walls, They speculated that permeability of hydrophilic solutes is restricted to intercellular regions. In the case of large molecules, lower levels of permeability were observed with increasing a_e [effective hydrodynamic radius calculated using the Stokes–Einstein equation ($D = RT/6\pi\eta a_e N$, where D is diffusion coefficient, R is the molar gas constant, T is the absolute temperature, N is the

Avogadro number and η is viscosity of the solvent)]. However, the steep fall in values of P (permeability coefficient) was limited by an extension of smaller slope, which indicated the presence of a separate transport mechanism, quantitatively insignificant for small molecules but important for large. Renkin suggested that part or all of the extension of permeability beyond the small pore might be attributed to transport by micropinocytotic vesicles; a phenomenon known as 'cytopempsis'.

Even the site of administration affects the absorption of drugs. The absorption of insulin and human growth hormone (hGH), for example, increased with subcutaneous injection in the abdomen in comparison to the thigh or upper arm (Berger et. al., 1982). Simonsen et al. (2003) observed significant regional differences in the washout rates of xenon (133Xe) from subcutaneous, abdominal adipose tissue. Among the various layers of subcutaneous tissue (viz. pre-peritoneal, superficial subcutaneous and deep subcutaneous), normal blood flows were found to range from 1.5-2.5 mL.100 (g minute)$^{-1}$ (Enevoldsen et. al., 2001). Sindrup et al. (1991) reported nocturnal variations (mean increase of 84%; upto 200% in some cases) along with postural changes (30–40% in the beginning of night period) in the subcutaneous blood flow rate in the lower leg of normal human subjects. Zuidema et al. (1988) reported that the subcutaneous adipose layer has important retarding effects on the absorption of drugs administered as an oily suspension or liposomes. These studies emphasize that several factors need to be considered in the mechanistic interpretation of drug release data from implants.

1.4 Method Development and Optimization

Traditionally, the flow-through apparatus has been operated at a flow rate of 16 mL/minute, although flow rates ranging from 12.5 to 50mL/minute have been investigated (Qureshi et. al., 1994a; Qureshi et. al., 1994b; Cammarn and Sakr, 2000). The rate of flow of 16 mL/minute, however, was chosen to be consistent with the compendial paddle or basket type apparatus for oral dosage forms, such that about 1 liter of the dissolution medium flows past the implant in 1 h. This does not represent an appropriate range of flow-rate when the apparatus is employed for controlled-release parenterals such as implants or depots, and may not be suitable for 'biorelevant' flow conditions. In an *in vivo* situation, the implant will be exposed to slow-moving fluid in the subcutaneous tissue with convection and/or diffusion processes predominating until the released drug reaches a vascular or cellular barrier (Figure 2).

Another important aspect in dissolution method development is the choice of a suitable medium (refer Table 2). As mentioned in the previous paragraph, much of the research activity has been focused, quite understandably, on oral dosage forms. The most common dissolution medium used for the study of non-oral dosage forms has been phosphate buffer saline (PBS) at pH 7.4. The report on the EUFEPS Workshop provides a general opinion that in order for *in vitro* data to achieve biorelevance, physiological variables at the site of implantation should be considered (Burgess et. al., 2004a). This includes, but is not restricted to, body temperature, blood flow, drug metabolism due to enzymes, muscle pH, buffer capacity, and osmolality.

Table 2. Factors influencing the selection of a 'biorelevant' medium for *in vitro* drug release studies

a) Solubility of drug and dose rate and influence of 'sink' conditions
b) Stability of the drug in media during the complete period of study
c) Well-characterized media components capable of maintaining its pH and osmolality over the entire study period
d) Stability of media components: Temperature effects depending on the type of study (real-time/accelerated study)
e) Economy for use during the entire period of study
f) Non-proprietary formula for ease of access

It was also recognized in the report that there is a need for examining the mechanisms of the *in vivo* release process while developing *in vitro* release methods. From this standpoint, various physiological buffers such as Hanks' Balanced Salts solution, Kreb's bicarbonate buffer, Earle's Balanced Salts solution, etc. with suitable modifications have potential for application to such studies (Waymouth, 1954 and 1970). Consideration of pH and osmolality changes of the media as a function of time, however, will be necessary (Lelong and Rebel, 1998). Method development for such dosage forms is an active area of research (Chidambaram and Burgess, 1999).

A very important aspect of method development in drug release testing is a consideration of the degradation of drugs and the dosage form matrix in dissolution media and under experimental conditions. Degradation of drugs might be observed due to maintenance of the release media for a prolonged period of time under temperatures above room temperature. This can be overcome by a frequent change of the media or by measurement of the concentration of the drug remaining within the product, rather than that in the release medium (Burgess et. al., 2004a). The latter approach, however, may be neither feasible, nor economical, during studies involving implants. The implant cannot be expected to retain its original shape when removed from the apparatus after a few months of study, and would be lost permanently for further release profiling if data indicate the presence of high drug content. Variability in dissolution data could arise from the degradation of protein formulations or from acidic byproducts generated from polymers (such as poly (lactic-co-glycolic) PLGA matrices) into the media (Burgess et. al., 2004a). Biodegradation of polymers has been described as being a bulk process

consistent with 'autocatalysis', whereby the liberated carboxylic end groups catalyze further ester group cleavage (Pitt, 1980 and 1990). This description supports the hypothesis that physicochemical changes in the implant matrix, as a function of time, influence drug release characteristics. Besides chemical degradation, polymers also undergo enzyme-catalyzed degradation *in vivo*. Even relatively inert polymers (including nylon, poly (ether urethane), poly (terephthalate), poly (hydroxybutyrate), poly (e-caprolactone) or poly (glycolic acid), are degraded by enzymes such as esterase, proteinase, papain and elastase (Park and Shalaby, 1993).

During normal subcutaneous wound healing with implants, the implant may be smoothed surfaced, and chemically inert. If this is the case, a densely fibrous and relatively avascular tissue capsule will form around the implant within a few weeks that effectively walls off the implant from its environment (Wisniewski et. al., 2001; Stenken et. al., 2002). This process, called 'fibrous encapsulation', must also be considered. The fibrous capsule potentially imposes both diffusion and perfusion transport limitations that may render the implanted device less effective.

An additional potential barrier to diffusion results from the infiltration of proteinaceous material into the pores of compressed implants. This has been termed 'ghost' formation by Folley (1942) and occurs due to infiltration, followed by deposition, of tissue protein inside the pores of the surface of the matrix. Folley observed that when a fragment of a 'ghost' was burned, it swelled first and then charred giving off the smell of burning wood. This suggested that it consisted primarily of scleroprotein formed as a result of the reaction of animal tissues to the presence of a foreign body. Although there

are conflicting opinions whether plugging of the pores of the implant matrix would significantly affect drug release, due consideration may be necessary when viewing *in vitro* dissolution data mechanistically (Bishop and Folley, 1944; Lewin and Huidobro, 1953; Davis, 1974). Factors such as fluid volume, viscosity, tissue barriers, phagocytosis, tissue inflammation, etc. can also affect *in vivo* release and absorption. A look into the mass balance for such *in vitro* release tests has been suggested (Burgess et. al., 2004a), although problems may arise while withdrawing the implant from the apparatus after a prolonged period of testing.

An important factor affecting drug release is the physicochemical properties of the drug itself. For example, a slower release is observed for the same polymer system with increasing drug hydrophobicity. Furthermore, basic drugs may behave as catalysts, which could potentially enhance the degradation rate and hence the release rate. This is in contrast to the neutralization of the polymer's terminal carboxyl residues by basic drugs, thereby reducing autocatalysis due to acidic end groups (Malmsten, 2002). The various considerations for developing 'biorelevant' *in vitro* drug release tests for implants are listed in Table 3.

A verification of standards that specify the apparatus/agitation rate, medium, study design, assay, and acceptance criteria is mandatory to satisfy the performance tests of the USP. Overall, the procedure must yield data to allow an accept/reject decision relative to the set acceptance criteria. However, there is a need to develop a general guideline/recommendation on how to develop and validate a dissolution procedure. To

Table 3. Considerations for method development of 'biorelevant' *in vitro* drug release tests for implants

a) Suitable apparatus
b) Appropriate flow-rate/agitation
c) Medium of physiological relevance (pH, buffer capacity, osmolality)
d) Degradation of drug and dosage form
e) 'Ghost' formation
f) Drug metabolism
g) Tissue response such as fibrous encapsulation, inflammation, etc.
h) Determination of sampling interval
i) Accommodation of methods for accelerated release testing

achieve this objective, the USP is in process of incorporating a new general chapter. Aspects of method development and validation for dissolution studies that had been addressed somewhat superficially in other general information chapters viz. 1088 and 1225 have been examined in greater detail in the new general chapter. The chapter had been available for public comment as a Pharmacopeial Preview (2004). It will serve as a guide during assay method development for the investigation of drug release, but the chapter lacks the specifics that can be applied directly to special dosage forms such as implants. The discussion on dissolution media, for example, is entirely focused on method development for oral dosage forms.

1.5 Development of IVIVC for Implants

An *In vitro-In vivo* Correlation (IVIVC) adds *in vivo* relevance to *in vitro* data. For solid oral dosage forms with immediate release characteristics, drugs that are classified as Class II (low solubility and high permeability class) according to the FDA's BCS Guidance document are likely to be good candidates for an IVIVC (Kasim et. al., 2004). This is because, in most cases, *in vitro* dissolution will be the rate-limiting step for absorption of a drug in this class, and subsequently its appearance in *in vivo* circulation. Naltrexone is a low solubility-high permeability drug, and would therefore be categorized in BCS Class II. IVIVCs also tend to decrease the regulatory burden by reducing the number of biostudies required in support of a drug product. This would enable a faster and more efficient processing of regulatory filings. As an additional benefit to the developers, these correlations can support more liberal *in vitro* dissolution specifications,

wherever justified. IVIVC for controlled-release dosage forms would be beneficial if utilized in one or more of the following ways: (a) as a surrogate to bioequivalency studies which might be required for Scale-up and Post Approval Changes (SUPAC), especially where minor post-approval changes may include site of manufacture, formulation or strength; (b) to support and/or to validate the use of dissolution testing and specifications as a quality control tool for process control since dissolution specifications may be shown to be relevant to *in vivo* data; (c) predict *in vivo* performance of a formulation based on *in vitro* dissolution data, which may be used in the justification of dissolution specifications and may aid in the design of formulation release-time profiles resulting in optimal plasma concentration-time profiles; (d) identify appropriate dissolution conditions for a formulation which result in data relevant to *in vivo* performance (Rohrs et. al., 1997). At this point, it is imperative to point out that although few reports for successful IVIVCs with implants are available (Lin et. al., 2001; Schlieker et. al., 2004), none of the *in vitro* tests can be considered to be physiologically relevant.

An animal model is considered appropriate for the exploration of a possible IVIVC during drug development, although it cannot be used for demonstration of bioavailability or bioequivalence in the regulatory setting (Burgess et. al., 2004a). According to the FIP Guideline issued in 1997, a rank order correlation is a sufficient verification of a specification under the assumption that no quantitative interpolation is necessary (Siewert et. al., 2004).

New methods for the establishment of dissolution specifications for controlled release formulations have now been proposed in two FDA Guidances released in 1997.

For a comparison of dissolution profiles, Moore and Flanner (1996) proposed the 'model-independent' approach using the f1 (a function of the average absolute difference between two dissolution curves, referred to as a 'difference' factor) and f2 (a function of the reciprocal mean square-root transform of the sum of square distances at all points, referred to as 'similarity' factor) factors. The f2 factor has been recommended as a simple measure for the comparison of profiles (Shah et. al., 1998). The other method involves a 'model-dependent' approach using Probit, Logistic or Weibull fitting to dissolution data. Sathe et al. (1996) have found the model dependent approach to be useful for a comparison of inter-lot *in vitro* dissolution profiles. IVIVC modeling may also involve a time scaling approach in which the scale of the ordinate axis is shifted to account for the lag time prior to drug release, provided the time scaling factor remains the same across all formulations tested (Burgess, 2004b). In the study reported by Rackley (1997), the linear regression of absorption versus dissolution indicated a fairly large negative y-intercept, although a strong linear relationship was evident. Based on the concepts presented by Hwang et al. (1993), a shift in absorption data by -1.35 hour resulted in an intercept value close to zero. A report of the 2004 Annual Meeting of the Controlled Release Society suggests that a multivariate model-dependent comparison of data can be useful for the flow-through apparatus (Gray, 2004b). For oral controlled-release dosage forms, a multidimensional approach involving pH as the primary factor has been reported to be a better predictor of *in vivo* performance (Skelly et. al., 1986a and 1986b). During the bioequivalence characterization of implants however, it may not bear relevance unless a significant change in the microenvironment at the implantation site occurs. Young et al.

(2004) presented an approach that involved modeling sections of the pharmacokinetic profile of a drug having multiple peaks in its *in vivo* profile after administration as a microsphere parenteral formulation. At this point in time however, no single method exists for handling data that would be applicable to most implantable dosage forms and a case-by-case analysis is often reported in most literature. Besides plasma drug levels, approaches involving tissue concentrations for surrogate markers may also be useful for IVIVC development for implants.

1.6 Accelerated *In Vitro* Release Testing

Dissolution analysis of extended release formulations is time-consuming since real-time data collection would require the study to be conducted over a period of weeks or months. This is disadvantageous in early research and unacceptable for effective process control (Shameem et. al., 1999). While real time *in vitro* release studies under physiological conditions is essential to evaluate and register these systems, an accelerated (short-term) *in vitro* release method would be helpful for a rapid assessment of formulation and processing variables (Zackrisson et. al., 1995). The benefit of utilizing an accelerated method is twofold: (1) to differentiate formulations prepared from a similar polymer varying in molecular weight, drug loading, particle size, and morphology, and (2) to correlate short-term release with real-time release in order to predict real-time release. Accelerated tests should also be biorelevant if possible and the mechanism of drug release should not be altered. In these tests, only the rate of drug release should increase (Burgess et. al., 2002).

Although accelerated dissolution rate analysis (ACDRA) has been used for tablets especially during at-line tests that are conducted by the use of process dedicated testing equipment on the production line (Quist et. al., 2002; Scott, 2002), an in-depth investigation of this technique for controlled release parenterals including implants has not been reported. Of the various parameters (temperature, solvent, ionic strength, pH, enzymes, surfactants and agitation rate) that can be altered, the easiest way to achieve accelerated release has been reported to be an increase in temperature (Makino et. al., 1985). During these studies, a variety of factors affect the dissolution stability of the drug however. These include processing factors, formulation variables, drug and excipient solubility/hygroscopicity/thermal behavior, and the product packaging itself in terms of its moisture barrier properties. Further insight can be gained by an investigation of the drug's stability under elevated temperatures. Verification of the validity of using these conditions could include an Arrhenius plot after obtaining release rate profiles from linearized release profiles (Makino et. al., 1985). The question remains, however, what constitutes a significant change in the dissolution profile, since defined limits for percent drug remaining in the medium after a designated test time are not available thus far in any guidance (Storey, 1996).

The specifications for accelerated release should include a determination of an early time, mid-point time and >80% of the cumulative amount released. For a prediction between accelerated and real time release, it has been suggested that the time to reach a cumulative release of approximately 100% be used to determine whether a relationship can be established for products with different real time release rates (Burgess et. al.,

2004a). Since most implants are designed for drug release over a period of several months, waiting for this time period for complete drug release is not practical during product development. When a controlled release delivery system produces an initial burst release (shown in Figure 3), the recommendation is to augment accelerated release tests by an initial real time study that allows adequate assessment of this burst. Mathematical modeling to predict long-term release from accelerated release profiles has been suggested (Burgess et. al., 2002).

1.7 Research Significance

Pharmaceutical development and manufacturing has evolved into an endeavor that is science and engineering based. Using available knowledge effectively in regulatory decisions that establish specifications and evaluate manufacturing processes can substantially improve the efficiency of both manufacturing and regulatory processes (FDA Report, 2004). Characterization of the release profile of any drug from an implant constitutes an important step towards using the resulting data for *in vitro-in vivo* correlation and accelerated release testing. A universal test is normally sought for most areas of regulated science, and dissolution testing is no exception (Wood, 1992). Furthermore, an acceptable experimental design for establishing specifications for modified release products has been a high priority of researchers to assure product sameness under specifications set following the Scale-Up and Post-Approval Changes Guidance document (FDA, 2000).

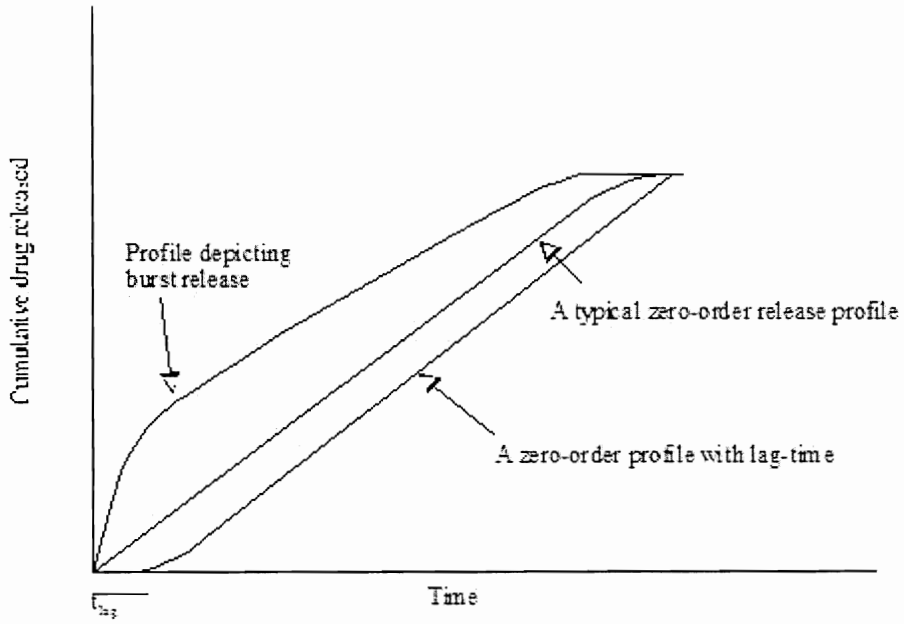


Figure 3. Schematic to show examples of different release profiles.

(Ref: Iyer et al., 2006).

In order to incorporate 'biorelevance' into implant drug release studies, this research has investigated an approach to apparatus design and media selection significantly different from conventional dissolution studies involving oral dosage forms. No published information of such work is currently available. As discussed previously, the constraints offered by maintenance of biorelevant conditions (apparatus and medium) provide less flexibility during method development. A real-time *in vitro* release study necessary to evaluate subcutaneous implant systems for physiological relevance has been described. This study also involved a comparison of drug release profiles obtained using the newly developed capillary cartridge device to that obtained using the commercially available flow-through apparatus. In addition, stability issues of the drug and dosage form have been addressed.

The development of an accelerated (short-term) *in vitro* release method has been another important goal of this project. This would enable a rapid assessment of formulation and processing variables during product development. An approach employing elevated temperatures has been investigated.

As Edward Teller, the Hungarian-born American nuclear physicist has well stated, "The science of today is the technology of tomorrow". Knowledge derived from this work will ultimately be useful in improvement of the biopharmaceutical characterization of drug products, and as a potential tool to assure consistent product and/or batch-to-batch quality within a defined set of criteria in the pharmaceutical industry. A standardization of the real-time drug release procedure for implants and an investigation of accelerated conditions will prove significant for drug product

development and dosage form rationalization. Thus, for the near future, the methods followed for drug release testing of an implant should be expected to vary considerably from that followed for an oral dosage form.

Chapter 2 of this dissertation describes a novel Liquid Chromatography - Tandem Mass Spectrometry (LC-MS-MS) method for the analysis of naltrexone in dog plasma. The LOQ of the method was established at 10 pg/mL. Based on a Molecular Modeling approach, a prediction has been made about the influence of the number and position of deuterium substitutions on isotopic internal standard matrix effects.

Chapter 3 describes optimization and characterization of a modified Hanks' Balanced Salts formula that was used as the drug release medium. It involves a detailed analysis of pH, osmolaity and spectral changes of the medium as a function of time and temperature. Also described is the development and validation of a stability-indicating High Performance Liquid Chromatography method employed for determination of the time periods for media replacement, and for the analyses of *in vitro* samples generated during release studies.

Chapter 4 describes the modification and assembly of a new miniature, capillary device to assess real-time drug release from a naltrexone implant. The results of this device were compared to those obtained using a flow-through cell. An implantation study in dog was conducted to evaluate *In vitro-In vivo* Correlation (IVIVC). A macroscopic

and histological assessment of changes in subcutaneous tissue at the site of implantation is also incorporated.

The investigation of accelerated drug release testing using an approach based on elevated temperature is provided in Chapter 5.

Chapter 6 summarizes this research and provides an overall conclusion.

CHAPTER 2 A Liquid Chromatography-Electrospray Tandem Mass Spectrometry Method for the Analysis of Naltrexone in Canine Plasma Employing a Molecular Model to Demonstrate the Absence of Internal Standard Deuterium Isotope Effects.

2.1 Introduction

Naltrexone (NAL), an opiate receptor antagonist, has been marketed as an oral tablet for the clinical management of opiate addiction and as adjunctive treatment for individuals with alcohol dependence (Gonzalez and Brogden, 1988; O'Malley et al., 1992; Volpicelli et. al., 1992). However, patient non-compliance to dosage schedules is a difficult obstacle to treatment. Two factors, namely, the complete abstinence of opioid-induced reinforcing effects and the absence of adverse consequences (unlike methadone) upon discontinuation of medication are primarily responsible for patient non-compliance (Mark et. al., 2003). To overcome this problem, dosage forms such as implants and depots are under different stages of development in order to have a long-acting alternative to the tablet (Comer et. al., 2002; Stinchcomb et. al., 2002; Bartus et. al., 2003).

An early report on bioanalytical methods for naltrexone is available as a National Institute on Drug Abuse (NIDA) monograph (Research Monograph Series Number 28, 1981). This report includes an electron-capture gas chromatographic assay developed by Reuning et al., Karl Vereby's Gas-Liquid Chromatographic assay in different matrices as well as a TLC procedure described by Wall and Brine. These methods involved large

sample volumes and complicated sample preparation techniques. The mid-90s saw the development of various methods on High Performance Liquid Chromatography (HPLC) with UV or amperometric detection (Peh et. al., 1997; Hurst et. al., 1999; Kambia et al., 2000).

In recent years, there is renewed interest among physicians in exploring treatments with low doses of naltrexone, as evident from the website on Low Dose Naltrexone (Bihari, 2004). In addition, through the use of a dual-detector system with positron-emitting radioactive drugs, Lee et al. (1988) established that naltrexone had a significantly longer half-life due to receptor occupancy as compared to its estimated plasma half-life. This has promoted a larger number of pharmacokinetic studies that pose additional challenges to the analytical chemist to develop more sensitive and selective methods for quantification. Publications on Gas chromatography-mass spectrometry (GC-MS) and Liquid chromatographic-mass spectrometry (LC-MS-MS) for estimation of naltrexone and its major metabolite, 6β-naltrexol, in various biological matrices report Lower Limits of Quantification (LLOQ) in the range of 0.1-2 ng/ml (Monti et al., 1991; Buggé et. al., 1996; Chan et. al., 2001; Naidong et. al., 2002). No report is available however for determination of naltrexone in the low pg/ml range in dog plasma. Therefore, to assess the pharmacokinetics of naltrexone in a dog study that was conducted, it was necessary to develop this method for analysis of dog plasma samples.

The use of stable isotope-labeled analogs as internal standards is highly recommended because similar relative efficiencies of ionization of the two molecular species would be expected to compensate for any matrix effect (Chavez-Eng et. al.,

2002). In an effort to better understand the persistence of matrix effects in spite of the use of deuterated internal standards, a molecular modeling approach to evaluate or predict isotope effects in a normal-phase chromatographic separation had been investigated (Iyer et al., 2004).

In this chapter, a study of naltrexone using a similar modeling approach is described as a means to evaluate whether or not an uncompensated matrix effect would be expected.

2.2 Experimental

2.2.1 Chemicals and Reagents

Naltrexone hydrochloride (working standard) was obtained from Sigma (St. Louis MO, USA). [15,15,16-^{2}H]-naltrexone was procured under license from the National Institute in Drug Abuse (NIDA), MD, USA, for use as an internal standard (IS). Figure 4 shows the structure of NAL. Analytical grades of formic acid, ammonium hydroxide and trifluoroacetic acid were purchased from Sigma (St. Louis, MO, USA). Methyl tert. Butyl ether (MTBE) and HPLC grades of Acetonitrile and Methanol were purchased from Burdick & Jackson (Honeywell International, Inc. MI, USA). Water was obtained in-house using the Nanopure Diamond water system (Barnstead International, IO, USA).

2.2.2 Stock Solution and Stock Dilutions

Approximately 10 mg. of working standard was accurately weighed and transferred into a 10 ml volumetric flask. It was dissolved in methanol:water (50:50, v/v)

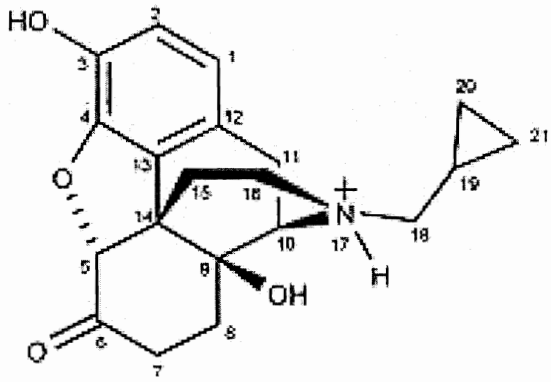


Figure 4.

Naltrexone, protonated under acidic conditions

to yield a stock solution, the exact concentration of which was corrected for naltrexone freebase. The stock solution was stored below 10°C in a refrigerator. A serial dilution was performed in the same solvent to yield stock dilutions ranging from 0.20 to 100.28 ng/ml, immediately prior to fortification into plasma.

2.2.3 Preparation of Calibration Curve (CC) and Quality Control (QC) Standards

Dog plasma from two batches (containing sodium heparin as anticoagulant) was thawed and pooled to provide the matrix for the study. Spiking (5%, v/v) of the Stock Dilutions yielded Calibration Curve standards at 10, 25, 50, 100, 501, 1003, 2507 and 5014 pg/ml in the plasma. Similarly, Quality Control samples were prepared at 10, 29, 361 and 4512 pg/ml representing LOQ-QC, Low (LQC), Middle (MQC) and High (HQC) controls, respectively. Aliquots were stored in capped tubes below -50°C until analysis. The storage duration was less than one week. Frozen plasma samples containing naltrexone are reported to be sufficiently stable (only 15% degradation observed after 576 days), hence a detailed evaluation of stability was not conducted for the purpose of the pilot pharmacokinetic study (Buggé et. al., 1996; Moody et. al., 1999).

2.2.4 Sample Extraction

Prior to analysis, the Calibration Curve standards & Quality Control samples were thawed at room temperature. 0.5 ml of each sample was transferred to a capped glass tube and 10 µl of Internal Standard dilution (20 ng/ml of NAL-d_3) was added, and vortex mixed. 5 ml of ammonium hydroxide (0.6%, v/v) in MTBE was then added. Basic

conditions were employed to maintain the analyte molecule in its non-ionized state, thereby preferentially partitioning the analyte into the organic phase. The samples were placed on a laboratory rotator and spun at 30 rpm for 15 minutes. After allowing approximately 2 minutes of standing to facilitate separation of the aqueous and organic phases, these were placed in a refrigerated circulator until the aqueous layer was frozen. The supernatant (organic layer) was poured into conical-bottomed tubes. These were evaporated to dryness at 50°C for 10 minutes under a stream of dry nitrogen at 10 psi. The residue was reconstituted in 100 µL of acetonitrile: water (50:50, v/v) and transferred into 200 µl silanized glass vials for analysis.

2.2.5 LC-MS-MS Conditions

The Shimadzu *VP* LC system consisted of a system controller (SCL-10A), two high-pressure pumps (SIL-10AD, designated as Pumps A and B) and an autosampler (SIL-10AD). Chromatography was carried out using a Polaris Silica column (5 µm; 2.1 x 50 mm, purchased from Thermo-Electron, Inc., PA, USA). The reservoir of Pump contained water with formic acid (1%, v/v) and trifluoroacetic acid (0.001%, v/v) added. Acetonitrile was employed as the reservoir of Pump B. The linear gradient employed at a flow rate of 0.35 ml/minute is described in Table 4. Acetonitrile: water (50:50, v/v) was used as a rinse solution for the injector, and the injection volume was fixed at 50 µl.

A TSQ 7000 triple-quadrupole mass spectrometer (Finnigan MAT, CA, USA) was used with Excalibur ver. 1.2 software for data acquisition and analysis. Electrospray

Table 4. Gradient Conditions for the Mobile Phase

Time (minutes)	% Pump A	% Pump B	Curve
0.10	5	95	0
1.25	100	0	1
2.50	100	0	0
2.51	5	95	0
3.00	5	95	0

Ionization (ESI) in the positive ion mode was employed. The Multi-Reaction Monitoring (MRM) transition at 342-324 for naltrexone was optimized for response, and fixed at 345-327 for [15,15,16-^{2}H]-naltrexone.

The two transitions were confirmed for the absence of any "cross-talk" before subsequent optimization steps. This was performed by infusion of a 1 μg/ml solution of one compound in methanol, and checking for the absence of the other transition, and vice-versa. The same drug solution was infused for an iterative, manual optimization of other instrument parameters to the following values - Source: 5 kV, extractor: 37 V, manifold temperature: 70°C, and capillary temperature and voltage were set at 275°C and 20V, respectively. Nitrogen was used as the sheath gas at 70 psi, and purified air (zero grade) optimized to 2 mT was employed in the collision cell.

A post-column infusion experiment was then conducted following the method of Bonfiglio et al. (1999). A 10 ng/ml solution of naltrexone was prepared in acetonitrile: water (50:50, v/v), and was continuously infused at 5 μL/minute into the mass spectrometer using a 'tee', the third end of which was connected to the injector. Upon stabilization of the baseline response, a processed sample of blank dog plasma was injected and the resulting profile was evaluated for any suppression of ionization.

2.2.6 Molecular Modeling

Molecular modeling investigations were carried out using the approach described in the earlier work (Iyer et. al., 2004). An updated version of SYBYL ver. 7.2 was used. For modeling the deuterated analyte (NAL-d_3), the force constant and bond length

parameters defining the C.3 (sp^3 hybridized carbon)-D bond in the Tripos force field were modified to 800 and 1.075 A°, respectively, as opposed to 662.4 and 1.100 A° for the C.3-H bond as reported previously. Furthermore, it was necessary to account for the charged, ionized state of the nitrogen at the 17^{th} position at the pH of the mobile phase. This was achieved in SYBYL by changing the hybridization state from N.3 to N.4. The two molecules were drawn and the energy minimized. Each analyte was rolled over the stationary phase surface and a 3-D visual examination was conducted to assess qualitatively the likelihood of analyte-stationary phase interactions. Twenty-five (25) models were created in such a manner. Bond length constraints were applied for some positions of hydrogen bonds but were removed prior to the final energy minimization step. This was again followed by energy minimization of the resulting complexes to a gradient step-size of 0.05 kcal/mole. In Figure 5, representative positions of naltrexone interacting with the stationary phase are depicted. Finally, energy calculations were performed for each position as described in the following section.

2.2.7 Binding Energy Calculations

Energy calculations used the Tripos force field in SYBYL (Force Field Manual *ver 6.6*, 1999). The total energy E_{total} for an arbitrary geometry of a molecule derived from a force field is given by the sum of energy contributions. For the Tripos force field, this can be represented as:

$$E_{total} = \Sigma E_{str} + \Sigma E_{bend} + \Sigma E_{oop} + \Sigma E_{tors} + \Sigma E_{vdw} + \Sigma E_{elec} + \Sigma E_{constraints} \quad \ldots\ldots (2)$$

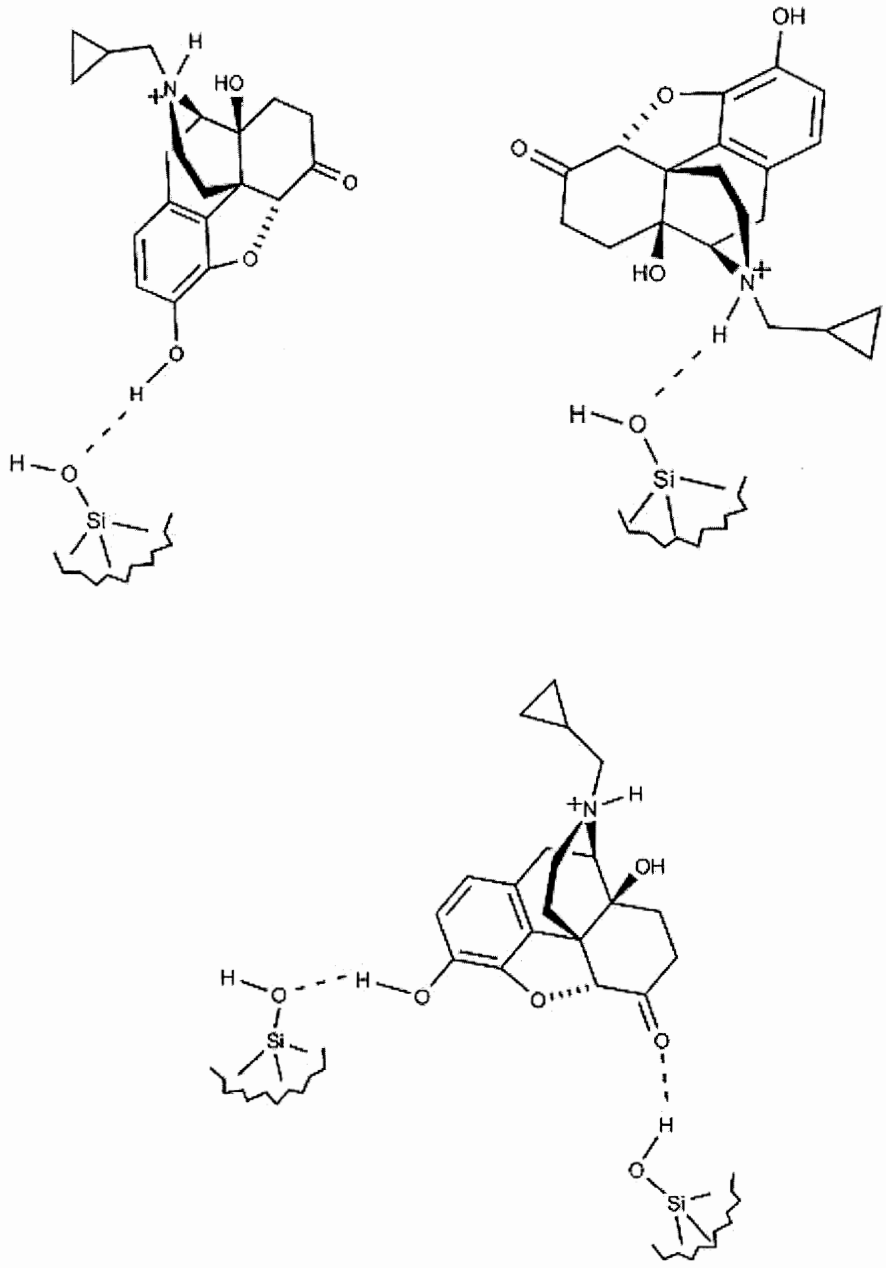


Figure 5.

Representative positions of naltrexone interacting with the stationary phase

where the sums extend all over the bonds, bond angles, torsion angles and non-bonded interactions between atoms not bound to each other or to a common atom, and,

E_{str} = Bond stretching energy term,

E_{bend} = Angle bend energy term,

E_{oop} = Out of plane bending energy term due to the bending of bonds from their natural values,

E_{tors} = Torsional energy term due to the twisting of bonds,

E_{vdw} = Van der Waals energy term arising due to non-bonded interactions,

E_{elec} = Electrostatic energy term,

and $E_{constraints}$ = an energy term for the artificially inserted constraints (if any).

The Tripos force field treats the hydrogen bonds as non-directional and electrostatic in nature. To accommodate this, calculations in which hydrogen bonds are expected to be important include partial charges and the electrostatic contributions. Hydrogen bond energies are included in the evaluation of the force field by scaling the Van der Waals interactions between N, O and F and hydrogens bonded to N, O or F.

Thus, the binding energy (ΔE) for each position can be calculated as:

$$\Delta E = (E_{analyte} + E_{stationary\ phase}) - (E_{analyte+stationary\ phase}) \quad \ldots\ldots (3)$$

where $E_{analyte}$, $E_{stationary\ phase}$ and $E_{analyte+stationary\ phase}$ represent the total energies of the free unbound analyte, unbound stationary phase and the bound analyte-stationary phase complex, respectively. During the calculation of average binding energy, any molecular

model for analyte-stationary phase complexes that exhibited positive ΔE values were omitted from further consideration, since these are not energetically possible.

2.3 Results and Discussion

Figure 6 shows a representative chromatogram each of blank, blank with internal standard and LOQ samples. The blank plasma sample was devoid of any interference at the retention time of naltrexone and internal standard. Also, no interference of naltrexone was observed from the internal standard in the blank sample fortified with IS. The post-column infusion experiment resulted in a relatively low amount (approximately 25%) of ion suppression at the retention time of the analyte (Figure 7). This indicated that optimal sample clean up and chromatography had been achieved.

The calibration curves were linear in the range of 10-5014 pg/ml ($r^2 > 0.98$) using a weighting factor of 1/concentration. The concentration residuals were between 92.4%-108.9% (RSD = 1.9%-9.8%). The data are summarized in Table 5. The LLOQ of the method was tested at 10 pg/ml by injection of plasma samples prepared and processed independent of the calibration curve. The deviation was found to be 9.8% of the nominal concentration (RSD = 4.8%; n = 6). The precision and accuracy of the method were calculated as the Relative Standard Deviation (%RSD) and percent of nominal value. The global inter-day precision and accuracy for 3 batches (n = 9) were found to be between 10.2%-14.7%, and 93.8%-101.5%, respectively [refer to Table 6].

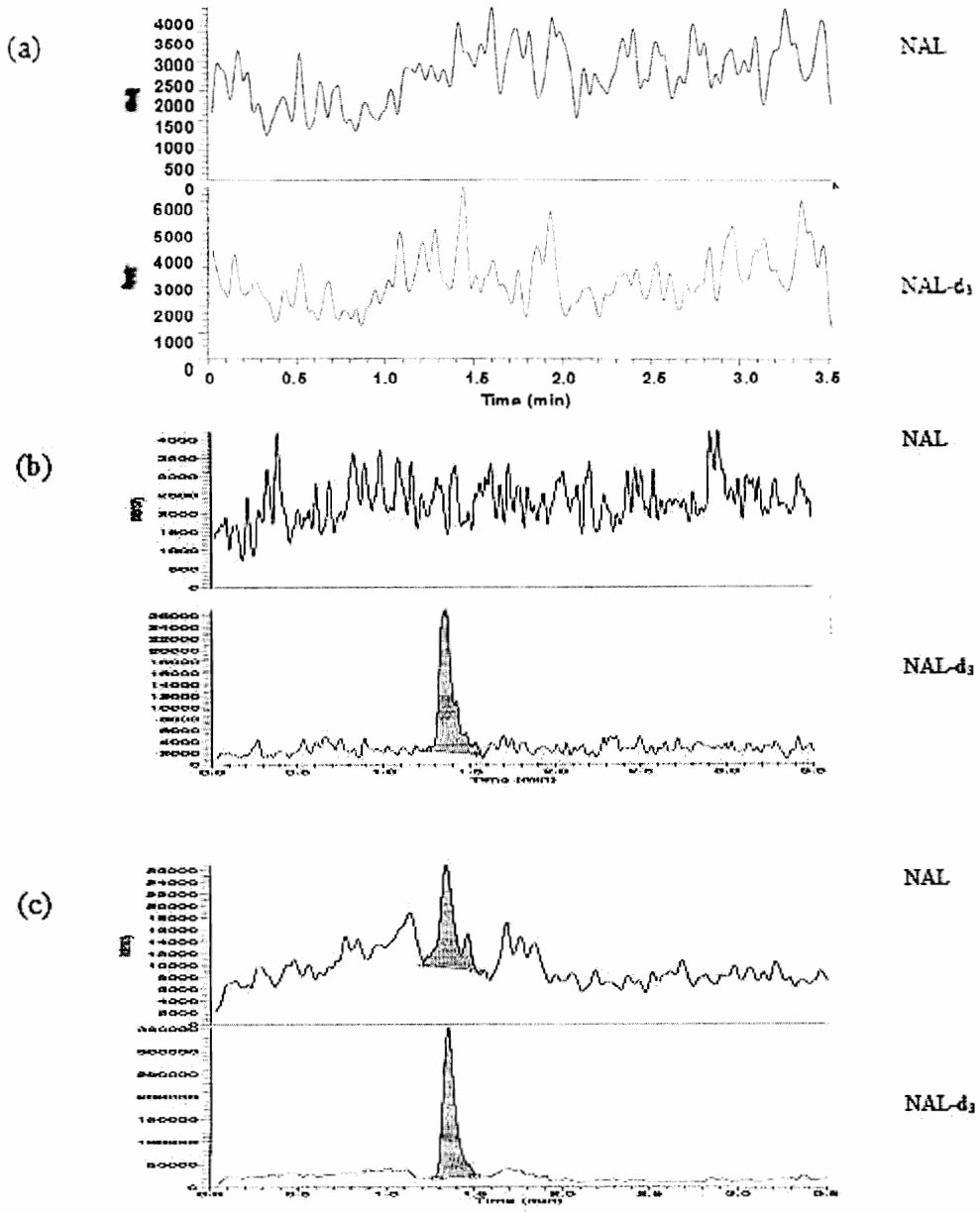


Figure 6. Representative chromatograms of (a) blank, (b) blank with internal standard and (c) a LOQ sample

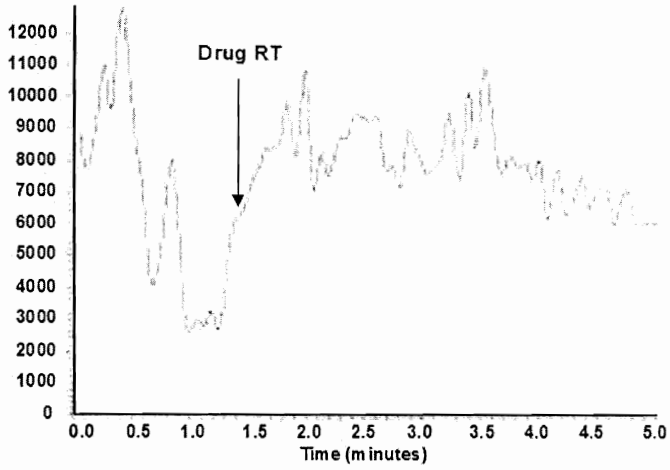


Figure 7

Ion suppression profile after post-column infusion of naltrexone

Table 5. Reverse predicted concentration residuals of naltrexone

Set #	Nominal Concentration (pg/mL)								r^2	Slope	Intercept
	10	25	50	100	501	1003	2507	5014			
1	10	23	54	95	502	1064	2165	5270	0.9880	0.0050	0.3067
2	12	27	45	98	451	979	2533	5143	0.9980	0.0216	0.1238
3	11	27	53	106	435	960	2275	5334	0.9923	0.0001	0.0168
Mean	11	25	51	100	463	1001	2325	5249			
SD	0.6	2.3	5.0	5.2	35.1	55.5	189.1	97.1			
CV (%)	5.6	9.2	9.8	5.3	7.6	5.5	8.1	1.9			
% nominal	108.9	101.5	101.9	99.7	92.4	99.8	92.7	104.7			

Table 6. Precision and accuracy data of naltrexone

Set #	Nominal Concentration (pg/mL)		
	29	361	4512
1	27	311	4989
	25	309	4384
	34	375	3591
Mean	29	332	4321
SD	4.3	37.6	701.5
CV (%)	15.0	11.3	16.2
% nominal	99.0	91.8	95.8
2	23	369	4069
	33	346	4719
	25	406	4103
Mean	27	374	4297
SD	5.2	30.3	365.7
CV (%)	19.2	8.1	8.5
% nominal	93.6	103.5	95.2
3	32	309	3952
	30	320	4583
	35	302	4668
Mean	32	310	4401
SD	2.6	9.0	391.2
CV (%)	8.1	2.9	8.9
% nominal	111.9	85.9	97.5
Global Calculation			
Mean	29	339	4340
SD	4.3	37.2	443.8
CV (%)	14.7	11.0	10.2
% nominal	101.5	93.8	96.2

The recovery of the method was determined by a comparison of peak heights of processed samples at the Middle Quality Control level to the mean peak height of samples spiked at the same concentration level in reconstitution solution. The mean recovery was 40.9% (RSD = 1.3%; n = 5).

Immediately at the end of one of the analytical batches, a blank sample was injected to check for carry-over from the previous injection. The carry-over level was less than 5% of the mean peak height of the LOQ indicating minimal attachment of the analyte to exposed chromatographic surfaces and tubing.

The molecular models, created as described above, were examined qualitatively for analyte-stationary phase interactions upon energy minimization. There are, in fact, nearly an infinite number of positions at which the analyte may have interactions with the stationary phase. One method of limiting the search however is to specify distance constraints between pairs of atoms. Assuming that a set of molecules presents a common range of distances between two particular molecular features that are chemically relevant, the search can be reduced to the exploration of the restricted conformational space defined by the acceptable range for this particular inter-atomic distance. For a hydrogen bond, this distance is between 1.7A^{o} and 2.3A^{o}. Different models of the bound analyte with the stationary phase revealed a different number and position of the hydrogen bonds. A few representative positions are shown in Figure 6. Out of 25 models created for naltrexone and [15,15,16-^{2}H]-naltrexone, 4 positions showed positive binding energies and were discarded as discussed in the previous section. The mean binding energies of the compounds along with their retention times are shown in Table 7 and Figure 8.

Table 7. A comparison of binding energies of the two compounds

Compound	Retention time (minutes)	Binding energy (kcal/mole)
Naltrexone	1.32 (0.01)	-10.22
[15,15,16-^{2}H] naltrexone	1.31 (0.01)	-10.26

* Values in parentheses represent standard deviation; n = 5.

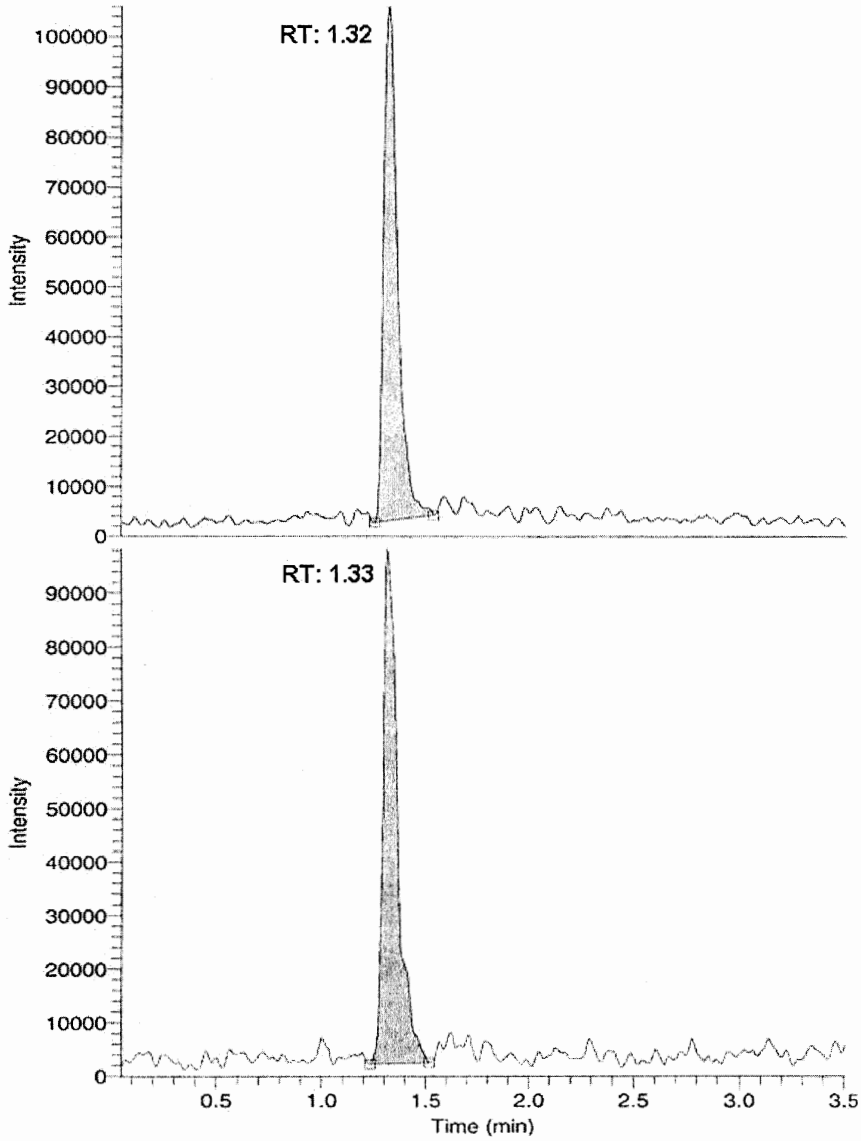


Figure 8

A representative chromatogram of a system suitability sample to demonstrate unresolved peaks for the drug and IS.

The calculated binding energy difference was found to be only 0.03 kcal/mole; thus it is expected that the two analytes should not exhibit different retention behaviors in the column. This calculation is consistent with the experimental observation.

Furthermore, upon a comparison of binding energy difference and chromatographic resolution of the isotopologs of naltrexone, olanzapine and des-methyl olanzapine (the latter two analytes had been employed in an earlier study (Iyer et. al., 2004), it was found that naltrexone had the lowest value for binding energy difference, a trend consistent with the resolution obtained experimentally. The data with a linear relationship are represented in Figure 9. Although both olanzapine and naltrexone have 3 hydrogen atoms substituted for deuterium, the major difference lies in the presence of quaternary nitrogen in naltrexone and the clustering of deuterium atoms near the charged nitrogen. These results are consistent with the findings of Zhang et. al. (2002). Studies with deuterium substituted analogs at multiple positions will be necessary to further validate this approach; nevertheless, it is speculated that during development of quantitative LC-MS-MS methods, the number and position of deuterium atoms on an isotopically labeled internal standard needs consideration for its ability to compensate for any matrix effect.

2.4 Application to a Pilot Implantation Study in Dog

The analytical method was employed for the analysis of dog plasma samples collected from a pilot study involving subcutaneous implantation of a biodegradable dosage form of naltrexone. This has resulted in an excellent pharmacokinetic profile. The

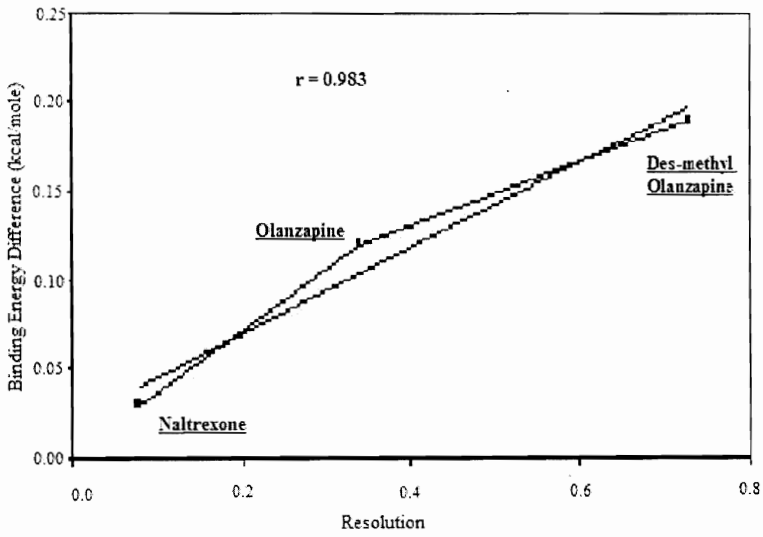


Figure 9.

Linear Relationship of the Binding Energy Difference between Isotopologs to their Chromatographic Resolution

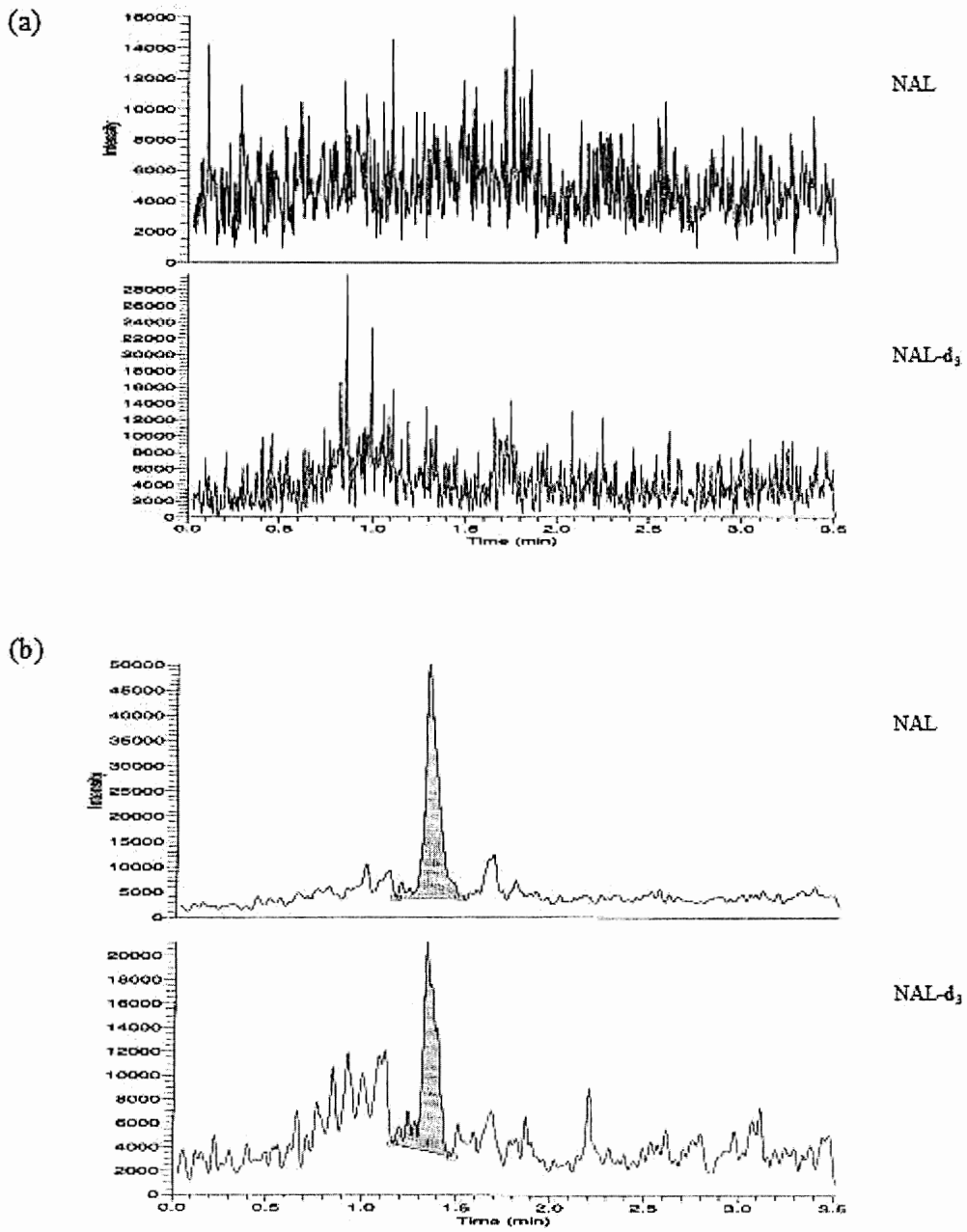


Figure 10.

Chromatograms of (a) pre-dose, and (b) Day-2 post implantation dog samples.

pre-dose plasma sample showed no interference at the retention time of naltrexone and [15,15,16-^{2}H]-naltrexone and the chromatogram of the Day-2 sample has been shown (Figure 10).

2.5 Conclusion

The LC-MS-MS method has been shown to be selective, sensitive, accurate and precise for quantification of naltrexone in dog plasma samples. It involves a simple, single step extraction procedure and therefore, is easily applicable to routine pre-clinical sample analyses.

Additional information obtained through molecular modeling provides a useful, semi-quantitative understanding of analyte-stationary phase interactions. The binding energies calculated from the models explain identical retention times of the deuterated and non-deuterated analogs observed experimentally. Further molecular modeling studies on a series of internal standards that have different number and positions of isotopic substitutions are necessary to create a basis for their selection in quantitative LC-MS-MS analyses.

CHAPTER 3. Characterization of a 'Biorelevant' Medium for *In Vitro* Release Testing of a Naltrexone Implant, Employing a Validated Stability-indicating HPLC Method

3.1 Introduction

The identification of appropriate media for *in vitro* studies of dosage forms is critical to the assessment of drug release (Burgess et. al., 2004). Corrigan et. al. (2003) observed that the sensitivity of *In vitro-In vivo* Relationships (IVIVR) to medium composition could be attributed to systematic shifts in release profiles as a function of changes in pH and buffer capacity. These workers also reported that difficulties exist in making a rational choice for a dissolution medium with well-characterized components capable of resisting changes in pH over the entire period of study.

Iyer et. al. (2006) had reviewed method development strategies for profiling *in vitro* drug release from subcutaneous implant dosage form. Among various factors listed, an understanding of physiological variables at the site of implantation was given priority for the design of drug release tests that provide more clinically meaningful specifications. Also, the potential for use of buffers that would mimic physiological condition *in vivo* was recognized. An assessment of the literature, however, revealed that most *in vitro* release methods to study implants did not utilize 'biorelevant' media (Halstead et. al., 1985; Okabe et. al., 2003; Ikegami et. al., 2003). In support of this underlying need, it was decided to employ the Hanks' Balanced Salts Solution (HBSS), with suitable modification, for the present *in vitro* release study of a naltrexone implant dosage form.

In this endeavor, it is important to consider the properties desired of an ideal drug release medium (Iyer et. al., 2006). Extracellular interstitial fluid differs from plasma because it contains fewer protein ions, primarily due to the impermeable character of a normal capillary membrane. Also, Na^+ and Cl^- form the most abundant ions in extracellular fluid (Tortora et. al., 1996). The role of Hanks' Balanced Salts Solution (HBSS) has been well documented in several papers on maintenance of tissue viability (Waymouth, 1954; Waymouth, 1970; Collier et. al., 1989; Stinchcomb et. al., 2002). Hanks established the formula for this Solution (Table 8) in 1948, an application for which was shown in tissue culture experiments the following year (Hanks, 1948; Hanks and Wallace, 1949). The phosphate and bicarbonate equilibria are responsible for maintenance of the pH in the physiological range (~ 7.4). Primary considerations for selection of HBSS as the release medium of choice for the present study were: (a) The simplicity of the formula and well defined components, (b) a comparable inorganic phosphate concentration to the human plasma level (Documenta Geigy, 1970; Krebs, 1950), and (c), adequate solubility of naltrexone (Stinchcomb et. al., 2002) to ensure 'sink' conditions throughout the period of study. Modifications to media composition were incorporated, as detailed in the Experimental section.

Naltrexone, an opiate receptor antagonist, has been identified as a candidate for formulation as an implant to overcome patient non-compliance to dosage schedules of the tablet (Brewer et al., 1988). Various research groups are currently investigating the development of dosage forms, such as implants and depots, to provide a long-acting alternative to the tablet (Comer et. al., 2002; Brewer, 2002; Hulse et. al., 2004).

Table 8.

Composition of the Hanks' Balanced Salts Solution with modifications performed for its application to an *in vitro* drug release study

Component*	g/l
$CaCl_2.2H_2O$	0.185
$MgSO_4$ (anhyd.)	0.09767
KCl	0.4
KH_2PO_4 (anhyd.)	0.06
NaCl	8.0
Na_2HPO_4	0.04788
Glucose	1.0

*Deletions: Phenol red and $NaHCO_3$
Additions: HEPES buffer (25 mM) for enhanced buffer capacity
Primocin (0.2%, v/v), an antimicrobial agent

**pH adjusted to 7.40 ± 0.05

Trissel reports the stability for a compounded oral product of naltrexone (Trissel, 2000). The analytical method employed for that evaluation had been developed by Fawcett et al. (1997), comprising a High Performance Liquid Chromatography (HPLC) procedure for detection of naltrexone at an absorbance detector wavelength of 214 nm. The compendial monograph cites a reversed-phase HPLC assay for naltrexone hydrochloride powder and tablets (USP28/NF23, 2005). Other workers have employed method modifications to HPLC assays to suit specific needs (Aungst et. al., 1987; Hussain et. al., 1998; Stinchcomb et. al., 2002). A selective and sensitive method is not available however, for quantification of naltrexone in Modified Hanks' Balanced Salts Solution as employed in this study.

This chapter describes a characterization of pH, osmolality, buffer capacity and spectral changes for Modified Hanks' Balanced Salts Solution for its suitability as a 'biorelevant' medium. The dosage form was a biodegradable, subcutaneous implant of naltrexone. Three temperature levels viz. 38°C (canine body temperature), 45°C (an intermediate level), and 55°C (determined by the melting point range of the polymeric implant matrix, 59-64°C) besides 25°C (room temperature) have been targeted for media characterization.

3.2 Experimental

3.2.1 Chemicals and Reagents

Naltrexone hydrochloride (working standard) was obtained from Sigma (St. Louis MO, USA). Hanks' Balanced Salts, 4-(2-hydroxy ethyl) piperazine-1-ethanesulfonic acid

(HEPES buffer, 1 mM), and analytical grades of triethylamine and orthophosphoric acid were purchased from Sigma (St. Louis, MO, USA). Primocin was procured from InvivoGen (CA, USA). Potassium phosphate (monobasic, anhydrous) and sodium hydroxide were purchased from Fisher Scientific (Fairlawn, NJ, USA). Acetonitrile (HPLC grade) 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).

3.2.2 Description of the Modified Hanks' Balanced Salts Solution

A modification of Hanks' Balanced Salts involved deletion of two components: phenol red and sodium bicarbonate. Phenol red is an indicator that potentially interferes in chromatographic separations (Chaplen et. al., 1996). Sodium carbonate provided additional buffer capacity through a continuous bubbling of CO_2, such that the O_2/CO_2 exchange processes in living tissue cells could be simulated. Since bubbling gas was not a viable option during this study, and because no cell culture was involved, 4-(2-hydroxy ethyl) piperazine-1-ethanesulfonic acid (HEPES) was employed as an enhancer of buffer capacity (Vega and Bates, 1976). A higher buffer capacity could be anticipated as a necessity because a future objective of the project was to investigate drug release under accelerated conditions. HEPES has a pk_a of 7.3 at 37°C, and is commonly used to arrest the drift in pH of physiological media (Shipman, Jr., 1969; Lelong and Rebel, 1998). In addition, since the intention was to use the medium for at least a month, it became essential to incorporate an anti-microbial agent to its formula. Most common antibiotics

have a shelf life not greater than one week at mammalian body temperature (Freshney, 1993). Therefore a new antimicrobial agent, Primocin (0.2%, v/v), was identified. Primocin has a broad-spectrum of activity against Gram (+) and Gram (-) bacteria, fungi and mycoplasma, and is stable for at least a month at 37°C (Product Specifications: Primocin™, Invivogen, CA, USA).

The following procedure was adopted for media preparation: 9.8 grams of the commercially available Modified Hanks' Balanced Salts was accurately weighed and dissolved in 975 ml of water. The resulting pH of 6.6 $\pm$ 0.2 was adjusted to 7.4 $\pm$ 0.1 with sodium hydroxide (10 mM) in water. 25 ml of HEPES solution (1M) was then added, followed by 2 ml of Primocin. The solution was mixed throroughly and filtered through a 0.45 μm filter. Finally, the pH was again adjusted to 7.40 $\pm$ 0.05, if required, at the designated study temperature.

3.2.3 Medium characterization

3.2.3.1 pH changes as a function of temperature

Changes in pH were monitored using a pH meter (Model 340; Corning Instruments, NY, USA) equipped with probe for temperature correction. 200 ml of medium in a stoppered flask was placed on a magnetic stirrer with temperature control. Continuous stirring at slow speed and a stepwise gradient increase of 1°C were employed. Temperature measurements were made with a thermocouple thermometer (Model 600-1040; Barnant Company, IL, USA) having a least count of 0.1°C. A time of 5 minutes was fixed for equilibration at each step before the stirring was stopped, and the pH

recorded immediately. Stirring influences pH measurements, at times, by affecting any existing junction potential adversely (Presley, 1999). The pH meter was also re-calibrated at each temperature level with standard buffers maintained at the same temperature. This ensured that the pH drift of the electrode had been corrected (Presley, 1999). Recordings of pH were made between 24°C and 56°C. When the highest temperature was attained, to crosscheck the readings, a downside gradient of 1°C step size was made to 24°C, while the pH was recorded as before. The complete procedure was repeated in triplicate to ensure reproducibility. An inert gas atmosphere was not used in the headspace of the flask. This was because the purpose of the study was to simulate real-time drug release, conditions under which the medium is expected to be in equilibrium with atmospheric carbon dioxide and oxygen.

3.2.3.2 Buffer Capacity changes as a function of temperature

An incremental addition of sodium hydroxide solution (10 µl, 10mM) was made to 200 ml of the medium, maintained at a fixed temperature. pH determinations were carried out at the temperatures, 38°C, 45°C, and 55°C as mentioned earlier, along with an assessment at room temperature. Also, the efficiency of HEPES was evaluated by testing HBSS alone.

3.2.3.3 Ultraviolet spectrum changes as a function of temperature and time

Absorption changes were evaluated using a UV/Visible spectrophotometer (Model Lambda 2S; Perkin-Elmer Instruments, MA, USA) in the wavelength range of

190-300 nm. Any potential interfering contamination due to naltrexone sticking to cuvettes was avoided by thorough rinses with water and methanol between consecutive measurements. A spectrum of the Modified Hanks' Balanced Salts Solution was recorded on the day the medium was prepared (Day-0), and compared to spectra obtained on days 15, 30, 60 and 90 post-storage of aliquots maintained at room temperature, 38°C, 45°C and 55°C. Prior to spectral evaluation, the aliquots were cooled to room temperature to prevent errors arising out of changes in absorptivity. A freshly prepared medium was treated each time as a reference along with test media to check for any baseline drift.

3.2.3.4 Osmolality Changes as function of temperature and time

Osmolality studies were conducted using an Osmometer (Model 3W; Advanced Instruments, Inc., MA, USA) based on depression in freezing point. The meter was calibrated at 100 and 900 mOsm using standards supplied by the vendor. Care was taken to set the temperature of standards and test solutions at the same values prior to testing. A 2.0 ml aliquot of solution was analyzed each time. Osmolality was recorded on the day medium was prepared (Day-0), and on days 7, 15, 30, 60 and 90 for aliquots stored at room temperature, 38°C, 45°C and 55°C. On each of the days, a freshly prepared medium was also tested to check for any instrument variations from day-0.

3.2.4 Validation of the HPLC analytical method

A reversed-phase HPLC method was developed and validated for analysis of the *in vitro* samples. It employed UV detection and involved minimal sample preparation time.

3.2.4.1 Preparation of stock solution

Approximately 10 mg. of naltrexone hydrochloride working standard was accurately weighed and transferred into a 10 ml volumetric flask. It was dissolved in methanol: water (50: 50, v/v) to yield a stock solution, the exact concentration of which was corrected for naltrexone freebase. The stock solution was stored below 10°C.

3.2.4.2 Preparation of Standards and Quality Control samples

From the stock solution, a serial dilution was performed in Modified Hanks' Balanced Salts Solution to yield Calibration Curve Standards ranging from 0.16 to 20.0 µg/ml. Similarly, Quality Control samples were prepared at 0.45, 4.0, 8.0 and 18.0 µg/ml, representing Low (LQC), Middle (MQC-1 and MQC-2) and High (HQC) controls, respectively. Aliquots were stored in capped polypropylene tubes below -20°C until analysis.

3.2.4.3 Sample Preparation

No sample filtration or extraction was required. The aliquots were thawed at room temperature, and transferred into 2 ml glass HPLC vials for analysis.

3.2.4.4 Chromatography

A modification to the method of Fawcett et al. (1997) was carried out. The Shimadzu *VP* LC system consisted of a system controller (SCL-10A), a high-pressure pump (SIL-10AD), an autosampler (SIL-10AD), and a diode array detector (SPD-M10A). The chromatographic separation was achieved using a Supelcosil C_{18} column (5 μm; 4.6 x 150 mm, Supelco Corporation, PA, USA) maintained at 50°C in a column oven (CTO-10AC). The mobile phase was premixed and contained 88 parts (by volume) of a solution containing monobasic potassium phosphate (40mM), triethylamine (0.06%, v/v) and orthophosphoric acid (for pH adjustment to 4.75) in water was mixed with 12 parts (by volume) of acetonitrile. An isocratic flow rate of 1.25 ml/min was found optimal. Acetonitrile: water (50:50, v/v) was used as a rinse solution for the injector, and the injection volume was fixed at 5 μl. Detection was carried out using a wavelength of 204 nm. Also, the peak purity for naltrexone was determined using diode-array detection.

3.2.4.5 Forced degradation

It is essential that the incorporated drug must have sufficient stability in the medium for either evaluation of real-time or accelerated release from a dosage form. This is because drug would accumulate over a period of time in the media reservoirs. For implants employed in this study, this time was estimated, based on preliminary data from a prototype, to be of the order of a few months. Therefore, a forced degradation experiment of naltrexone in Modified Hanks' Balanced Salts Solution was necessary. Based on this investigation, it is possible to stipulate a time interval before complete

media replacement should be conducted during release testing at any given temperature. This procedure enables further fresh drug released from the dosage form to accumulate in the reservoir.

3.2.4.5.1 Solution Preparation and Storage Condition

From the stock solution of naltrexone, dilutions were prepared at 0.45, 4, 8 and 20 μg/ml to cover the entire dynamic range of the method. Aliquots of all solutions were stored in capped borosilicate glass vials (to simulate the reservoir material used for actual studies) at room temperature, 38°C, 45°C and 55°C. An analysis of aliquots after 2, 4, 8 and 16 days was conducted by HPLC, and naltrexone concentrations at each level were compared to Day-0 values.

3.2.4.5.2 Qualitative LC-MS Support

To identify peaks of potential degradation products observed through HPLC, a Waters Micromass Quattro mass spectrometer (Waters Corporation, Manchester, UK) with Mass Lynx ver. 3.4 software for data acquisition was used. A high-pressure pump (Shimadzu DGU-14A), a system controller (Shimadzu SCL10ADVP) and an HTS-PAL autosampler (Carrboro, NC, USA) was configured with the mass spectrometer (MS). Since the HPLC-UV analysis employed phosphate buffer and a high mobile phase flow rate, this mobile phase could not be used for mass spectroscopy. Therefore, a mobile phase comprised of water with ammonium acetate (20 mM): acetonitrile (20:80, v/v) was employed. A soft, Electrospray Ionization (ESI) technique in the positive ion mode was

used, and a moderate optimization of response for naltrexone in the Q1 mode was carried out by an infusion of a 1 μg/ml solution in methanol. The following values were obtained and set for the analysis - Capillary: 3.5kV, Cone 40V, Extractor 3V, Source temperature 150°C, and ESI probe temperature 300°C. Nitrogen was used as the sheath gas at 300 L/hr. Once the system had been optimized, a scan range of m/z 100-1000 was fixed for sample analyses. The same analytical column at a flow rate of 0.25 ml/min was employed for both HPLC and MS to check for corresponding retention times of drug and degradation products.

3.3 Results and Discussion

In this study, a Modified Hanks' Balanced Salts Solution was characterized for various parameters, including pH, buffer capacity, osmolality, spectra and the stability of naltrexone in the medium. Stability testing was conducted with a validated HPLC procedure to establish the time interval before complete media replacement was necessary during release testing studies.

3.3.1 Media Characterization

3.3.1.1 pH and buffer capacity

The temperature dependence on pH of the medium is represented in Table 9. The pH was found not to vary beyond 0.02 between repeated measurements (n =3) at any given temperature. There was a decrease in pH with an increase in temperature, and a corresponding increase in pH when the temperature was lowered back to 24°C.

Table 9.

Temperature dependence on pH

Temperature (°C)	pH (increasing temperature)		pH (decreasing temperature)	
	Mean	S.D.	Mean*	S.D.
24	7.58	0.01	7.59	0.01
25	7.56	0.00	7.57	0.01
26	7.55	0.01	7.55	0.01
27	7.53	0.00	7.52	0.00
30	7.50	0.01	7.50	0.01
35	7.41	0.01	7.42	0.01
37	7.38	0.01	7.38	0.00
38	7.37	0.01	7.37	0.01
39	7.36	0.01	7.36	0.01
40	7.34	0.00	7.34	0.01
44	7.28	0.01	7.28	0.01
45	7.26	0.01	7.27	0.01
46	7.25	0.01	7.25	0.01
50	7.20	0.02	7.20	0.02
54	7.14	0.01	7.14	0.01
55	7.13	0.00	7.13	0.00
56	7.12	0.01	7.12	0.01

*Difference not significant ($p > 0.05$)

This was justified thermodynamically from the positive value of enthalpy change ($\Delta H = +3.6$ kJ/mole) for phosphate equilibrium, based on LeChatelier's principle (Cox et. al., 1989). For every 1°C, the observed pH decreased by 0.015, which concurs with the published value of 0.014 (Shipman, 1973). This decrease was best described by a second-order polynomial fit as: pH = 7.52E-05.[temperature (°C) - 40.94]^2 - 1.46E-02.[temperature (°C)] + 7.724, with an r^2 of 0.99. The pH changes at elevated temperatures necessitated addition of different amounts of sodium hydroxide for maintenance of the medium within the physiological range. This could in turn have an influence on osmolality changes, a description of which is provided in the following section.

The Van Slyke equation was used for calculation of buffer capacity, and the values are presented in Table 10. An addition of HEPES to Hanks' Balanced Salts solution resulted in a 38-fold increase in buffer capacity at room temperature. The variation observed following an increase in temperature from 38°C to 55°C was relatively small (1.36-fold decrease).

3.3.1.2 Spectral Evaluation

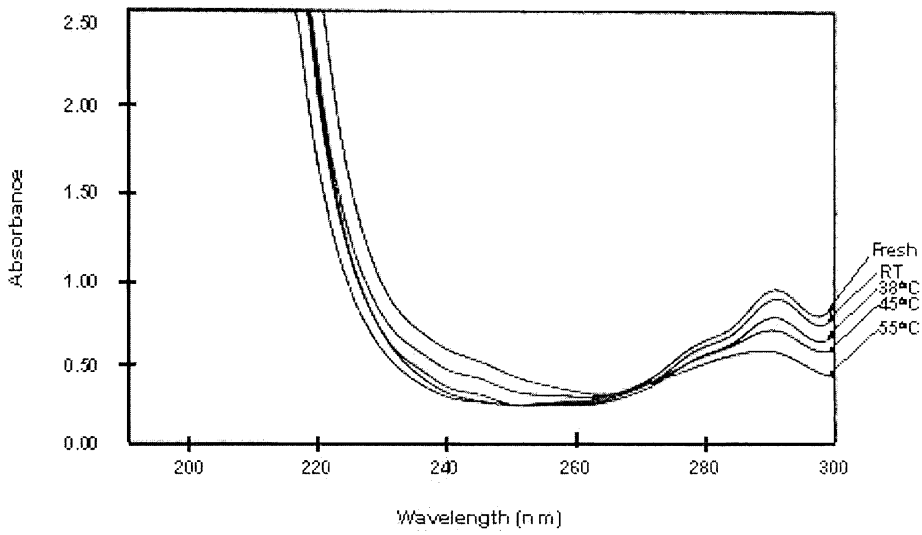
The changes in UV absorption of the medium as a function of temperature and time are better understood when the day 90 spectra collected are represented overlaid (Figure 11a). Figure 11b is a spectrum of the Hanks' Balanced Salts Solution with HEPES added, but without Primocin. Clearly, the contribution of the antimicrobial agent in the UV absorption spectrum can be observed.

Table 10.

Buffer capacity (β) of medium under different conditions

Condition	β
25°C	9.1×10^{-4}
38°C	9.8×10^{-4}
45°C	7.7×10^{-4}
55°C	7.1×10^{-4}
25°C (HBSS alone)	2.4×10^{-5}

(a)



(b)

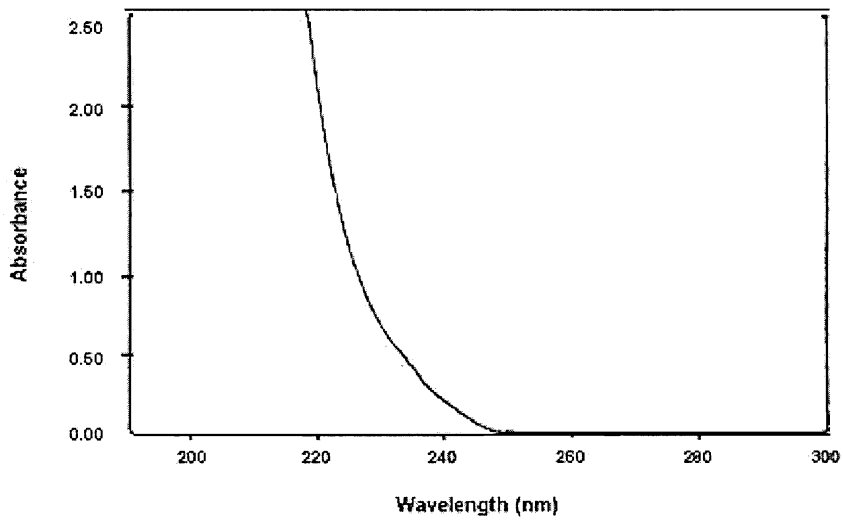


Figure 11.

a) Absorption spectra of the Modified Hanks' Balanced Salts Solution at Day-90, with the spectrum of freshly prepared solution overlaid for comparison
b) Absorption spectrum of the release medium with out added antimicrobial agent.

Also, the spectrum of the medium stored at 55°C was distinctly different from that obtained for the medium maintained at 38°C. Although there was no difference in absorption up to approximately 260 nm, absorption due to the antimicrobial agent seems to have decreased, representing a possible degradation of its constituents. The additional moieties could result in a change in colligative properties of the medium, the most pertinent being a variation in osmolality. This further indicated the study of osmolality to be a required characteristic for suitability of the medium.

3.3.1.3 Osmolality

The previously described experiments indicated a degradation of components that could potentially cause the medium to exceed the normal iso-osmolar range [270-330 mOsm. (Waymouth, 1970; Documenta Geigy, 1970)]. Table 11a summarizes osmolality values of the medium as a function of temperature and time following the day of preparation (day-0). In Table 11b, the osmolalities of a freshly prepared Hanks' Balanced Salts Solution without added HEPES, HEPES in water (25 mM), and Primocin in water (0.2%, v/v) are provided for comparison.

Although the difference in osmolality was significant ($p < 0.05$) at day-90, values were within the normal physiological range. This provided further substantiating data for physiological relevance of the medium.

Table 11.

a) Osmolality changes of medium as a function of temperature and time

Day	Condition		
	38°C	45°C	55°C
7	282.3	284.7	286.2
15	282.7	289.1	291.0
30	284.9	292.3	301.8
60	285.4	303.4	308.9
90	288.1	305.2	321.6

* Values within a RSD of 1% (n =5)

b) Contribution of the individual components of a freshly prepared medium

Component Description	Osmolality (mOsm.)
HBSS	267.1
HEPES (25 mM)	14.8
Primocin (0.2%, v/v)	-*
Total	281.9
Total osmolality (observed)	281.3

*Dip in osmometer readings observed indicating negligible values

3.3.2 HPLC Method Validation

The system suitability parameters were found to be within acceptable limits (refer Table 12 and Figure 12). An analytical run time of 8 minutes was optimized for each sample because of elution of a peak at 11 minutes. This peak was later attributed to a medium component.

3.3.2.1 Linearity

The calibration curves were linear in the range of 0.16-20 μg/ml ($r^2 > 0.99$). The data are presented in Table 13. The concentration residuals ranged from -1.9%-2.7% (RSD = 1.9%-4.8%; n=6). Figure 13 shows representative chromatograms of blank medium, the lowest standard and a QC sample. The LLOQ of the method, 0.16 μg/ml, was calculated by injection of samples prepared independent of the calibration curve from its slope. . The deviation was found to be 1.4% of the nominal concentration (RSD = 1.7%; n = 6).

3.3.2.2 Precision and accuracy

The precision and accuracy of the method were reported as the Relative Standard Deviation (%RSD) and percent deviation from the nominal value (%DFN). The global inter-day precision and accuracy for 3 batches (n = 9) were found to be between 1.4%-1.7%, and -1.0%-0.1%, respectively (Table 14). These values are well within acceptance criteria (ICH Guideline, Topic Q2A; Shah et. al., 1992).

Table 12.

System suitability parameters for the HPLC-UV method

Parameter	Value
Retention Time (min.)	4.8
Capacity factor (k')	1.5
Tailing (T)	< 2.0
Theoretical Plates (N)	2452
Separation factor (α)	1.6
Resolution (R)	2.1

Table 13

Reverse predicted concentration residuals of naltrexone

Set #	Nominal Concentration ($\mu g.ml^{-1}$)								r^2	Slope	Intercept
	0.16	0.31	0.63	1.25	2.50	5.00	10.00	20.00			
1	0.16	0.30	0.62	1.33	2.54	4.64	9.10	21.28	0.9929	15869	-0.074
2	0.17	0.30	0.62	1.20	2.57	5.03	10.05	19.91	0.9999	15378	0.102
3	0.16	0.31	0.58	1.17	2.43	4.85	9.87	20.46	0.9992	16353	0.060
Mean	0.16	0.30	0.61	1.23	2.52	4.84	9.67	20.55		15867[#]	0.03[*]
SD	0.00	0.01	0.02	0.09	0.07	0.20	0.51	0.69		487	
% RSD	2.2	2.9	4.1	7.1	2.9	4.1	5.2	3.3		3.1	
% DFN	2.5	-2.3	-3.6	-1.4	0.6	-3.3	-3.3	2.7			

[#]C.I. (14656, 17078)

[*]Not significantly different from zero ($p > 0.05$)

Table 14.

Precision and accuracy data of naltrexone

Set #	Nominal Concentration ($mg.ml^{-1}$)			
	0.45	4.00	8.00	18.00
1	0.45	3.94	7.93	17.94
	0.46	4.06	7.87	17.70
	0.44	3.93	7.78	17.65
Mean	0.45	3.98	7.86	17.76
SD	0.01	0.07	0.08	0.16
% RSD	2.0	1.7	1.0	0.9
% DFN	-0.4	-0.6	-1.8	-1.3
2	0.46	4.07	8.14	18.13
	0.45	4.05	7.86	17.53
	0.44	4.04	8.17	17.61
Mean	0.45	4.05	8.06	17.76
SD	0.01	0.02	0.17	0.33
% RSD	1.5	0.4	2.1	1.8
% DFN	0.2	1.3	0.7	-1.3
3	0.44	4.09	7.89	17.81
	0.44	3.92	7.83	18.25
	0.45	3.94	7.95	17.83
Mean	0.44	3.98	7.89	17.96
SD	0.00	0.09	0.06	0.25
% RSD	0.5	2.3	0.8	1.4
% DFN	-1.2	-0.5	-1.4	-0.2
Global Calculation				
Mean	0.45	4.00	7.93	17.83
SD	0.01	0.07	0.13	0.24
% RSD	1.5	1.7	1.7	1.4
% DFN	-0.5	0.1	-0.8	-1.0

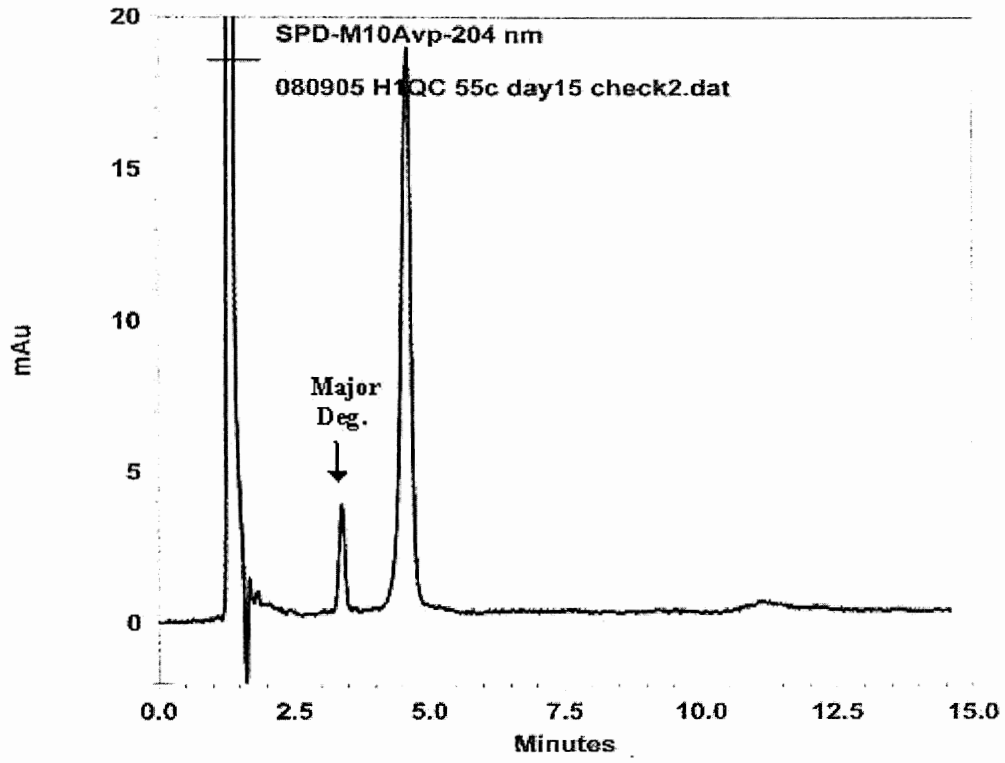


Figure 12.

A chromatogram of a degraded sample to show resolution of naltrexone from its degradation product. The analysis time was optimized during validation to 8 minutes to avoid carryover of the peak at 11 minutes to subsequent injections.

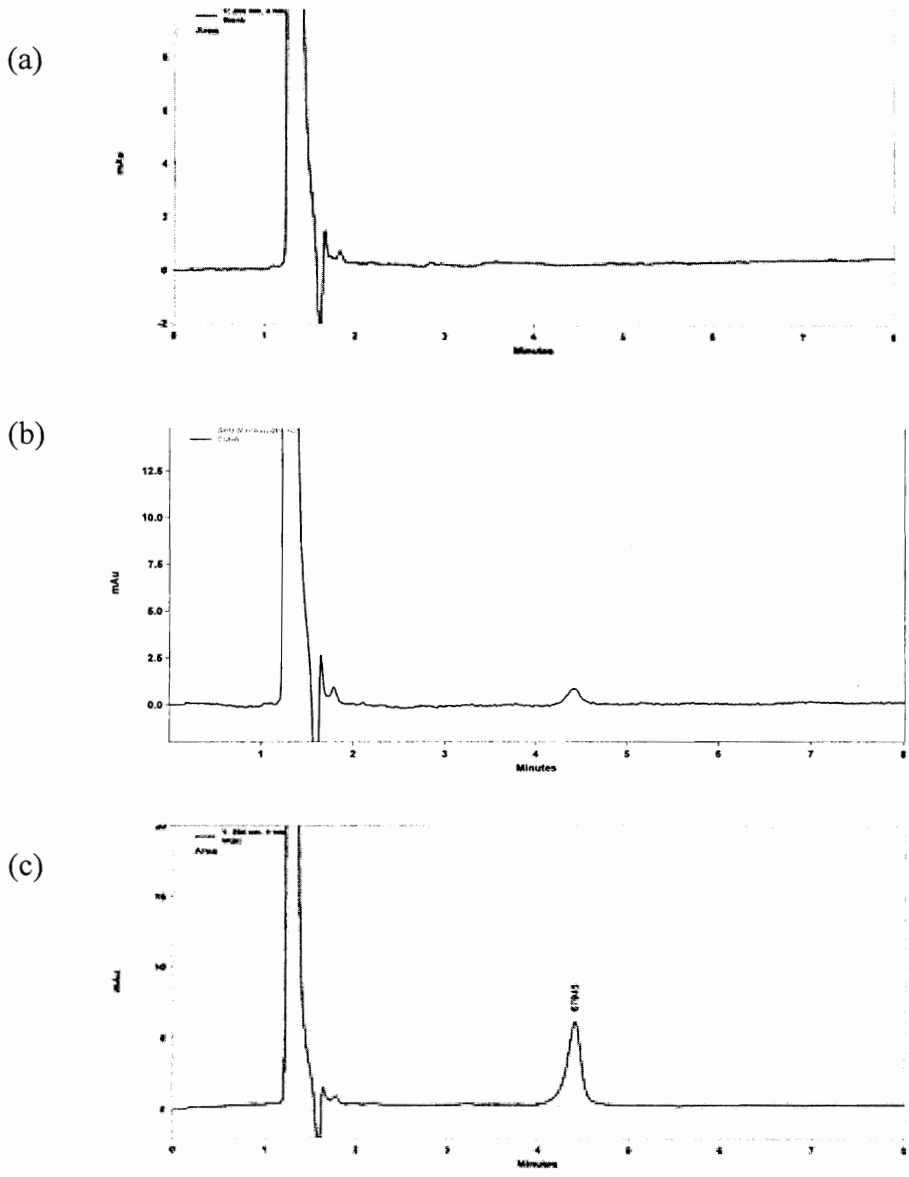


Figure 13.

Representative chromatograms of (a) blank medium, (b) a 0.16 μg/ml sample and (c) a 4 μg/ml sample

3.3.3.3 Autosampler stability

Autosampler stability was validated by a comparison of the peak areas of QC samples injected at 4 and 24 hr to the mean peak areas of samples obtained at 0-hr. Table 15 presents the results. The mean values of autosampler stability up to 24 hours was 100.1-106.7% of initial QC concentration at 0-hr (RSD = 0.3-3.4%).

3.3.3.4 Freeze-thaw stability

The freeze-thaw was assessed by a comparison of the peak areas of QC samples injected after 1 and 2 cycles of freeze-thaw to the mean peak area of freshly prepared QC samples. A storage duration of 6 days below -20°C was allowed between each cycle. The mean values up to 2 freeze-thaw cycles ranged from 97.8-105.9% of the initial QC concentration at 0-hr (RSD = 0.5-2.3%). The results also ensured that the study samples could be collected for at least 12 days prior to analysis.

3.3.3.5 Drug Stability

3.3.3.5.1 Determination of time interval for medium replacement

The stability of naltrexone was tested at 0.4, 4, 8 and 20 μg/ml. Figure 14 represents the loss of drug in solution as a function of temperature and time. With an increase in temperature, the degradation of naltrexone increased. The degradation was observed to follow an Arrhenius relationship [$\ln(k) = -2852.4 \cdot (1/T) + 4.76$; $r^2 = 0.94$], where k is a reaction rate coefficient calculated from the time taken for the drug concentration to reach 90% ($t_{90\%}$) of its initial value, and T represents absolute

Table 15.

Autosampler stability of naltrexone

Time		LQC	MQC	HQC	H1QC
4-hr	Mean*	106.7	102.0	100.3	100.9
	% RSD	3.4	1.4	1.2	1.2
24-hr	Mean*	101.6	100.6	100.1	102.2
	% RSD	1.2	0.5	0.3	0.9

* Values reported as percentage of mean 0-hr value; n = 3

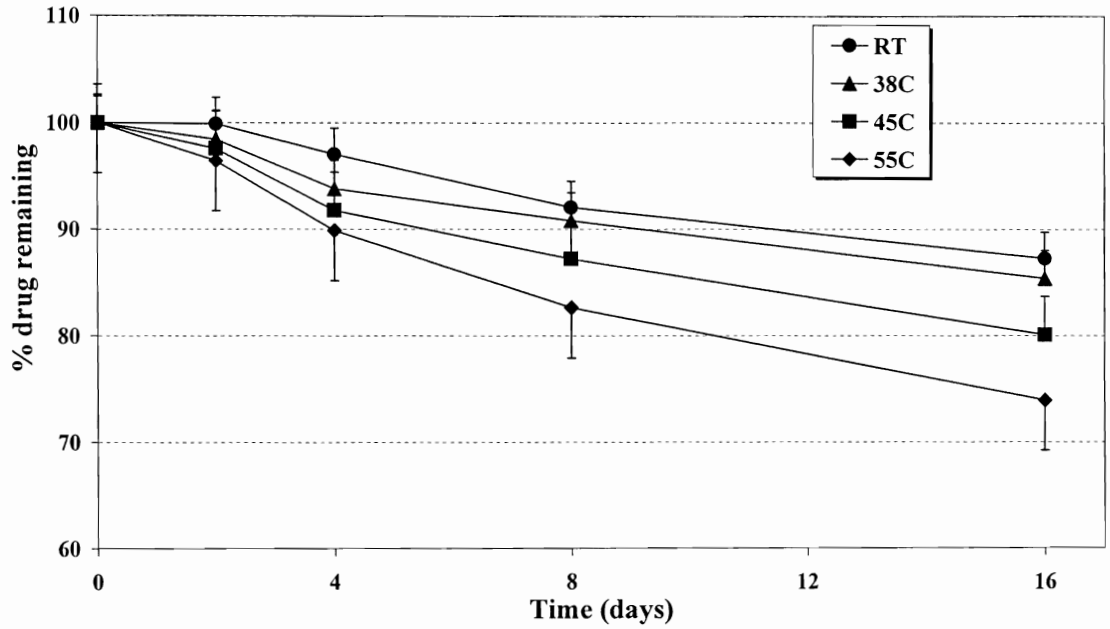


Figure 14.

Loss of naltrexone in drug release medium subjected to different temperature conditions

temperature (Arrhenius, 1889). The activation energy (E_a) calculated from the slope was 5.65 kcal/mole. This E_a value of less than 10 kcal/mole implies the possible involvement of oxidation as a potential mechanism for degradation (Banker and Rhodes, 1995; Menzinger and Wolfgang, 1969). Furthermore, for evaluation of the Arrhenius relationship in making predictions, a technique based on product plots was investigated. As shown in Figure 15, for naltrexone at 18 μg/ml level, the % appearance of the degradation product plotted against the extent of substrate degradation resulted in almost superimposable lines. Although the plot represents a single concentration level, it shows that for the temperature range investigated, minimal prediction errors are likely from results of the Arrhenius equation.

Also, the $t_{90\%}$ values of 10.0, 7.1 and 5.1 days, respectively, at 38°C, 45°C and 55°C were recorded. Based upon these values, more conservative time intervals of 7, 4 and 3 days, respectively, were fixed for media replacement during studies at those temperatures

3.3.3.5.2 Qualitative Mass Spectral Information

Drug degradation was also indicated by the appearance of an additional peak at 3.5 minutes, found only in the chromatograms of samples following forced degradation (Figure 12). This peak increased in area as a function of temperature and time. A diode-array spectral scan revealed an absorption wavelength maximum (λ_{max}) of 209 nm for the peak. Another peak was observed at 11 minutes having a λ_{max} of 225 nm. Figure 16 depicts the scheme followed for identification of the peak using mass spectroscopy.

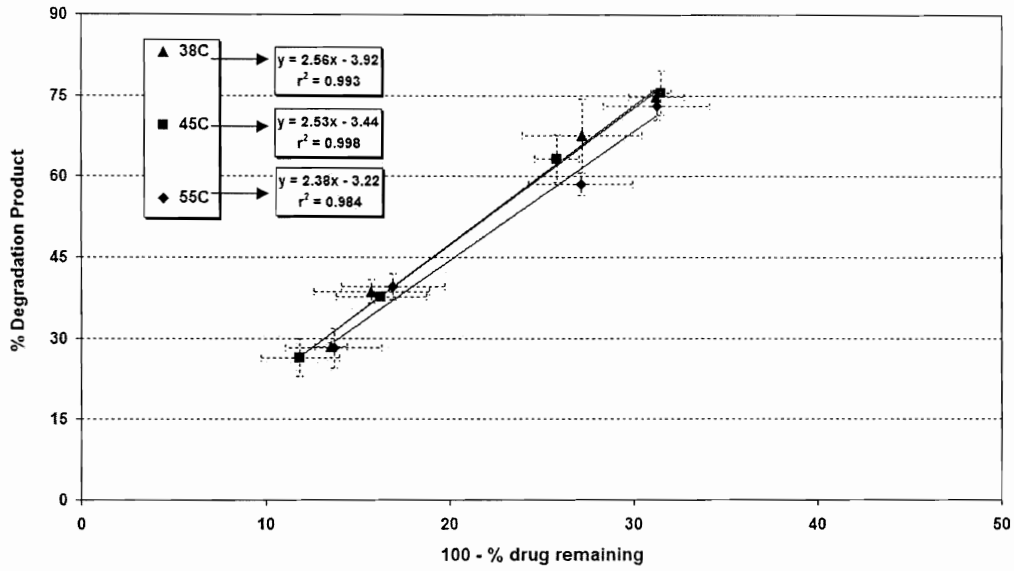


Figure 15.

Product plot of naltrexone at 18 μg/ml in drug release medium

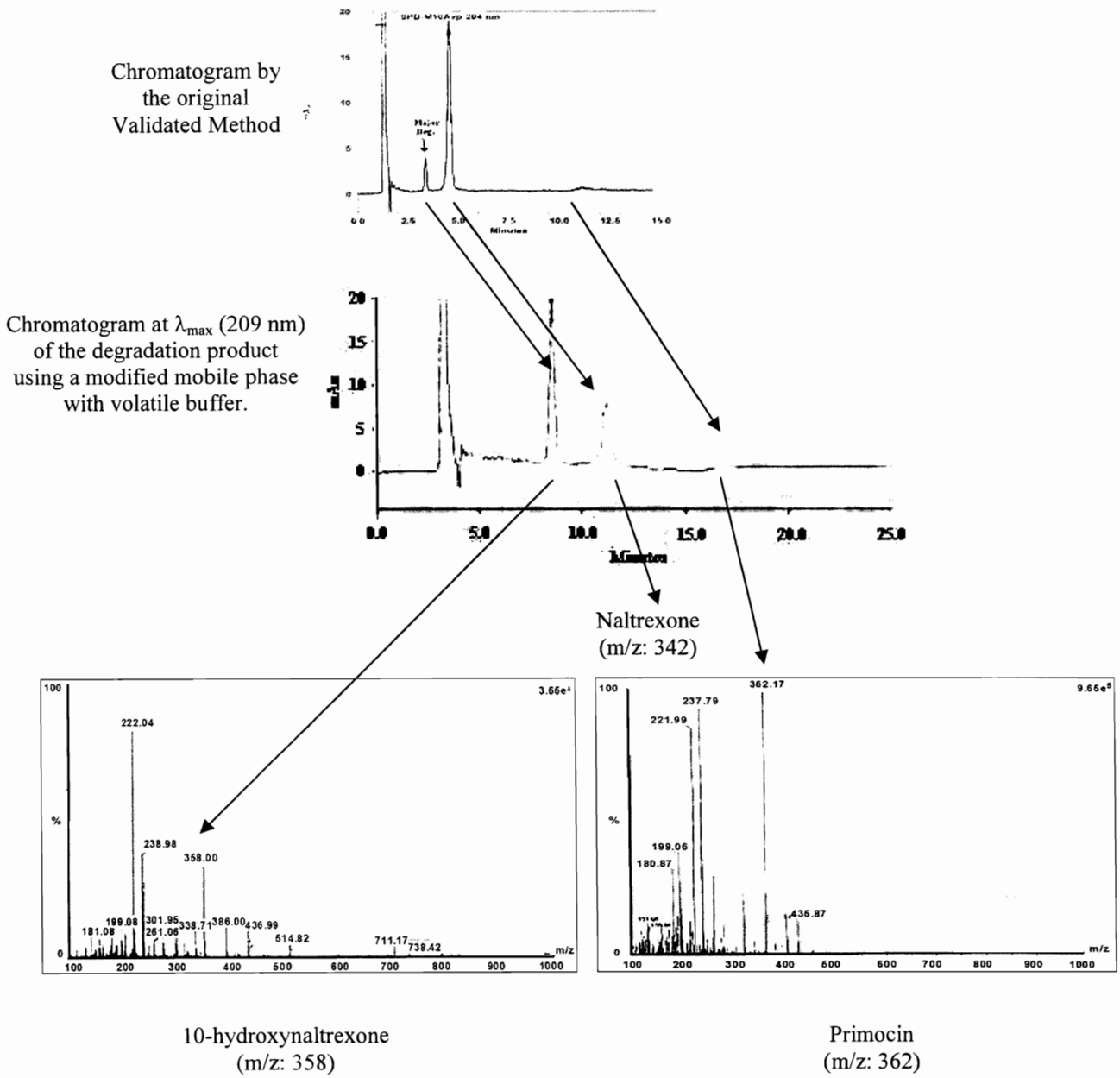


Figure 16.

Scheme for qualitative mass spectral analysis of a degraded 18 µg/ml sample with a comparison of retention times to the validated HPLC method

The figure shows a chromatogram of a degraded 18 μg/ml sample obtained on HPLC-UV, using the modified mobile phase comprised of water with ammonium acetate (20 mM): acetonitrile (20:80, v/v). The chromatograms with corresponding peaks using both methods have been provided for reference.

Mass spectra yielded peaks with m/z of 358, 342 and 362 at retention times of 8.4, 11.3 and 17.1 minutes, respectively. An m/z ratio of 358 may be attributable to 10-hydroxy naltrexone which, based on a relative retention time of 0.74 is comparable to the value of 0.7 specified in the USP Monograph (USP28/NF23, 2005). The m/z of 362 is attributable to Primocin, as (crosschecked with its solution in water). Other m/z ratios of 239, 261 and 278 were observed and found to correspond to HEPES and its sodium and potassium adducts, respectively. Insignificant intensities of m/z 364 and 380 were also observed which corresponded to sodium and potassium adducts of naltrexone.

3.3.3.5.3 Quantification of degradation product

For quantification of the degradation product in the absence of a reference standard, a method based on absorptivity changes was employed. A degraded high quality control sample was diluted 2-fold and 4-fold with drug release medium, and injected into the HPLC. The resulting areas of naltrexone and its degradant were plotted against drug concentration and the slopes were compared. The ratio of slopes was 0.954, which was then used as a correction factor so that naltrexone could also be employed for quantification of the degradant. The concentration of degradation product increased with an increase in temperature (Figure 17) and for the 18 μg/ml sample, a maximum

degradation of 1.67 μg/ml was measured at 16 days of forced degradation at 55°C. This accounted, in molar terms, for about 40% of the total degradation of the drug at that temperature, indicating the possible existence of multiple pathways for drug degradation that had not been detected at the low concentration of drug employed.

The purpose of development of the HPLC method however, was to provide a reliable and selective quantification of naltrexone in the presence of medium components, and to determine the time interval prior to media replacement during release testing. Therefore further degradation studies to establish mass balance were not conducted.

3.4 Application to samples of an *in vitro* release study

The Modified Hanks' Balanced Salts Solution has been applied for investigation of real-time *in vitro* naltrexone released from a biodegradable implant supplied by Durect Corporation, CA, USA. The validated HPLC method was used for quantification of these samples. A chromatogram of the Day-2 sample is shown in Figure 18. Approximately 1500 samples have been analyzed thus far using the HPLC method proving its robustness. No chromatographic interference was observed from any degradation products. Also, the peak purity for naltrexone was 99.9%, as estimated using diode-array detection.

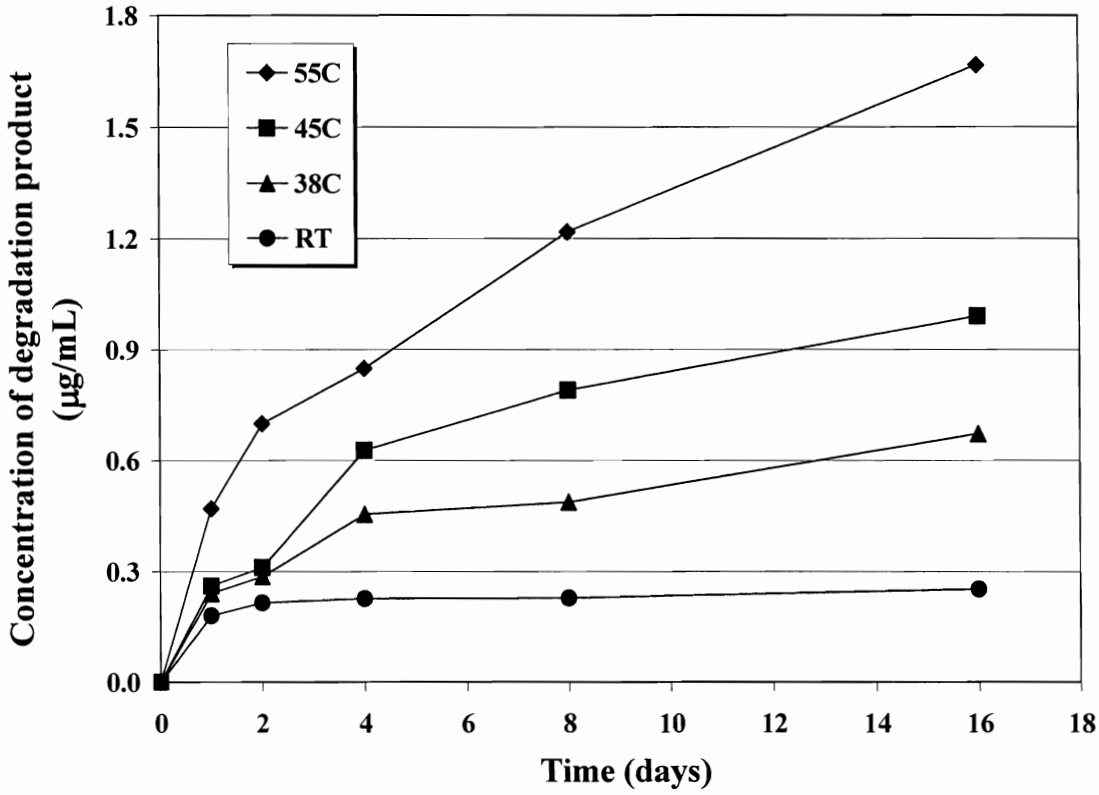


Figure 17.

Quantification of the degradation product formed in a 18 µg/ml solution of naltrexone in drug release medium when subjected to different temperatures

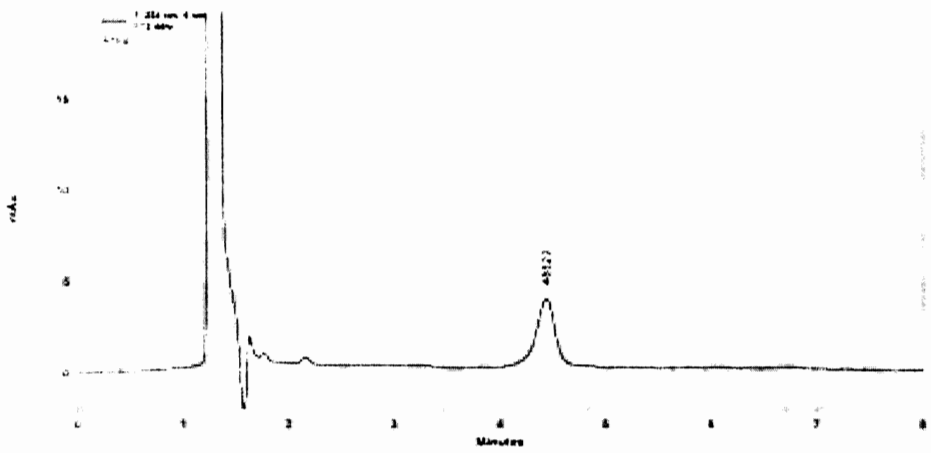


Figure 18.

Chromatogram of Day-2 sample of a real-time *in vitro* release study

3.5 Conclusion

The Modified Hanks' Balanced Salts Solution has proven to be a suitable 'biorelevant' medium for *in vitro* release testing of a naltrexone implant. The physicochemical parameters evaluated for its characterization were within acceptable limits. Application of this medium for further studies involving other types of implants may be carried out to extend this approach. Many other factors, such as the physicochemical properties of the drug etc. also influence media selection since it has to be sufficiently stable in the medium until analysis. The HPLC method when applied to real-time samples has generated expected data.

CHAPTER 4. A 'Biorelevant' System for *In vitro* Investigation of Drug Released from a Naltrexone Implant

4.1 Introduction

The development of subcutaneous pellets and implants as drug delivery devices had been necessary 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 management 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 et al., 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 et al., 2003). The majority of research studies have been focused on incorporation of the drug into biodegradable polymers, such as polylactic acid, poly glycolic acid and polycaprolactone. During formulation of these drug delivery systems, manipulation of 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 et al., 2004). 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 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 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 chapter 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.

4.2 Materials and methods

4.2.1 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 x 10 liter) 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).

4.2.2 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 $\pm$ 0.07 cm, 3.57 $\pm$ 0.16 mm, 1.14 $\pm$ 0.04, and 471.8 $\pm$ 44.8 mg, respectively. Each implant contained approximately 280 mg of naltrexone. Figure 19 is a photograph of the implant having a homogenous matrix core covered by a smooth outer coating.

4.2.3 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.

4.2.3.1 '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 (Chapter 3). The selection of a media volume between 900-1000 ml for compendial dissolution Apparatus 1 and 2 is based on the volume required to ensure adequate 'sink' conditions for most drugs.

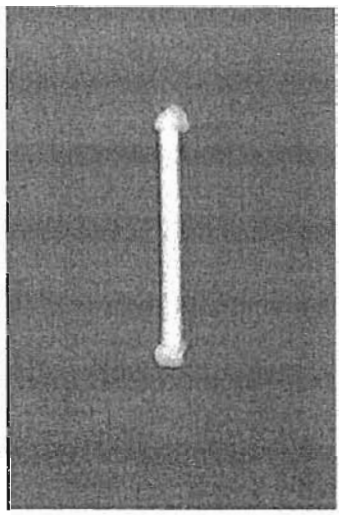


Figure 19.

The biodegradable naltrexone implant

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. 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):

$$Q = \frac{SS.\ \pi r^3}{4\mu} \qquad \ldots\ldots (4)$$

where Q is the flow rate (ml/s), SS is the shear stress (dyne/cm^2), r is 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., 2000). The shear stress range that the endothelial wall of subcutaneous capillaries is exposed to is 0.07-20 dyne/cm^2 (Davies, 1989). This translated to a flow rate range from 2.47 x 10^{-5} to 7.06 x 10^{-3} ml/s/capillary. For this study, a shear stress of 1 dyne/cm^2 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 x 10^{-4} ml/s/capillary, or 1.06 ml/min for the cartridge with 50 capillaries.

4.2.3.2 Calculation of 'sink' conditions

The solubility of naltrexone freebase in Hanks' Balanced Salts Solution at 32°C is 5.42 mM (Stinchcomb et al., 2000). 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.

4.2.3.3 Modeling the Capillary Diffusion Process

In their seminal papers on the pore theory of capillary permeability, Renkin (1954, 1964 and 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, also described in Chapter 1, is given below:

$$A_x/A_o = \left[2\,(1 - a/r)^2 - (1 - a/r)^4\right].\left[1 - 2.104\,(a/r) + 2.09\,(a/r)^3 - 0.95\,(a/r)^5\right] \quad \ldots\ldots (1)$$

where A_x/A_o is the ratio of effective area of the opening to the total cross-sectional area of pore, 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 A° (RSD = 10%), as determined using a molecular modeling approach described earlier (Chapter 2). 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. in 1951, and found to be 24 A^{o}. Based on these values, the ratio, A_x/A_o, was calculated separately for *in vitro* [defined as $(A_x/A_o)^{vitro}$], and *in vivo* [defined as $(A_x/A_o)^{vivo}$] scenarios.

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

$$R_{diff} = \frac{(A_x/A_o)^{vivo}}{(A_x/A_o)^{vitro}} \quad \ldots\ldots (5)$$

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* as compared to the barrier simulated by the *in vitro* capillaries.

Also, a pharmacokinetic model is proposed for subcutaneous absorption as shown in Figure 20. 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 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-

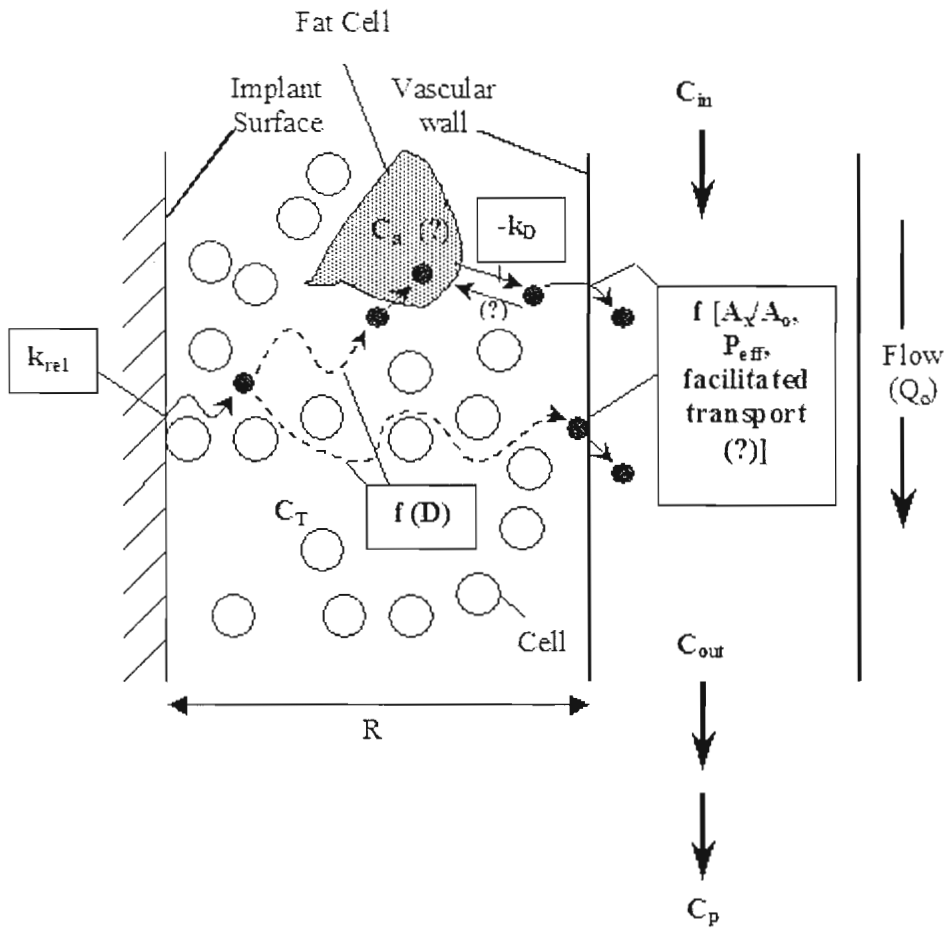


Figure 20.

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.)

distribution rate. This could lead to formation of a localized depot, represented by the drug concentration, C_a, in adipose. 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 gm/minute (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.

4.2.4 System Components and Assembly

A schematic diagram for the *in vitro* system is given in Figure 21. 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).

4.2.4.1 Glass beads and filters

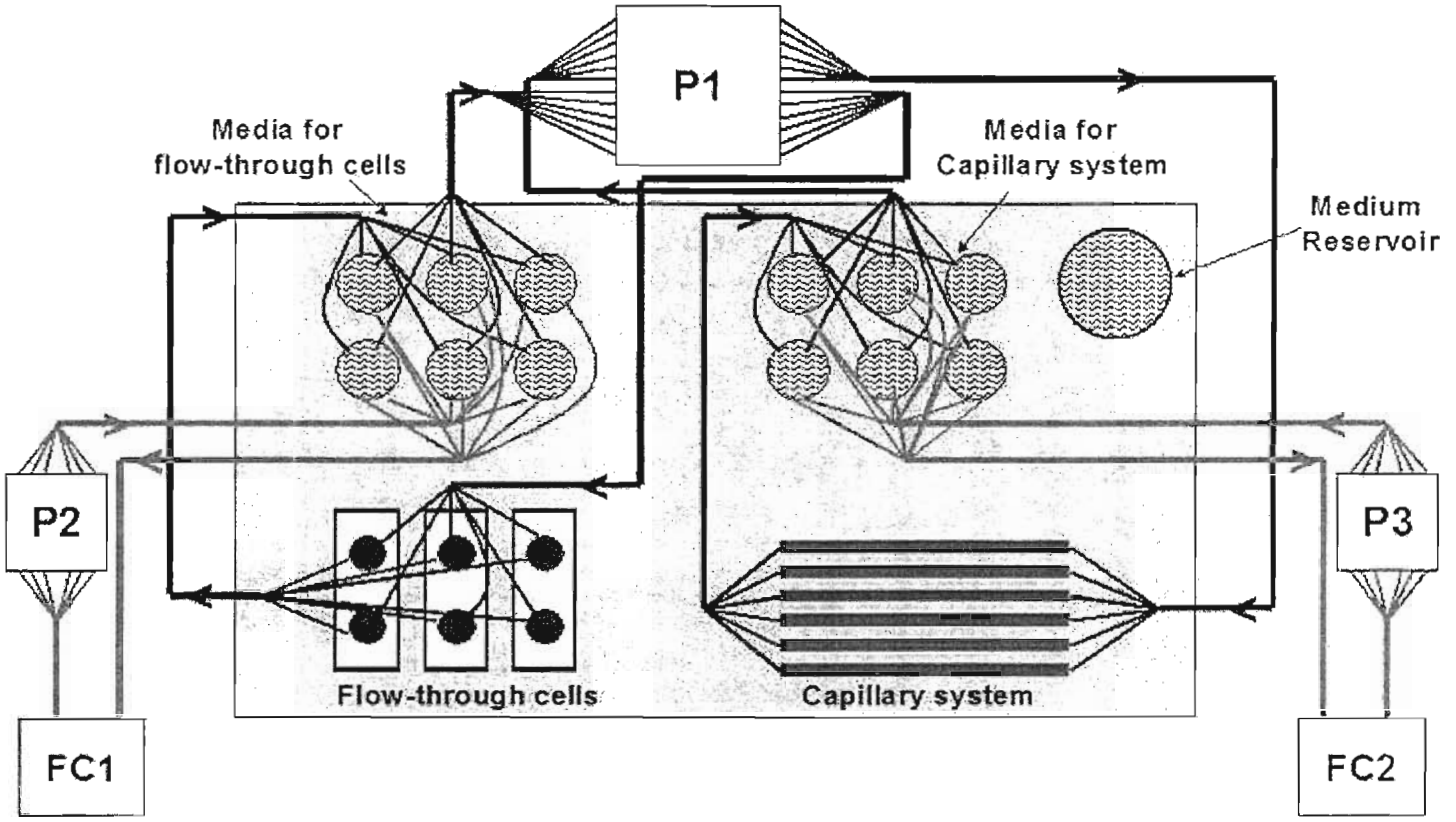


Figure 21.

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 controlling 6-channel peristaltic pumps, P2 & P3.

Borosilicate glass beads (Chemglass, Inc., NJ, USA) having a mean diameter of 1 mm. (RSD = 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 the porosity of the beads. The porosity was determined 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 gm 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 the extracapillary space with no possibility of fragmentation that might penetrate through the membrane wall and reach the media reservoir.

4.2.4.2 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 to 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. 6 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.

4.2.4.3 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. Figure 22 represents the flow-through cell and each of its components.

4.2.4.4 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 Figure 23a. 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 x 4.2 cm was carefully made. The slot cover was retained. A layer of glass beads was placed into the opening 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. Figure 23b is a schematic representation of the implant placed inside the extracapillary space of the device. 12 hours was allowed following placement of the implant and the filled cartridge was positioned vertically with clamps prior to use.

4.2.4.5 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^{o}C prior to analysis.

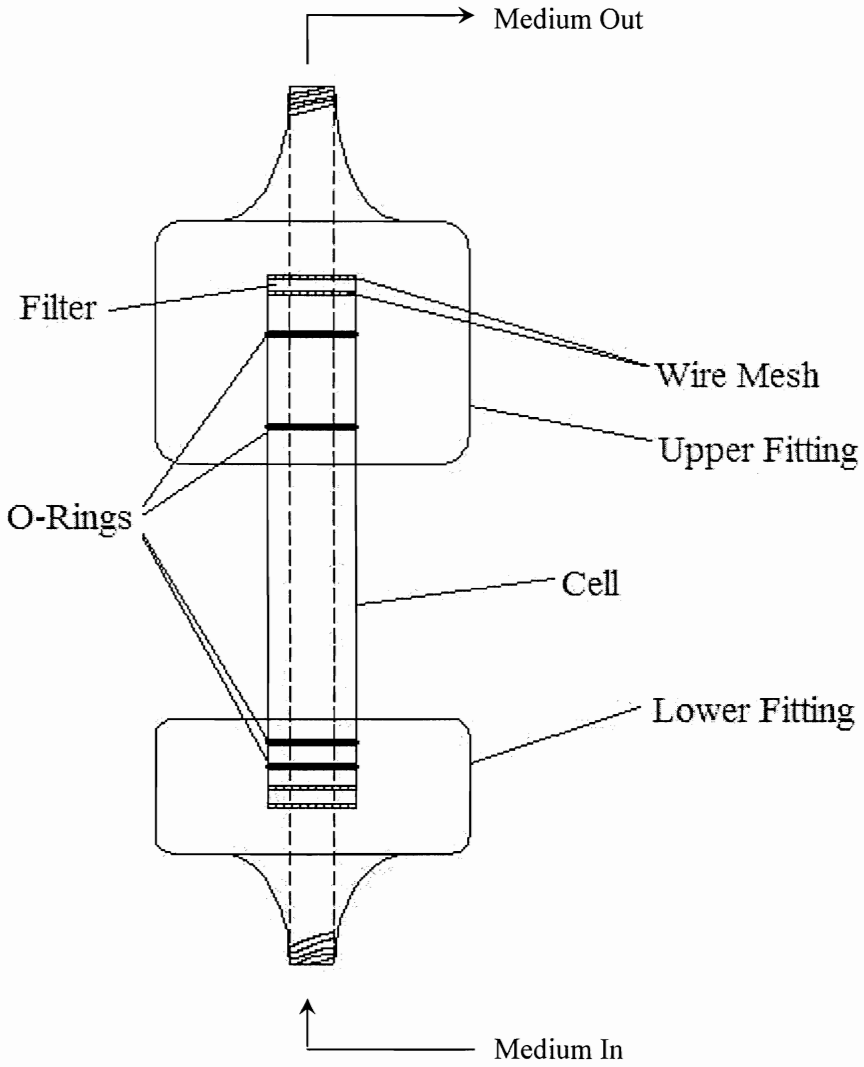


Figure 22.

The modified USP flow-through cell.

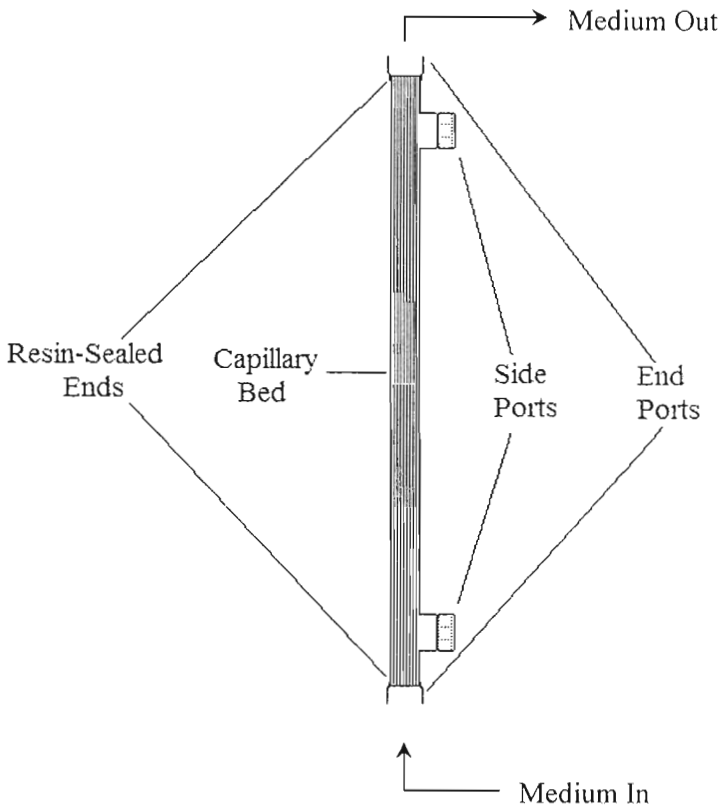


Figure 23a.

The new capillary device identified for 'biorelevance'.

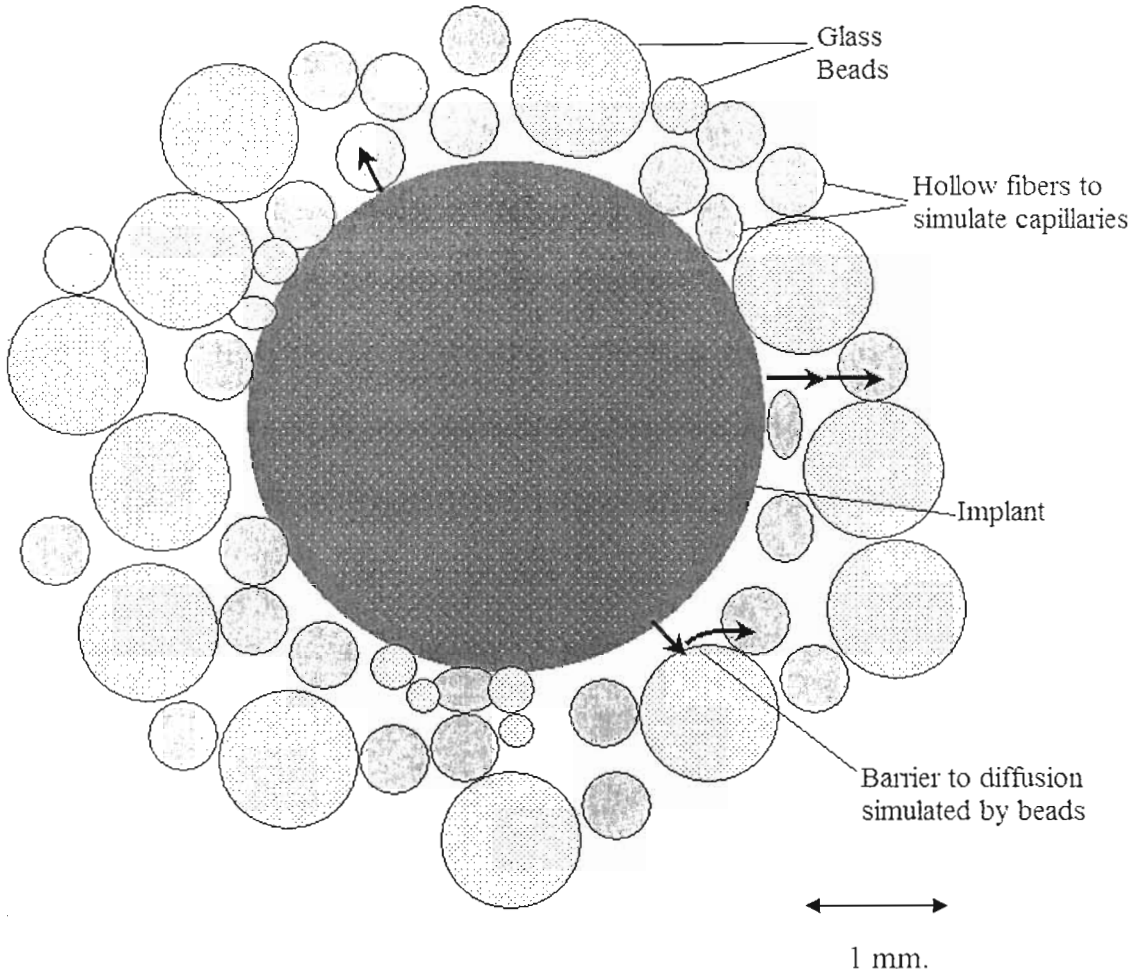


Figure 23b.

Diagram of a transverse section of capillary device with the implant positioned ready for testing.

4.2.4.6 Medium Reservoir

Cylindrical borosilicate glass reservoirs (250 ml) were used with tight fitting silicone stoppers (50x43x25 mm) from Fisher Scientific, GA, USA. Perforations of 1 mm were drilled for ports as depicted in Figure 24. 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. An additional orifice, 7 mm in diameter was drilled for insertion of temperature and sample probes, as and when required, and for media replenishment. The system was kept closed with another silicone stopper (10x6x20 mm). Filters were fixed at the end of needles intended for sample withdrawal to prevent any particulate matter from entering the tubing of the fraction collector.

4.2.5 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 extra-capillary 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 the 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 (Chapter 3).

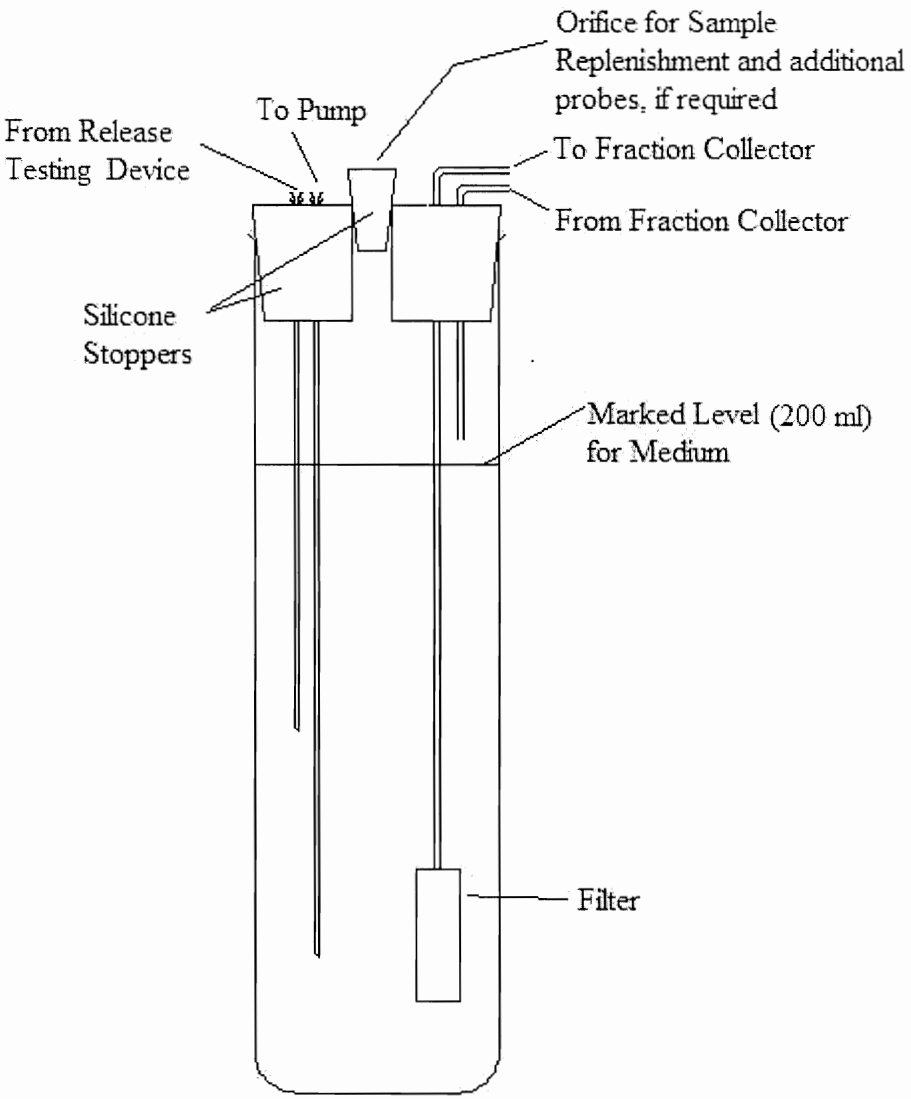


Figure 24.

Schematic of the reservoir

Temperature measurements inside the vessel were made periodically. Samples were collected every 12 hours 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 minutes of time in which the pumps were stopped. No fluctuations in flow rate were observed, possibly because no clogging of filters occurred. At the end of 90 days, the implants were taken out of the release devices, allowed to dry at room temperature and the weights were recorded.

4.2.6 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 (35x) and high (2000x) magnification.

4.2.7 *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.

4.2.7.1 Protocol Development

Since the main 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.

4.2.7.2 Test Material

Athanasiou 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 the 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 celophane tape, and carried to the surgical suite ready for implantation.

4.2.7.3 Surgical Procedure for Implantation

The dog was premedicated 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.

4.2.7.4 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.

4.2.7.5 Blood Collection and Plasma Storage

The VCU Division of Animal Resources Standard Operating Procedure of the 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^{o}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.

4.2.7.6 Sample Analysis

The plasma samples were analyzed using a validated Liquid Chromatography-Mass spectrometry (LC-MS/MS) method described elsewhere (Chapter 2). The peak height

ratio of naltrexone to [15,15,16-^{2}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.

4.2.7.7 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 4.2.6.3). 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 and evaluated using the SEM technique as described earlier, for a comparison of *in vivo* changes to those that had occurred *in vitro*.

4.3 Results and Discussion

4.3.1 *In vitro* release study

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

4.3.1.1 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-minute pump stoppage time. The pH was observed to vary up to only $\pm$ 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.

4.3.1.2 Sample Analysis

The samples were analyzed by a validated High Performance Liquid Chromatography (HPLC) method described elsewhere (Chapter 3). 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 μg/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 RSD of 22.51% (n=6) was obtained for mean concentrations of naltrexone in samples withdrawn 12 hours 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.

4.3.1.3 *In vitro* Release Data Analysis

Figure 25 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 hr (95% CI: 0.105, 0.124), and 0.06 mg/12 hr. (95% CI: 0.050, 0.064), respectively, for flow-through and capillary devices between 3-90 days. The rate of release was significantly less ($p < 0.0001$) for the capillary device than the flow-though 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 hr and 0.01-0.16 mg/12 hr 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 the 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

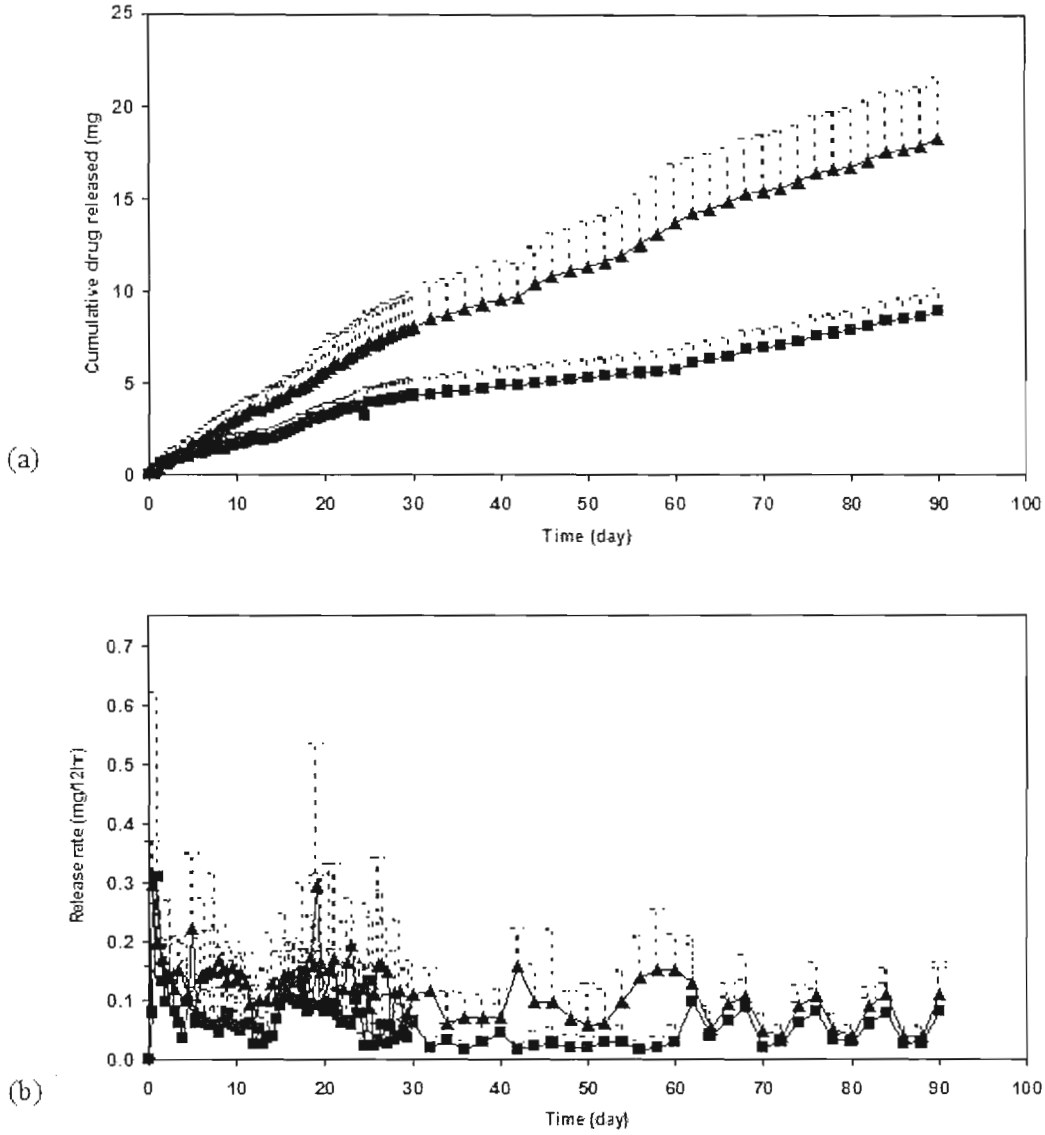


Figure 25.

(a) Comparison of real-time *in vitro* release profiles and, b) Release rates of naltrexone using both devices (n=6).
Key: ▲, flow-though cell; ■, capillary device.
(Error bars only representing positive standard deviations are shown)

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 many 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/12hr) is 12 hours for the flow-through cell versus 24 hours for the capillary system. Since other known variables (Huang et al, 2001) related to the dosage form were similar in both devices and since the polymeric matrix was non-swellable, the reason for the longer time required for the capillary device could be speculated based on the percolation-limited diffusion theory (Tzafriri, 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.

4.3.2 *In vivo* study

4.3.2.1 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.

4.3.2.2 Plasma Data Analysis

The plasma concentration-time profile of naltrexone in the dog is shown in Figure 26. 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-hour 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 (RSD: 16.2%; 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 ($t_{½}$) 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.

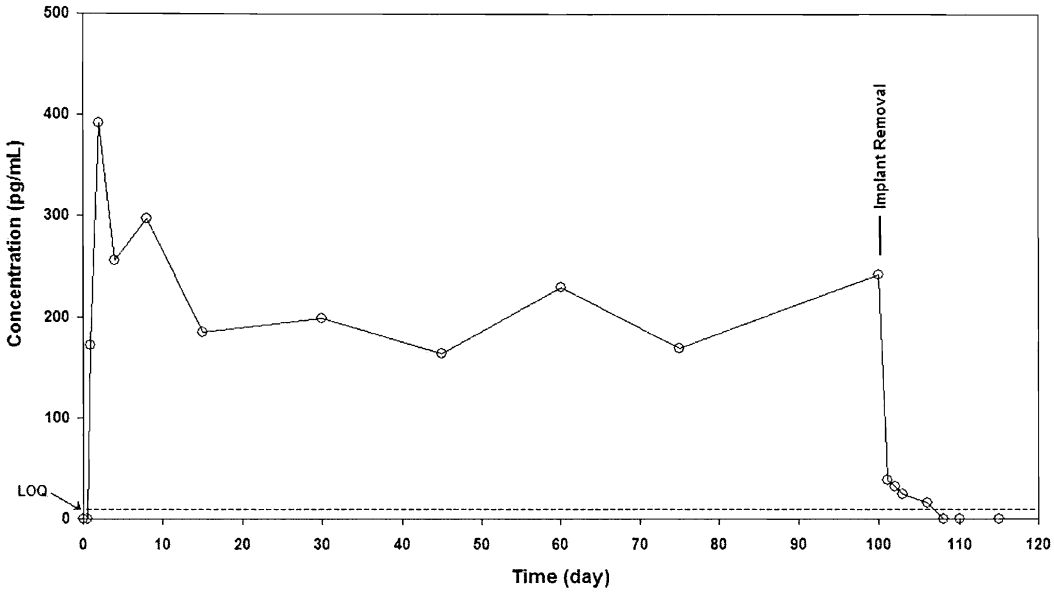


Figure 26.

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.

4.3.2.3 Macroscopic Evaluation

The scores of the three parameters as described in Section 2.6.7 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.

4.3.2.4 Histological Evaluation

As shown in Figure 27, 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).

4.3.3 Scanning Electron Microscopy

In Figure 28, the electron micrographs of the implant taken initially are compared to 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 (Figures 10b and 10d), 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 Figures 10c and 10e, were formed at random but perpendicular to

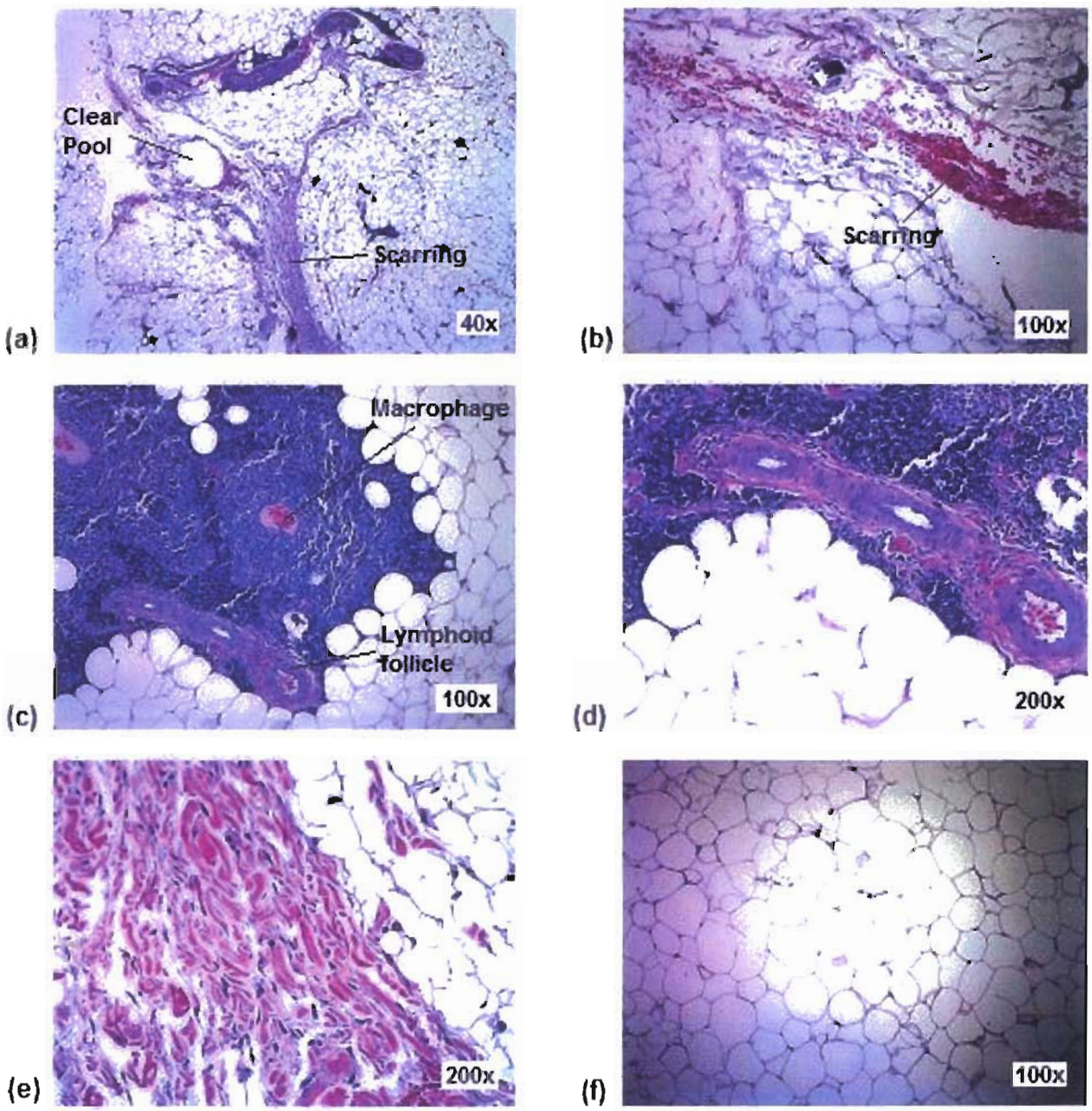


Figure 27. Tissue slides depicting changes after 3 months of implantation.
(a) Clear pool indicating presence of 'foreign material', (b) Scarring caused due to washed out implant material, (c) Chronic inflammation with lymphoid follicle, (d) View of lymphoid follicle showing engulfed material with a thin, crudely organized membrane, (e) Loose connective tissue, and (f) control slide showing normal fat at regions further away from the implantation zone.

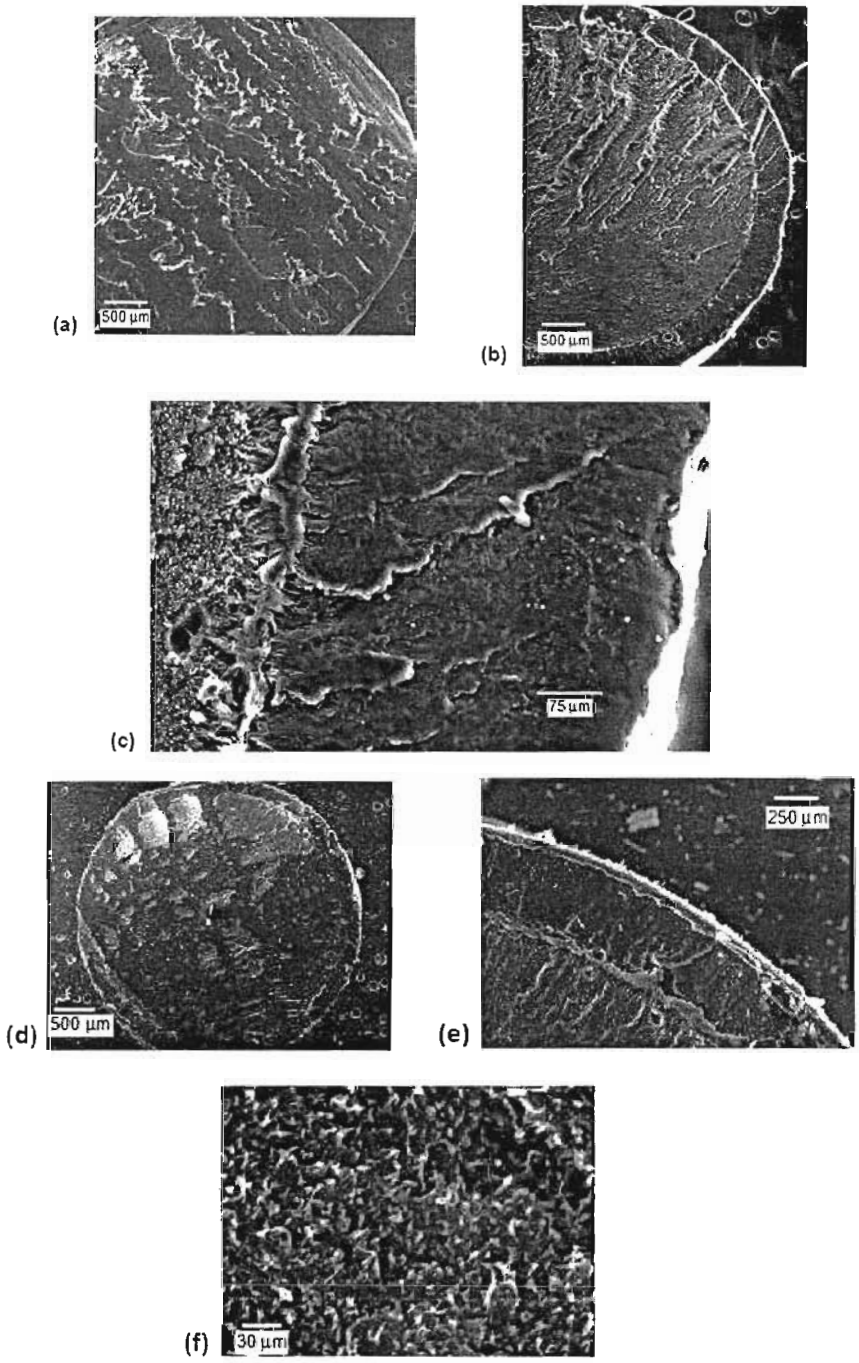


Figure 28. Scanning electron micrographs of transverse sections of implant.
(a) Initial Condition (35x), (b) Post Day-90 *in vitro* release (35x),
(c) Channels in membrane sheath after 90 days of *in vitro* release (500x),
(d) Post Day-90 *in vivo* study (35x), (e) Channels in sheath after *in vivo* study of 90 days similar to the *in vitro* observation (350x), and (f) Intact homogenous core after *in vivo* study (2000x)

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 (Figure 28f).

4.3.4 *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.

The Wagner-Nelson approach 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 the curve (AUC_{0-100}). The value of β was estimated from the terminal portion of semi-log plot of the plasma concentration-time profile (attributed earlier to receptor binding of naltrexone), and found to be 0.00751 hr^{-1}. This was used for estimation of $AUC_{0-\infty}$, the area under the curve to time infinity. Following this, the

percentage absorbed *in vivo* was calculated and the existence of an *In vitro-In vivo* Relationship was quantitatively assessed (Figure 29). The curves were superimposable, indicating the likelihood of a good correlation.

Finally, the percent absorbed *in vivo* was plotted versus the percent released *in vitro* to yield the correlations represented in Figure 30. Overall, the data obtained with both devices could be well described by a linear relationship with an r^2 of 1.0. It appeared that both devices had an excellent correlation. The slopes of the lines were not different from each other. However, the intercepts had low positive values, although those were not significantly from zero ($p = 0.92$). Also, the value of the intercept for the capillary device (0.53) was lower than that for the flow-through cell (2.33). To evaluate this observation further, the data up to 48 hr. were examined (refer Figure 31). The difference in slopes and intercept during the period of burst release could be clearly demonstrated. The capillary device was able to simulate the lag-time in absorption more effectively than the flow-through cell, as indicated by its intercept of -2.18 hr.

A further substantiation of difference between the two devices to simulate the overall lag of absorption relative to *in vitro* drug was obtained from the Levy's plot (Figure 32). The time required for *in vivo* absorption of 0-10%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80% and 80-90%, was plotted versus the time for *in vitro* release of the same amounts of naltrexone using both devices.

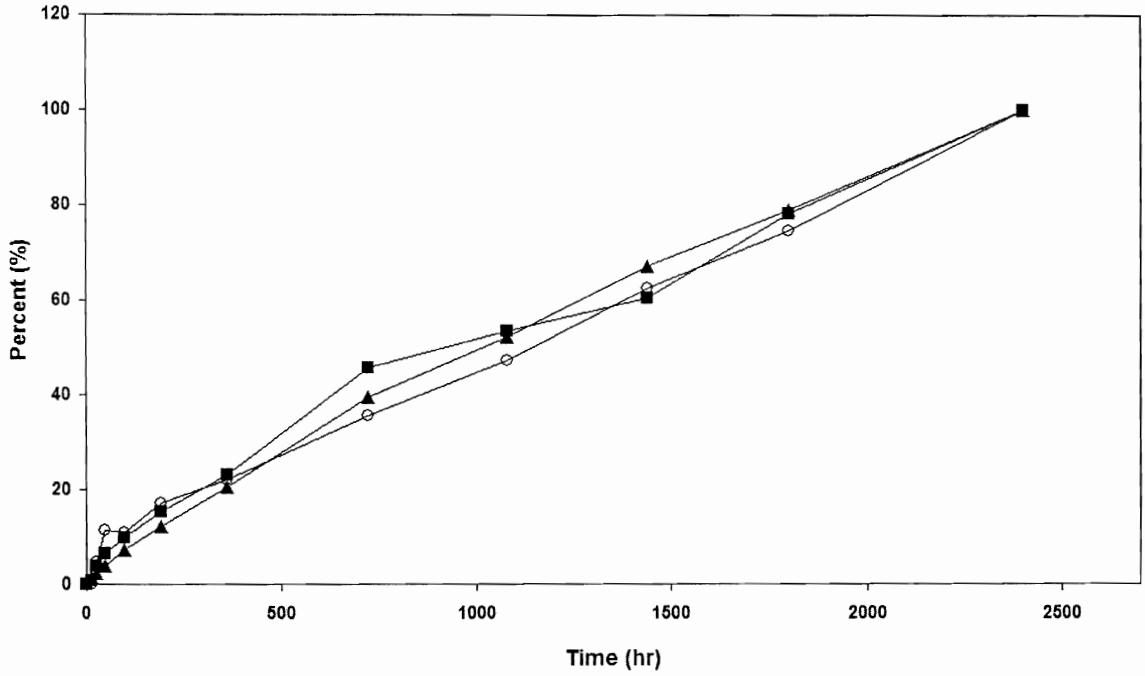
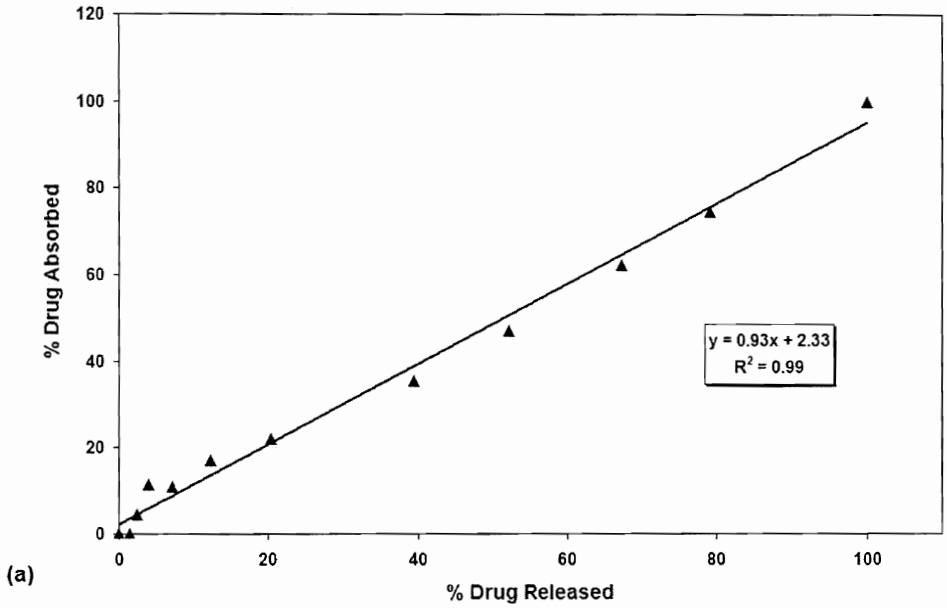


Figure 29.

Qualitative Visualization of an *In vitro-In vivo* Relationship.
Key: ▲, flow-though cell; ■, capillary device; ○, *in vivo*.



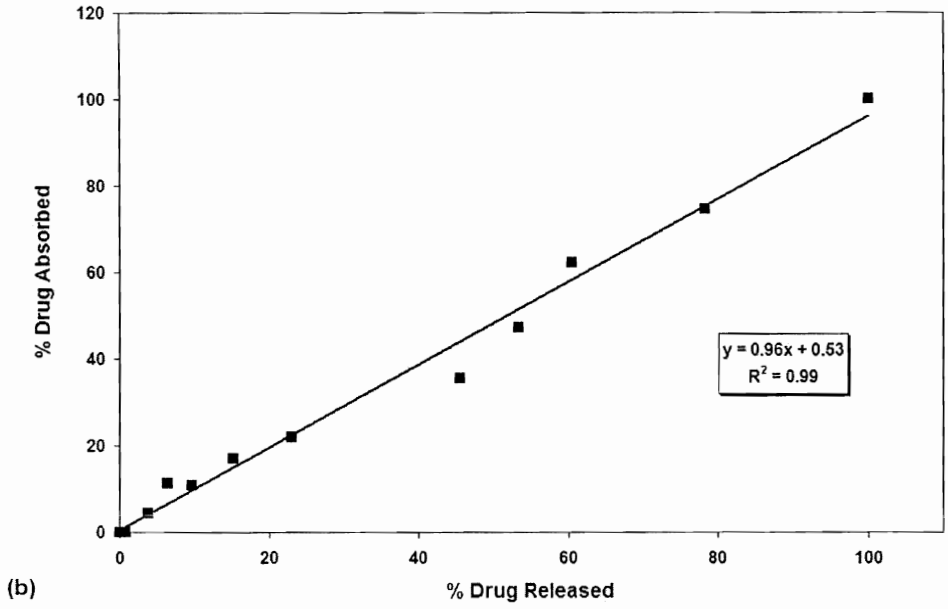


Figure 30.

Plots to show *In vitro-In vivo* Correlations using
(a) the flow-through cell, and (b) the capillary device.
Key: ▲, flow-though cell; ■, capillary device.

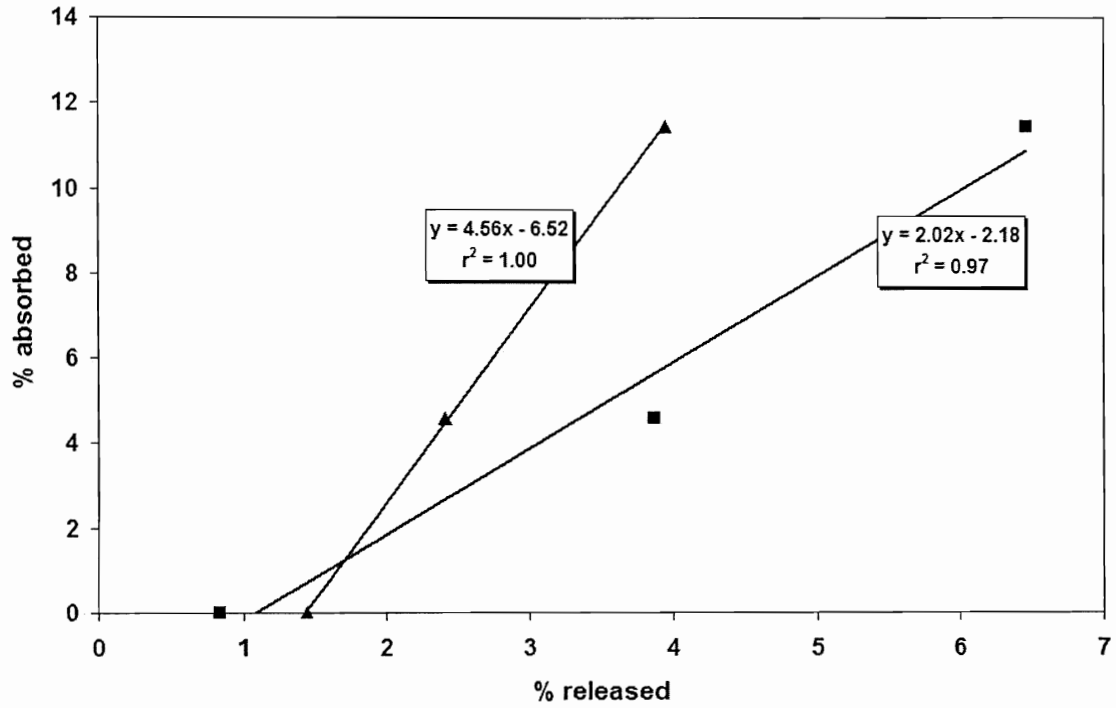


Figure 31.

Comparison of IVIVC using the flow-through cell and the capillary device during the first 48 hours of release to show difference in slopes and lag-times.

Key: ▲, flow-though cell; ■, capillary device

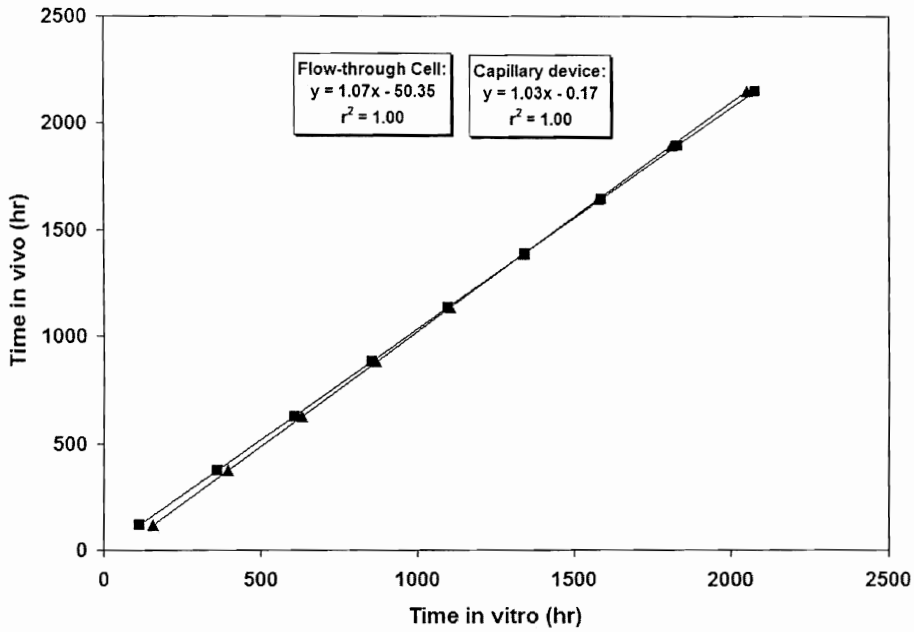


Figure 32.

Levy's Plot to demonstrate a closer simulation of *in vivo* lag time using the capillary device.
Key: ▲, flow-though cell; ■, capillary device.

The relationship showed that the lines for the two devices had significantly different intercepts from zero ($p < 0.0001$). As expected, however, the value of the intercept for the capillary device was close to zero, indicating that the capillary device was better able to simulate the barrier to diffusion than the flow-through cell.

The statistical significance of the intercept (-0.17 hr) being different than zero can be ignored at this stage. Also, it is important to understand that margin of error for *in vivo* data has not been considered in this study (n=1).

4.4 Conclusion

A new 'biorelevant' approach to *in vitro* release testing of a naltrexone implant has been described this chapter. The Modified 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 linear correlation was obtained with both the devices. As expected from the flow characteristics and barriers to diffusion however, the capillary device was able to simulate the lag-time in absorption more effectively than the flow-

through cell. Therefore, the capillary device could 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. This chapter demonstrated the potential of the 'biorelevant' approach in further investigations of real-time *in vitro* drug release from implant dosage forms.

CHAPTER 5. A 'Biorelevant' Approach to Accelerated *In vitro* Drug Release Testing of a Biodegradable, Naltrexone Implant

5.1 Introduction

Most depot formulations made from biodegradable polymers release the incorporated drug over an extended period of time (Eldrige et. al., 1991). An estimation of the real-time release rate is critical for characterization of these dosage forms. The process however, consumes significant time spanning weeks or months for sustained-release parenteral dosage forms. This is disadvantageous in early research, and therefore not conducive for efficient management of product development. Accelerated *in vitro* release tests are also desirable for quality control, particularly in establishment of specifications for releasing product batches.

Accelerated stability testing has been employed to predict the shelf life of drugs and dosage forms (Baertschi and Jansen, 2005). Method applications to accelerate dissolution rates of oral dosage forms have been published (Scott, 2002). Several parameters such as changes in temperature, pH, solvent, ionic strength, surfactants, agitation rate, enzymes, and the application of microwaves, alter the rate of *in vitro* dissolution/release (Iyer et. al., 2006). The primary consideration during application of these tests is that only the rate of drug release should increase as a function of changes in the selected parameter; the mechanism of drug release, however, should not be altered

(Burgess et. al., 2002). Furthermore, the accelerated test should mimic physiological conditions at the site of administration ('biorelevance') to the extent possible.

For biodegradable matrices, temperature is most often selected as the parameter of choice since it has been proven to induce maximum effects on release rates (Scott, 2002). The application of accelerated tests for *in vitro* release of subcutaneous implants requires prior consideration of several factors (Table 16). These factors represent changes that could be induced as a result of employing stressed conditions for the test. As an example, variables that control the release rate from matrix based drug delivery systems are the composition of the polymer and porosity changes (Bergsma et. al., 1994). The variables are influenced at elevated temperature to shorten degradation time for polylactides (Aso et. al., 1994), and to demonstrate drug-release mechanisms (Cha et. al., 1989; Kaniwa et. al., 1999). However, as a function of the composition of the polymer, drug release is also accompanied by a softening of the polymer due to enhanced hydration and degradation (Pitt, 1990). Therefore, it is important to select and adjust the parameter only to an extent such that the integrity of the dosage form matrix is not compromised.

Stabilities of the medium and the drug are also significant factors that need consideration. The components of the medium are likely to undergo degradation under the stress conditions employed. The degradation products formed would accumulate in the medium if a closed system, in which the same medium is kept recirculating through the release apparatus into the reservoir for the complete duration of study, is used. Several confounding events can then occur in the system. Drug release may be influenced by possible deviations in pH, buffer capacity or osmolality from initial values of a

Table 16

Factors influencing the application of stress conditions for accelerating *in vitro* drug release from subcutaneous implants

---

a) Selection of an appropriate parameter for stress induction

b) The mechanism controlling the release of drug from the dosage form

c) The stability of the medium

d) The stability of the drug in the dosage form, and in the medium once released

e) The robustness of the *in vitro* release apparatus to withstand the applied stress

f) Determination of the sampling interval and the duration of the study

g) The solubility of the drug, the dose rate and the influence of 'sink' conditions

---

medium freshly prepared. In addition, a faster degradation of the dosage form matrix may be induced due to bulk processes such as 'autocatalysis', whereby the liberated free carboxylic end groups catalyze further cleavage of ester groups of polycaprolactones. This could result in complicated release mechanisms, deviating from the objective of keeping the mechanism consistent with real-time studies.

Another important factor for consideration during method optimization for accelerated release is the robustness of the test apparatus to withstand the applied stress conditions. This requires that the apparatus must have well-defined components that do not undergo deterioration. Depending upon the exposure of parts to the flow of medium, all pieces of tubing, connectors, and filters, O-rings etc. have to be checked for abrasion under test conditions.

Further insight can be gained by an investigation of the drug's stability under elevated temperatures. Verification of the validity of using these conditions might include an Arrhenius plot after obtaining release rate information from linearized release profiles (Makino et. al., 1985). The question that remains, however, is what constitutes a significant change in the dissolution profile, since defined limits for the consideration of percent drug remaining in the medium after a designated test time are not available in any guidance (Storey, 1996).

Naltrexone, an opiate receptor antagonist (Resnick et. al. 1974), has been recognized as a good candidate for formulation as an implant to increase patient compliance and ultimately improve treatment effectiveness (Brewer et. al., 2002; Hulse et. al., 2004). In earlier studies (Chapters 2 and 4), an investigation of a 'biorelevant'

approach to real-time drug release testing of a biodegradable, subcutaneous implant of naltrexone had been carried out. Design modifications of a capillary device and a flow-through cell were employed for release rate investigations, and the data demonstrated that a better representation of the initial phase of burst release *in vivo* could be achieved *in vitro* using the capillary device. In addition, a detailed characterization of the Modified Hanks' Balanced Salts Solution had established its potential for *in vitro* drug release studies. Although the use of accelerated methods for dissolution rate studies of oral dosage forms (Quist et. al., 2002) are available, no published information is available on application of this technique for subcutaneous implants.

In this chapter, an approach based on elevated temperature to accelerate naltrexone release from the implant is investigated. This is the first study on 'biorelevant' accelerated release testing for a subcutaneous implant dosage form, although published approaches for tablets are available. Two elevated temperatures, 45°C and 55°C, were investigated for increasing the rate of drug release. Modified flow-through cells and previously separated capillary devices for real-time release were utilized.

5.2 Materials and methods

5.2.1 Materials

Naltrexone hydrochloride (USP Grade, working standard) for the assay was obtained from Sigma (St. Louis MO, USA). The capillary device (Cellmax™) was procured from Spectrum Labs., CA, USA. The sagittal saw for the study was generously provided by Stryker Corporation, MI, USA. Analytical grades of triethylamine,

ammonium hydroxide and trifluoroacetic acid, and Hanks' Balanced Salts (1 x 10 liter) 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).

5.2.2 Selection of temperature as the parameter for accelerating *in vitro* release rate

The Modified Hanks' Balanced Salts Solution was pumped at the same flow rate as employed in the real-time study (Chapter 4). The 'biorelevant' flow rate had been calculated to be 1.06 ml/min on the basis of shear stress (0.07-20 dyne/cm^2) that endothelial cells are exposed to *in vivo* (Redmond et. al., 1995). Although an enhancement of flow rate within the biorelevant range was possible as an option to accelerate drug release using the flow-through cell, it could not be expected to result in significant changes in release rates with the capillary device. This was because the flow of medium entering a capillary device would be split between 50 capillaries within the device. Also, since the implant was placed in the extracapillary space (representative of the subcutaneous interstitium) as compared to the flow-through cell, the medium would not flow directly across its length. Any increase of flow rate in the circulating loop would not have translated into a corresponding flow rate increase at the site in which the implant was positioned. Therefore, flow rate was not considered as a useful parameter. pH and ionic strength of the medium could also be potentially modified to accelerate drug

release. However, a reduced ionic strength would denote a reduction in buffer capacity of the medium and could result in the medium to deviate from physiological pH range when used over a prolonged period of time. Polycaprolactone and poly (glycolic acid) are degraded by enzymes, such as esterase and elastase (Park and Shalaby, 1993). The enhanced degradation of the polymer could increase the release rate of drug. However, the addition of enzymes could potentially lead to other challenges, such as development of selective analytical methods capable of detecting a larger number of degradation products generated by the enzyme itself. Furthermore, a difficulty exists in maintaining enzyme activity constant throughout the period of study. The use of surfactants would also suffer from similar disadvantages as enzymes, in terms of maintenance of exact concentration and detection of additional degradation products.

Therefore, temperature was selected as the parameter of choice. Two elevated temperatures, 45°C and 55°C, were utilized for this purpose, and a comparison of data obtained using the modified flow-through cell and capillary device were compared with the real-time release rates at 38°C. 55°C was selected as the highest temperature based upon the melting point range of the polymer, 59-64°C (Pitt et. al., 1980; Pitt, 1990).

5.2.3 Calculation of 'sink' conditions

The solubility of naltrexone freebase in Hanks' Balanced Salts Solution at 32°C is 5.42 mM (Stinchcomb et. al., 2000). 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 for a departure from sink conditions at 32°C would be 1.63 x

10^{-2} M or 5.54 mg/ml. Since release rates from implants were expected to be much slower and assuming that the solubility of naltrexone does not vary much as a function of temperature, the value indicated fair flexibility provided for accumulation of drug in the release medium prior to replacement.

5.2.4 Additional Modifications to System Assembly

All components of the system used previously for investigation of real-time release rate were employed also for this study. No changes were required, except that out of the 6 flow-through cells and 6 capillary devices used previously at 38°C, 3 each were utilized at the two elevated temperatures investigated. Two water baths calibrated to provide temperatures of 45°C and 55°C inside the media reservoirs were utilized for this purpose. The flow path and location of the system components (pump, tubing etc.) relative to each other were modified suitably. This involved providing new connections to all 12 channels of the pump in order to divert them to the desired devices.

The other system components that including reservoirs, fraction collectors, glass beads, and filters were the same as employed in the previously (Chapter 4). The pump was calibrated for a flow rate of 1 ml/min.

5.2.5 Test Material

The naltrexone implants employed earlier in the real-time release study (Chapter 4) were utilized for this investigation. Important information about the persistence of an intact membrane sheath and a homogenous core after 90 days of real-time testing had

been obtained through Scanning Electron Microscopy. These implants were obtained from Durect Corporation, CA, USA. The monolithic implant consisted of a biodegradable core of naltrexone: polycaprolactone enclosed in a sheath of poly-(DL-lactide: caprolactone). The implant itself was fabricated by melt-extrusion and the ends of the cylinder were sealed with the same polymer that had been used for the membrane sheath. The implants retained their dull grey appearance following the real-time *in vitro* study. Figure 33 is a photograph of the implant after the real-time *in vitro* release study.

5.2.6 Accelerated *In vitro* Drug Release Experiments

The flow-through and capillary systems were modified to accommodate the implants as described earlier (Chapter 4). The flow-paths were assembled as described in Section 5.2.4. A final check of the flow rate was conducted before reservoirs containing fresh media pre-heated to the selected temperatures were placed in the water bath, and the flow path closed. For the accelerated study, three flow-through and three capillary devices containing an implant were studied at 45°C and 55°C. Since one implant had been sacrificed for microscopic evaluation following real-time studies, it was decided to employ 2 implants for the flow-through cell at 55°C. 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 its extra-capillary space to escape. As soon as the medium made its way up, the ports were closed to enable the medium to exit via the end port into the reservoir. The system was operated continuously except that the pumps were stopped during replacement of the buffers. Samples were collected every 24 hours for 45 days, and were

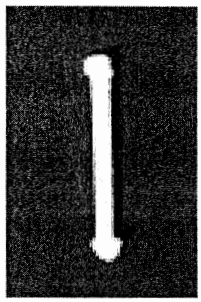


Figure 33.

Photograph of the biodegradable naltrexone implant after real-time release investigation of 90 days.

stored below -20°C until analysis. Periodic checks were conducted for the temperature inside the vessel. For reservoirs at 45°C and 55°C, the flow rate and pH of the media were checked every 4th and 3rd day, respectively, as determined in Chapter 2. Media replacement was also conducted on those days. 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. Reservoirs containing fresh medium pre-heated to 45°C or 55°C, as the case may be, was placed in the water bath, 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 minutes of pump stoppage time. No fluctuations in flow rate were observed throughout the study, because no clogging of the filters occurred. At the end of 45 days, the implants were taken out of the release devices, and allowed to dry at room temperature and the weights were recorded.

It was decided to cross-validate the approach in terms of changes in the implant matrix during the accelerated study that could potentially influence the mechanism of release. This was based on the assumption that if any matrix changes had taken place, a reversal of the temperature back to the real-time condition (38°C) would result in a different release profile from that reported initially (Chapter 4). Therefore using both types of devices, the study was extended into a real-time investigation at 38°C to crosscheck if satisfactory release profiles were still obtained. A single water bath was used for this purpose, as described earlier (Chapter 4).

5.3 Results and Discussion

5.3.1 System Performance

The system worked efficiently throughout the study period. At the end of Day-20, the peristaltic pump tubing was checked for abrasions to avoid possible leakage. Replacement of tubing was not required during the study. No visible contamination of microorganisms was observed indicating 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 fluctuations due to clogging of filters in the flow-through device were observed, also indicating that the dosage form had retained its integrity. This was further verified at the end of the study by removal of the dosage form from the devices and measuring its dimensions.

On one occasion while the capillary devices was running at 55°C, a minor leak (approximately less than 2 ml of medium had been lost) was detected at the spot where the system had been resealed. This was effectively sealed within ½ hour however, and a concentration factor for the lost amount of naltrexone was employed in further calculations for that particular device.

Media replacement proceeded smoothly with a 10-minute pump stoppage time. The pH was observed to vary up to only $\pm$ 0.05 units as measured for the medium immediately after each replacement; thereby indicating that the Modified Hanks' Balanced Salts Solution had adequate buffer capacity for accelerated study of the implant. A visible examination of media performed periodically indicated the absence of microbial contamination.

5.3.2 Sample Analysis

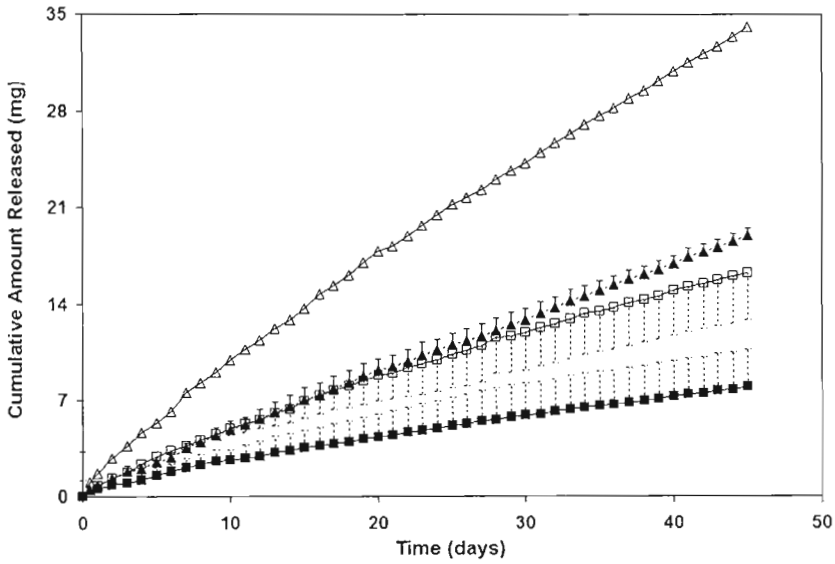
The samples were analyzed by a validated High Performance Liquid Chromatography (HPLC) method described elsewhere (Chapter 3). The peak areas at 204 nm for naltrexone were used for quantification. No chromatographic interference was observed from any degradation product. The calibration curves were linear in the range of 0.16-20 μg/ml ($r^2 > 0.99$) using a weighting factor of 1/concentration, and the precision and accuracy of quality control samples processed and analyzed along with the samples were all 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.

A RSD of 19.43% (n = 6) was obtained for mean concentrations of naltrexone in samples withdrawn 12 hours after each consecutive replacement of medium. Any additional peaks that would have represented degradation of the drug, dosage form, or capillary material, were not observed. Assuming drug release to follow zero-order kinetics, this result is consistent with the fact that: a) solid-state degradation of the drug in the implant, if any, was negligible, and b) no carryover in terms of non-specific binding of naltrexone to the flow path existed.

5.3.3 Analysis of Accelerated *In vitro* Release Data

A comparison of the accelerated release profiles obtained for 45 days using the modified flow-through and capillary devices is made in Figure 34. Overall, the rates of

(a)



(b)

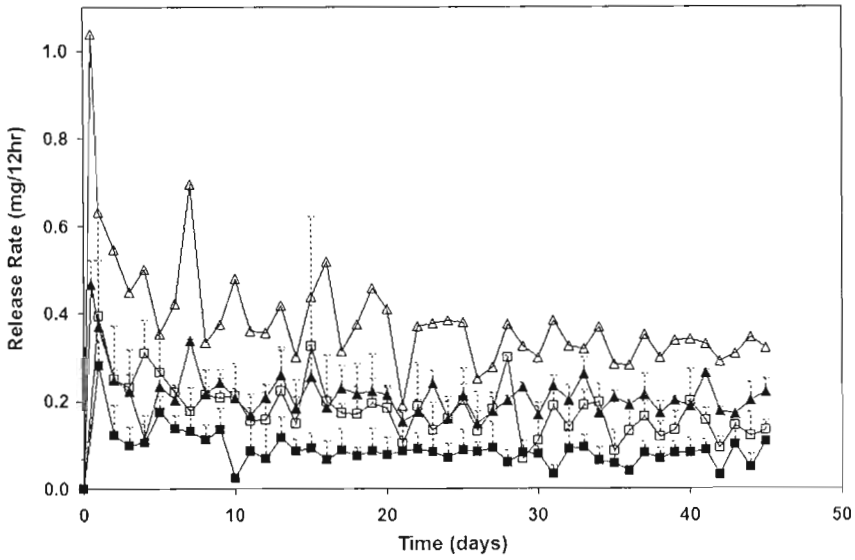


Figure 34.

(a) Cumulative release plot, and b) Release rate plot for naltrexone implants under accelerated conditions using the modified flow-through and capillary devices (n=3).

Key: Δ, flow-though cell at 55°C*; ▲, flow-though cell at 45°C; □, capillary device at 55°C ■, capillary device at 45°C.
(Error bars represent standard deviations; *n = 2)

release of naltrexone using the flow-through cell were 0.22 mg/12 hr (95% CI: 0.198, 0.233), and 0.39 mg/12 hr. (95% CI: 0.348, 0.429), respectively, at 45°C and 55°C. The rates of release of naltrexone using the capillary device were 0.09 mg/12 hr (95% CI: 0.077, 0.102), and 0.18 mg/12 hr. (95% CI: 0.161, 0.201), respectively, at 45°C and 55°C. The capillary device resulted in lower rates of release at corresponding temperature levels, consistent with those observed in real-time data. This can be explained by a lower rate of flow of the medium in the extracapillary space of the capillary model, thus effectively simulating a barrier to diffusion that would exist *in vivo*.

For both the devices and at both temperatures, the values mentioned above represented a significant ($p < 0.0001$) increase in the overall rate of release from the corresponding real-time rates. This proved that the increased temperature had succeeded in accelerating the release rate from the implant *in vitro*.

Table 17 represents mean release rates of naltrexone from the implants using both devices at 38°C, 45°C and 55°C, in periods following initial burst when steady zero-order profiles were observed. At 45°C, the release rate using the flow-through cell and capillary device increased 2.2-fold and 2.0-fold, respectively, as compared to the corresponding rates at 38°C; whereas at 55°C, the rates increased 3.9-fold and 4.0-fold with the corresponding devices. These observations suggest that, irrespective of the type of device used, temperature was the sole critical parameter that determined the increase in release rate.

Furthermore, an initial period of burst release was observed consistent with the phenomenon observed during real-time release. The percolation-limited diffusion theory

(Tzafriri, 2000) described in Chapter 4 offers a possible explanation for this phenomenon. A pool of mobile drug molecules would exist on the dried polymer surface. As soon as fresh medium flows into the pool, naltrexone release would follow immediately and account for the phase of burst release. For the flow-through cells, mean peak release rates of 1.04 and 0.47 mg/12 hr were observed at 55°C and 45°C, respectively; whereas for the capillary device, the rates were 0.37, and 0.28 mg/12 hr, respectively, at the two temperatures. The time for peak burst release at both temperatures, however, remained 0.5 and 1 day with the flow-through and capillary decices, respectively, as comparable to corresponding observations in real-time release profiles. This observation shows that although the magnitudes of release rates were different, no indication of a change in release mechanism had occurred.

To demonstrate the validity of the temperatures used, an Arrhenius relationship was investigated for both devices using the zero-order rate constants. A good linear relationship (Figure 35) was found for both devices. The energy of activation (E_a), as calculated from the slopes was 16.62 kcal/mole and 17.84 kcal/mole, for the flow-through cell and capillary device, respectively. The similar activation energy values obtained for both the devices demonstrated that the release mechanism had been consistent; and that the rates of release could be used for long-term prediction. Also, the E_a values were greater than 5.65 kcal/mole, the activation energy earlier reported (Chapter 3) for naltrexone in the same medium under similar conditions. Although activation energies cannot be assumed to be perfectly additive, it can be speculated that

Table 17.

Comparison of mean release rates (mg/12hr) of naltrexone at different temperatures

Condition	Type of Device		Statistical Significance
	Flow-through	Capillary	
38°C (Data beyond Day-25)	0.09 (0.04)	0.04 (0.03)	$p < 0.0001$
45°C (Data beyond Day-10)	0.20 (0.03)	0.08 (0.02)	$p < 0.0001$
55°C (Data beyond Day-10)	0.35 (0.07)	0.16 (0.05)	$p < 0.0001$

*Values in parentheses represent standard deviations

the higher values of E_a are a result of contributions from degradation and/or polymer erosion.

It is recommended that the specifications for accelerated release should include a determination of at least 80% of the cumulative amount released (Burgess et. al., 2002). Also, for a prediction between accelerated and real time release, it has been suggested that the time to reach a cumulative release of approximately 100% be used to determine whether a relationship can be established for products with different real time release rates. A major objective for any product is to provide a thorough characterization of the release profile *in vitro*, the practical aspects of the recommendation are questionable, especially when sustained release dosage forms with drug release over a period of months are investigated. Furthermore, results from the Arrhenius plot showed that a prediction for long-term accelerated release from this implant can be made. Therefore, it was decided to terminate the study after 45 days, during which the release rates were determined to be consistent with zero-order kinetics.

Figure 36 is a representation of the profiles obtained after reverting the test temperature back to 38°C. A phase of burst release more prominent for the flow-through cell was observed. Release rates decreased correspondingly with the flow-through cell [0.07 ($\pm$ 0.013) mg/12 hr] and capillary device [0.04 ($\pm$ 0.004) mg/12 hr]. This observation demonstrates that the integrity of the dosage form was maintained throughout the studies and the release rate mechanism followed zero-order kinetics after an initial stage of burst release.

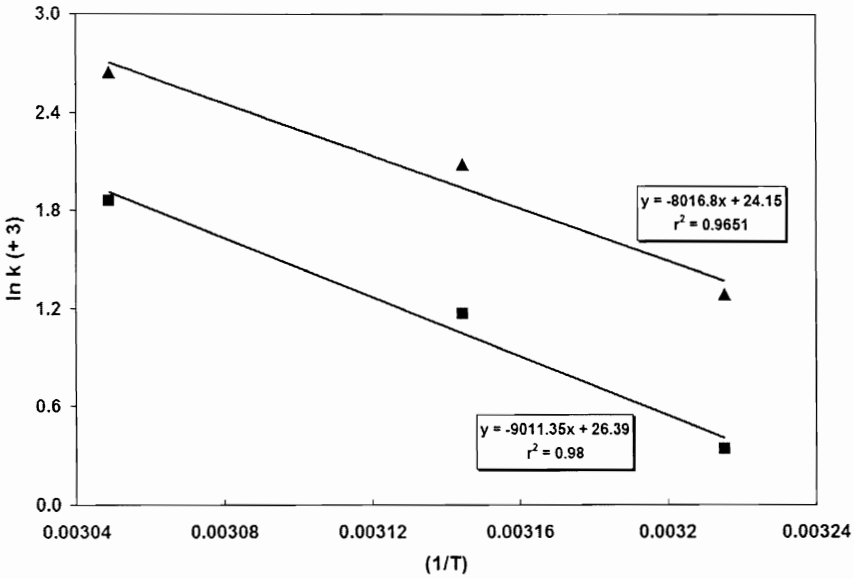
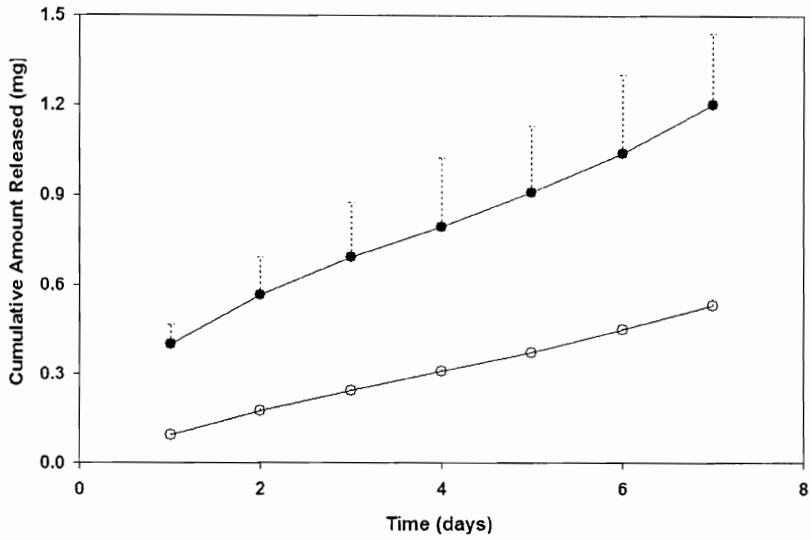


Figure 35.

Arrhenius relationships of release rates using the modified flow-through (n=5) and capillary devices (n=6) at 38°C, 45°C and 55°C.

Key: ▲, flow-though cell; ■, capillary device.

(a)



(b)

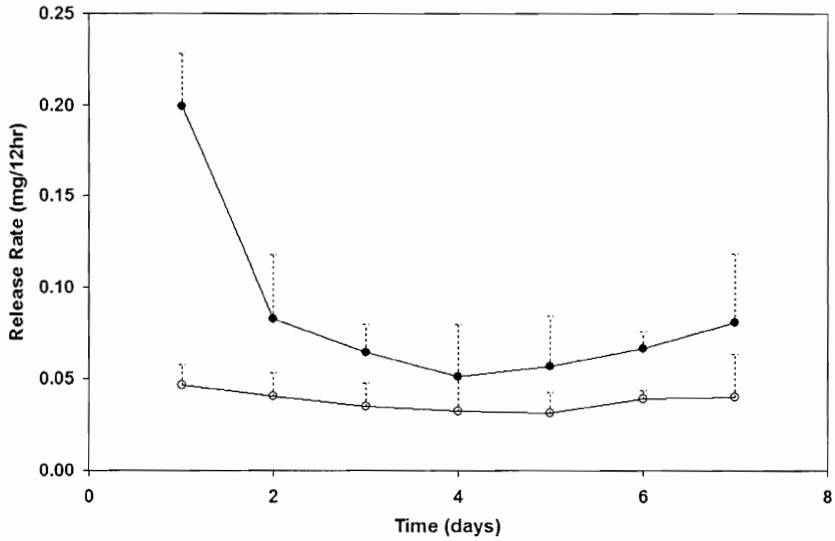


Figure 36.

(a) Cumulative release plot, and b) Release rate plot for naltrexone implants under real-time conditions using the modified flow-through (n=5) and capillary devices (n=6) to cross-validate the accelerated release study

Key: ●, flow-though cell; ○, capillary device.

5.4 Conclusion

The application of a 'biorelevant' approach to accelerate naltrexone release from a biodegradable implant has been described in this chapter. The capillary system was employed without any major modification from that used for real-time study and remained stable throughout the investigation at elevated temperatures. The Modified Hanks' Balanced Salts Solution has been shown to be suitable for accelerated release rate investigation of the implant. Sample analyses using a validated stability-indicating HPLC method resulted in zero-order *in vitro* release profiles. Drug release using both devices increased by identical magnitudes without affecting the rate mechanism. The results demonstrated the controlled use of temperature to be a useful parameter to investigate accelerated drug release from implant dosage forms. Decisions regarding the duration for which accelerated release studies need to be conducted in order to allow adequate characterization of drug release need to be made on a case-by-case basis.

CHAPTER 6. Summary and General Conclusions

An estimation of the real-time *in vitro* drug release rate is critical for characterization of dosage forms. To obtain data that are more meaningful clinically however, it is essential that the *in vitro* test should simulate the environment at the site of administration (a condition termed 'biorelevance'). This research investigated a novel application of a miniature, capillary bioreactor for *in vitro* release testing of a subcutaneous, biodegradable implant of naltrexone. The Hanks' Balanced Salts Solution was modified for testing as a physiologically relevant medium for subcutaneous implant release studies. A comparison of the *in vitro* profile generated using the capillary device, was carried out versus the profile generated using the flow-through cell.

In vitro release profiling of sustained release parenteral dosage forms however, consumes significant time spanning weeks or months. This is disadvantageous in early research, and therefore not conducive to efficient management of product development. An accelerated *in vitro* release test is therefore desirable for quality control, particularly in establishment of specifications for releasing product batches. An approach for accelerating *in vitro* drug release from the implant using elevated temperature conditions has also been shown to be predictive in this study.

The development and validation of analytical methods in Modified Hanks' Balanced Salts Solution and in dog plasma for the analyses of *in vitro* and *in vivo*

samples, respectively, has been described. The Modified Hanks' Balanced Salts Solution was shown to be a suitable 'biorelevant' medium for *in vitro* release testing of the naltrexone implant. The physicochemical parameters of the medium that were evaluated included pH, buffer capacity, osmolaity and spectral changes as a function of time and temperature. These were found to be within acceptable limits. The calibration curves of the stability-indicating HPLC-UV method were linear in the range of 0.16-20 μg/ml ($r^2 >$ 0.99). The global inter-day precision and accuracy for 3 batches (n = 9) were found to be between 1.4%-1.7%, and -1.0%-0.1%, respectively. The HPLC method was robust during more than 1500 real-time *in vitro* sample analyses. Information obtained on stability of naltrexone in the medium at different temperatures was employed to determine time intervals for media replacements. A novel Liquid Chromatography - Tandem Mass Spectrometry (LC-MS-MS) method for the analysis of naltrexone in dog plasma was also developed and validated. The LOQ of the method was established at 10 pg/ml. This was found to be suitable for quantification of low concentrations in samples of the terminal phase of decline following implant removal. The calibration curves of the method were linear in the range of 10-5014 pg/ml ($r^2 > 0.98$) using a weighting factor of 1/concentration. The concentration residuals were between 92.4%-108.9% (RSD = 1.9%-9.8%). The global inter-day precision and accuracy for 3 batches (n = 9) were found to be between 10.2%-14.7%, and 93.8%-101.5%, respectively. The method employed a deuterated analog of naltrexone as the internal standard. This was because in quantitative LC-MS-MS methods, the similar relative efficiencies of ionization of the two molecular species would be expected to compensate for any matrix effect. A molecular modeling

approach was evaluated to predict whether or not an uncompensated matrix effect would be expected. The calculated binding energy difference from the model was found to be only 0.03 kcal/mole; thus it was expected that the two analytes should not exhibit different retention behaviors in the column.

This work also describes modification, assembly and comparison of data of a new miniature, capillary device and a flow-through cell in order to assess real-time drug release from a naltrexone implant. Zero-order *in vitro* release profiles were observed with both devices after an initial period of burst release. An implantation study in a dog was conducted to evaluate *in vitro-in vivo* correlation (IVIVC). Macroscopic and histological assessments of changes in subcutaneous tissue at the site of implantation revealed no signs of toxicity after 3 months of implantation. Although good IVIVCs were obtained overall with both devices, the capillary system was able to simulate the lag-time in absorption more effectively than the flow-through cell. Data from a Levy's plot further demonstrated this difference in the correlations. An investigation of accelerated drug release testing using an approach based on elevated temperature yielded encouraging results. At 45°C, the release rate using the flow-through cell and capillary devices increased 2.2-fold and 2.0-fold, respectively, as compared to the corresponding rates at 38°C; whereas at 55°C, the rates increased 3.9-fold and 4.0-fold with the respective devices. These observations suggest that, irrespective of the type of device used, temperature was the sole parameter that determined the increase in release rate. Similar activation energy values of 16.62 kcal/mole and 17.84 kcal/mole for the flow-through cell and capillary device, respectively, were obtained from Arrhenius plots. This

demonstrated that the release mechanism was consistent in both devices; and that the rates of release could be used for long-term predictions.

The research described in this dissertation has provided new tools that may prove useful in drug development and production. There have also been a number of issues that will benefit from further investigation. An important extension of this work would involve testing of a larger number of deuterated analogs using the molecular modeling approach to evaluate potential matrix effects. The analogs should vary in the number and position of deuterium substitution. Linear relationships of the differences in binding energies of the analogs to the stationary phase, versus their chromatographic resolution, needs to be further documented and validated.

Although a direct extrapolation of the plasma concentrations could not be made to humans because of interspecies differences, naltrexone concentrations at steady-state indicated that therapeutic levels (1-2 ng/ml) are not likely to be achieved using the implant employed for this study. Further product development based on modifications of the dosage form matrix and/or implantation of multiple unit dosage forms needs to considered prior to dose rationalization.

A particularly exciting area for further research would involve making improvements to the capillary device itself. The average radius of pores for the *in vitro* capillary material was 0.5 μm, whereas the average radius of pores *in vivo* is 24 A^{o}. The factor (R_{diff}) describing this additional restriction to diffusion *in vivo* indicated that, possibly, testing capillary modules having lower pore diameters might simulate the *in vivo* condition more accurately. However, it would be difficult to manufacture capillaries

with smaller pore sizes reproducibly. Further, if blood flow were the rate-limiting step, it would be very easy to simulate this condition using the capillary device. Variations in blood flow, if known, can be easily reproduced using the peristaltic pump. Information obtained using microdialysis experiments and intravenous administration of the drug would be useful to gain an understanding of the *in vivo* input rates. Based on the Renkin equation described in Chapter 4, it can be speculated that for larger molecules (e.g. proteins and biologics that have sufficient diffusional resistance provided by pores in the capillary wall), better *in vitro-in vivo* relationships might be obtained with permeation rate across the capillary wall, rather than with the release rate from the delivery system. Studies involving molecular modeling and permeability would be very useful to simulate these conditions. A predictable acceleration of drug release resulted when the temperature was elevated for both the capillary and flow-through devices. Other parameters for accelerating drug release should also be investigated especially for compounds that are found to be thermally labile. These other parameters would be subject to the limitations as described in Chapter 5.

This study demonstrated that strategies for development of 'biorelevant' *in vitro* release methods must consider important variables, such as the properties of the drug and the dosage form, the release medium, and the physiology at the site of implantation. This research has provided valuable information on development and optimization of a 'biorelevant' *in vitro* release testing system for drug implants. Further studies using this 'biorelevant' device with implants that have different release characteristics are necessary to completely validate and broaden the scope of applicability of this system.

List of References

Anderson, J.M., 1994. In vivo biocompatibility of implantable delivery systems and biomaterials. Eur. J. Pharmacol., 40, 1-8.

Agrawal, C.M., Athanasiou, K.A., 1997. Technique to control pH in vicinity of biodegrading PLA-PGA implants. J. Biomed. Mater. Res., 38, 105-114.

Allababidi, S., Shah, J.C., 1998. Kinetics and mechanism of release from glyceryl monostearate-based implants: Evaluation of release in a gel simulating in vivo implantation. J. Pharm. Sci., 87, 738-744.

Athanasiou, K.A., Niederauer, G.G., Agrawal, C.M., 1996. Sterilization, toxicity, biocompatibility and clinical applications of polylactic acid/polyglycolic acid copolymers. Biomaterials, 17, 93-102.

Arrhenius, S., 1889. Z. Phys. Chem. 4, 226-248.

Aso, Y., Yoshiyoka, S., Wan Po, L., Terao, T., 1994. Effect of temperature on mechanisms of drug release and matrix degradation of poly (D,L-lactide) microspheres. J. Controlled Rel., 31, 33-39.

Aukland, K., Reed, R.K., 1993. Interstitial lymphatic mechanisms in the control of extracellular fluid volume. Physiol. Rev., 73, 1-78.

Aungst, B.J., Myers, M.J., Shefter, E., Shami, E.G., 1987. Int. J. Pharm. 38, 199-209.

Baertschi, S.W., Jansen, P.J., 2005. Stress testing: a predictive tool. In Baertschi, S.W. (Ed.), Pharmaceutical stress testing. Predicting drug degradation. Drugs and the Pharmaceutical Sciences, volume 153. Marcel Dekker Inc., New York, pp. 13-50.

Banakar, U.V. Dissolution Testing Devices. In Pharmaceutical Dissolution Testing, Swarbrick J (ed). Marcel Dekker Inc., New York, 1992, pp. 53-105.

Banker, G.S., Rhodes, C.T., Modern Pharmaceutics. second ed. Marcel Dekker, New York, 1995.

Ballard, B.E., 1961. The physicochemical properties of drugs that control their absorption rate after implantation. Ph.D. Thesis, University of California, San Francisco, USA.

Bartus, R.T., Emerich, D.F., Hotz, J., Blaustein, M., Dean, R.L., Perdomo, B., Basile, A.S., 2003. Vivitrex®, an injectable, extended-release formulation of naltrexone, provides pharmacokinetic and pharmacodynamic evidence of efficacy for 1 month in rats. Neuropsychopharmacol., 28, 1973-1982.

Barzilay, R.B., Hersey, J.A., 1968. Dissolution rate measurement by an automated dialysis method. J. Pharm. Pharmac. 20 Suppl., 232S-238S.

Benet, L.Z., 1990. The effect of route of administration and distribution on drug action. In Banker, G.S., Rhodes, C.T. (ed.), Modern Pharmaceutics, second edition. Drugs and the Pharm. Sci. Series, Vol. 40, Marcel Dekker Inc., New York, pp. 181-207.

Berger, M., Cuppers, H.J., Hegner, H., Jorgens, V., Berchtold, P., 1982. Absorption kinetics and biologic effects of subcutaneously injected insulin preparations. Diabetes Care, 5, 77-91.

Bergsma, J.E., Rozema, F.R, Bos, R.R.M., Boering, G., De Bruijn, W.C., Pennings, A.J., 1994. In vivo degradation and biocompatibility study of in vitro pre-degraded polymerized polylactide particles. Biomaterials, 16, 267-274.

Bert, J.L., Mathieson, J.M., Pearce, R.H., 1982. The exclusion of human serum albumin by human dermal collagenous fibres and within human dermis. Biochemical. J., 201, 395-403.

Bihari, B. Online website: http://www.lowdosenaltrexone.org/ (May 8, 2006).

Bishop, P.M.F., Folley, S.J., 1944. Implantation of testosterone in cast pellets. Lancet, 1, 434-435.

Bonfiglio, R., King, R.C., Olah, T.V., Merkle, K.. The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. Rapid Commun. Mass Spectrom. 13: 1175-1185 (1999).

Borst, I., Ugwu, S., Beckett, A.H., February 1997. New and extended application for USP drug release apparatus 3. Dissolut. Technol., 1-6.

Brewer, C., Rezae, H., Bailey, C., 1988. Opioid withdrawal and naltrexone induction in 48-72 hours with minimal drop-out, using a modification of the naltrexone-clonidine technique. Br. J. Psychiatry., 153, 340-343.

Brewer, C., 2002. Serum naltrexone and 6-beta-naltrexol levels from naltexone implants can block very large amounts of heroin: a report of two cases. Addict. Biol., 7, 321-323.

Buggé, C.J.L., Grün, I.U., Stanford, K., Tan, M.S., Garcia, D.B., 1996. Determination of naltrexone and 6β-naltrexol in human plasma by LC-MS-MS. Cedra Corporation, Austin, TX, USA, 1996.

Burgess, D.J. Presentation: New Apparatus Design: Dispersed systems, stents and other implantable devices. AAPS Workshop on Dissolution: New Technologies and Regulatory Initiatives. March 2004; Bethesda MD.

Burgess, D.J., Hussain, A.S., Ingallinera, T.S., Chen, M.L., 2002. Assuring Quality and Performance of Sustained and Controlled Release Parenterals: Workshop Report. AAPS Pharm. Sci., 4, article 7.

Burgess, D.J., Crommelin, D.J.A., Hussain, A.S., Chen, M.L., 2004. Assuring Quality and Performance of Sustained and Controlled Release Parenterals: EUFEPS Workshop Report. AAPS Pharm. Sci., 6, article 11.

Cammarn, S., Sakr, A., 2000. Predicting dissolution via hydrodynamics: salicylic acid tablets in flow through cell dissolution. Int. J. Pharm., 201, 199-209.

Cha, Y., Pitt, C.G., 1989. The acceleration of degradation-controlled drug delivery from polyester microspheres. 8, 259-265.

Chan, C.F., Chiswell, G.M., Bencini, R., Hackett, L.P., Dusci, L.J., Ilett, K.F., 2001. Quantification of naltrexone and 6β-naltrexol in plasma and milk using gas chromatography-mass spectrometry: Applications to studies in lactating sheep. J. Chrom B., 761, 85-92.

Chaplen, F.W., Fahl, W.E., Cameron, D.C., 1996. Method for determination of free intracellular and extracellular methylglyoxal in animal cells grown in culture. Anal. Biochem., 238, 171-178.

Chavez-Eng, C.M., Constanzer, M.L., Matuszewski, B.K., 2002. High-performance liquid chromatographic–tandem mass spectrometric evaluation and determination of stable isotope labeled analogs of rofecoxib in human plasma samples from oral bioavailability studies. J. Chromatogr. B., 767, 117-129.

Chien, Y.W., 1978. Methods to achieve sustained drug delivery. In Robinson, J.R. (ed.), Physical Approach: Implants, sustained and controlled release drug delivery systems. Marcel Dekker Inc., New York, pp. 211-349.

Collier, S.W., Sheikh, N.M., Sakr, A., Lichtin, J.L., Stewart, R.F., Bronaugh, R.L., 1989. Maintenance of skin viability during in vitro percutaneous absorption/metabolism studies. Toxocol. Appl. Pharmacol., 99, 522-533.

Comer, S.D., Collins, E.D., Kleber, H.D., Nuwayser, E.S., Kerrigan, J.H., Fischman, M.W., 2002. Depot naltrexone: long-lasting antagonism of the effects of heroin in humans. Psychopharmacol., 159, 351.

Cone, E.J., Gorodetzky, C.W., Yeh, S.Y., 1974. The urinary excretion profile of naltrexone and metabolites in man. Drug Metab. Dispos., 2, 506-512.

Corrigan, O.I., Devlin, Y., Butler, J., 2003. Influence of dissolution medium buffer composition on ketoprofen release from ER products and in vitro-in vivo correlation. Int. J. Pharm., 254, 147-154.

Cox, J.D., Wagman, D.D., Medvedev, V.A. Key Values for Thermodynamics, Committee on Data for Science and Technology (CODATA), Hemisphere Publishing Corp., New York, 1989.

Chidambaram, N., Burgess, D.J., 1999. A novel *in vitro* release method for submicron sized dispersed systems. AAPS Pharm. Sci., 1, article 11.

Chien, Y.W., 1978. Methods to achieve sustained drug delivery. In Robinson, J.R. (Ed.), Physical Approach: Implants, sustained and controlled release drug delivery systems. Marcel Dekker Inc., New York, pp. 211-349.

Cukierski, M.J., Johnson, P.A., Beck, J.C., 2001. Chronic (60-week) toxicity study of Duros leuprolide implants in dogs. Int. J. Toxicol., 20, 369-381.

Davies, P.F., 1989. How do vascular cells respond to flow? NIPS, 4, 22-25.

Davis, B.K., 1974. Diffusion in polymer gel implants. Proc. Nat. Acad. Sci., 71, 3120-3123.

Documenta Giegy, Scientific Tables. Diem, K., Lentner, C. (Eds.), seventh ed., Giegy Pharmaceuticals, New York, 1970.

Dunne, A. O'Hara, T., Devane, J., 1997. Approaches to IVIVR modeling and statistical analysis. Adv. Exp. Med. Biol., 423, 67-86.

Eckenhoff, B., Theeuwes, F., Urquhart, F., 1987. Osmotically actuated dosage forms for rate-controlled drug delivery. Pharm. Tech. 11, 96-105.

Eldrige, J.H., Staas, J.K., Meulbroek, J.A., Tice, T.R., Gilley, R.M., 1991. Biodegradable and biocompatible poly(dl-lactide-co-glycolide) microspheres as an adjuvant for staphylococcal enterotoxin B toxoid which enhances the level of toxin neutralizing antibodies. Infect. Immun., 59, 2978-2986.

Enevoldsen, L.H., Simonsen, L., Stallknecht, B., Galbo, H., Bülow, J., 2001. In vivo human lipolytic activity in preperitoneal and subdivisions of subcutaneous abdominal adipose tissue. Am. J. Physiol. Endocrinol. Metab., 281, E1110-E1114.

Fawcett, J.P., Morgan, N.C., Woods D.J., 1997. Formulation and stability of naltrexone oral liquid for rapid withdrawal from methadone. Annals Pharmacother., 31, 1291-1295.

Fédération Internationale Pharmaceutique. Guidelines for Dissolution Testing of Solid Oral Products, 1997.

Folkman, J., Long, D.M., 1964. The use of silicone rubber as a carrier for prolonged drug therapy. J. Surg. Res., 4, 139-142.

Folley, S.J., 1942. 'Ghost' formation in subcutaneously implanted tablets of synthetic estrogens. Nature, 150, 403-404.

Force Field Manual *ver 6.6*, chapter 3. Tripos Inc., St. Loius, MO (USA), 1999, p. 81.

Freed, L.E., Langer, R., Martin, I., Pellis, N.R., Vunjak-Novakovic, G., 1997. Tissue engineering of cartilage in space. Proc. Natl. Acad, Sci., 94, 13885-13890.

Freshney, R.I. (ed.) Culture of animal cells, a manual of basic technique, third ed., Wiley-Liss, New York, 1993.

Fyfe, C.A., Grondey, H., Blazek-Welsh, A.I., Chopra, S.K., Fahie, B.J., 2000. NMR imaging investigations of drug delivery devices using a flow-through USP dissolution apparatus. J. Control. Rel., 68, 73-83.

Gao, J., Szymanski, A., Stowe, N., Haaga, J., 2001. Monitoring drug release and distribution in vivo by computed tomography. BED, Bioengineering Conference, Am. Soc. Mech. Eng., 95-96.

Gonzalez, J.P., Brogden, R.N., 1988. Naltrexone: a review of its pharmacodynamic and pharmacokinetic properties and therapeutic efficacy in the management of opioid dependence. Drugs, 35: 192-213.

Goiss, G., Parreira, D.R., Suzigan, S., 2003. A study on the biocompatibility and integration of acellular polyanionic collagen:elastin matrices by soft tissue. Brazilian J. Biomed. Eng., 19, 167-173.

Göpferich, A., 1996. Polymer degradation and erosion: mechanisms and applications. Eur. J. Pharm. Biopharm., 42, 1-11.

Gray, V. Dissolution testing: An Overview and a Summary of Recent Trends and Regulatory Activities. V. A. Gray Consulting, Inc., DE; Website: http://www.tabletscapsules.com/free/tc_20031001_20.pdf [15 April, 2004a].

Gray, V., November 2004. Controlled Release Society Annual Meeting Report: Dissolution Highlights. Disso. Tech., 30-31.

Halstead, G.H., Theis, D.L., 1985. Drug release testing of a prostaglandin containing controlled-release vaginal device: development of a semi-automated method. J. Pharm. Sci., 74, 1086-1090.

Hanks, J.H., 1948. The longevity of chick tissue cultures without renewal of the medium J. Cellular Comp. Physiol., 31, 235.

Hanks, J.H., Wallace, R.E., 1949. Relation of oxygen and temperature in the preservation of tissues by refrigeration. Proc. Soc. Exp. Biol. Med., 71, 196-200.

Hollingshead, M.G., Alley, M.C., Camalier, R.F., Abbott, B.J., Mayo, J.G., Malspeis, L., Grever, M.R., 1995. In vivo cultivation of tumor cells in hollow fibers. Life Sci., 57, 131-141.

Huang, X., Brazel, C.S., 2001. On the importance and mechanisms of burst release in matrix-controlled drug delivery systems. J. Controlled Rel., 73, 121-136.

Hulse, G.K., Arnold-Reed, D.E., O'Neil, G., Chan, C.-T., Hansson, R., O'Neil, P., 2004. Blood naltrexone and 6-β-naltrexol levels following naltrexone implant: comparing two naltrexone implants. Addict. Biol., 9, 59-65.

Hulse, G.K., Stalenberg, V., McCallum, D., Smit, W., O'Neil, Morris, N., Tait, R.J., 2005. Histological changes over time around the site of sustained release naltrexone-poly(DL-lactide) implants in humans. J. Controlled Rel., 108, 43-55.

Hurst, W.J., Zagon, I.S., Aboul-Enein, H.Y., 1999. A rapid sample preparation method for the HPLC determination of opioid antagonist naltrexone in serum. Pharmazie, 54, 595-596.

Hussain, M.A., Koval, C.A., Myers, M.J., Shami, E.G., Shefter, E., 1987. Improvement of the oral bioavailability of naltrexone in dogs: a prodrug approach. J. Pharm. Sci., 76, 356-358.

Hussain, M.A., Aungst, B.J., Koval, C.A., Shefter, E., 1988. Improved buccal delivery of opioid analgesics and antagonists with bitterless prodrugs. Pharm. Res., 5, 615-618.

Hwang, S.S., Bayne, W., Theeuwes, F., 1993. *In vivo* evaluation of controlled-release products. J. Pharm. Sci., 82, 1145-1150.

ICH, Topic Q2A, Validation of Analytical Procedures: Methodology, PCM/ICH/281/95.

Ikegami, K., Tagawa, K., Kobayashi, M., Osawa, T., 2003. Prediction of in vivo drug release behavior of controlled-release multiple-unit dosage forms in dogs using a flow-through type dissolution test method. Int. J. Pharm. 258, 31-43.

Iyer, S.S., Zhang, Z.-P., Kellogg, G.E., Karnes, H.T., 2004. Evaluation of deuterium isotope effects in normal-phase LC-MS-MS separations using a molecular modeling approach. J. Chrom. Sci., 42, 383-387.

Iyer, S.S., Barr, W.H., Karnes, H.T., 2006. Profiling in vitro drug release from subcutaneous implants: A review of current status and potential implications on drug product development. Biopharm. Drug Dispos., 27, 157-170.

Kalkwarf, D.R., Sikov, M.R., Smith, L., Gorden, R., 1972. Release of progesterone from polyethylene devices in vitro and in experimental animals. Contraception, 6, 423-431.

Kambia, K., Bah, S., Dine, T., Azar, R., Odou, P., Gressier, B., Luyckx, M., Brunet, C., Ballester, L., Cazin, M., Cazin, J.C., 2000. High-performance liquid chromatographic determination of naltrexone in plasma of hemodialysis patients. Biomed. Chrom., 14, 151-155.

Kaniwa, N., Shameem, M., Katori, N., Aoyagi, N., Kojima, S., 1995. The suitable temperature for the evaluation of controlled release products in vitro. Pharmazie, 50, 53-55.

Kasim, N.A., Whitehouse, M., Ramachandran, C., Bermejo, M., Lennernäs, H., Hussain, A.S., Junginger, H.E., Stavchansky, S.A., Midha, K.K., Shah, V.P., Amidon, G.L., 2004. Molecular properties of WHO essential drugs and provisional biopharmaceutical classification. Mol. Pharm., 1, 85-96.

Krebs, H.A., 1950. Chemical composition of blood plasma and serum. Ann. Rev. Biochem., 19, 409-430.

Langenbucher, F., 1969. In vitro assessment of dissolution kinetics: Description and evaluation of a column-type method. J. Pharm.Sci., 58, 1265-1272.

Langer, R., Peppas, N.A., 2003. Advances in biomaterials, drug delivery, and bionanotechnology. AIChE. J. 49, 2990-3006.

Langevin, H.M., Cornbrooks, C.J., Taatjes, D.J., 2004. Fibroblasts form a body-wide cellular network. Histochem. Cell. Biol., 122, 7-15.

Lee, M.C., Wagner Jr., H.N., Tanada, S., Frost, J.J., Bice, A.N., Dannals, R.F., 1988. Duration of occupancy of opiate receptors by naltrexone. J. Nucl. Med., 29, 1207-1211.

Lelong, I.H., Rebel, G., 1998. pH drift of "physiological buffers" and culture media used for cell incubation during in vitro studies. J. Pharmacol. Toxicol. Methods, 39, 203-210.

Levy, G., 1963. Effect of certain tablet formulation factors on dissolution rate of the active ingredient I: Importance of using appropriate agitation intensities for *in vitro* dissolution rate measurements to reflect *in vivo* conditions. J. Pharm. Sci., 52, 1039-1046.

Lewin. J., Huidobro, F., 1953. On the absorption mechanism of pellets. Acta Physiol. Latinoamericana., 3, 17-24.

Li, S.M., Garreau, H., Vert, M., 1990. Structure-property relationships in the case of degradation of massive poly(α-hydroxy acids) in aqueous media: Part I. Poly(DL-lactic acid). J. Mater. Sci.: Mater. Med., 1, 123-130.

Lickorish, D., Chan, J., Song, J., Davies, J.E., 2004. An in vivo model to interrogate the transition from acute to chronic inflammation. Eur. Cells Mater., 8, 12-20.

Lin, S., Chao, P.Y., Chien, Y.W., Sayani, A., Kumar, S., Mason, M., West, T., Yang, A., Monkhouse, D., 2002. In vitro and in vivo evaluations of biodegradable implants for hormone replacement therapy: Effect of system design and PK-PD relationship. AAPS PharmSciTech., 2, article 16.

Makino, K., Arakawa, M., Kondo, T., 1985. Preparation and in vitro degradation properties of polylactide microcapsules. Chem. Phar. Bull., 33, 1195-1201.

Malmsten, M. Degradation of surfactants and polymers in drug delivery. In Surfactants and polymers in drug delivery. Swarbrick J (ed). Marcel Dekker, Inc., New York, 2002, pp. 293-317.

Mark, T.L., Kranzler, H.R., Song, X., 2003. Understanding US addiction physicians' low rate of naltrexone prescription. Drug Alcohol Depend., 71, 219-228.

Martin, A., 1995. Polymer Science. In Physical Pharmacy, fourth ed., B.I. Waverly Pvt. Ltd., New Delhi, India, pp. 556-594.

Menzinger, M., Wolfgang, R., 1969. The meaning and use of Arrhenius activation energy. Angew. Chem. Internat. Edit., 8, 438-444.

Miclau, T., Dahners, L.E., Lindsey, R.W., 1993. In vitro pharmacokinetics of antibiotic release from locally implantable materials. J. Orthopaed. Res., 11, 627-632.

Mikac, U., Demšar A., Serša, I., Demšar, F., 2001. Electric density imaging of a tablet dissolution. Proc. Int. Conf. Life Sci., 11.

Mohl, S., Reithmeier, H., Winter, G., 2002. Poster presentation: Sustained Release of BSA from Implant Systems by using Physiological Lipids. *Colorado Protein Stability Conference*, Breckenbridge, CO, USA. Website: http://www.chemie.uni-muenchen.de/pb/aks/winter/Mohl_poster_denver_072002.pdf [17 October 2003].

Monti, K.M., Foltz, R.L., Chinn, D.M., 1991. Analysis of naltrexone and 6-beta-naltrexol in plasma and urine by gas chromatograph/negative ion chemical ionization mass spectrometry. J. Anal. Toxicol., 15, 136-140.

Moody, D.E., Monti, K.M., Spanbauer, A.C., Hsu, J.P., 1999. Long-term stability of abused drugs and antiabuse chemotherapeutical agents stored at -20°C. J. Anal. Toxicol., 23, 535-540.

Moore, J.W., Flanner, H.H., 1996. Mathematical comparison of curves with an emphasis on *in vitro* dissolution profiles. Pharm. Tech., 20, 64-74.

Naidong, W., Bu, H., Chen, Y.-L., Shou, W.Z., Jiang, X., Halls, T.D.J., 2002. Simultaneous development of six LC-MS-MS methods for the determination of multiple analytes in human plasma. J. Pharm. Biomed. Anal., 28, 1115-1126.

Narasimhan, B., Snaar, J.E.M., Bowtell, R.W., Morgan, S., Melia, C.D., Peppas, N.A., 1999. Magnetic Resonance Imaging analysis of molecular mobility during dissolution of poly(vinyl alcohol) in water. Macromolecules, 32, 704-710.

National Aeronautics and Space Administration, NASA Technology Spinoffs: Bringing space down to earth. FS-1995-08-004JSC, 1995.

Nicolaides, E., Hempenstall, J.M., Reppas, C., February 2000. Biorelevant dissolution tests with the flow-through apparatus? Disso. Tech., 8-15.

Okabe, J., Kimura, H., Kunou, N., Okabe, K., Kato, A., Ogura, Y., 2003. Biodegradable intrascleral implant for sustained intraocular delivery of betamethasone phosphate. Invest. Ophthalmol. Vis. Sci., 44, 740-744.

Okumu, F.W., Cleland, J.L., 2003. Implants and Injectables. In Rathbone, M.J., Hadgraft, J., Roberts, M.S. (Ed.), Modified-Release Drug Delivery Technology, Marcel Dekker Inc., New York, pp. 633-638.

O'Malley, S., Jaffe, A.J., Chang, G., Scottenfeld, R.S., Meyer, R.E., Rounsaville, B., 1992. Naltrexone and coping skills therapy for alcohol dependence. A controlled study. Arch. Gen. Psychiatry., 49, 881-887.

Pace, N.L., Parrish, R.G., Lieberman, M.M., Wong, K.C., Blatnick, R.A., 1979. Pharmacokinetics of naloxone and naltrexone in the dog. J. Pharmacol. Exp. Ther., 208, 254-256.

Pappanheimer, J.R., Renkin, E.M., Borrero, L.M., 1951. Filtration, diffusion and molecular sieving through peripheral capillary membranes: A contribution to the Pore Theory of capillary permeability. Am. J. Physiol., 167, 13-46.

Park, K., Shalaby, W.S.W., Biodegradable hydrogels for drug delivery. *Technomic* 1993.

Peh, K.K., Billa, N., Yuen, K.H. 1997. Simple liquid chromatographic method for the determination of naltrexone in human plasma using amperometric detection. J. Chrom B., 701, 140-145.

Pharmacopeial Previews, 2004. <1092> The Dissolution Procedure: Development and Validation, Pharm. Forum., 30, 351-363.

Pitt, C., Marks, T.A., Schindler, A., 1980. Biodegradable drug delivery systems based on aliphatic polyesters: Application to contraceptives and narcotic antagonists. In Baker, R. (Ed.), Controlled Release of Bioactive Materials, Academic Press, Inc., New York, pp. 19-43.

Pitt, C, 1990. Poly-ε-caprolactone and its copolymers. In Chasin, M., Langer, R. (Ed.), Biodegradable Polymers as Drug Delivery Systems, Drugs and the Pharm. Sci. Series, Vol. 45, Marcel Dekker Inc., New York, pp. 71-120.

Polli, J.E., Crison, J.R., Amidon, G.L., 1996. Novel approach to the analysis of in vitro-in vivo relationships. J. Pharm. Sci., 85, 753-760.

Porter, C.J.H., Charman, S.A., 2000. Lymphatic transport of proteins after subcutaneous administration. J. Pharm. Sci., 89, 297-310.

Presley, R, 1999. The sometimes maddening science of pH measurement. Am. Lab. News, 6, 10-11.

Quist, P.O., Östling, G., 2002. Accelerated dissolution testing for improved quality assurance. J. Pharm. Biomed. Anal., 28, 1081-1089.

Qureshi, S.A., Caillé, G., Brien, R., McGilveray, I.J., 1994. Drug release characterization of extended-release nifedipine products using a flow-through dissolution apparatus. Proc. Int. Symp. Control. Rel. Bioact. Mater., 21, 668.

Qureshi, S.A., Caillé, G., Brien, R., Piccirilli, G., Yu, V., McGilveray, I.J., 1994. Application of flow-through dissolution method for the evaluation of oral formulations of nifedipine. Drug Dev. Ind. Pharm., 20, 1869-1882.

Redmond, E.M., Cahill, P.A., Sitzmann, J.V., 1995. Perfused transcapillary smooth muscle and endothelial cell co-culture-a novel in vitro model. In vitro Cell Dev. Biol., 31, 601-609.

Redmond, E.M., Cahill, P.A., Sitzmann, J.V., 1997. Flow-mediated regulation of G-Protein expression in cocultured vascular smooth muscle and endothelial cells. Circulation, 75-83.

Redmond, E.M., Cullen, J.P., Cahill, P.A., Sitzmann, J.V., Stefansson, S., Lawrence, D.A., Okada, S., 2001. Endothelial cells inhibit flow-induced smooth muscle migration: Role of Plasminogen Activator Inhibitor-I. Circulation, 597-603.

Renkin, E.M., 1954. Filtration, diffusion and molecular sieving through porous cellulose membranes. J. Gen. Physiol., 38, 225-243.

Renkin, E.M., 1964. Transport of large molecules across capillary walls. Physiol., 7, 13-28.

Renkin, E.M., 1977. Multiple pathways of capillary permeability. Circ. Res., 41, 735-743.
Research Monograph Series Number 28 (Part-II: Analytical Methods). Narcotic Antagonists: Naltrexone pharmacochemistry and sustained-release preparations. National Institute on Drug Abuse (NIDA), U.S. Department of Heath and Human Services, 1981.

Rackley, R.J. Examples of *In Vitro-In Vivo* Relationships with a diverse range of quality. Figure 3. In *In Vitro-In Vivo Correlations*, Young, D., Devane, J.G., Butler, J. (ed). Plenum Press, New York, 1997, pp. 1-15.

Resnick, R.B., Volavka, J., Freedman, A.M., Thomas, M., 1974. Studies on EN-1639A (naltrexone): a new narcotic antagonist. Am. J. Psychiatry, 131, 646-650.

Reuning, R.H., Batra, V.K., Ludden, T.M., Jao, M.Y., Morrison, B.E., McCarthy, D.A., Harrigan, S.E., Ashcraft, S.B., Sams, R.A., Bathala, M.S., Staubus, A.E., Malspeis, L.,

1979. Plasma naltrexone kinetics after intravenous bolus administration in dogs and monkeys. J. Pharm. Sci., 68, 411-416.

Resnick, R.B., Volavka, J., Freedman, A.M., Thomas, M., 1974. Studies on EN-1639A (naltrexone): a new narcotic antagonist. Am. J. Psychiatry, 131, 646-650.

Rohrs, B.R., August 2001. Dissolution method development for poorly soluble compounds. Disso. Tech., 1-5.

Rohrs, B.R., Skoug, J.W., Halstead, G.W. Dissolution Assay Development for *In Vitro-In Vivo* Correlations. In *In Vitro-In Vivo Correlations*, Young, D., Devane, J.G., Butler, J. (ed). Plenum Press, NY, 1997, pp. 17-30.

Rothen-Weinhold, A., Besseghir, K., Vuaridel, E., Sublet, E., Oudry, N., Kubel, F., Gurny, R., 1999. Injection-molding versus extrusion as manufacturing technique for the preparation of biodegradable implants. Eur. J. Pharm. Biopharm., 48, 113-121.

Sathe, P., Tsong, Y., Shah, V.P., 1996. In vitro dissolution profile comparison: statistics and analysis, model dependent approach. Pharm. Res., 13, 1799-1803.

Schliecker, G., Schmidt, C., Fuchs, S., Ehinger, A., Sandow, J., Kissel, T., 2004. In vitro and in vivo correlation of buserelin release from biodegradable implants using statistical moment analysis. J. Cont. Rel., 94, 25-37.

Scott, P., 2002. Process Analytical Technology: Applications to the Pharmaceutical Industry. Dissol. Tech., 6-9.

Shah, J. Presentation: In vitro-in vivo correlation (IVIVC) for parenteral sustained release formulations. AAPS Annual Meeting and Exposition: Sunrise school session 'IVIVC: A useful tool in the development of drug delivery systems', November 2004; Baltimore, MD.
Shah, S.S., Cha, Y., Pitt, C., 1992. Poly (glycolic acid-*co*-DL-lactic acid: Diffusion or degradation controlled drug delivery? J. Controlled Release, 18, 261-270.

Shah, V.P., Siewert, M., Dressman, J., Moeller, H., Brown, C.J., February 2002. Dissolution/In Vitro Release Testing of Special Dosage Forms. Disso. Tech., 1-5.

Shah, V.P., Tsong, Y., Sathe, P., Liu, J.P., 1998. In vitro dissolution profile comparison--statistics and analysis of the similarity factor, f2. Pharm. Res., 15, 889-896.

Shameem, M., Lee, H., DeLuca, P.P., 1999. A short-term (accelerated release) approach to evaluate peptide release from PLGA depot-formulations. AAPS Pharm. Sci., 1, article 7.

Shipman Jr., C.H., 1969. Evaluation of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) as a tissue culture buffer. Proc. Soc. Exp. Biol. Med., 130, 305-10.

Shipman, C., In: Kruse Jr., P.F., Patterson, M.K. (Eds.). Tissue Culture: Methods and applications, Academic Press, New York, 1973, p. 709.

Siewart, M., Dressman, J., Brown, C., Shah, V.P., February 2003. FIP/AAPS Guidelines for Dissolution/*In Vitro* Release Testing of Novel/Special Dosage Forms. Disso. Tech., 6-15.

Siewert, B., Siewert, M. Dissolution tests for ER products. Dissolution Technologies website: http://www.dissolutiontech.com/Dtresour/1198art/Nov98Art1.html (4 February 2004).

Simonsen, L., Enevoldsen, L.H., Bülow, J., 2003. Determination of adipose tissue blood flow with local 133Xe clearance. Evaluation of a new labeling technique. Clin. Physiol. Funct. Imaging, 23, 320-323.

Sindrup, J.H., Kastrup, J., Jorgensen, B., Bülow, J., Lassen, N.A., 1991. Nocturnal variations in subcutaneous blood flow rate in lower leg of normal human subjects. Am. J. Physiol. Heart Circ., Physiol., 260, H480-H485.

Sirisuth, N., Eddington, N.D., 2001. In Vitro-In Vivo Correlation Definitions and Regulatory Guidance. Int. J. Generic Drugs. Website: http://www.locumusa.com/pdf/members/ivivc-01.pdf (21 January 2004).

Skelly, J.P., Yau, M.K., Elkins, J.S., Yamamoto, L.A., Shah, V.P., Barr, W.H., 1986. Topographical dissolution characterization for controlled release products - A new technique. Drug Dev. Ind. Pharm., 12, 1159-1175.

Skelly, J.P., Yau, M.K., Elkins, J.S., Yamamoto, L.A., Shah, V.P., Barr, W.H., 1986. *In vitro* topographical characterization as a predictor of *in vivo* controlled release quinidine gluconate bioavailability. Drug Dev. Ind. Pharm., 12, 1177-1201.

Stenken, J.A., Reichert, W.M., Klitzman, B., 2002. Magnetic resonance imaging of a tissue/implanted device biointerface using *in vivo* microdialysis sampling. Anal. Chem., 74, 4849-4854.

Stevenson, C.L., Theeuwes, F., Wright, J.C., 2000. Osmotic implantable delivery systems. In Wise, D.L. (Ed.), Handbook of Pharmaceutical Controlled Release Delivery, Marcel Dekker Inc., New York, pp. 225-254.

Stinchcomb, A.L., Swaan, P.W., Ekabo, O., Harris, K.K., Browe, J., Hammel, D.C., Cooperman, T.A., Pearsall, M., 2002. Straight-chain naltrexone ester prodrugs: Diffusion and concurrent esterase biotransformation in human skin. J. Pharm. Sci., 91, 2571-2578.

Storey, D.E., 1996. The role of dissolution testing in the design of immediate release dosage forms. Drug Info. J., 30, 1039-1044.

Sunkara, G., Chilukuri, D.M. IVIVC: An important tool in the development of drug delivery systems. *Drug Delivery Technology*. Website: http://www.drugdeliverytech.com/cgi-bin/articles.cgi?idArticle=144 (7 February 2004).

Therin, M., Christel, P., Li, S.M., Garreau, H., Vert, M., 1992. In vivo degradation of massive poly(α-hydroxy acids): validation of in vitro findings. Biomaterials, 13, 594-600.

Trissel, L.A., Stability of Compounded Formulations, second ed., American Pharmaceutical Association, Washington, D.C., 2000.

Tzafriri, A.R., 2000. Mathematical modeling of diffusion-mediated release from bulk degrading matrices. J. Control. Rel., 63, 69-79.

Uppoor, V.R.S., 2001. Regulatory Perspectives on *In vitro* (dissolution)/*In vivo* (bioavailability) Correlations. J. Contr. Rel., 72, 127-132.

US Food and Drug Administration, Guidance for Industry: Dissolution Testing of Immediate Release Solid Oral Dosage Forms, 1997.

US Food and Drug Administration, Guidance for Industry: Extended Release Oral Dosage Forms: Development, Evaluation and Application of In Vitro/In Vivo Correlations, 1997.

US Food and Drug Administration, Draft Guidance for Industry: Waiver of *In Vivo* Bioavailability and Bioequivalence Studies for Immediate Release Solid Oral Dosage Forms containing certain Active Moieties/Active Ingredients based on Biopharmaceutical Classification System, 1999.

US Food and Drug Administration, Summary Progress Report: Pharmaceutical cGMPs for the 21st Century: A Risk-Based Approach.
Website: http://www.fda.gov/cder/gmp/21stcenturysummary.htm (31 August 2004).

US Food and Drug Administration. Trexan (Naltrexone Tablets). New Drug Application 18-932/S-010, December 30, 1994.

United States Pharmacopeia 28/National Formulary 23. Official Monographs. The United States Pharmacopeial Convention, Inc., Rockville, MD, 2005, pp. 1329-1331.

United States Pharmacopeia 28/National Formulary 23. <711> Dissolution. The United States Pharmacopeial Convention, Inc., Rockville, MD, 2005, pp. 2412-2414.

United States Pharmacopeia 28/National Formulary 23. <724> Drug Release/Physical Tests. The United States Pharmacopeial Convention, Inc., Rockville, MD, 2005, pp. 2415-2422.

United States Pharmacopeia 28/National Formulary 23. <1088> *In vitro* and *In vivo* Evaluation of Dosage Forms. The United States Pharmacopeial Convention, Inc., Rockville, MD, 2005, pp. 2636-2641.

United States Pharmacopeia 28/National Formulary 23. <1225> Validation of Compendial Methods. The United States Pharmacopeial Convention, Inc., Rockville, MD, 2005, pp. 2748-2751.

Vega, C.A., Bates, R.G., 1976. Buffers for the physiological pH range: thermodynamic constants of four substituted aminoethanesulfonic acids from 5 to 50 degrees C. Anal. Chem., 48, 1293-1296.

Verebey, K., Mule, S.J., 1975. Naltrexone pharmacology, pharmacokinetics and metabolism: Current status. Am. J. Drug Alc. Abuse, 2, 357-363.

Verebey, K., Volavka, J., Mule, S.J., Resnick, R.B., 1976. Naltrexone: disposition, metabolism and effects after acute and chronic dosing. Clin. Pharmacol. Ther., 20, 315-328.

Volpicelli, J.R., Rhines, K.C., Rhines, J.S., Volpicelli, L.A., Alterman, A.I., O'Brien, C.P., 1997. Naltrexone and alcohol dependence. Role of subject compliance. Arch. Gen. Psychiatry, 54, 737-742.

Volpicelli, J.R., Alterman, A.L., Hayashida, M., O'Brian., C.P., 1992. Naltrexone in the treatment of alcohol dependence Arch. Gen. Psychiatry, 49, 876-880.

Wall, M.E., Brine, D.R., Perez-Reyes, M., 1981. Metabolism and disposition of naltrexone in man after oral and intravenous administration. Drug Metab. Dispos., 9, 369-375.

Wagner JG. Biopharmaceutics and Relevant Pharmacokinetics. Drug Intelligence Publications: IL, 1971, pp. 110-114.

Wagner, J.G., Nelson, E., 1964. Kinetic analysis of blood levels and urinary excretion in the absorptive phase after single doses of drug. J. Pharm. Sci., 53, 1392-1403.

Waymouth, C., 1954. The nutrition of animal cells. Int. Rev. Cytol., 3, 1-68.

Waymouth, C., 1970. Osmolality of mammalian blood and of media for culture of mammalian cells. In Vitro, 6, 109-127.

Watson, P.D., Grodrins, F.S., 1978. An analysis of the effects of interstitial matrix on plasma-lymph transport. Microvasc. Res., 16, 19-41.

Weir, N.A., Buchanan, F.J., Dickson, G.R., 2004. Degradation of poly-L-lactide. Part 1: in vitro and in vivo physiological temperature degradation. Proc. Instn. Mech. Engrs. Part H: J. Engineering in Medicine, 218, 307-319.

Whang, J.M., Quinn, J.A., Graves, D.J., Neufeld, G.R., 1989. Permeation of inert gases through human skin: modeling the effect of skin blood flow. J. Appl. Physiol., 67, 1670-1686.

Wisniewski, N., Klitzman, B., Miller, B., Reichert, W.M., 2001. Decreased analyte transport through implanted membranes: Differentiation of biofouling from tissue effects. J. Biomed. Mater. Res., 57, 513-521.

Wood, J.H. Dissolution Rediscovered. In Pharmaceutical Dissolution Testing, Swarbrick, J. (ed.). Marcel Dekker, Inc., NY, 1992, 413-426.

Young, D., Chilukuri, D., Becker, R., Bigora, S., Farrell, C., Shepard, T. Approaches to developing a Level-A IVIVC for injectable dosage forms. Website: www.globomaxservice.com/resource/LevelAIVIVCPoster.pdf (30 October 2004).

Yu, L.X., Wang, J.T., Hussain, A.S., 2002. Evaluation of USP Apparatus 3 for Dissolution Testing of Immediate-Release Products. AAPS Pharm. Sci., 4, article 1.

Zackrisson, G., Östling, G., Skagerberg, B., Anfält, T., 1995. Accelerated dissolution rate analysis (ACDRA) for controlled release drugs. Application to Roxicam. J. Pharm. Biomed. Anal., 13, 377.

Zhang, R., Sioma, C.S., Thompson, R.A., Xiong, L., Regnier, F.E., 2002. Controlling deuterium isotope effects in comparative proteomics. Anal. Chem., 74, 3662-3669.

Zhao, S., Pinholt, E.M., Madsen, J.E., Donath, K., 2000. Histological evaluation of different biodegradable and non-biodegradable membranes implanted subcutaneously in rats. J. Cranio-Maxillofacial Surgery, 28, 116-122.

Zuidema, J., Pieters, F.A.J.M., Duchateau, G.S.M.J.E., 1988. Release and absorption rate aspects of intramuscularly injected pharmaceuticals. Int. J. Pharm., 47, 1-12.

Appendix 1.

Project Title: Investigation of naltrexone release from a novel implant dosage form in dog.

Protocol No.: 0511-3406

Principal Investigator: William H. Barr, Pharm. D., Ph.D.
Second Investigator: Sunil S. Iyer, M. Pharm.

March 1st, 2006

Virginia Commonwealth University
Instututional Animal Care and Use Committee (IACUC)

IACUC NO. 0511-3406

VIRGINIA COMMONWEALTH UNIVERSITY
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)
PROTOCOL FOR USE OF VERTEBRATE ANIMALS IN RESEARCH

PROJECT TITLE: Investigation of naltrexone release from a novel implant dosage form in dog.

PROJECT STATUS: New X Pilot Teaching Renewal

Modified Existing IACUC No. (for 3-Year Renewal or Modification)

****

FUNDING: Department Chair's signature is required prior to initiating work on any protocol which has not received scientific review from a recognized extramural body (i.e. NIH Study Section, VAMC Review Board, NSF Review, American Heart Association, etc.). The Chair's signature indicates that he takes responsibility for scientific review of the protocol.

Intramural Dr. Barr Budget or OSPA No. 534845

Extramural (Sponsor's Name) Proj. Dates 12/05 to 02/06 ; Deadline for submission:

Dept. Chair's Signature (or Dean, if Chair is PI): TYPED NAME: Ron E. Polk, PharmD SIGNATURE:

****

PRINCIPAL INVESTIGATOR: William H. Barr, PharmD., Ph.D. Title: Professor and Executive Director, CDS
Department: Pharmacy Box # 980533

SECOND INVEST: Sunil S.Iyer Title: Graduate Student
Department: Pharmaceutics Box # 980533

Assistance and training will be provided by the DAR veterinary staff (i.e., Dr. Dance and/or Ms. Gerber).

****

TYPE OF STUDY: (Check all applicable categories and complete appropriate pages; remove pages "not applicable".

X COLLECTION OF BLOOD/TISSUE - COMPLETE APPROPRIATE SECTIONS OF APPENDIX B

_____ NON-INVASIVE STUDY (i.e., physiological responses to materials administered) - COMPLETE APPROPRIATE SECTIONS OF APPENDIX B

HAZARDOUS MATERIALS Check all that apply and complete appropriate sections of APPENDIX C

Biological Hazards ____ (Any fungi (live or dead), bacteria (live or dead), viruses, prions, clinical samples, known infected human tissue samples, or any biotoxins (ie. Tetrodotoxin, botulinum toxin) that pose a health hazard to healthy adult humans. Also include any replicative deficient viruses. If you are using any CDC Select Agents (http://www.cdc.gov/od/sap/docs/salist.pdf) additional registration requirements must be filled out. Please contact OEHS with questions regarding the use of Select Agents

Recombinant DNA ____ (as specified by the NIH Guidelines: http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html. These include use of fungi, bacteria, viruses or gene transfers, which may have and adverse affect on healthy adult humans)

Chemical Hazards ____ (please circle any that apply: Highly/Acutely Toxic, Highly/Acutely Carcinogenic, Highly/Acutely Mutagenic)

Radiological Hazard____ (radiolabeled material injected into live animals)

Irradiation Hazard _____ (exposure of animals to ionizing radiation using a research irradiator or other radiation-producing devices)

_____BEHAVIORAL STUDY - COMPLETE APPENDIX D

_____SURGICAL-ACUTE (surgical procedures in which the animal is euthanized prior to recovery from anesthesia) - COMPLETE APPROPRIATE SECTIONS OFAPPENDIX E

_X_ SURGICAL-SURVIVAL (surgical procedures in which the animal is allowed to recover from anesthesia) - COMPLETE APPENDIX E

_____ FIELD STUDIES/BIOLOGICAL SURVEYS - COMPLETE APPENDIX F

****

DESCRIPTION OF ANIMAL SUBJECTS:

Protocols are approved for a three-year period. Please specify numbers of animals to be used for the first year and total for three years for each species. Space is provided for three species provided experimental procedures are similar for all three. One protocol form may be used for rodents and rabbits, but separate protocol forms must be completed for higher species.

Techniques for euthanasia shall follow current guidelines established by the American Veterinary Medical Association Panel on Euthanasia (2000; available at http://www.avma.org/resources/euthanasia.pdf). Other methods must be reviewed and approved by the IACUC (consult http://www.research.vcu.edu/dar/dar_guides/Acceptable_Methods_for_Euthanasia_of_Animals.pdf for additional information). If other than approved methods are needed, include justification in the summary.

****

1. SPECIES A: canine 2. Strain Mixed Hound

3. Sex M 4. Age/Weight >6months/>6kg 5. #/1st Year 1 6. Total # for 3 Years NA

Will animals be held more than 12 hours outside the vivarium? YES_____NO X (If yes, justify in Summary).

INDICATE USDA PAIN CATEGORIES: (SEE http://www.aphis.usda.gov/ac/policy/policy11.pdf - weblink FOR DEFINITION OF PAIN CATEGORIES)

No distress/ Non Research Activity Pain Category B - # 1st Year = _____ Total # for 3 Years =_____

No Distress/No Anesthesia Pain Category C - # 1st Year = _____ Total # for 3 Years =_____

Alleviated Distress Pain Category D - # 1st Year = 1 Total # for 3 Years = NA

Unrelieved Distress Pain Category E*- # 1st Year = _____ Total # for 3 Years =_____

***If Category "E" applies, Appendix A on page X must be completed.**

EUTHANASIA: (Describe method(s) of euthanasia of animals including dose (mg/kg) and route of administration of applicable agent. If utilizing CO_2 for euthanasia, please include location of CO_2 exposure system and means of ensuring termination (see new guidelines for CO_2 euthanasia at http://grants.nih.gov/grants/guide/notice-files/NOT-OD-02-062.html).

The subject in this study will not be euthanized at the end of the project but transferred to another approved protocol. However, in the unlikely event of unexpected illness/morbidity occur, the dog would be euthanized with i.v. euthasol (pentobarbital 390 mg/ml; phenytoin 50 mg/ml) at a dose of 2 ml solution/10 kg body weight to provide approximately 80 ml pentobarbital/kg.

****

SUMMARY: Describe your proposed protocol, emphasizing the use of animals and including a brief statement of the overall purpose of the study. Do not submit an abstract of your grant proposal. **Write in terminology understandable to educated lay persons, not scientific specialists, and avoid or define abbreviations.** Describe specifically what will be done with the animals, and indicate the expected results. Discuss the procedures in the order they are performed and give time intervals (use tables to indicate uses of animals in complex protocols) occurring between procedures. The Committee needs to understand what happens to each animal. Use additional sheets if necessary. Useful assistance may be found at http://www.research.vcu.edu/dar.htm.

Overview:

The purpose of this study is to investigate the release of naltrexone from a novel, biodegradable implant dosage form in a beagle dog. Implants are dosage forms that are subcutaneously placed and are designed to release drugs over a prolonged period of time. The drug candidate, naltrexone, is an opioid antagonist, and has been used commonly used in addiction treatment centers. However, patient non-compliance to oral tablets is an issue that led to the development of implants as an alternate route of administration. This novel biodegradable dosage form procured from Durect Corp., CA, is designed to release the drug at a uniform rate after an initial period of burst release.

Guidelines for *In vitro-In vivo* Correlations (IVIVC) laid down by the US Food and Drug Administration (FDA) are very useful during drug product development. Typically, the dosage form is characterized for *in vitro* drug release under conditions that mimic physiological conditions to the maximum extent possible. To date, however, there is no defined apparatus to study drug release from implants, although the recommendation is to use a modified flow-through type of device. Furthermore, to mimic physiological conditions, we have tested in the laboratory a novel capillary cell culture module with appropriate modifications. The implants have been characterized for *in vitro* drug release using both the apparatuses for more than 2 months. The question remains, however, which of the two apparatuses is more representative of an *in vivo* system? To address this question, it is essential to obtain *in vivo* data. The general scientific opinion favors the use of larger animals for implant dosage form studies to minimize interspecies differences compared to small laboratory animals; hence the selection of dog as the animal model.

Procedure:

The dog will be premedicated in its home cage with atropine 0.04 mg/kg, S.C. and acepromazine 0.1 mg/kg, S.C. It will be moved to the DAR provided surgical suites (4th floor of Sanger) where it will be fixed with an indwelling I.V. catheter in a cephalic or saphenous vein. Lactated ringers solution will be administered via the catheter at a slow rate throughout the procedure (1ml/min). Anesthesia will be induced and maintained with I.V. xylazine. Following induction, an implant will be aseptically placed in the dorsal subcutaneous space. Blood samples will be collected in vacutainers/tubes with an anticoagulant, pre-dose and at 0.5, 0.75, 1, 2, 4, 8, 15, 30, 45, 60, 75 and 90 days post-dose. These samples will be centrifuged to obtain plasma that will be separated in a tube and stored below -50°C, prior to analysis. Following collection of these samples on Day-90, the implant will be surgically removed from its site (following the same pre-medication schedule as during implantation) and the affected area will be sutured aseptically and dressed up. (Although the implant is biodegradable, its surgical removal is necessary because at the end of the study period (Day-90), biodegradation cannot be expected to be complete). The dog will be kept under observation until complete recovery. Based on the interesting results that we have obtained thus far upto Day-45, it is necessary to collect blood samples (3 mL) each at 1, 2, 3, 4, 5, 7, 10, 15, 20 and 30 days (may be omitted depending on prior blood levels) following removal of the implant to characterize the terminal phase in the plasma concentration-time profile. Also, from a pharmacokinetic standpoint we need to have an input function to use the data for modeling purposes. Therefore, a sterile aqueous solution of naltrexone hydrochloride (equivalent to 1 mg/kg free base) will be administered intravenously to the same dog and blood samples (3 mL each) will be drawn at 0 (pre-dose), 0.083, 0.25, 0.5, 1, 2, 4, 8, 24 hours and at 2, 3, 4, 5, 7, 10, 15, 20 and 30 days post-dose. We do not expect any adverse effect from this dose based on available literature.

Assistance and training will be provided by the DAR veterinary staff (i.e., Dr. Dance and/or Ms. Gerber). Also insertion of the implant into the subcutaneous tissue will be done or directed by Dr. Peter Coleman, a physician who is trained and experienced in inserting naltrexone implants into addicted patients. This is to assure that the implant will adequately simulate the way the implant would be used in a patient.

The *in vivo* release profile will be generated from the analysis of dog plasma placed subcutaneously for a period of 3 months and blood samples will be collected over this period of time for the assay of naltrexone released. The concentration of drug will be estimated in dog plasma will be correlated with the *in vitro* release data obtained using both the devices mentioned in the previous section.

Post procedural care: Given the minimally invasive nature of this procedure, it is unlikely that post operative pain will occur. Regardless, the dog will be monitored for signs of postoperative pain and administered a non-narcotic analgesic, e.g., aspirin, 200 mg, every 4 hours as needed. The dog will be fed sufficient amounts to ensure maintenance of an anabolic state and receive supplemental vitamins with iron daily. The dog will also be monitored daily for possible complications following the subcutaneous implantation including hematoma formation (although this is an uncommon complication with implants). Procedures outlined in Appendix-A (based upon Cukierski, *et al.*, Int. J. Toxicol., 20: 369-381; 2001) will be used. The scores for capsule formation, increased vascularity and presence/absence of fluid will comprise the total score for a minimal to extreme complications.

PERSONNEL QUALIFICATIONS: (It is an institutional obligation to ensure that professional and technical personnel and students who perform animal anesthesia, surgery, or other experimental manipulations are qualified through training or experience to accomplish these tasks in a humane and scientifically acceptable manner, Guide, pg. 13, 1996; http://books.nap.edu/books/0309053773/html/13.html)

Indicate personnel who will be performing the animal procedures and indicate the type(s) of training they have received and the number of years of experience of each person for the specific types of animal procedures proposed for each species. Personnel who will be irradiating experimental animals must be trained and have approval from the Radiation Safety Section of the Office of Environment Health & Safety. **Please notify the committee by memorandum of any changes in personnel after approval of protocol.**

William H. Barr, PharmD., Ph.D. - Has been an Investigator in animal studies over the past 30 years at MCV. Received training in animal surgery in graduate school. Has worked with dogs, rabbits and rodents. It is anticipated that surgical procedures will be done with the assistance of surgically trained individuals at VCU.

Sunil S. Iyer, M.Pharm. - Has been working as a Graduate student in VCU Dept. of Pharmaceutics since 2002. Prior to joining VCU, he has had 4 years professional bio-analytical experience in the Department of Pre-clincal Pharmacokinetics and Metabolism at a major pharmaceutical company in India. Will provide assistance in surgical preparation, daily observation and care of animals and collection of blood samples.

JUSTIFICATION FOR THE USE OF PROPOSED ANIMAL MODEL

1. What are the probable benefits of this work to human or animal health, the advancement of knowledge, or the good of society?

The purpose of this study is to evaluate the release of naltrexone from an implant dosage form in a beagle dog. Data obtained from the analysis of blood samples will be correlated to the *in vitro* release profiles already generated using two apparatuses. This is essential for validation of a better *in vitro* predictor model out of the two devices. To the best of our knowledge, this is the first work attempting to develop a "biorelevant" approach to study *in vitro* release of an implant dosage form. Furthermore, a good IVIVC will be useful to minimize *in vivo* studies during drug product development since better information on characterization of the dosage form will be available.

2. Justify the selection of the proposed animal species, strain, and numbers **(include statistical or other criteria for animal numbers).** Cost is not a valid justification. Please provide a justification of animal numbers across different procedures justifying animal usage for each year, species/strain and type of animal (e.g., adult, dam, pup, etc.). Include a table if it will help simplify your explanation. For useful calculation sites, you may consult:
http://altweb.jhsph.edu/publications/statistics.htm. Useful, inexpensive programs include StatMate (http://www.graphpad.com/statmate/statmate.htm) and SAS (http://www.at.vcu.edu/faq/statistics.html).

Primary considerations in selecting the species for use included the blood volume and growth curve of various animals. Either a large dog or a sheep was considered. However, the sheep would likely require surgical cut downs to visualize all but ear arteries. In addition, monitoring of the implantation site after the procedure, including blood flow distal to the site and tissue oxygenation requires a subject amenable to frequent hands-on manipulation without sedation. Therefore, a canine model was selected. The subject will not undergo any overly invasive procedures nor be permanently altered in any way. It will be determined if the subject could be transferred to another IACUC approved protocol either at VCU or the McClean VA Hospital in order to conserve this valuable resource. At this time, 1 subject has been requested. Performing the procedure over 3 months should provide sufficient information to ensure the accuracy of drug delivery from the dosage form. Based on the data obtained, additional samples may be required to sufficiently characterize the absorption profile.

3. What databases or services have you used to determine that alternative methods would not be acceptable? The Animal Welfare Act dictates that the investigator must provide written documentation that alternatives were not available. **An alternative is not limited to replacement with in vitro methodologies but includes any procedure which results in the reduction in the numbers of animals used, refinement of techniques, or replacement of animals.** If the project involves teaching, explain why films, videotapes, demonstrations, etc. would not be acceptable. A minimum of two databases is required. Also required is the date of the search and date range for both databases. Acceptable databases include Entrez-PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi), AGRICOLA (http://www.nal.usda.gov/ag98/), and multiple engines available via the VCU library (http://www.library.vcu.edu/cfapps/webdb/ulsdbactezp.cfm?SubjectID=5) and the IACUC home page (http://www.orsp.vcu.edu/iacuc/index.html). Some additional assistance with search strategies can be found at http://www.frame.org.uk/Useful.htm. This question can be completed on the "Database Search Form on the following page.

4. Does this experiment duplicate previous experiments (other than control data)? Yes____ No X
If yes, explain why duplication is necessary for your research.

DATABASE SEARCH FORM

The IACUC requests that investigators complete and submit the following form:

Date search conducted: June-Aug, 2005

Databases searched:

(1) Medline/Pubmed

(2)

Keywords and/or search strategy used: IVIVC, Implant, dosage form

Publication year(s) covered: ___1964_____ **to** ___Current____

Other sources consulted:

Search Results as pertains to the three R's:

1. REDUCTION: Did you find any ways to reduce animal numbers? _Yes___ **If so, describe why you can or cannot use them:**
The aim of this study is to determine which of the two *in vitro* devices developed can serve to be a better predictor of drug absoption from the implant *in vivo*. Thus, data generated from a single animal should suffice, although no information about inter-individual variability can be obtained.

2.REFINEMENT:Did you find any methods that minimize pain or distress? _No___ **If so, describe why you can or cannot use them:**

3. REPLACEMENT: Can you replace your animal model with a non-animal model or less sentient species? __No__ **Why or why not?**
The main purpose of this study is to generate *in vivo* data that in turn will be used for correlation with the *in vitro* data. Non-animal data cannot be a true reflection of what would happen in a complete living individual, and hence selection of an appropriate animal model is key to this study. This is also in concurence with the current scientific opinion of experts in the field.

Additional Comments:

FOR ALL EXPERIMENTAL PROCEDURES:
Are procedures to be used that are **intended to study pain?** Yes_____ No__X

If the answer is YES, what criteria will be used to assess pain/discomfort and what will be done to minimize or relieve pain/discomfort? (If analgesics can not be used and pain/discomfort is going to be minimized by early euthanasia of the animals rather than using analgesics, describe the monitoring schedule and the criteria that will determine the time of euthanasia). For assistance, consult http://www.research.vcu.edu/dar/dar_guides/Assessing_pain_and_distress.pdf.

VIRGINIA COMMONWEALTH UNIVERSITY
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

ASSURANCE FOR THE HUMANE CARE AND USE OF ANIMALS
USED FOR TEACHING AND RESEARCH

1. I agree to abide by all the federal, state and local laws and regulations (http://www.research.vcu.edu/dar/dar_lareg.htm) governing the use of animals in research. I understand that emergency veterinary care will be administered to animals showing evidence of pain or illness.

2. I have considered alternatives to the animal models used in this project and found other methods unacceptable.

3. I affirm that the proposed work does not unnecessarily duplicate previous experiments.

4. I affirm that all experiments involving live animals will be performed under my supervision or that of another qualified biomedical scientist. Technicians involved have been trained in proper procedures in animal handling, administration of anesthetics, analgesics and euthanasia to be used in this project.

5. I further affirm the information provided in the accompanying protocol is accurate to the best of my knowledge. Any proposed revisions to the animal care and use procedures will be promptly forwarded in writing to the Committee for approval.

I have read and understand the above statements.

William H Barr, PharmD., PhD
Typed Name

Signature of Principal Investigator

March 1st, 2006
Date

SIGNATURE FOR
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

Signature of Chairman, IACUC

APPENDIX B (REMOVE IF NOT APPLICABLE)

EXPERIMENTAL PROCEDURES (COLLECTION OF BLOOD OR TISSUE AND/OR NON-INVASIVE)
This section includes antibody production, blood/tissue collection, or any non-invasive study.
NOTE: Include below: expected rate of growth of tumors or ascites, monitoring schedule, criteria for assessment of distress, and earliest point at which animals in distress will be euthanized. Consult http://www.research.vcu.edu/dar/dar_guides/Guidelines_for_the_production_of_tumors_and_cancer_research_in_animals.pdf for additional information.

Materials to be Administered to Animals as Part of Experimental Protocol (do not include hazardous materials which you have listed and described on page 4):
For dose volumes, consult http://www.research.vcu.edu/dar/dar_guides/Dose_volumes.pdf.

Species	Antigen/Drug	Dose(mg/kg)	Route	Frequency	Total Number of Animals Treated

Describe potential effects of material(s) administered on the animals:

How long will individual animals be on the study?

Location where experimental procedures will be conducted: Building_____Room_or_____

Blood or Tissue Collection:
Describe technique used to collect blood or tissue (include route of collection and anesthetic, sedative or tranquilizing agents administered prior to specimen collection). For appropriate scheduling and amounts of blood withdrawal, consult http://www.research.vcu.edu/dar/dar_guides/SOP_for_blood_sampling.pdf :

The DAR SOP for Blood Sampling Amounts & Techniques for Various Laboratory Animal Species will be followed. Blood (5 mL) will be collected from the cephalic or saphenous vein at pre-dose and at 0.5, 0.75, 1, 1.5, 2, 4, 8, 15, 30, 45, 60, 75 and 90 days post-dose, for generation of a pharmacokinetic profile. No anesthesia will be required.

Species	Blood/Tissue	Amount/Size	Frequency	Total No. of Animals Used
Dog	Blood	70-100 ml	As described above	1

If the nature of your project makes it difficult to complete the above table, include a bleeding or collection schedule:

Indicate methods for the prevention of anemia:
We do not perceive, based on the proposed blood withdrawal volume, that anemia will occur. Between procedures, the dog will be fed sufficient amounts to ensure maintenance of an anabolic state and receive supplemental vitamins with iron daily to facilitate red blood cell replacement.

Location where collection procedures will be conducted: Building Sanger Hall Room 4th floor surgical suite for blood draws.

APPENDIX E (REMOVE IF NOT APPLICABLE)

SURGICAL PROCEDURES

Name(s) and qualifications of surgeons (include office and emergency phone numbers, if not previously given):
Surgeon to be named.

Location where surgical procedures will be conducted:

Building Sanger Room 4TH Floor DAR Surgical Suite

What is the expected duration of anesthesia and surgery? Approximately 2 hours

PREOPERATIVE CARE:

Describe preoperative care (include physical examinations, lab tests, preconditioning to apparatus, and fasting or withholding of water):

Dogs will be faster overnight prior to anesthesia and have water removed the morning of the procedure.

Preoperative medications (preanesthetic agents, antibiotics, etc.):

Species	Drug	Dose	Route	Frequency	No. of Days
Dog	Atropine	0.04 mg/kg	s.c.	once	two
Dog	Acepromazine	0.1 mg/kg	s.c.	once	two

Surgery:
Specify both initial and supplemental anesthetic regimens (consult http://www.research.vcu.edu/dar/dar_guides/Guide_to_laboratory_animal_anesthesia.pdf):

Species	Agent	Dose/%	Route	Frequency
Dog	Xylazine	4 mg/kg	i.v.	once
Dog	Yohimbine	0.1 mg/kg	i.v.	once

(If gas anesthesia will be used, indicate precautions (i.e., hood, scavenger units, masks) taken to protect personnel from anesthetic fumes):

Will paralyzing drugs be used? Yes___ No__X

If yes, describe (include drug, dose, route of administration, justification and monitoring methods used to ensure that the animal does not experience pain): Note: The law states that paralytic drugs may not be used alone; only when covered by adequate anesthesia.

SURGICAL PROCEDURES (CON'T)

Supportive Care and Monitoring:
NOTE: ALL ANESTHETIZED ANIMALS MUST BE OBSERVED BY THE INVESTIGATOR OR HIS/HER STAFF UNTIL FULLY RECOVERED AND RETURNED TO THE ANIMAL FACILITY STAFF.

How will the level of anesthesia be monitored and how often? (e.g. absence of toe pinch or corneal reflex at 15 min intervals)

Prior to surgery, depth of anesthesia is monitored by eye blink response to corneal stimulation and a muscle twitch to a strong pinch of the skin between the toes. During surgery, we monitor respiration, heart rate, blood pressure, body temperature, pO2 and the corneal response minimally every 10-15 min. If anesthesia appears to be becoming insufficient, supplemental anesthesia is given immediately (4mg/kg xylazine i.v.) through the i.v. line we have in place.

What method will be used to prevent dehydration and hypothermia during surgery?
The dog will be maintained on a circulating water heating pad and body temperature monitored throughout. To maintain hydration, lactated ringers solution will be administered at a slow rate (1 ml/min) throughout the procedure as well as at a higher rate (4 to 5 ml/min) between time of the final blood sampling and recovery of the subject to equal total volume of blood extracted.

Surgical Manipulation:
Describe surgical procedures (sterile instruments and aseptic surgical techniques **MUST** be used in all survival surgeries).
The area over the implantation site will be surgically prepared (clipping of fur, alternating betadine scrub/alcohol scrubbing x 3). The area around the surgical site will be draped with a sterile drape to avoid contamination. The dogs will be aseptically implanted using a syringe or a trocar and stylet type of device. The skin incision will be closed with a subcutaneous layer of 4-0 dexon (simple continuous) and a skin closure with 2-0 nylon (simple interrupted). Following the final blood sampling, the implant will be surgically removed from its site (following the same premedication schedule as during implantation) and the affected area will be sutured aseptically and dressed up.

Multiple Surgeries:
(Multiple major survival surgical procedures, i.e., those involving opening of body cavities, on a single animal are discouraged. However, under special circumstances they might be permitted with the approval of the committee; e.g. when the surgeries are related components of a research project. Cost savings alone is not acceptable (Guide, pg. 11, 1996; http://books.nap.edu/books/0309053773/html/11.html#pagetop)).

Will multiple major survival surgeries be performed? Yes__ No_X

If yes, describe and justify:

SURGICAL PROCEDURES (CON'T)
Postoperative Care (Survival Studies Only)

Animals must be held in a postoperative area until recovered from anesthesia.
Post surgical care should include observing the animal to ensure uneventful recovery from anesthesia and surgery: administering supportive fluids, analgesics, and other drugs as required; providing adequate care for surgical incisions; and maintaining appropriate medical records. Consult http://www.research.vcu.edu/dar/dar_guides/Guidelines_for_rodent_survival_surgery.pdf or http://www.research.vcu.edu/dar/dar_guides/Postoperative_surgical_care_guidelines.pdf for additional information.

Postanesthesia Recovery: Describe frequency and type of observations that will assure that the animals are stable and have returned to a safe level of recovery from anesthesia:
The subject will be monitored for heart rate, respiratory rate, mucosal color, pulse strength and capillary refill throughout recovery. Subject will remain intubated until laryngeal reflexes return (swallowing ability). The dog will be continuously observed until able to maintain sternal recumbency and then minimally every 15 minutes until able to ambulate.

Supportive Care (Postoperative recovery: Include frequency of examination, frequency and type of lab tests, monitoring and management of pain when indicated, observations and management of potential experimentally-related disease, wound care, parenteral fluids, special diet, etc):

The condition of the surgical site is examined at least once a day during the post-surgical recovery period until wound healing occurs. We have developed an evaluation scale for monitoring post-surgical progress. Occurrence of infection at the catheterization/ surgical/implantation sites is extremely uncommon based on our experience in humans, non-human primates and rats. Indication of implantation site complications may include appearance of capsule, increased vascularity or presence of fluid. These will be assigned scores based on criteria mentioned in Appendix-A. A rare occurrence is hematoma formation as indicated by swelling, bruising and/or tenderness; infection of incision as indicated by swelling and inflammation with or without exudate; clot disruption of blood flow distal to the implantation site as indicated by altered blood flow, tissue oxygenation and temperature. Hematomas will generally dissipate without intervention. Infection would necessitate antibiotic therapy, most likely trimethoprim/sulfmethoxazole or amoxicilli/clavulinic acid combinations.

Describe criteria for the assessment of post surgical pain:

Given the minimally invasive procedure, it is not expected that the subject will require any postoperative medications. If the animal shows signs of pain or distress (alterations in normal behavior, poor appetite, lying down in cage, painful response on palpation of implantation site, etc) we would initiate an analgesia regimen with a non-narcotic analgesic, and evaluate (CBC, physical exam, temperature) for signs of infection.

Postoperative medications (analgesics, anti-inflammatory drugs, antibiotics, etc.)

Species	Drug	Dose	Route	Frequency
Dog	aspirin	200 mg total	P.O	every 4 hours as needed

What is expected duration of anesthesia and surgery?
Up to 2 hours

Indicate the length of time the animal will be kept alive postoperatively: Indefinitely - the dog will be maintained on this protocol for 2-3 weeks after the final blood sampling session to ensure complete recovery of red blood cell number and lack of adverse events. It will then be transferred to an IACUC approved study at VCU or McClean VA Hospital.

Person(s) responsible for postoperative care records: Dr Dance and/or Ms. Gerber
Location of Records (Room number): Sanger B02-XXX (dog housing room in vivarium)

Describe long-term care of chronically instrumented animal(s): NA

Appendix-A

Macroscopic evaluation scale

Score	Capsule	Vascularity	Fluid
None (0)	No visible proliferation of connective tissue	No visible increase in vascularity	No fluid present
Minimal (1)	Slight proliferation of connective tissue	Slight increase in vascularity	None to barely perceptible amount of fluid—clear
Mild (2)	Transparent connective tissue <1 mm thick surrounds implant	Slight, but distinguishable increase in vascularity—a small "halo" of blood vessels may be present	Slight, but distinguishable amount of clear or straw-colored fluid present
Moderate (3)	Translucent capsule, 1–3 mm thick surrounds implant	Red "halo" (increased vascularity) <3 mm thick surrounding capsule and/or reaction in adjacent tissue barely perceptible	Distinguishable amount of straw-colored and/or red fluid present
Severe (4)	Translucent-to-opaque capsule >3 mm thick	Vascularity extends >3 mm beyond site and/or reaction involving adjacent tissue is distinctly visible (well-defined)	Pus or >5 ml of red or straw-colored fluid present

Total score: capsule + vascularity + fluid	Category
0–1.0	Minimal
1.1–4.0	Mild
4.1–7.0	Moderate
7.1–10.0	Severe
10.1–12.0	Extreme

Vita

Sunil S. Iyer was born on December 28, 1973 in Ahmedabad, Gujarat, India. He graduated from the K.L.E. Society's College of Pharmacy with a Bachelor of Pharmacy degree in 1996. In 1998, he received his Master of Pharmacy degree from Birla Institute of Technology, Ranchi, India. He was working as a Senior Research Scientist at Ranbaxy Laboratories Limited, New Delhi, India, prior to joining the Department of Pharmaceutics, Virginia Commonwealth University in 2002.

Sunil has co-authored 4 research papers, 4 abstracts and 1 review article. 2 research papers related to his Ph.D. project have been submitted recently; 2 more research papers related to his Ph.D. project and 1 paper on another research topic are in preparation. He was an invited speaker at the 8th International Addiction Conference, Berlin, Germany, in March 2006, and co-author to invited speaker at the 16th International Symposium for Pharmaceutical and Biomedical Analysis (PBA), Baltimore MD, 2005. He gave presentations at the 2003 and 2004 International Symposia for Pharmaceutical and Biomedical Analysis, and at the 2004 Annual Meeting of American Association of Pharmaceutical Scientists, besides several in-house presentations. He was awarded a Gold Medal for being the Best Outgoing Student in Bachelor of Pharmacy program. In 1996, he received a Junior Research Fellowship for Master of Pharmacy program from the University Grants Commission, Government of India. At VCU, he was awarded the 2004 Jyotsna and Mavji Thacker Award, for academic excellence in Department of Pharmaceutics; the 2005 University Leadership Award; the 2005 Phi Kappa Phi Scholarship Award; the 2005-06 Dissertation Fellowship Award by the School of Graduate Studies; the 2006 Who's Who Recognition Among Students in America's Universities and Colleges; the 2006 John Wood Award in Department of Pharmaceutics; and the 2006 Charles T. Rector and Thomas W. Rorrer, Jr. Dean's Award for Graduate Studies, in recognition of demonstrated excellence in scholarship, research, teaching and service at the School of Pharmacy. He served as the first appointed Graduate Student Liason in the Executive Committee of AAPS - Analysis and Pharmaceutical Quality (APQ) Section, 2004-05, and has been actively involved in the 2004 and 2007 Programming Committees of the APQ Section. He was awarded the AAPS Annual Meeting Graduate Travel Award in 2004, the Travel Fund Award, APQ Section Executive Committee, 2005, and has been nominated for the 2006 AAPS Graduate Student Symposium Award in Analysis and Pharmaceutical Quality (co-sponsored by UCB Pharma, Inc.).