TOWSON UNIVERSITY
COLLEGE OF GRADUATE STUDIES AND RESEARCH

DEVELOPMENT OF A BIODEGRADABLE SUBCUTANEOUS IMPLANT CONTAINING ACYCLOVIR FOR THE LONG-TERM SUPPRESSION OF HSV-1 REOCCURENCES

by

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THESIS APPROVAL PAGE

This is to certify that the thesis prepared by Ashley N. Nelson entitled Development of a Biodegradable Subcutaneous Implant containing Acyclovir for the Long-Term Suppression of HSV-1 Reoccurrences has been approved by the thesis committee as satisfactorily completing the thesis requirements for the degree of Masters of Science in Biology in the department of Biological Sciences.

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ABSTRACT

Development of a Biodegradable Subcutaneous Implant Containing Acyclovir for the Long-Term Suppression of HSV-1 Reoccurrences

Ashley N. Nelson

Herpes simplex virus-1 (HSV-1) is a ubiquitous human pathogen that is usually associated with causing lesions on the dermal epithelium. Acyclovir (ACV) is commonly used to treat initial infections and suppress reactivations. Due to acyclovir's low oral bioavailability, HSV-1-infected individuals need multiple daily doses of the drug. An ideal alternative to oral delivery would be to design a long term drug delivery system that is subject to biodegradation. This study aims to construct a biodegradable implant using polycaprolactone (PCL) for the long-term delivery of ACV. A series of implants were constructed with different polymer: drug ratios (60:40, 65:35, 70:30, and 75:25) and *in vitro* studies were conducted to determine which ratio exhibits the best antiviral activity. The implant's release kinetics of ACV under various temperatures (4 °C, 25 °C, and 37 °C) and pH (6, 7, and 8) was examined. We found that temperature, but not pH had a significant effect on release kinetics.

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Chapter I:

Introduction

The *Herpesviridae* family encompasses a large group of double-stranded DNA viruses that infect both humans and animals (1, 2). Currently, there are eight herpesviruses known to infect humans: herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), cytomegalovirus, Epstein-Barr virus, varicella-zoster virus (chicken pox), human herpesvirus 6, human herpesvirus 7, and Kaposi's sarcoma herpes virus (3, 4). All herpesviruses are structurally similar. They have an icosahedral nucleocapsid that is surrounded by an amorphous layer of proteins, called a tegument, and outside the tegument is the envelope which contains a dozen or more virus glycoproteins (4, 5).

The Herpesviridae family is divided into three subfamilies: alphaherpesvirinae, betaherpesvirinae, and gammaherpesvirinae (4, 5). Classification of viruses into these subfamilies is based on biological properties and host range. Herpes simplex virus type- 1 (HSV-1) is a member of the alphaherpesvirinae subfamily. Members of this subfamily were classified based on their broad host range, short reproductive cycle, efficient destruction of infected cells, and ability to establish latency primarily in sensory ganglia (4, 5). They are also one of the most studied groups of herpesviruses because of their ability to infect a wide variety of cultured cells and to spread rapidly in those cells (4).

Viral Growth and Replication

To initiate infection, the virus must first attach to cell surface receptors. The envelope of HSV-1 contains more than a dozen glycoproteins, four of which are

important for viral entry into its host (4, 6). These glycoproteins utilize glycosaminoglycans (usually heparin sulfate), nectins, and herpes virus mediator (HVEM) receptors on the cell surface to promote binding and fusion of the virion envelope with a cell (4, 5, 7). The nucleocapsid is then transported to the nuclear pore through which the viral DNA is released into the nucleus (4, 8). Once in the nucleus, the viral DNA is transcribed in three phases. The immediate early (α) genes are expressed first; these genes have mainly regulatory functions and signal the activation of early (β) genes (4, 8). The early genes are mostly involved in DNA replication and signal the expression of the late (γ) genes, which encode for structural proteins, and are required for virion assembly (4).

After the primary infection of an individual with an alphaherpesvirus (HSV-1, HSV-2, or VZV), the virus enters nerve termini and is transported up the axons to the sensory neurons where the viral genome is retained in a non-replicating state, known as latency (3, 4, 9-11). During latency, specific and limited viral RNA expression occurs and very few viral proteins are made (12). Periodically, reactivation of the virus can be initiated through a variety of local and systematic stimuli such as physical or emotional stress, fever, UV light exposure, tissue damage or immune suppression (8, 12-15). During reactivation, the HSV genome switches to a lytic pattern of expression that results in the delivery of infectious virions from axons to epithelial cells (12, 13). Thus, a person with a reactivated infection can serve as the source of infection even when no lesions are present due to the virus still being actively produced (4).

HSV-1: Transmission and Pathology

HSV-1 is usually acquired during childhood via nonsexual contact; it can also be transmitted during young adulthood by sexual contact (16). In 2002, a study showed that in the U.S., the overall HSV-1 seroprevalence was 31.1% in children ages 6 to 13 years (17). Furthermore, data from the National Health and Nutrition Examinations (NHANES) III study showed that HSV-1 seroprevalence in the U.S. increased from 41% in young adults age 12-19 years old to about 90% among those >70 years of age (17, 18). These statistics show that HSV-1 seroprevalence and exposure tends to increase with age.

Primary infection of HSV-1 is established when mucosal surfaces or abraded skin of a seronegative individual comes into contact with infected secretions (4, 5, 12). Primary infections usually appear 2 to 20 days after inoculation and typically last 10 to 14 days (4, 16, 19). Clinical manifestations can vary and depend on the portal of entry, the immune system of the host, and whether the infection is primary or recurrent (13). Primary oral infection is usually associated with vesicle formation on the oral mucosa or lips (herpes labiallis), but has recently been shown to be present in some genital infections. Usually, clinical symptoms range from being completely asymptomatic to any combination of fever, sore throat, malaise, ulcerative and vesicular lesions, and gingivostomatitis (5, 13, 16).

HSV-1 invades and replicates in neurons, as well as epidermal and dermal cells (16). Following primary infection and local replication at mucosal surfaces, the virus enters sensory nerve endings and is then transported by retrograde axonal transport to the neuronal cell bodies and results in latent infection of these neurons (20). In the case of

HSV-1, latency is typically established in the trigeminal ganglia (9, 10). When reactivation occurs, there is usually an appearance of skin vesicles or ulcers around the vermillion border of the lip (5). In recurrent herpes labialis, symptoms of tingling, pain, itching, and burning usually precede the lesions (16). Healing occurs within 1–10 days of the onset of initial symptoms (8, 20).

There are other less common diseases that are caused by HSV-1. Herpetic keratitis is an HSV infection of the eye. Common symptoms include eye pain, light sensitivity, and discharge in the eye (16). Herpetic whitlow occurs when vesicular lesions appear on the hands or digits (16). This infection is typically seen in children who suck their thumbs or in medical/dental workers exposed to HSV-1 while not wearing gloves (13, 16). Herpetic sycosis is a follicular infection with HSV that causes vesicular lesions in the beard area, often caused by autoinoculation from shaving (16). More serious disease is seen in neonatal infections and infections in immunocompromised hosts (4, 13). HSV infections of neonates frequently leads to a disseminated infection accompanied by encephalitis (4). Also, infections or reactivations in these individuals are usually severe and can lead to fatality (4).

Treatment

There is currently no cure for herpes infections; however, a number of treatments are now available for primary and recurrent disease. Two of the most common drugs used for HSV therapy are acyclovir and valacyclovir, the latter a second generation drug derived from acyclovir. A series of clinical trials proved the acyclic guanosine derivative, acyclovir (ACV), to be effective in shortening the course of and suppressing the

occurrence of symptomatic HSV-1 and HSV-2 infections in normal and immunocompromised patients (21). Acyclovir works as a selective inhibitor of the replication of HSV (22). Acyclovir targets the viral thymidine kinase, which converts acyclovir to its monophosphate derivative; in uninfected cells this event does not occur as readily (22, 23). The subsequent diphosphorylation and triphosphorylation of acyclovir are catalyzed by cellular enzymes, and result in a 40- to 100- times higher concentration of acyclovir triphosphate in HSV-infected cells than in uninfected cells (22, 23). Acyclovir triphosphate inhibits viral DNA synthesis by competing with deoxyguanosine triphosphate for viral DNA polymerase (22). Also, since acyclovir triphosphate lacks a 3'-hydroxyl group required for DNA elongation, the synthesis of viral DNA is terminated (22). Although acyclovir is an effective therapy for HSV suppression, it has low oral bioavailability (14, 22).

When taken orally, acyclovir is slowly and incompletely absorbed from the gut, leading to an oral bioavailability of about 10-20% (13, 22). Furthermore, the absorbed drug is rapidly degraded in the kidneys, and has a short, 3-hour half-life in vivo (22). Acyclovir can be used for the treatment of initial HSV episodes, but the single oral dose that is usually administered to treat initial infections is not effective in reducing the frequency of recurrences (22). The preferred dosage for oral acyclovir therapy typically requires either 200 mg three times daily or 400 mg twice daily to maintain a constant level of drug in the body (8, 19). Due to this need for multiple daily doses of ACV for suppressive therapy, patient compliance is another issue with lifelong oral dosing of acyclovir for long term therapy (14).

There have been some attempts to improve the absorption of acyclovir. Valacylcovir, the L-valyl ester of acyclovir, has about a 50% oral bioavailability and is rapidly converted to acyclovir in the liver (13). The typical dosing regimen for initial infection is 1000 mg twice a day for seven days, and for chronic suppressive therapy is 500-1000 mg once a day (19). However, for HSV-2 therapy and immunocompromised individuals, the dosing schedule is more frequent, and thus still requires multiple daily doses.

Controlled Release Devices

Controlled release systems are typically used to deliver drugs via a single dose, over a long period of time, and with the intention of a particular drug being released at levels within its therapeutic index (24). They are also viewed as potentially advantageous over oral dosing because they (i) improve localized delivery of the drug; (ii) increase the preservation of medications that are rapidly destroyed by the body; (iii) reduce the need for follow-up care; (iv) increase comfort; and (v) improve patient compliance (24). The use of polymers is very common in many controlled release systems. The most common release mechanism using these types of systems is diffusion, in which the drug migrates from its initial position in a polymer matrix to the surface, where it can then be delivered into the body (24).

Silicone polymer-based devices have been very successful in controlled release systems. For example, Norplant® is a silicone based implant that was developed to deliver levonorgestrel over a three-year period for long-term contraception (14, 25). Additionally, ganciclovir has been formulated into a polymer-based intraocular implant

(Vitrasert®) that slowly releases drug over a five- to eight-month time period for the treatment of cytomegalovirus retinitis (14, 26). A previous study showed that this implant was a more effective treatment compared to intravenous ganciclovir (26). Although these systems tend to be successful for long-term drug delivery and don't depend on patient compliance, they do require surgical intervention for implantation and removal of the device. Furthermore, there are concerns with these devices migrating from their original site, which makes implant retrieval even more difficult (27).

Biodegradable Drug Delivery Devices

Technological advances have brought about many new drug delivery systems for commercial use. Among these, biodegradable polymers have become important in the pharmaceutical industry as both drug encapsulates and as controlled release devices for drug delivery. The most commonly studied biodegradable polymers for controlled drug delivery are the aliphatic polyesters: poly(lactide), poly(glycolide), polycaprolactone (PCL), and their copolymers (28). Polycaprolactone (PCL) is a hydrophobic, semi-crystalline material with a melting range from 59 °C to 64 °C (29, 30). PCL is soluble in chloroform, dichloromethane, carbon tetrachloride, benzene, toluene, cyclohexanone, and 2-nitropropane at room temperature (29). It has a low solubility in acetone, 2-butanone, ethyl acetate, dimethylformamide, and acetonitrile (29).

PCL is subject to biodegradation by outdoor living organisms, such as bacteria and fungi, but not in animal and human bodies due to a lack of suitable enzymes (29). However, PCL is bioresorbable, meaning that it can be hydrolytically degraded by the human body and its byproducts can be eliminated via natural pathways with no residual

side effects (29). More specifically, degradation studies have been performed which showed that PCL undergoes a two-stage degradation process. PCL first goes through non-enzymatic hydrolytic cleavage of its ester groups, which produces ε-hyproxycaproic acid (29, 30). Once the molecular weight reaches 3000 or less, the polymer is phagocytosed by macrophages and further degraded via the citric acid cycle (29, 31).

PCL has become a popular candidate for long-term drug delivery due to its high permeability to many drugs, non-toxic properties, and slow degradation rate of about two to four years (28). Additionally, it is a commercially available product that has been approved by the FDA for human application. For example, PCL is used in the commercially available monofilament suture Monocryl® (32). Studies showed that this suture is able to be used successfully in multiple types of surgeries without any cytotoxic effects, irritation, and minimal tissue reaction (32, 33).

Polycaprolactone has also been used experimentally in many studies to construct biodegradable microspheres for the long-term delivery of drugs and proteins, such as taxol to inhibit angiogenesis, cisplatin, ribozymes, heparin, and insulin (28, 34). This polymer has also been formulated into microspheres containing the contraceptive steroids levonorgestrel and ethinyl estradiol (35). Additionally, Rodrigues and colleagues were able to encapsulate naproxen in a PCL nanoparticle for use as an implantable sustained release system for the treatment of chronic inflammatory diseases (36). *In vitro* and *in vivo* release kinetics studies showed that the PCL nanoparticles increased naproxen plasma levels and drug release was sustained for 42 days in male Wistar rats (36). Thus,

PCL has been extensively investigated for use as an implantable or injectable biodegradable carrier for the controlled release of active agents.

Research Focus

Due to the low oral bioavailability of acyclovir and its short *in vivo* half-life, both of which require that HSV-infected individuals take multiple daily doses, the Towson University Herpes Virus Laboratory (TUHVL) has developed a silicone implant containing acyclovir designed for long term ACV drug delivery for HSV-infected individuals. Our studies have shown that ACV is released from these silicone-based implants with near-zero order release kinetics of 1 µg drug/ day, regardless of temperature or pH (14). These ACV-silicone implants were found to protect Vero cells from HSV-1-induced cytopathic effect (CPE). Most importantly, these implants suppressed HSV-1 reactivation in infected mice during repeated attempts to induce active replication over a ten-week period (14). Although this antiviral drug delivery system is effective at suppressing recurring HSV-1 outbreaks, it would require continuous surgical intervention for implant replacement because silicone is not biodegradable. An ideal solution to the repeated surgeries would be to design a long-term drug delivery system that is subject to biodegradation.

The goal of this research was to construct a subcutaneous biodegradable implant that would function in the controlled release of acyclovir to prevent HSV-1 reactivations. Additionally, we conducted a 60-day kinetics study to determine the rate of drug release from these implants at three different temperatures and three different pHs, and investigated the antiviral efficacy of these biodegradable implants *in vitro*.

CHAPTER II:

MATERIALS AND METHODS

Implant Development

Implants were constructed using polycaprolactone (PCL) (Sigma-Aldrich, St. Louis, MO) and powdered acyclovir (ACV) (Advanced Scientific & Chemical Inc., Ft. Lauderdale, FL). PCL was dissolved in acetone under continuous stirring conditions at 37 °C. Once the PCL dissolved, ACV was added to develop drug:polymer ratios of 75:25, 70:30, 65:35, or 60:40 in a 50% w/v solution. Stirring was continued until the acetone completely evaporated, and the residual drug: polymer mixture hardened into large pieces. The resultant chunks were transferred to a 10-mL Eppendorf® Combitip (Fisher Scientific, USA) and heated in a 76 °C water bath for approximately 20 min, after which the chunks melted into a viscous mixture. The Combitip was then immediately removed from the hot water bath and the viscous mixture was extruded into 12-gauge steel needles (Painful Pleasures, Inc., Hanover, MD). The polymer:drug mixture was allowed to solidify in the needles for 24 hours and was then extruded. This method resulted in a rod-shaped implant with a 2.1-mm diameter. Implants were cut to be approximately 15 mm long (Figure 1).

PCL-only rod-shaped implants were constructed by transferring polymer into a 25-mL Combitip then heating and processing exactly as explained above.

Implant Sterilization

The process used to sterilize the implants has been cited in previous literature (31). The implants were first soaked in a reagent reservoir dish filled with approximately 10 mL of 70% ethanol for 30 minutes. They were then moved to a new dish and submerged in 70% ethanol for an additional 30 minutes. Four more dishes were filled with 10 mL of phosphate-buffered saline (PBS) (Mediatech, Inc., Herndon,VA); implants were submerged into the first three dishes for 2 h each and the last PBS rinse was for 1 h. Following the final rinse, implants were removed from the dish and allowed to dry in a laminar flow hood overnight.

Antiviral Efficacy of Implants at Different Polymer: Drug Ratios

An *in vitro* assay was performed to determine which polymer:drug ratio of implant exhibited the best antiviral activity. Vero cells (African green monkey kidney cells) were maintained in 75 cm^2 flasks in Dulbecco's modified Eagle medium (DMEM) (Mediatech, Inc., Herndon,VA) containing 10% fetal bovine serum (Thermo Fisher Scientific, Inc., USA), 1% Glutamax (Invitrogen, Inc., Carlsbad, CA), and 1% Antibiotic/Antimycotic solution (Mediatech, Inc., Herndon,VA) at 37 °C. For this assay, Vero cells were transferred from flask and cultured in a 24-well plate. In each well, 1 mL of freshly split Vero cells was added and allowed to grow for 24 h to about 60% confluence (approx. 1.2 x 10^5 cells). The cells were then treated with a single implant of each polymer:drug ratio. For comparison purposes we also treated cells with either a silicone implant containing ACV (MED 4750-ACV) (14), 10 µL of sterile ACV in PBS (25 mg/mL), a PCL only implant, a silicone-only implant, or no treatment. All cells were treated for 48 hours and all implants were tested in triplicate (one implant per well). After

48 hours, 1.9 x 10^2 pfu of HSV-1 (KOS) strain was added to each well (except for the non-infected control). Three days post-infection pictures were taken with a Sony® CyberShot attached to an AccuScope® 3032 microscope. Images were enhanced using the program Zerene Stacker (Zerene Systems, LLC).

Drug Release Kinetics Study

We conducted a 60-day release kinetics study to determine the effects of various temperatures and pHs on drug release from the implants. A set of implants was made for each parameter (temperature/pH) and consisted of eleven implants: five PCL-ACV (60:40), three PCL-only, and three MED 4750-ACV (silicone). Each implant was placed into a 1.5 mL microcentrifuge tube with 1 ml of phosphate- buffered saline (PBS). For the temperature study, one complete set of eleven implants was placed in each environment at 4 °C, 25 °C, or 37 °C. For the pH study, sets of implants were placed in 1.5 mL microcentrifuge tubes filled with 1 mL of PBS with a pH level of 6, 7, or 8. The pH of the PBS solutions was adjusted by adding 1 M HCl or 1 M NaOH. Every 24 h each implant was moved from its current microcentrifuge tube to a new 1.5 ml microcentrifuge tube with fresh PBS; this process was repeated for 60 days.

High-Performance Liquid Chromatography

The amount of drug released into solution from each implant was determined using high performance liquid chromatography (HPLC). Samples (50 µL from each 1 mL of each sample) were prepared for HPLC analysis with 450 µL of acetonitrile (Fisher Scientific, USA). Samples were run on a Shimadzu HPLC system equipped with an LC-20AT pump, a photo diode array detector (SPD-M20A), and a fluorescence detector (RF-

10Axl). Some samples were also run on an Agilent 1200 series HPLC system. All HPLC procedures were performed with the assistance of Dr. Ryan Sours in the Towson University Department of Chemistry.

Samples were run at a constant flow rate of 0.4 mL/min through a Phenomenex® Luna HILIC column (100 x 2 mm, 3 micron) (Phenomenex, Torrance, CA). The mobile phase was a mixture of 90% acetonitrile and 10% of a 0.1% formic acid solution. ACV concentrations for each sample were calculated based on a standard curve derived from area peaks of reference for known samples of 0.625, 1.25, 2.5, 5, 10, 25, 50, 75, or 100 µg/mL ACV. All reference samples also contained known concentrations of penciclovir (PCV) as an internal control. All solvents used for this analysis were HPLC grade.

CHAPTER III:

RESULTS

Effect of Implant Polymer: drug Ratio on Antiviral Efficacy

To determine which polymer:drug ratio exhibited the best antiviral activity, Vero cells were either mock-infected or infected with HSV-1 (KOS) strain. Each sample of HSV-1-infected cells were then treated with a PCL-ACV implant with a polymer:drug ratio of 75:25, 70:30, 65:35, or 60:40. Also, for comparison purposes infected cells were treated with either ACV in solution (0.25 µg/µL), a PCL only implant, a MED 4750 (silicone)-ACV implant, or received no treatment. All PCL-ACV implants, despite their different polymer:drug ratios, exhibited some antiviral activity 72 hours post-infection (Figure 2). There was almost no cytopathic effect (CPE) exhibited in infected cells that were treated with PCL-ACV implants with a ratio of 65:35 or 60:40 (Figure 2). However, infected cells treated with the 75:25 and 70:30 implants exhibited moderate levels of CPE 72 hours post-infection (Figure 2). All mock-infected cells remained healthy while cells that received no treatment exhibited severe CPE (Figure 2). Cells treated with the MED 4750-ACV implant and ACV in solution exhibited no CPE and were comparable to the mock-infected cells (Figure 2). Cells treated with blank implants (PCL only) exhibited severe CPE (Figure 2).

Effect of Temperature on Release Kinetics

We conducted a 60-day release kinetics study to determine the effects of various temperatures on drug release from the PCL:ACV 60:40 implants. The implants with a

60:40 ratio were chosen (i.e., as opposed to using the 70:30 or 75:25 implants) to ensure that there was enough drug to last throughout the 60-day trial period without compromising antiviral activity. Implants were placed into 1.5 mL microcentrifuge tubes with 1 mL of PBS and moved to a fresh tube with fresh PBS every 24 hours for 60 days. Each set of implants were stored at 4 ° C, 25 °C, or 37 °C for the duration of the study. Despite the temperature range, all implants containing drug exhibited a burst period of 14 days (Figure 3). This phenomenon is typical upon administration of most controlled release devices and is characterized by having unpredictable and varying release rates. After 14 days the drug release levels become more consistent and thus the days following the burst period were used for statistical analysis.

The PCL-ACV implants maintained at 4 °C and 25 °C exhibited a steady decline in the amount of drug released daily over the 60-day trial period. After the initial burst period, the PCL-ACV implants that were kept at 4 °C released an average of 53.87 ± 13.10 µg per day (Figure 3A), while the implants that were maintained at 25 °C released an average of 173.31 ± 56.35 µg per day over the trial period (Figure 4A). However, the implants that were maintained at 37 °C had the most variation and inconsistent release kinetics: from days 15-40 these implants released an average of 227.12 ± 154.42 µg per day, while the average release from days 41-60 was 21.85 ± 14.59 µg per day (Figure 5A). A summary of average ACV released every 24 hours at each temperature parameter after the initial burst period is provided in Table 1. A repeated measures ANOVA with a Huynh-Feldt correction determined that there was a significant interaction seen between temperature and time ($F= 6.65$, $P < 0.0001$). Thus, although both temperature ($F= 13.44$,

$P < 0.0001$) and time ($F=28.24$, $P < 0.0001$) had significant effects on the average release rate of ACV, these variables appear to work synergistically, rather than independently, of each other.

Each temperature set also contained three MED 4750-ACV implants in which the release kinetics are already known (14). The average amount of ACV released per day was much lower from these implants when compared to the PCL-ACV release rate. Also, the burst period was seven days for these implants, as previously documented (14). After the seven day burst period, the MED 4750-ACV implants that were kept at 4 °C released an average of 3.54 ± 2.87 µg/mL per day (Figure 3B), at 25 °C the average release was 2.10 ± 1.65 µg/mL per day (Figure 4B), and at 37 °C the average release of ACV was 1.61 ± 1.23 µg/mL per day (Figure 5B). The nine PCL implants which contained no drug (three for each temperature parameter) had no observable release of ACV.

Effect of pH on Release Kinetics

Just as with temperature, we conducted a 60-day release kinetics study to determine the effects of various pHs on drug release from the implants. Experiments were performed essentially as described for temperature dependence, except each set of implants was kept in 1 mL of PBS buffered to a pH of 6, 7, or 8 at 37 °C. The initial burst period was 14 days despite the pH level, and thus statistical analysis was performed for only the days following the burst period.

Similar to the temperature study, the PCL-ACV implants exhibited a steady decline in their drug release rates over the 60-day trial period regardless of the pH. Following the initial burst period, the implants that were kept in pH 6 solutions released

an average of 283.02 ± 128.82 µg of ACV per day from days 15-30, while the average release from days 31-60 was 33.01 ± 15.31 µg per day (Figure 6A). The implants that were kept in pH 7 solutions released an average of 263.34 ± 112.49 µg of ACV per day from days 15-30, while the average release from days 31-60 was 54.93 ± 26.80 µg per day (Figure 7A). Finally, the implants that were kept in pH 8 solutions released an average of 276.90 ± 199.05 µg of ACV daily from days 15-30, while the average release from days 31-60 was 27.48 ± 7.69 µg per day (Figure 8A). A summary of average ACV released every 24 hours at each pH level after the initial burst period is provided in Table 2. A repeated measures ANOVA with a Huynh-Feldt correction determined that the average amount of ACV released from the implants was not significantly affected by pH ($F= 0.21$, $P = 0.82$). However, time did significantly affect the average release rate ($F=42.81$, $P < 0.0001$). There was no interaction between time and pH ($F=0.67$, $P=0.72$).

Each pH set also contained three MED 4750-ACV implants (14). The average amount of ACV released per day was much lower from these implants compared to the PCL-ACV release rate. After the seven day burst period, the MED 4750-ACV implants that were kept at pH 6 released an average of 1.88 ± 0.89 µg/mL daily (Figure 6B), at pH 7 the average release was 1.97 ± 0.80 µg/mL daily (Figure 7B), and at pH 8 the average release of ACV was 1.87 ± 0.91 µg/mL per day (Figure 8B). The nine PCL implants which contained no drug (three for each pH level) had no observable release of ACV.

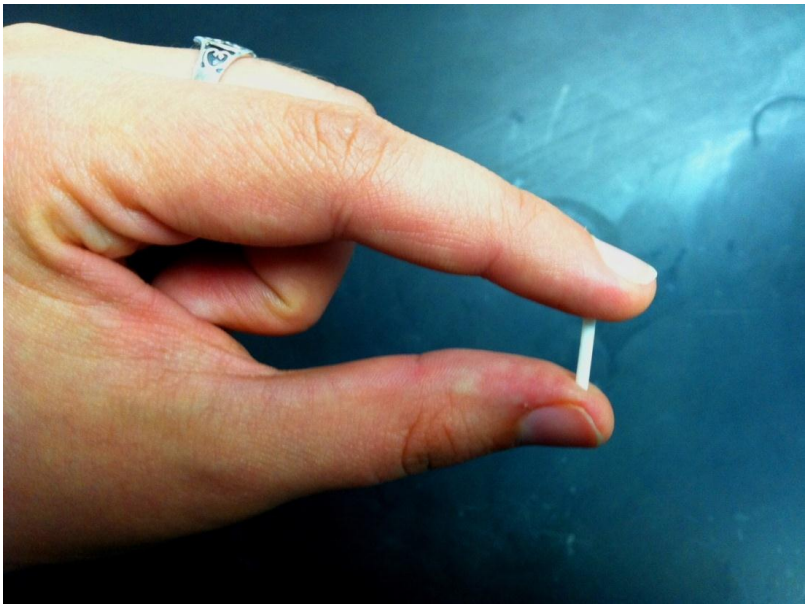


Figure 1: A rod-shaped PCL implant containing the drug ACV was constructed by using a solvent evaporation technique in conjunction with melt-casting. The designed implant is approximately 15 mm in length and 2.1 mm in diameter.

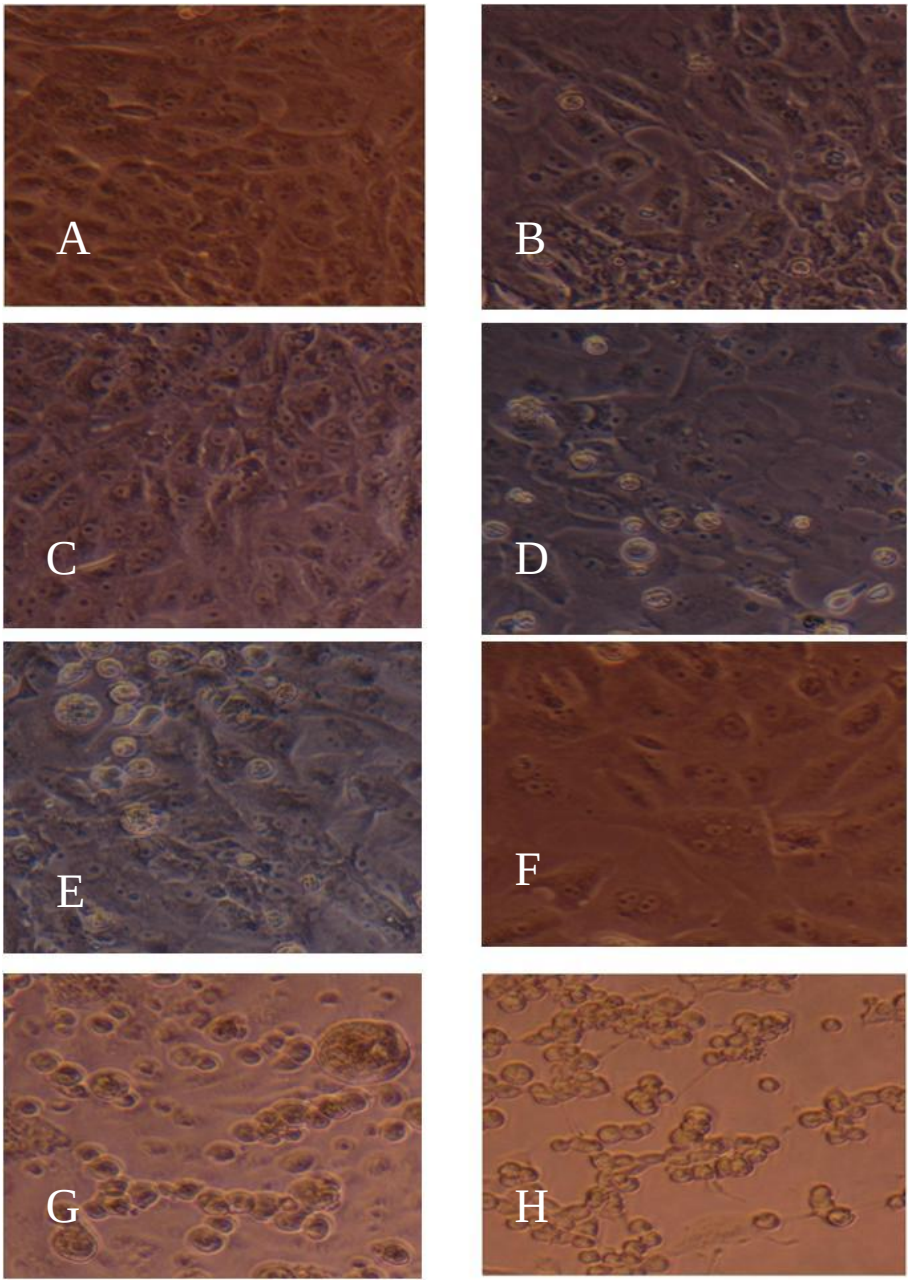
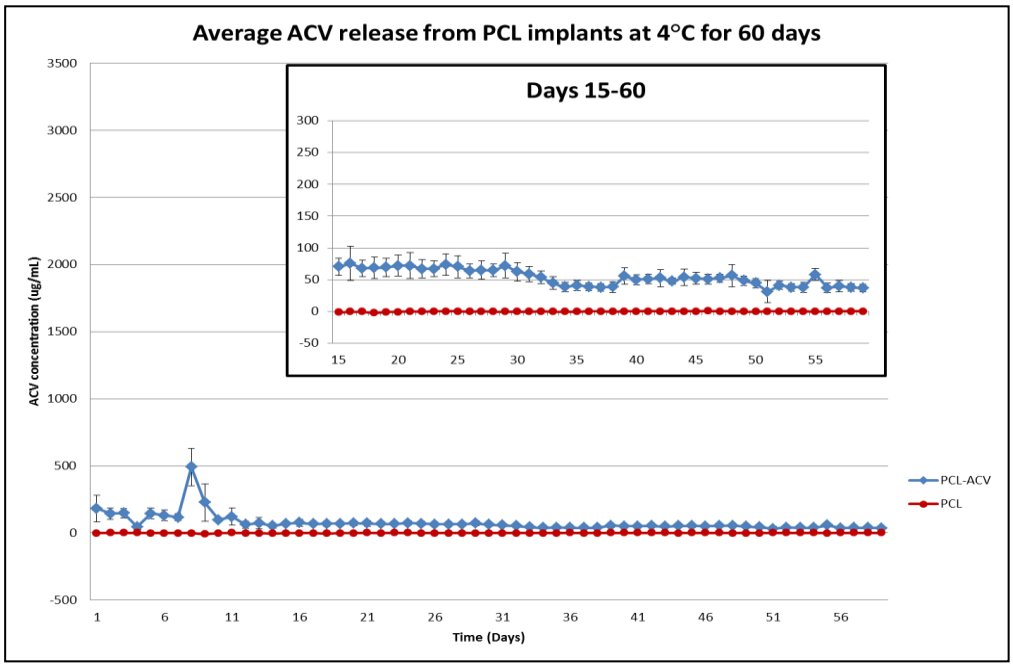


Figure 2: An *in vitro* assay was used to determine which polymer:drug ratio exhibited the best antiviral activity. Vero cells were either mock-infected (A) or infected with HSV-1 (KOS) strain and treated with (B) PCL:ACV in a 60:40 implant (C) PCL:ACV 65:35 implant (D) PCL:ACV 70:30 implant (E) PCL:ACV 75:25 implant (F) MED-4750 silicone implant with ACV (G) PCL-only implant or (H) no treatment. Photos were taken with a Sony® CyberShot attached to an AccuScope® 3032 microscope. Multiple images were taken, stacked, and compressed using Zerene Stacker® software.

A.



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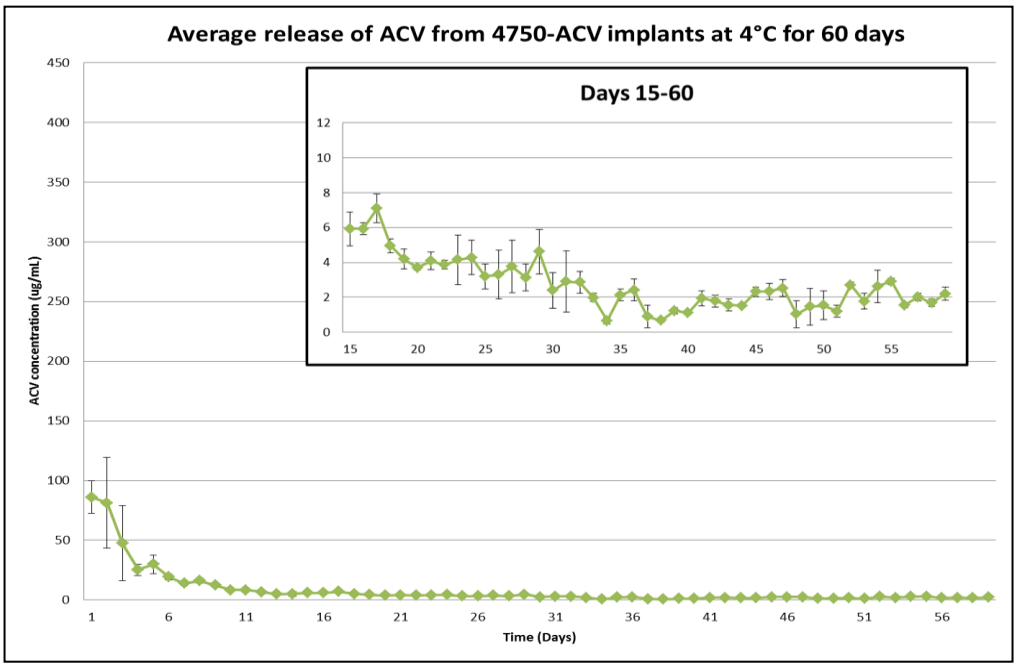
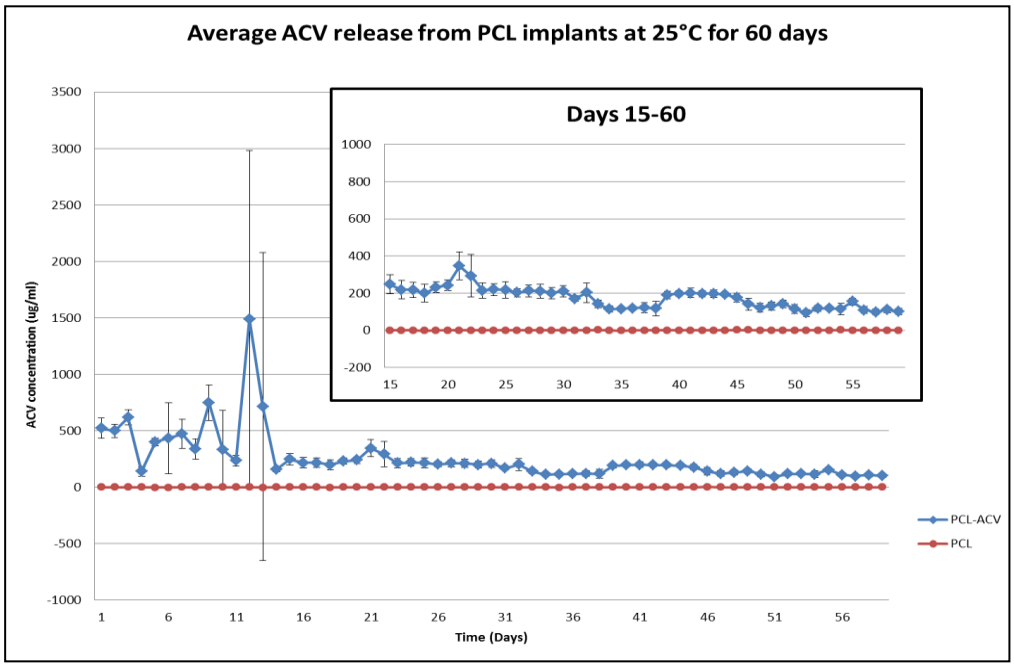


Figure 3: Average release of ACV (µg/mL) every 24 hours from implants that were kept at 4 °C. The inset to each graph shows the release kinetics from these implants following the initial 14 day burst period. (A) Average of five replicates of PCL implants' release kinetics (B) Average of three replicates of MED 4750 (silicone)-ACV implants' release kinetics. Implants were moved to fresh medium every 24 hours. HPLC analysis was performed to determine concentration of ACV in each sample.

A.



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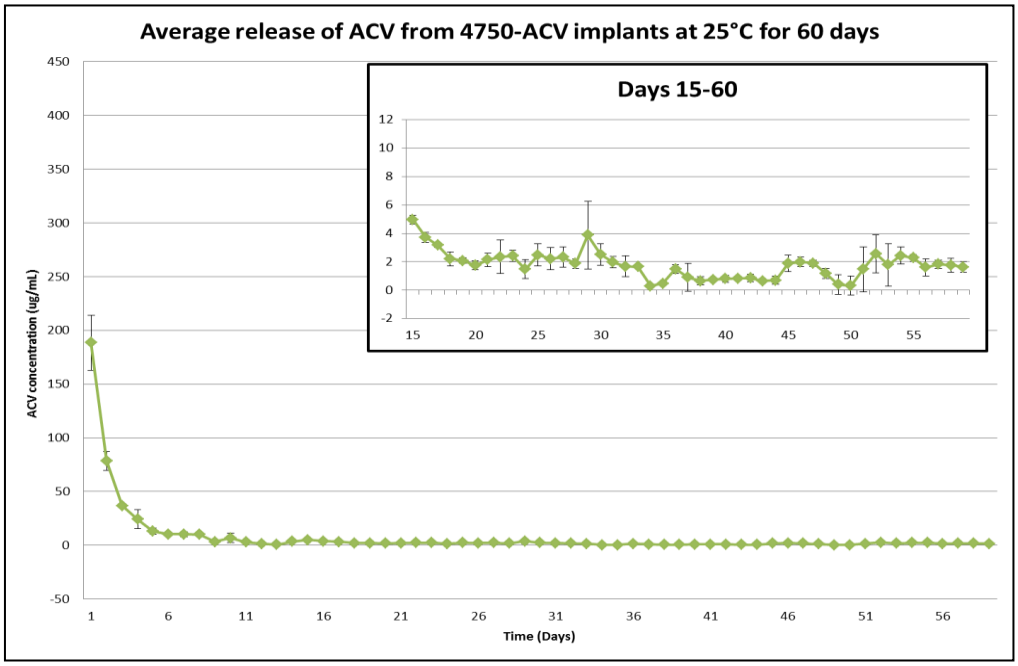
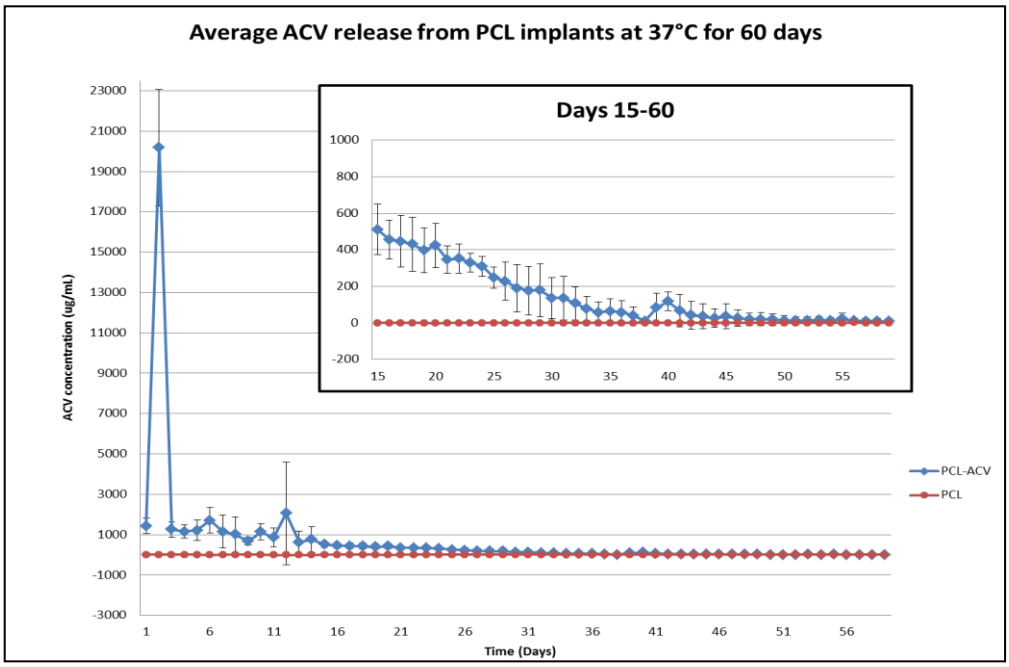


Figure 4: Average release of ACV (µg/mL) every 24 hours from implants that were kept at 25 °C. The inset to each graph shows the release kinetics from these implants following the initial 14 day burst period. (A) Average of five replicates of PCL implants' release kinetics (B) Average of three replicates of MED 4750 (silicone)-ACV implants' release kinetics. Implants were moved to fresh medium every 24 hours. HPLC analysis was performed to determine concentration of ACV in each sample.

A.



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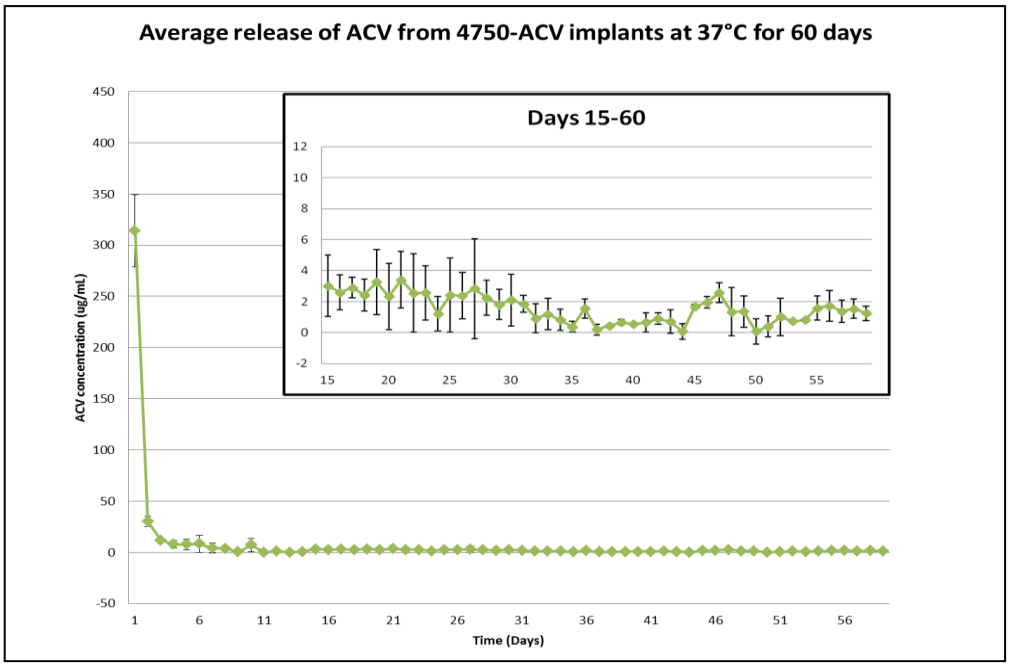
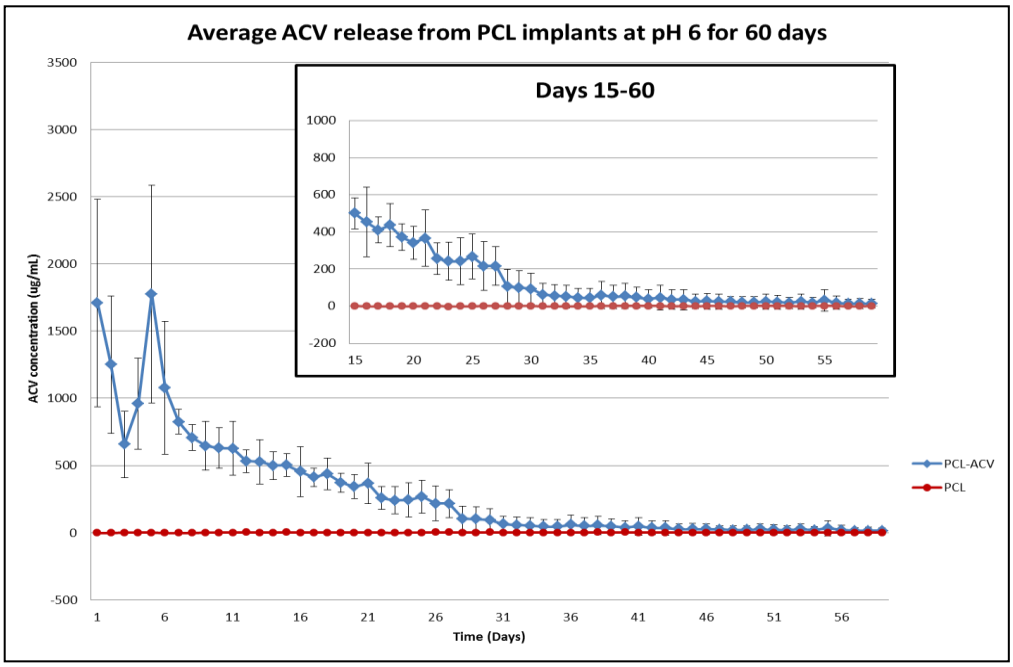


Figure 5: : Average release of ACV (µg/mL) every 24 hours from implants that were kept at 37 °C. The inset to each graph shows the release kinetics from these implants following the initial 14 day burst period. (A) Average of five replicates of PCL implants' release kinetics (B) Average of three replicates of MED 4750 (silicone)-ACV implants' release kinetics. Implants were moved to fresh medium every 24 hours. HPLC analysis was performed to determine concentration of ACV in each sample.

A.



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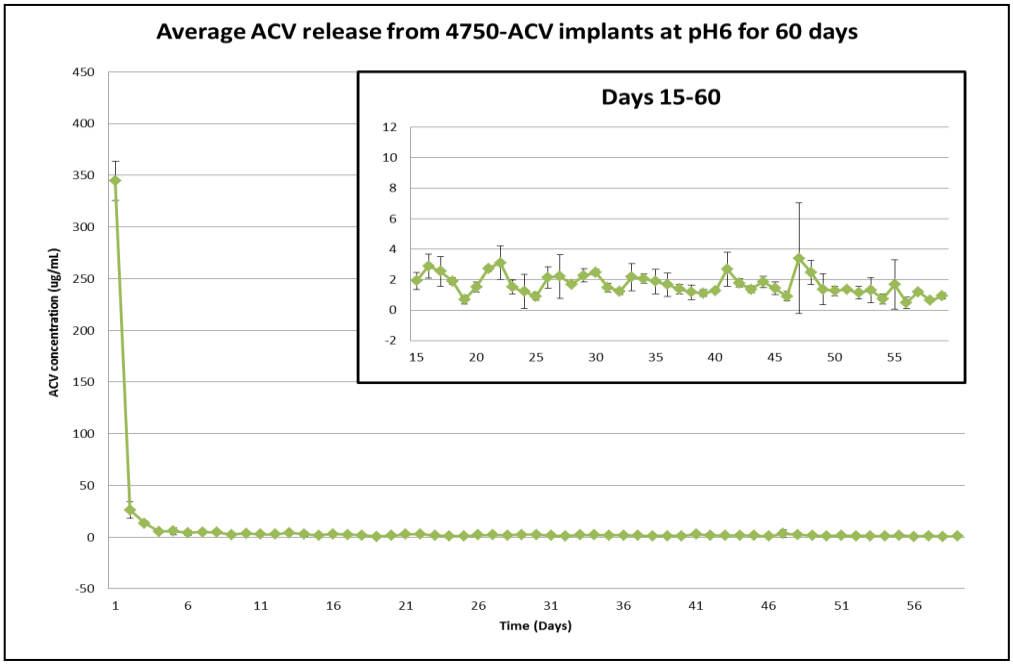
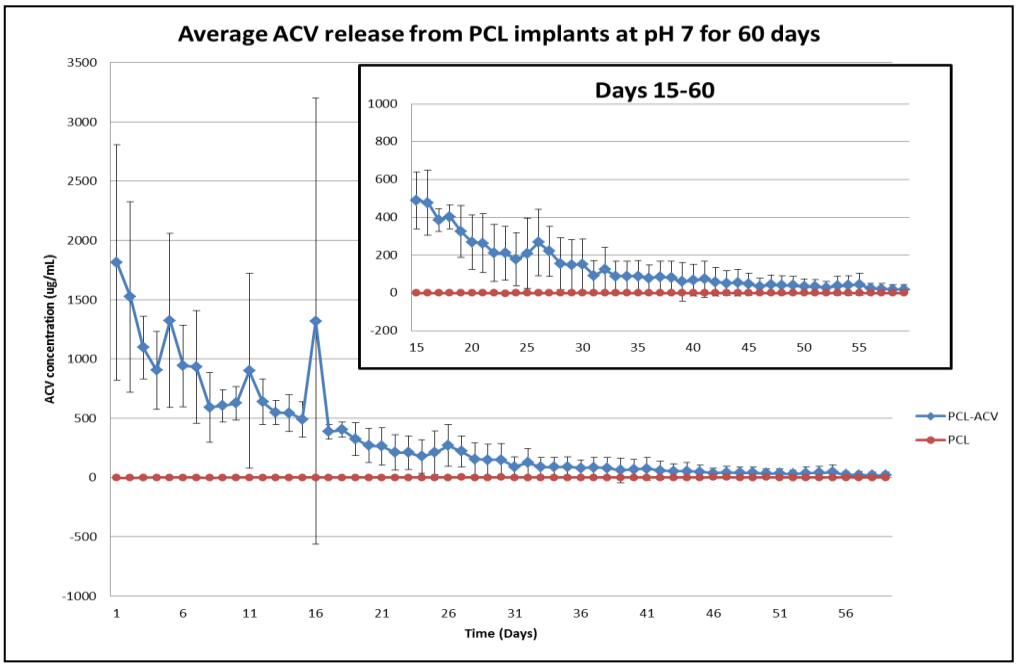


Figure 6: Average release of ACV (µg/mL) every 24 hours from implants that were kept at pH 6. The inset to each graph shows the release kinetics from these implants following the initial 14 day burst period. (A) Average of five replicates of PCL implants' release kinetics (B) Average of three replicates of MED 4750 (silicone)-ACV implants' release kinetics. Implants were moved to fresh medium every 24 hours. HPLC analysis was performed to determine concentration of ACV in each sample.

A.



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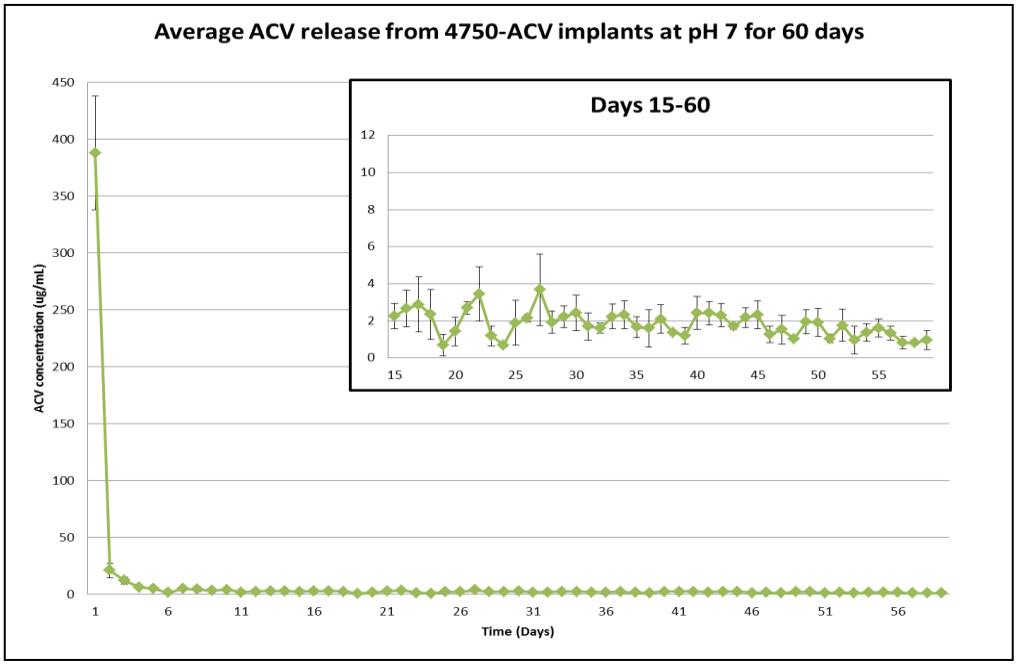
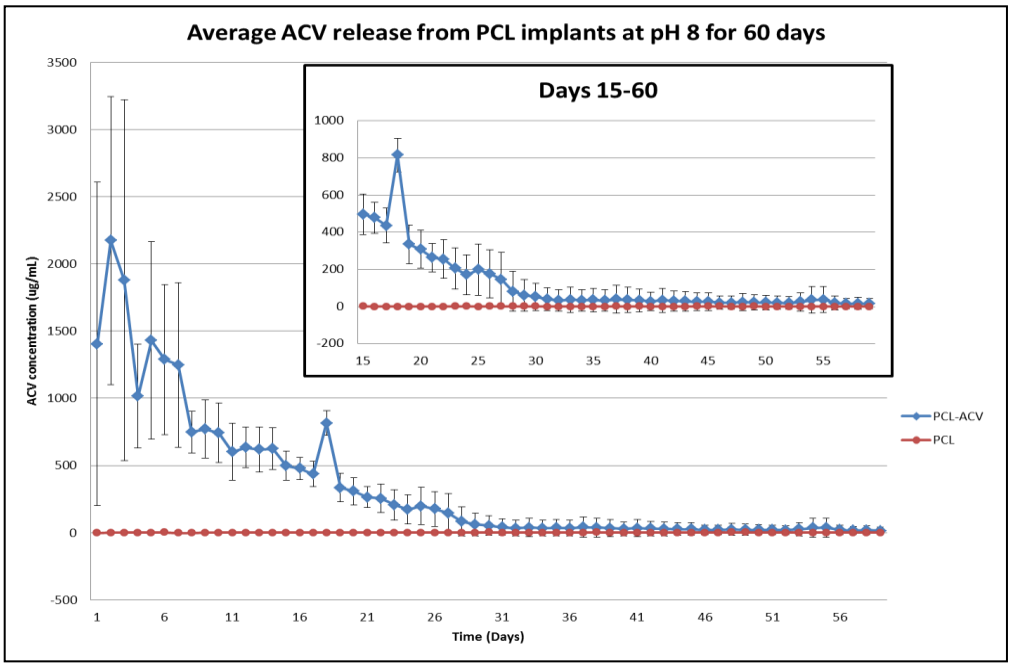


Figure 7: Average release of ACV (µg/mL) every 24 hours from implants that were kept at pH 7. The inset to each graph shows the release kinetics from these implants following the initial 14 day burst period. (A) Average of five replicates of PCL implants' release kinetics (B) Average of three replicates of MED 4750 (silicone)-ACV implants' release kinetics. Implants were moved to fresh medium every 24 hours. HPLC analysis was performed to determine concentration of ACV in each sample.

A.



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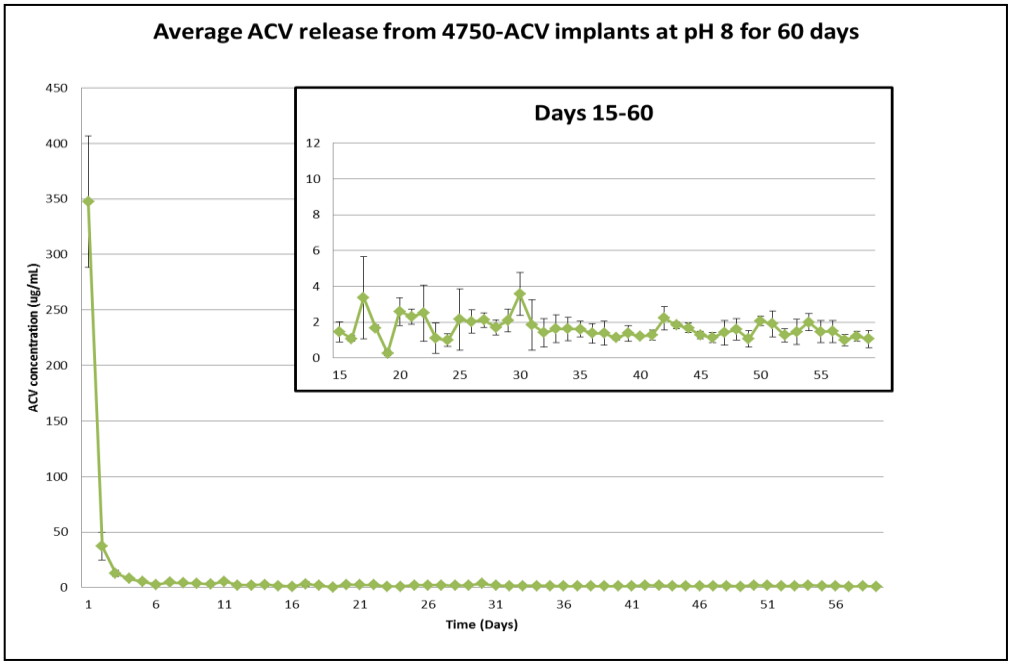

Figure 8: Average release of ACV (µg/mL) every 24 hours from implants that were kept at pH 8. The inset to each graph shows the release kinetics from these implants following the initial 14 day burst period. (A) Average of five replicates of PCL implants' release kinetics (B) Average of three replicates of MED 4750 (silicone)-ACV implants' release kinetics. Implants were moved to fresh medium every 24 hours. HPLC analysis was performed to determine concentration of ACV in each sample.

Table 1. The average amount of ACV released every 24 hours at various temperature levels after burst period subsided (day 14) (n=5).

Temperature (°C)	Average total release (µg/mL)
4	53.87 ± 13.10
25	173.31 ± 56.35
37	227.12 ± 154.42 (Days 15-40) 21.85 ± 14.59 (Days 41-60)

Table 2. The average amount of ACV released every 24 hours at various pH levels after burst period subsided (day 14) (n=5).

pH	Average total release (µg/mL) Days 15-30	Average total release (µg/mL) Days 31-60
6	283.02 ± 128.82	33.01 ± 15.31
7	263.34 ± 112.49	54.93 ± 26.08
8	276.90 ± 199.05	27.48 ± 7.69

CHAPTER IV:

DISCUSSION

Herpes simplex viruses (HSV) are ubiquitous human pathogens whose clinical manifestations can range from asymptomatic disease to life-threatening illness in immunocompromised hosts and neonates (13, 37). Acyclovir (ACV), a nucleoside analog commonly used to treat initial infections and suppress reactivations is slowly and incompletely absorbed following oral administration. Therefore, infected individuals need multiple daily doses of the drug for it to be effective, and thus results in some problems with poor patient compliance.

We have described a method involving the use of a solvent mixing/extrusion technique in conjunction with melt-casting in the development of a rod-shaped PCL implant containing the drug ACV for long-term drug delivery. Also, we determined that these implants are able to suppress primary HSV-1 infection *in vitro*. We conducted a 60-day kinetics study to examine the effects of temperature and pH on the drug release activity of the PCL-ACV 60:40 implants. We found that temperature did influence the average amount of ACV released over time. The PCL-ACV implants that were maintained at 4 °C and 25 °C exhibited a single near-zero order release kinetics phase, while the implants maintained at 37 °C exhibited a near zero-order release kinetics phase and then rapidly declined to a second near zero-order phase. We saw no significant effect of pH on release kinetics. However, the implants in this pH experiment did exhibit biphasic near zero-order release kinetics, presumably due to all samples being maintained at 37 °C. We also saw that time significantly influenced release kinetics; this is most

likely due to the fact that polymer breakdown occurs as time increases. Despite the effects of the previously stated parameters, ACV levels were never below the recommended therapeutic index. The 50% inhibitory dose of ACV in the plasma against HSV-1 was determined to be 0.03 µg/ml (38). Therefore, the average release rates observed from the PCL-ACV implants is well above the necessary inhibitory dose for HSV-1 infection and reactivation.

The general trend of release kinetics exhibited from the implants followed a steady decline over time. There was also a high amount of within-group variability seen in the implants, a phenomenon that is common in many clinically deployed controlled release devices. For example, the *in vitro* release kinetics of a 2 cm long levonorgestrel loaded PCL implant averaged 17.6 ± 3.1 µg/day/cm (39). Additionally, the non-biodegradable NuvaRing® releases an average of 1,578 ± 408 pg/mL of estonogestrel and 19.1 ± 4.5 pg/mL of ethinyl estradiol in the first week of use, 1,476 ± 362 pg/mL and 18.3 ± 4.3 pg/mL after two weeks, and by the third week an average release of 1,374 ± 328 pg/mL and 17.6 ± 4 pg/mL of each hormone respectively (NuvaRing® prescribing information). Therefore, the kinetics of ACV release from our PCL-ACV implants easily matches the variability seen in FDA-approved drug implants, implying that our experimental devices may see a useful future in true clinical deployment.

Although this study allowed us to examine the overall trend of drug release from the biodegradable implants, the process of moving the implants every 24 hours seemed to affect the structure of the implants; many of the implants could not withstand the day-to-day handling. In future studies it would be ideal to manufacture a set-up that doesn't

require physical handling of implants by the investigators because this could affect release kinetics and result in increased variability. The use of a mass production manufacturing process will allow for increased uniformity among implants. Since all the implants from this study were hand-made, some of the variability observed between implants in the average release kinetics profile may have been due to the lack of structural consistency.

We also used silicone implants (MED 4750-ACV) as a positive control in our kinetics study. These implants have shown an average release rate of 1 µg of ACV per day over a 60 day trial period (14). Compared with the PCL-ACV implants, the MED 4750-ACV implants released ACV at a much lower rate. However, the MED 4750-ACV implants were still able to exhibit antiviral activity *in vitro* and *in vivo* (14). Current studies are being undertaken to determine the efficacy of the PCL-ACV implant at suppressing HSV-1 reactivations *in vivo* using a murine model for infection. Based on the previously mentioned results from the MED 4750-ACV implants, the PCL-ACV implants should also be able to protect against attempted reactivations in a mouse model due to their higher average release rate.

Further studies are required to determine the clinical antiviral efficacy of these implants. Before this is done, it is important to understand more about the pharmacokinetics of drug delivery in a whole organism. Thus, it would be ideal to utilize a whole animal imaging system that would allow for the visualization of labeled drug being released from the implant and throughout the organism. Having this information will allow us to be able to determine the ideal placement for the implant in the human

body. If high concentrations of the drug are achieved systemically, then the implant can be administered subcutaneously in the arm or leg. Thus, we would be seeing concentrations of the drug being delivered at systemic levels that are high enough such that the implant can be administered essentially anywhere. However, if drug delivery from the implant results in high concentrations locally (around the site of implantation), then it would be more advantageous to administer the implant near the site of reactivation.

Also, before clinical implementation can begin it is important to determine whether there is enough ACV being released from a single implant per kilogram of tissue to suppress HSV-1 reoccurrences in the human body. If this is not the case, treatment using this implant may require the patient to receive a series of implants in order to increase drug load as opposed to a single implant. For example, the contraceptive implant Norplant® is administered as a set of six implants that sum up to a total drug load of 216 mg of levonorgestrel (40).

Although these implants were designed to suppress HSV-1 infection, it should be noted that ACV is also effective against the human alpha herpes viruses HSV-2 (known for causing genital herpes) and VZV (known for causing chicken pox and shingles), which allows us to potentially expand the use for our implants even further (23). A small trial performed in 1984 found that daily administration of ACV (200 mg three times a day) for 125 days significantly decreased the number of genital herpes recurrences (4, 19). Currently, the recommended dosage for chronic suppressive therapy of HSV-2 is 400-800 mg two to three times a day (19). Studies have shown that giving ACV at the

previously mentioned dosing regimen for one year allows 43% to 50% of patients to remain recurrence free (19).

We also recognize that these implants have a potential for veterinary application. Feline herpesvirus-1 is a common viral pathogen of domestic cats worldwide that causes severe conjunctivitis, keratitis, corneal ulceration, and even mortality (41). Famciclovir is the oral form of the anti-herpetic drug penciclovir (PCV). Famciclovir is commonly prescribed as an oral medication to treat cats with feline herpesvirus-1 (FHV-1), since acyclovir has shown to be toxic to cats (41). The recommended dosage for treatment in infected cats is 62.5 mg twice a day or 125 mg three times a day (42). Therefore, we are currently attempting to formulate PCL-PCV implants for veterinary application as an alternative to the oral dosing (and systemic drug distribution) of famciclovir and as an effective suppressive therapy for cats infected with FHV-1.

The successful development of this subcutaneous implant provides insight into a novel drug therapy for suppression of HSV-1 reoccurrences. Although intended for therapeutic usage, it is possible that implementation of this treatment could lead to a decrease in HSV-1 transmission and in turn decrease the seroprevalence rate. Additionally, it serves as an ideal alternative to using a non-biodegradable polymer implant that requires removal from the body and will serve to help with the issue of patient compliance observed with oral dosing.

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ABSTRACTS

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