Evaluation of Antibiotic Release from Polymeric Prodrugs

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Abstract

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Melioidosis is a bacterial lung infection caused by a pathogen capable of invading alveolar macrophages, where it gains protection from the immune system and antibiotic treatment. These complications necessitate an extensive 2-phase antibiotic treatment regimen that is often cost prohibitive to peoples in many endemic regions. Furthermore, there are currently no FDA approved methods to specifically target the intracellular bacteria populations. To meet this unmet medical need to treat intracellular bacterial infections, we have designed synthetic polymeric prodrugs with cleavable linkers bearing an antibiotic drug. Previously, *in vitro* experiments using percent drug release to characterize the release kinetics of the prodrugs have been performed. However, acid and base hydrolysis have been used to define complete release of drug from the polymer. The use of two different methods to define percent release prevents certain experiments from being directly compared. Therefore, the overall aim of my research was to validate these methods of quantification to ensure robust methodology and allow for interoperability.

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Chapter 1. INTRODUCTION

1.1 Respiratory Intracellular Infections

Lower respiratory infections (LRIs) were the 4th leading cause of death globally and the 2nd leading cause of death in low socio-demographic index countries in 2017 [1]. In children under 5 year of age, LRIs caused an estimated 808,902 death in 2017 [2]. Furthermore, in recent events, the COVID-19 pandemic caused by SARS-CoV-2 has led to an estimated 3,776,263 global deaths and an estimated 599,023 deaths in the United States as of June 11th, 2021 [3]. LRIs that are caused by pathogens capable of invading host cells, especially immune cells, are particularly difficult to treat. For example, approximately 1.5 million deaths occurred globally in 2018 due to tuberculosis (TB) caused by bacteria *Mycobacterium tuberculosis* [4]. The ability of *M. tuberculosis* to invade alveolar macrophages and other phagocytic immune cells contributes to the virulence of TB and potentially promotes the emergence of drug resistant TB amid increasing concern over multi-drug resistant (MDR), extensively drug resistant (XDR), extremely drug resistant (XXDR), and total drug resistant (TDR) TB [5]. Furthermore, intracellular pulmonary pathogens *Francisella tularensis* and *Burkholderia psuedomallei* have been classified as a Tier 1 agents by the Federal Select Agent Program [6]. This demonstrates the potential for these LRI causing bacteria to be deliberately misused as bioterrorism agents and their threat to public health and safety.

1.2 Melioidosis

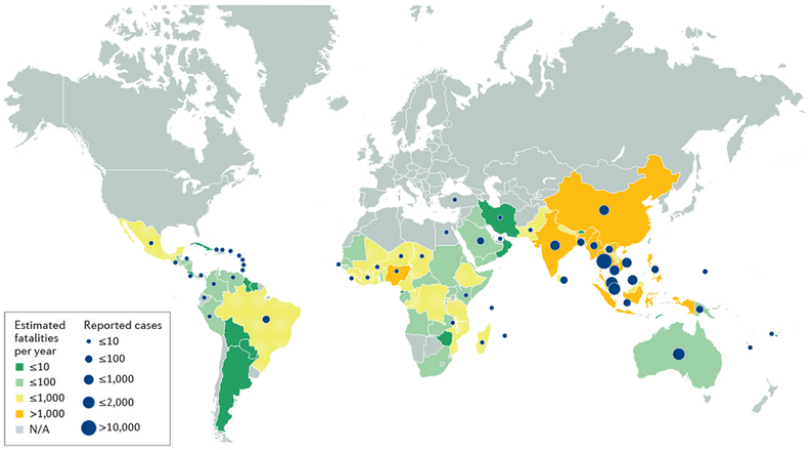

Figure 1: Incidence map for melioidosis. World map illustrating the estimated mortality and reported cases of melioidosis [7].

Melioidosis is a bacterial lung infection often misdiagnosed as TB or more common forms of pneumonia [8]. *B. psuedomallei*, the organism responsible for melioidosis, infects an estimated 165,000 patients and causes 89,000 deaths every year [9]. Furthermore, 99% of these deaths occur in low- and middle- income countries (see Figure 1).

By invading alveolar macrophages, *B. psuedomallei* gains protection from the immune system and antibiotic treatment, allowing it to multiply and eventually re-infect the patient (see Figure 2) [7]. This contributes to its virulence and high mortality rate. Furthermore, the broad antimicrobial drug resistance of *B. psuedomallei* adds to the challenge of treating melioidosis. These complications necessitate an extensive 2-phase antibiotic treatment regimen. The initial phase lasts for a minimum of 10-14 days and consists of intravenous therapy of ceftazidime or meropenem. This is followed by the second eradication phase, which consists of 3 to 6 months of oral antibiotics. However, this treatment strategy is cost prohibitive, making in inaccessible in many endemic regions. For instance, a 10-day course of ceftazidime can cost US$220 in Laos, which is 17.5% of the per capita income [10]. Currently, there are no FDA approved methods to specifically target the intracellular bacteria populations. Furthermore, as melioidosis primarily affects low-income countries, it is not a well-known disease in the US and not a prioritized subject for research, despite having a high mortality rate and high treatment burden. There is therefore an unmet need for improved treatment for melioidosis.

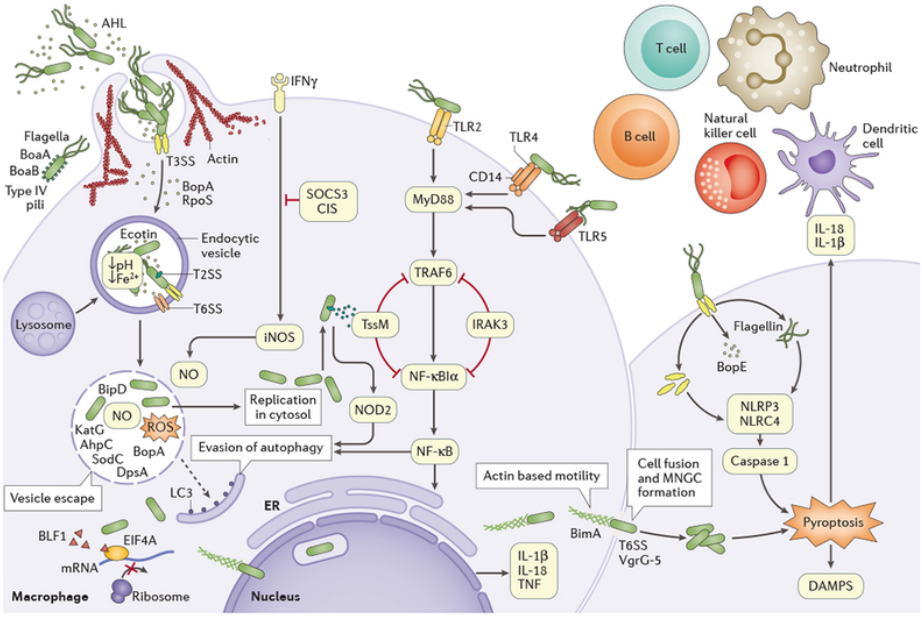

Figure 2: Schematic illustrating host-pathogen interactions and pathophysiology of melioidosis [7].

1.3 DRUGAMERS: POLYMERIC PRODRUGS

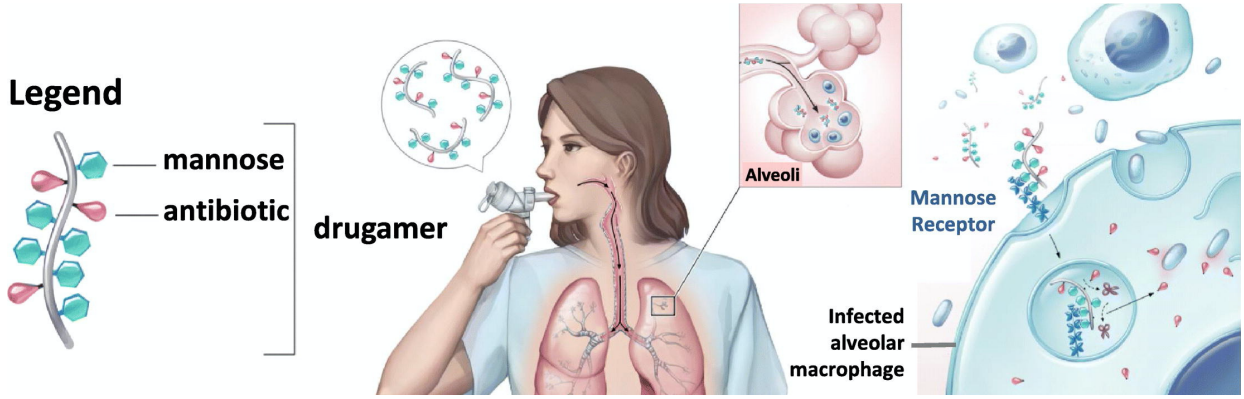

Figure 3: Schematic showing the delivery intracellular mechanism of drugamers [11].

The Ratner Lab and our collaborators aimed to fill this gap and designed synthetic polymeric prodrugs, termed "drugamers" (see Figure 3) [11]. Drugamers incorporate mannose residues and a cleavable linker bearing an antibiotic drug. The mannose residues confer biocompatibility and aids in cellular targeting, as they bind to and are internalized by mannose membrane receptors on macrophages. After internalization, lysosomal enzymes cleave the valine-citrulline (VC) linker which activates the antibiotic (see Figure 4). The dipeptide VC linker with a self-immolative spacer is stable in plasma and has been demonstrated to have efficient intracellular delivery [12]. Furthermore, this linker has been used in FDA approved antibody-drug conjugates such as Brentuximab vedotin. The ciprofloxacin drugamer was designed to be administered as an aerosol in order to achieve high local concentration at the target organ of the lung.

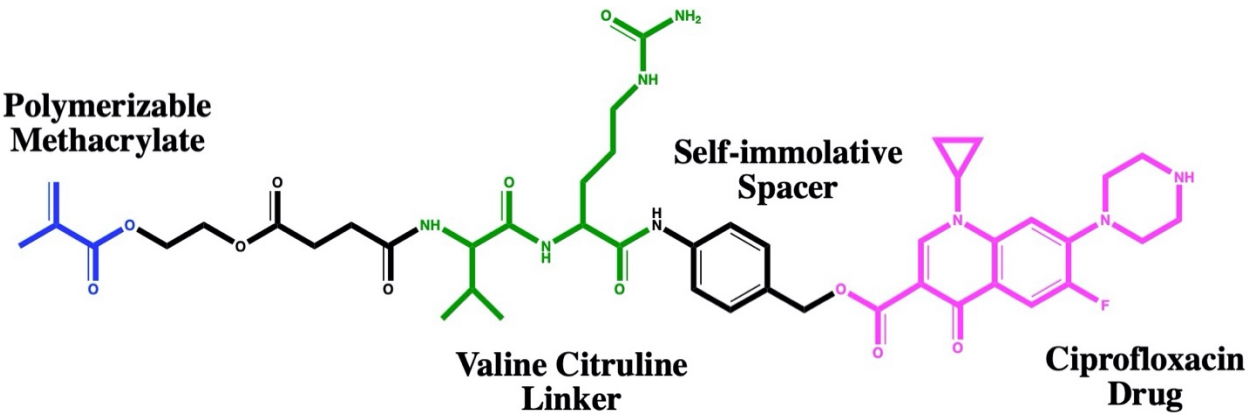

Figure 4: Structure of the valine-citrulline (VC) ciprofloxacin monomer.

1.4 DRUGAMER: NON-SPECIFIC RELEASE

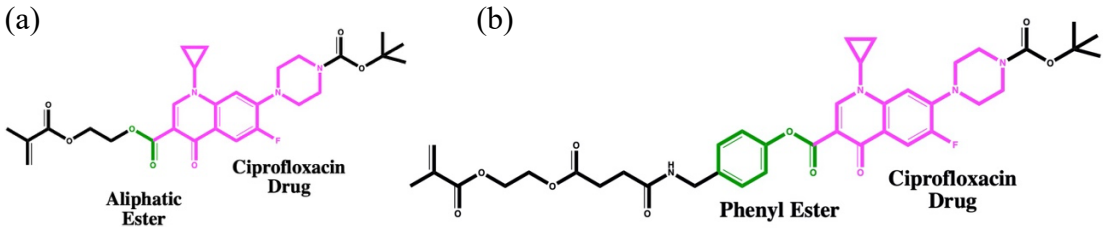

Figure 5: Structure of the ester linker ciprofloxacin monomers with (a) an aliphatic ester linker (HBC monomer) and (b) phenyl ester linker (CPM monomer).

The first generation of drugamers contained an ester linker rather than the dipeptide VC linker (see Figure 5) [13]. These drugamers were shown to be relatively unstable in human serum, with 100% of the ciprofloxacin released within 2 weeks from the phenyl ester co-polymer and over 75% of the ciprofloxacin released by day 35 from the aliphatic ester co-polymer (see Figure 6b). Additionally, the phenyl ester co-polymer showed release of ciprofloxacin even when incubated in buffer (see Figure 6a). These results demonstrate the non-specific release of ciprofloxacin from the drugamer. The efficacy of these ester linker drugamers were tested *in vivo* against *F. tularensis* [14]. When 20 mg/kg of treatment was provided on day -1, 0, and 1 days after infection, the drugamer with the phenyl ester linker provided 40% survival after 14 days, while the drugamer with the aliphatic ester and phosphate-buffered solution (PBS) provided 0% survival. When 40 mg/kg of treatment was provided on day 0, 1, and 2 days after infection, the drugamer with the phenyl ester linker provided 75% survival after 14 days, while the drugamer with the aliphatic ester and PBS provided 0% survival.

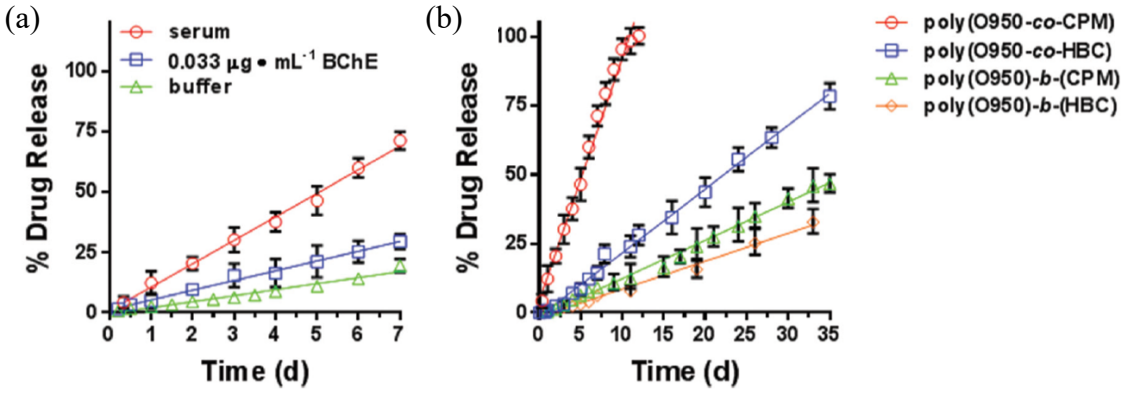

Figure 6: Drug release kinetics from ester linker drugamers over time [13]. (a) Phenyl ester polymer (poly (O950-co-CPM)) incubated in serum, in buffer, and with esterase. (b) Copolymers and diblock copolymers made with either HBC monomers (aliphatic ester linker) or CPM monomers (phenyl ester linker) incubated in 100% serum.

1.4 DRUGAMER: ENZYMATIC RELEASE

The VC-mannose ciprofloxacin drugamer is designed for enzymatic release intracellularly via lysosomal proteases. To demonstrate the specificity of the VC linker to protease mediated release, the VC-mannose ciprofloxacin was separately incubated with human liver cathepsin B, a lysosomal protease, and butyrylcholinesterase (BChE), a model human esterase [11]. As a control, a ciprofloxacin drugamer with a phenyl ester linker (Man-co-CTM) instead of a VC linker (Man-co-VC) was also incubated separately with cathepsin B and BChE. When incubated with cathepsin B, majority of the ciprofloxacin was released from the VC drugamer within four hours (see Figure 7a). On the other hand, no release of ciprofloxacin was observed from the CTM drugamer (see Figure 7a). When incubated with BChE, ~2.7% and ~15.1% of ciprofloxacin was released from the VC and CTM drugamers, respectively (see Figure 7b). This demonstrated the specificity of the VC drugamer to protease mediated release of ciprofloxacin.

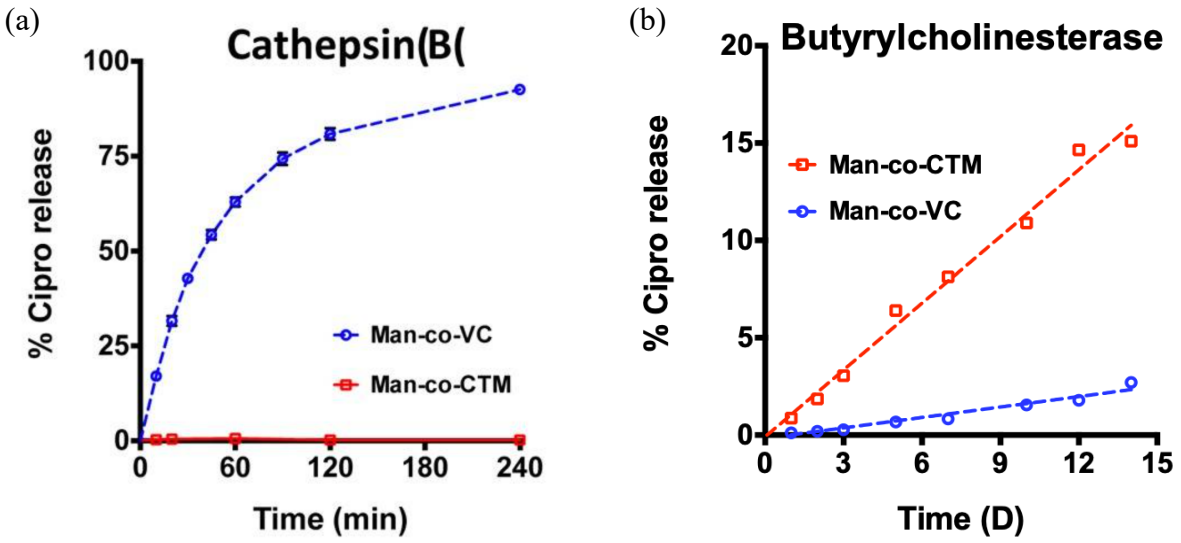

Figure 7: Protease mediated release of ciprofloxacin from VC-mannose drugamer [11]. (a) Ciprofloxacin release kinetics from 0.3 μg/mL drugamer with a dipeptide linker (Man-co-VC) or a phenyl ester linker (Man-co-CTM) incubated with 2.1 μg/mL cathepsin B. (b) Ciprofloxacin release kinetics from 0.3 μg/mL drugamer with a dipeptide linker (Man-co-VC) or a phenyl ester linker (Man-co-CTM) incubated with 0.5 μg/mL butyrylcholinesterase.

1.5 DRUGAMER: MACROPHAGE TARGETED RELEASE

The VC-mannose drugamer is also designed for targeted intracellular delivery of ciprofloxacin to macrophages. To demonstrate the intracellular release of ciprofloxacin in macrophages, 250

μg/mL[1] VC or CTM drugamer were incubated with RAW 264.7 cells (murine macrophage cells) [11]. After 2 hours of incubation, the cells were washed to remove any extracellular drugamer. The cells were then incubated in cell culture media for various periods of time. At each time point the cells were lysed and the concentration of release intracellular ciprofloxacin was quantified. The VC drugamer demonstrated a time-dependent release of ciprofloxacin, which was not observed for the CTM drugamer (see Figure 8). Additionally, the intracellular concentration was significantly greater than with the VC drugamer compared to the CTM drugamer. This demonstrates the uptake and internalization of the VC-mannose ciprofloxacin drugamer by macrophages, as well as the intracellular release of the ciprofloxacin.

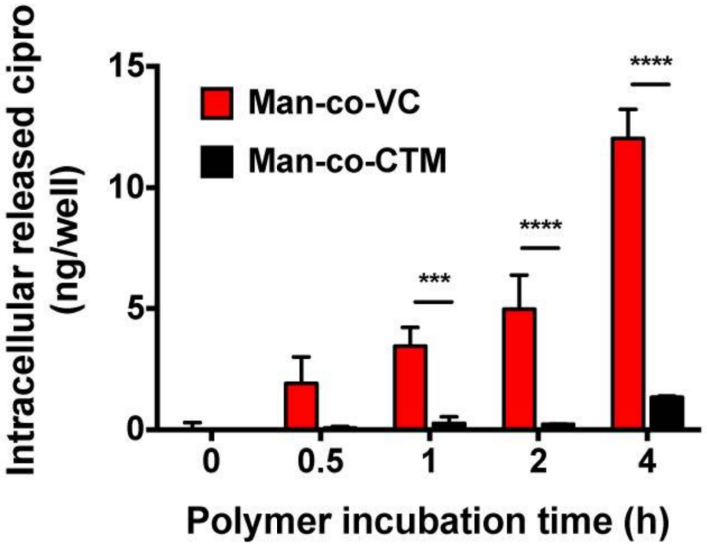

Figure 8: In vitro release kinetics of ciprofloxacin from VC-mannose drugamer [11]. RAW 264.7 cells were incubated with 250 μg/mL of VC or CTM drugamer for 2 hours. After being washed and lysed, the intracellular concentration of release ciprofloxacin was quantified at multiple time points.

1.6 DRUGMER: BIODISTRIBUTION

Given the clinical application of respiratory lung infections, the localization of the drugamer within the lungs was demonstrated through a biodistribution study [15]. C57bl/6 mice were administered with 40 mg/kg of rhodamine labeled drugamers with mannose residues and a

[1] All concentration and doses of polymeric prodrug written in this thesis represent the total concentration of ciprofloxacin incorporated in the polymer based on the percent weight of drug. For all experiments described in Chapters 2 through 7, the 11% drug weight of ciprofloxacin on the VC-mannose drugamer was used to calculate concentrations of ciprofloxacin.

phenolic ester linker. The drugamer was administered intratracheally to the mice via a Microsprayer Aerosolizer (PennCentury). The mice were euthanized at various times points and lungs, liver, kidney, spleen and blood were collected immediately after death. The concentration of polymer was determined by comparing the fluorescence from samples to a standard curve. The polymer was detectable in the lung tissue up to 72 hours (see Figure 9). In the lungs, the distribution half-life ($t_{1/2,\alpha}$) of the polymer was determined to be 11.12 minutes and the elimination half-life ($t_{1/2,\beta}$) of the polymer was determined to be 14.2 hours.

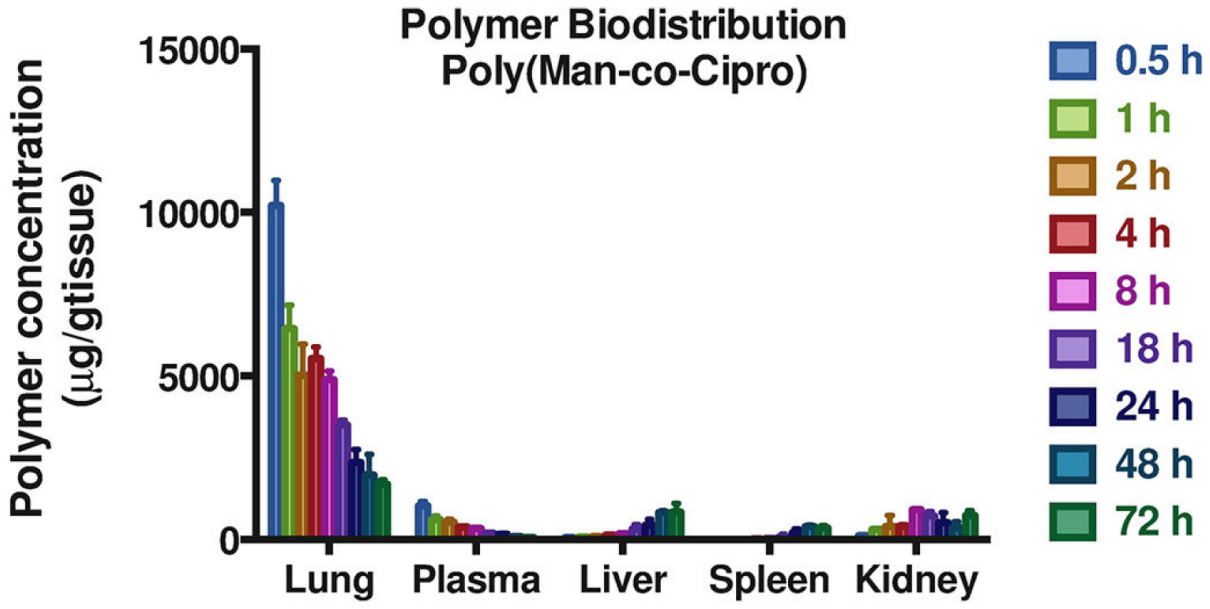

Figure 9: Biodistribution of drugamer with mannose residues in mice [15]. Mice were intratracheally administered 40 mg/kg ciprofloxacin drugamer and euthanized at various time points to determine the biodistribution of the polymer (n = 3).

1.7 Drugamer: Efficacy

The efficacy of the VC-mannose ciprofloxacin drugamer has been evaluated against *F. novicida*, the model pathogen for *F. tularensis* that, as mentioned above, has been classified as a Tier 1 agents by the Federal Select Agent Program [6]. Treatment with 20 mg/kg of free or polymeric ciprofloxacin was given to mice (n = 8) via intratracheal administration on day 0, 1 and 2 after infection using a MicroSprayer [11]. Two weeks after infection, treatment with the polymer led to 100% survival compared to 0% survival in mice treated with free ciprofloxacin.

Similarly, the efficacy of the VC-mannose ciprofloxacin drugamer has also been evaluated against model pathogen *B. thailandensis* [16]. Treatment with 20 mg/kg of free or polymeric ciprofloxacin was given to mice (n = 8) via intratracheal administration on day 0, 1 and 2 after infection using a MicroSprayer. Two weeks after infection, treatment with the polymer led to 100% survival compared to 25% survival in mice treated with free ciprofloxacin.

Furthermore, the prophylactic efficacy of the VC-mannose ciprofloxacin drugamer has also been evaluated against model pathogen *B. thailandensis* and the human pathogen *B. pseudomallei* [16]. Treatment with 20 mg/kg of free or polymeric ciprofloxacin was given to mice via intratracheal administration on day -2, -1 and 0 prior infection using a MicroSprayer. Treatment with the polymer provided full protection against both pathogens, with 100% survival compared to 0 to 12.5% survival in mice treated with free ciprofloxacin, for *B. pseudomallei* and *B. thailandensis* respectively (See Figure 10). Prior to our work, ciprofloxacin, whether by itself or in combination therapy, has not been efficacious against *Burkholderia*. These exciting results highlight the potential of this drug delivery strategy and its use for targeted delivery of other antibiotics and other drug types.

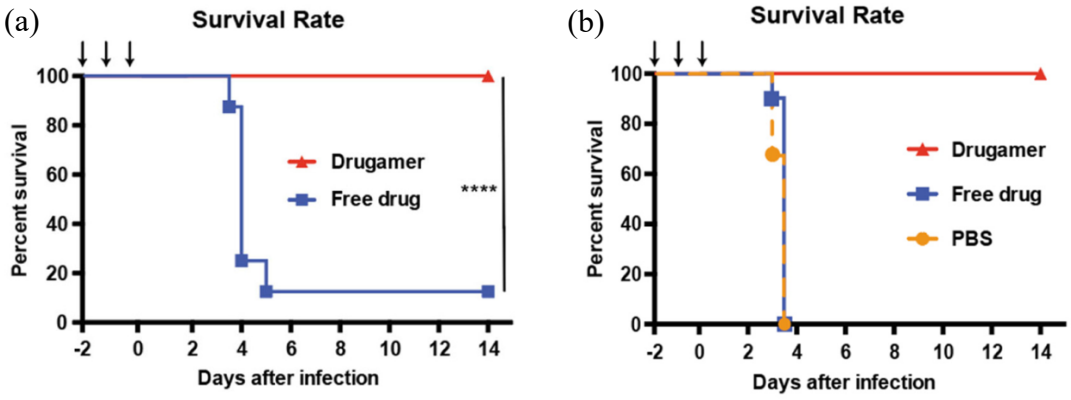

Figure 10: Pre-exposure prophylactic efficacy of ciprofloxacin drugamers against (a) model pathogen *B. thailandensis* (n = 8 for each treatment group) and (b) the human pathogen *B. pseudomallei* (n = 10 for each treatment group) demonstrated in a survival plot of mice [16].

1.8 Drugamers As A Drug Delivery Platform

This drugamer platform has also been adapted for the targeted liver delivery of antimalarial drugs 8-aminoquinolines (primaquine or tafenoquine) using N-Acetylgalactosamine (GalNAc) as

the targeting ligand [17]. Currently no challenge studies have been conducted and only pharmacokinetic *in vivo* studies have taken place. However, promising results were seen with delivery of 8-aminoquinolines to the liver with tuned pharmacokinetic properties. These results are novel as targeted liver release kinetics with 8-aminoquinolines have not been proven before.

1.9 Overall Objective

In some of the above-mentioned *in vitro* experiments, percent drug release, rather than drug concentration, was used to describe the pharmacokinetics. Percent drug release is defined by the ratio between the concentration of a drug in a given sample and the concentration of drug when all of the ciprofloxacin had been released from the drugamer (i.e., the concentration of drug at 100% release). Percent drug release is helpful in determining the amount of ciprofloxacin left on the polymer, which is significant as ciprofloxacin gets cleared from the body fairly quickly with a half-life of 3 to 4 hours [18].

Previously, two different methods have been used to define this 100% released from the drugamer. The first method uses acid hydrolysis to define 100% release and measures the concentration of free ciprofloxacin after a 48-hour incubation in 10% aqueous sulfuric acid [13]. The second method uses base hydrolysis to define 100% release and measures the concentration of free ciprofloxacin after a 24-hour incubation in 0.1 N sodium hydroxide [11]. This use of two widely different method to define 100% release prevents certain experiments from being directly compared. For instance, two similar experiments were conducted to characterize the release of ciprofloxacin from a phenyl ester linker drugamer when incubated with model human esterase BChE (see Figure 11a and 12a). The experiment that used acid hydrolysis to define percent release found that ~25% release of ciprofloxacin from the polymer with phenyl ester linker (poly(O95-co-CPM)) after 6 days incubation with 0.033 μg/mL BChE (see Figure 11b) [13]. On the other hand, the experiment that used base hydrolysis to define percent release found only ~15.1% release of ciprofloxacin from the polymer with phenyl ester linker (Man-co-CTM) after 14 days incubation with 0.5 μg/mL BChE (see Figure 12b) [11]. Comparing the ciprofloxacin release from the phenyl ester polymers, there is a ~10% greater release in the experiment using acid hydrolysis to define percent release despite the shorter time frame and lower concentration of BChE.

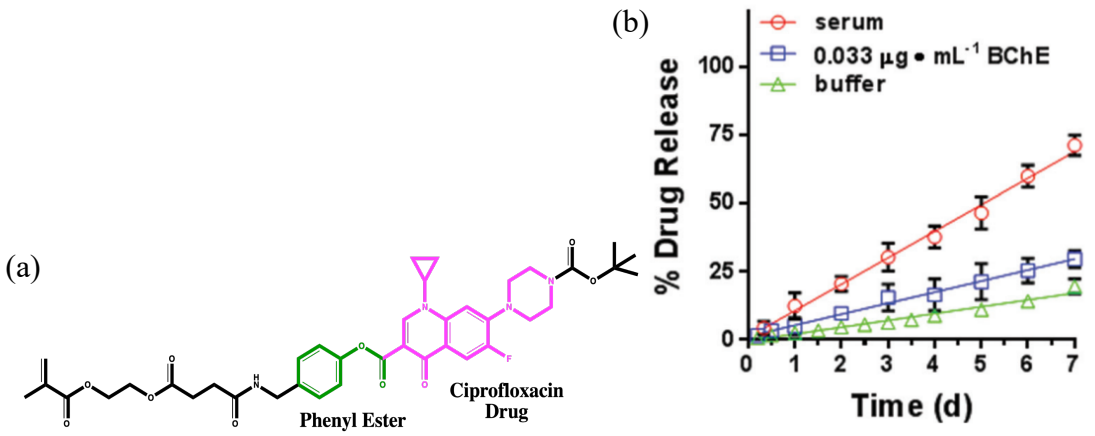

Figure 11: Monomer structure and release of poly(O95-co-CPM) [13]. (a) Structure of the ciprofloxacin monomer with phenyl ester linker used in the synthesis of poly(O95-co-CPM). (b) Release kinetics of poly(O95-co-CPM) when incubated in serum and with butyrylcholinesterase.

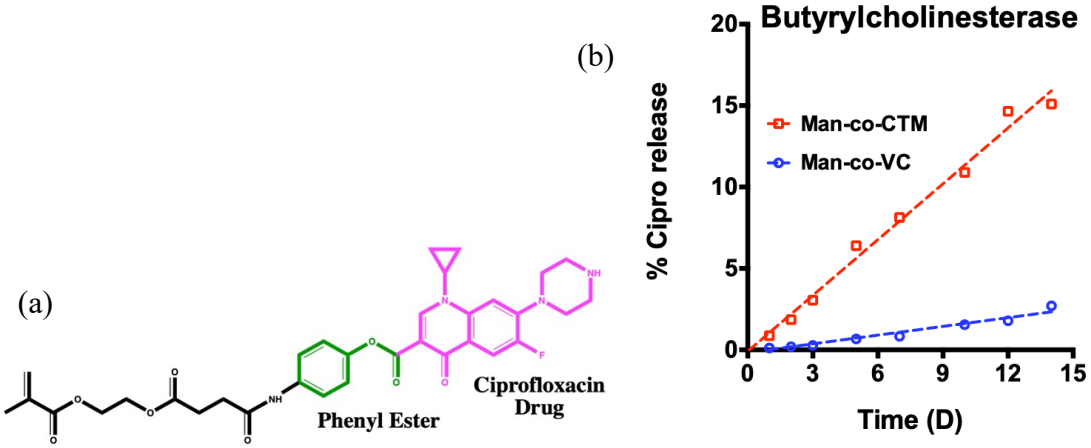

Figure 12: Monomer structure and release kinetics of Man-co-CTM [11]. (a) Structure of the ciprofloxacin monomer with phenyl ester linker used in the synthesis of Man-co-CTM. (b) Release kinetics of polymer with phenyl ester linker (Man-co-CTM) and polymer with protease-cleavable linker (Man-co-VC) when incubated with butyrylcholinesterase.

This brings forward important questions about the ability of acid and base hydrolysis to completely release the drug from the polymer, as well as questions on the stability of the polymer in acidic and basic environments. Furthering our understanding of the stability and release of free and polymeric ciprofloxacin in acidic and basic environments is therefore important for future pharmacokinetics characterization of the drugamer. Therefore, the overall aim of my research was to validate these methods of quantification to ensure robust methodology and allow for interoperability. The long-term stability and release of free and polymeric ciprofloxacin will also be investigated for further characterization of the ciprofloxacin drugamer.

Chapter 2. HPLC METHOD DEVELOPMENT

2.1 INTRODUCTION

High performance liquid chromatography (HPLC) is system used to separate and quantify components in a liquid sample. HPLC works by passing a liquid solvent (i.e., mobile phase) through a solid column (i.e., solid or stationary phase) at high pressure. There are two main types of HPLC: normal phase and reverse phase. Normal phase HPLC uses a polar solid phase and a non-polar mobile phase. On the other hand, reverse phase HPLC uses a non-polar solid phase and a polar mobile phase. The components of the liquid sample are eluted at distinct and separate times based on their interaction with the stationary phase. Gradient elution, a steady change in mobile phase overtime, can be used to further define difference in elution times between components of interest in the sample.

HPLC was selected as the technique for quantification of free and polymeric ciprofloxacin because its ability to separate components, allowing us to clearly differentiate between free and polymeric ciprofloxacin. A previous lab member had developed a HPLC gradient elution method for ciprofloxacin (see Table 1). This method was developed on the Agilent 1200 HPLC in MolES at the University of Washington. Analysis on this instrument was performed with a Zorbax RX-C18 analytical 4.6 x 150 mm 5-micron column. This chapter will focus on the further optimization of the initial HPLC gradient elution method for ciprofloxacin on a different HPLC instrument, as well as the development of the standard curve and determining the linear range of free and polymeric ciprofloxacin using the optimized method.

Table 1: HPLC gradient elution method for free ciprofloxacin developed by a prior lab member. Mobile phase A is 2% aqueous acetic acid and mobile phase B is 100% acetonitrile.

Time (minute)	Flow Rate (mL/min)	A (%)	B (%)	Max Pressure (bar)
0.00	0	98	2	400
0.01	1.0	98	2	400
15.00	1.0	0	100	400
25.00	1.0	0	100	400
30.00	1.0	98	2	400
37.00	1.0	98	2	400
37.01	0	98	2	400

2.2 Materials and Method

The HPLC method developed by the previous lab member for free ciprofloxacin was used as a starting point for the development of the final optimized HPLC method. This initial method was adapted for the slower flow rate and mobile phases that were standard at the Bagley Mass Spectrometry Facility. Furthermore, the method was adapted for a shorter column and to have a shorter runtime. Finally, the method was changed to move the elution time of the free ciprofloxacin farther way from the solvent front.

The finalized HPLC method was run on the Bruker Esquire Ion Trap LCMS in the Mass Spectrometry Facility in Bagley Hall (Department of Chemistry, University of Washington). The LC portion of the instrument is a Hewlett Packard (HP) 1100 Series HPLC system. This analysis was performed with a Zorbax SB-C18 narrow bore RR 2.1 x 100 nm 3.5-micron column. The mobile phase A was 5% acetonitrile and 1% acetic acid, aqueous. The mobile phase B was 99% acetonitrile and 1% acetic acid. UV absorption was used to quantify the standards and samples. The utilized signal wavelength was 274 nm with a reference wavelength of 380 nm. This method also has a post-time of 1 minute to allow the column to re-equilibrate and a standard injection volume of 10 μL was used in the analysis.

The final optimized method was used to create a standard curve and to determine the linear range for free ciprofloxacin. Free ciprofloxacin was dissolved in HPLC grade water to create standards with concentrations ranging from 0.39 to 200 μg/mL. The standards were then run on the HPLC instrument using the final method. The concentration and corresponding area under the curve (AUC) were then plotted to create the standard curve, as well as to determine the linear range and the limit of detection (LOD).

The final optimized method was also used to create a standard curve for the VC-mannose ciprofloxacin drugamer, as well as to determine the LOD. Polymeric ciprofloxacin was dissolved in HPLC grade water to create standards with concentrations ranging from 0.78 to 100 μg/mL. The standards were then run on the HPLC instrument from lowest to highest concentration using

the final method. The concentration and corresponding area under the curve (AUC) were then plotted to create the standard curve.

2.3 Results and Discussion

The final optimized ciprofloxacin HPLC gradient elution method, named NLPCIP11[2], is described in Table 2. Method NLPCIP11 provided sufficient separation between free and polymeric ciprofloxacin, symmetrical peaks, and an elution time distinct from the solvent front (see Figure 13).

Table 2: Method NLCIP11 – the final optimized ciprofloxacin HPLC gradient elution method. Mobile phase A is 5% acetonitrile and 1% acetic acid, aqueous, and mobile phase B is 99% acetonitrile and 1% acetic acid. Post time of 1 minute. UV absorbance: signal wavelength of 274 nm with a reference wavelength of 380 nm.

Time (minute)	Flow Rate (mL/min)	A (%)	B (%)	Max Pressure (bar)
0	0.2	100	0	400
12	0.2	48	52	400
14	0.2	0	100	400
16	0.2	0	100	400
19	0.2	100	0	400
21	0.2	100	0	400

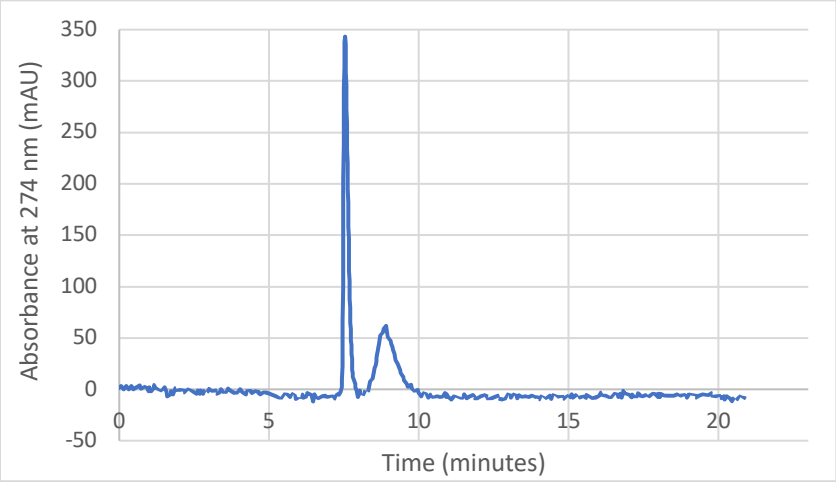

Figure 13: Chromatograms for aqueous free and polymeric ciprofloxacin standard using method NLCIP11. The concentration was 25 μg/mL for both free and polymeric ciprofloxacin. The sharp and stout peak correspond to free ciprofloxacin and polymeric ciprofloxacin, respectively.

[2] On the Bagely instrument, once mass spectrometry capabilities are enabled on a LC method they cannot be disabled. Therefore, there are two methods are saved on the computer. The LC method with mass spectrometry enabled is saved as NLPCIP11.M and same LC method with mass spectrometry disabled is saved as NLPCIP99.M.

Based on the free ciprofloxacin standards with concentrations ranging from 0.39 to 200 μg/mL, the LOD is 0.78 μg/mL and the linear range is from 0.78 to 100 μg/mL. This was determined based on the signal-to-noise ratio for the 0.39 µg/mL absorption signal (Figure 14a, b) and the asymmetrical peak shape for the 200 µg/mL absorption signal, indicating the saturation of the column (Figure 14c, d). A standard curve was created with standards with concentrations ranging 0.78 to 100 µg/mL (Figure 15). The resulting R^2 value was 0.9997 and the equation was $y = 179.18\,x + 42.815$, where y is the AUC and x is the concentration of ciprofloxacin in µg/mL.

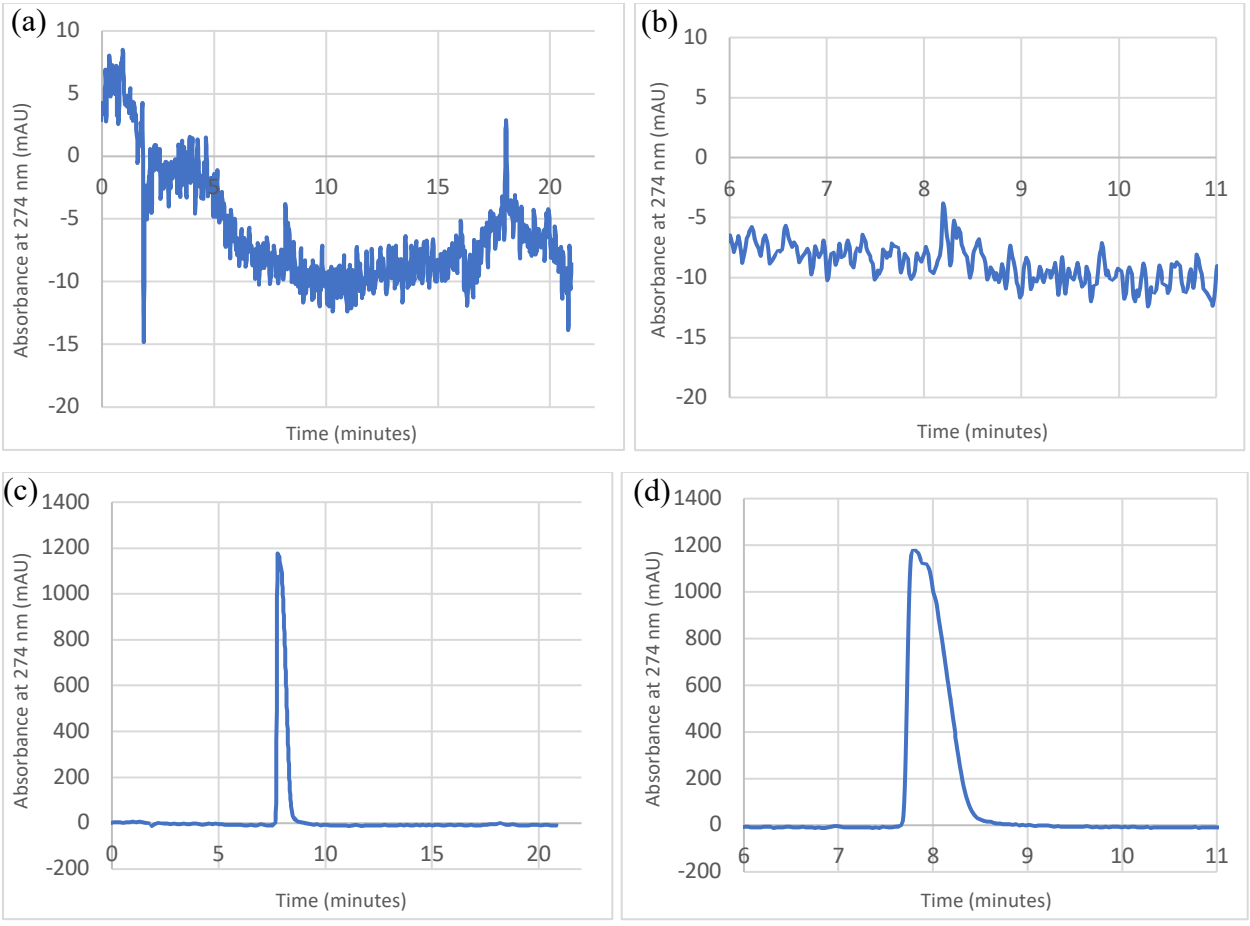

Figure 14: Chromatograms for aqueous free ciprofloxacin to determine LOD and linear range. (a) Full HPLC trace of 0.39 µg/mL free ciprofloxacin standard, and (b) HPLC trace of 0.39 µg/mL free ciprofloxacin standard from 6 to 11 minutes. (c) Full HPLC trace of 200 µg/mL free ciprofloxacin standard, and (d) HPLC trace of 200 µg/mL free ciprofloxacin standard from 6 to 11 minutes. (a, b) shows a low signal-to-noise ratio indicating that the concentration of 0.39 µg/mL is below the limit of detection. (c, d) shows an asymmetrical peak shape indicating that a concentration of 200 µg/mL saturates the column and will be outside of the linear range.

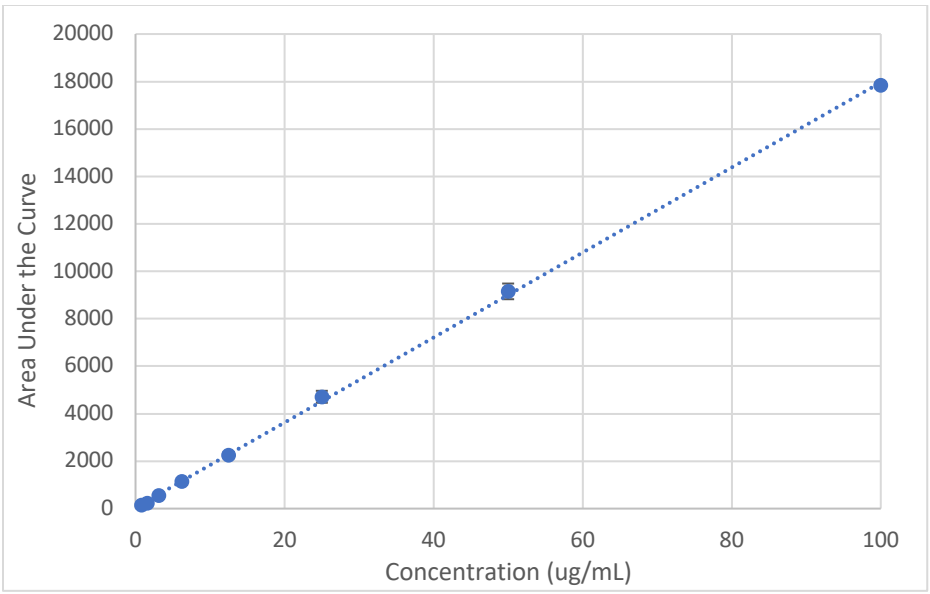

Figure 15: Standard curve for free ciprofloxacin analyzed using method NLPCIP11. Aqueous free ciprofloxacin standards' concentration ranges from 0.78 to 100 µg/mL (n = 3). AUC is the integrated area from the UV absorbance at 274 nm. Error bars represent standard deviation.

Based on the polymeric ciprofloxacin standards with concentrations ranging from 0.78 to 100 µg/mL, the LOD is 3.125 µg/mL. This was determined based on the signal-to-noise ratio for the 0.78 and 1.56 µg/mL absorption signal (Figure 16). A standard curve for polymeric ciprofloxacin was created with concentration 3.125 to 100 µg/mL (see Figure 17). The resulting R^2 value was 0.9988 and the equation was $y = 146.39\,x + 107.42$, where y is the AUC and x is the concentration of polymeric ciprofloxacin in µg/mL.

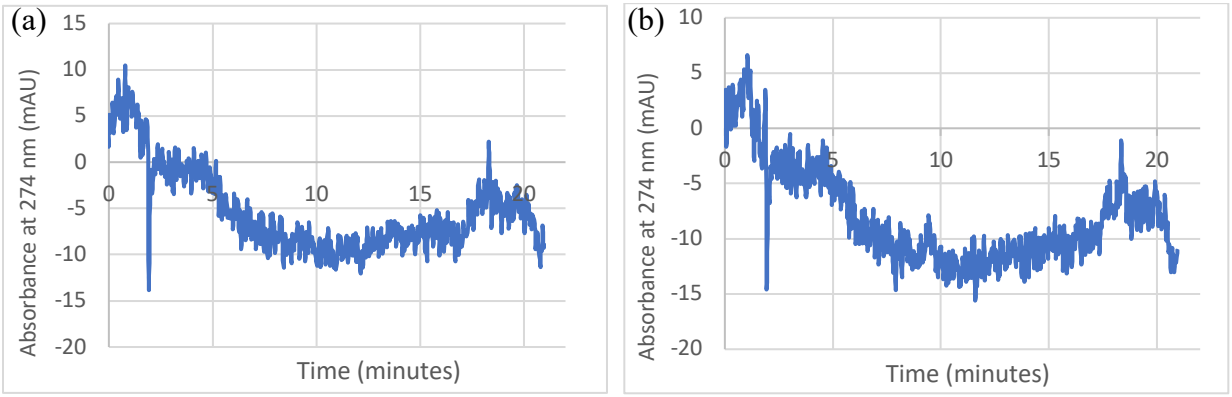

Figure 16: Chromatograms for aqueous polymeric ciprofloxacin for determining LOD. (a) Full HPLC trace of 0.78 µg/mL free ciprofloxacin standard and (b) full HPLC trace of 1.56 µg/mL free ciprofloxacin standard. Both (a, b) show a low signal-to-noise ratio indicating that the concentration of 0.78 and 1.56 µg/mL is below the limit of detection.

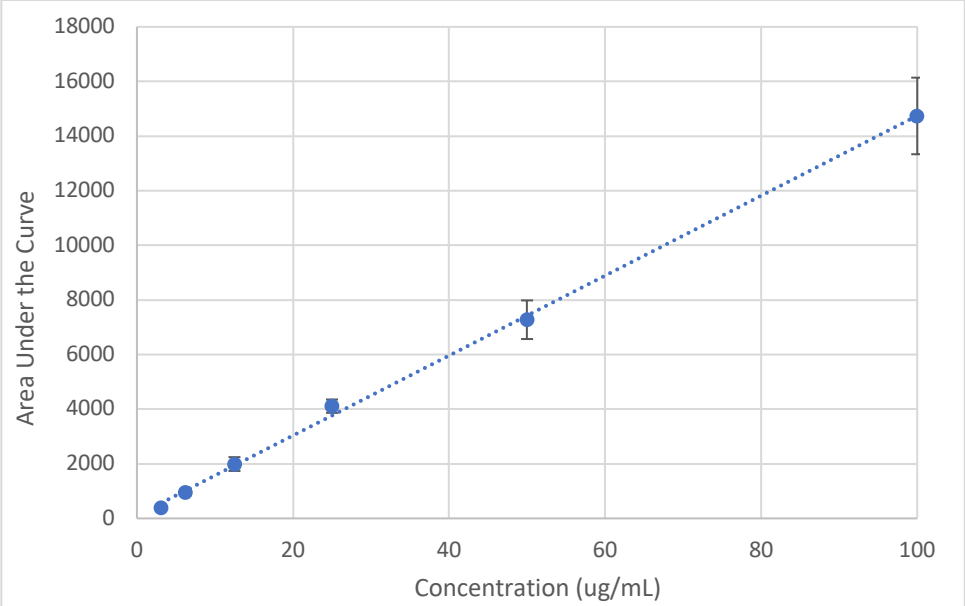

Figure 17: Standard curve for VC-mannose ciprofloxacin polymer analyzed using method NLPCIP11. Aqueous polymeric ciprofloxacin standards' concentration ranges from 3.125 to 100 µg/mL (n = 3). AUC is the integrated area from the UV absorbance at 274 nm. Error bars represent standard deviation.

Chapter 3. DRUGAMER CARRY OVER ANALYSIS

3.1 Introduction

When creating the polymeric ciprofloxacin standard curve (see Chapter 2), free ciprofloxacin standards were also run in the same sequence as the polymeric ciprofloxacin. Free ciprofloxacin standards run immediately after polymeric ciprofloxacin standards showed two peaks (see Figure 18). This suggest that there was "carry over" drugamer signal. This observed carry over could help to explain the relatively large standard deviation seen in the standard curve for polymeric ciprofloxacin (see Figure 17) compared to standard curve for free ciprofloxacin (see Figure 15).

3.2 Materials and Methods

First, the identity of the carry over peak (i.e., the second peak seen in Figure 18) was confirmed by looking at the full UV absorption spectrum. This was done by running a blank sample (HPLC water) immediately after a polymeric ciprofloxacin standard using the NLPCIP11 method. This was performed to confirm that the contaminant was indeed the VC-mannose drugamer and not another molecule.

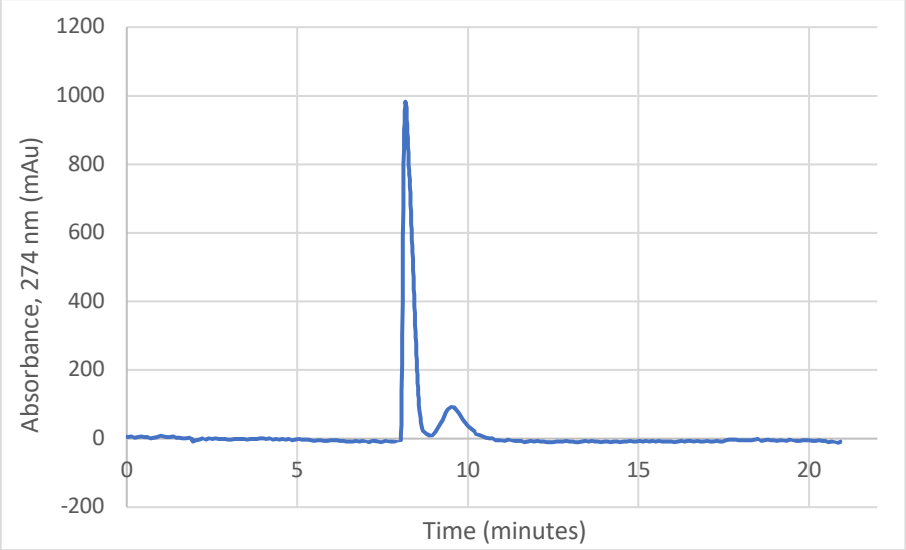

Figure 18: Free ciprofloxacin standard of 100 µg/mL concentration run immediate after a polymeric ciprofloxacin standard of 100 µg/mL concentration. The first peak at roughly 8 minutes corresponds to the elution time of free ciprofloxacin and the second peak at roughly 9.5 minutes corresponds to the elution time of polymeric ciprofloxacin.

Our initial hypothesis was that source of contamination was the injection needle. In order to test this hypothesis, we created another HPLC method (NLPCIP12) with the same gradient as NLPCIP11 but that washed the exterior of the needle after each injection and a post-time of 2 minutes. The needle was washed in a solution of water, acetonitrile, methanol, and isopropanol (1:1:1:1).

Next, we wanted a way to run the HPLC method immediately after a polymeric ciprofloxacin standard without using the injection needle. Therefore, we created a method called NLPCIP13 that was the NLPCIP11 back-to-back.

Lastly, we created a HPLC called NLPCIP14 with the same gradient as NLPCIP11 but directed the mobile phase and injected sample around the column (i.e., the mobile phase and sample do not pass through the column).

3.3 Results and Discussion

The UV absorption spectrum was used confirm the identity of the carry over peak. In the blank (HPLC grade water) run immediately after the polymeric ciprofloxacin standard, a signal was seen at the same elution time as the polymeric ciprofloxacin standard (see Figure 19). Furthermore, based on the 3D shape of the peak seen in the UV absorption spectrum, it was concluded that the carry over peak was polymeric ciprofloxacin. The AUC of the peak seen in the blank run was roughly 24% of the AUC seen in the polymeric ciprofloxacin standard.

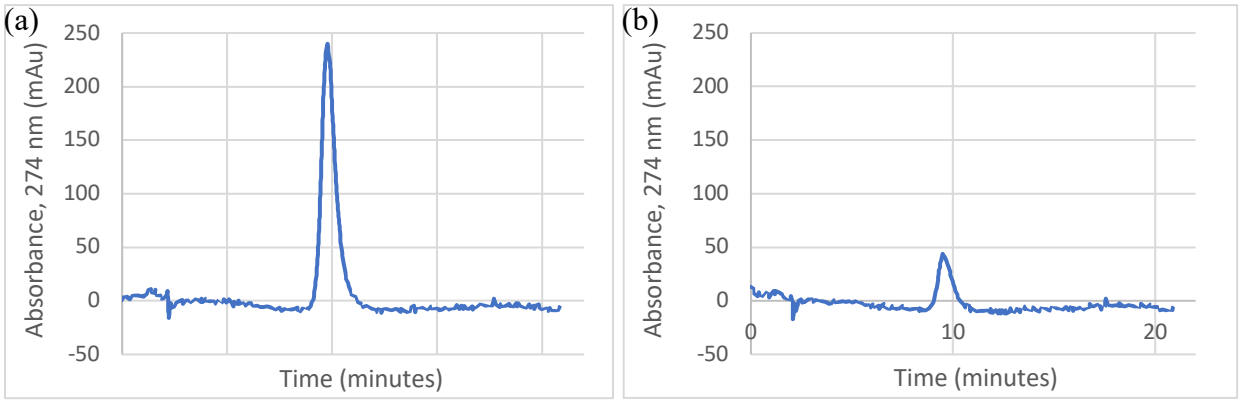

Figure 19: Chromatograms confirming the identity of the carry peak. Chromatograms for (a) aqueous polymeric ciprofloxacin of concentration 100 µg/mL and (b) subsequent blank sample of HPLC grade water run. Both samples were run with method NLPCIP11.

Using method NLPCIP12, which washes the exterior of the injection needle, a blank sample was run immediately after the polymeric ciprofloxacin standard and the carry over peak at the same elution time was observed (see Figure 20). The AUC of the peak seen in the blank run was roughly 27% of the AUC seen in the polymeric ciprofloxacin standard. This suggested that the source of the contamination was not the exterior of the injection needle.

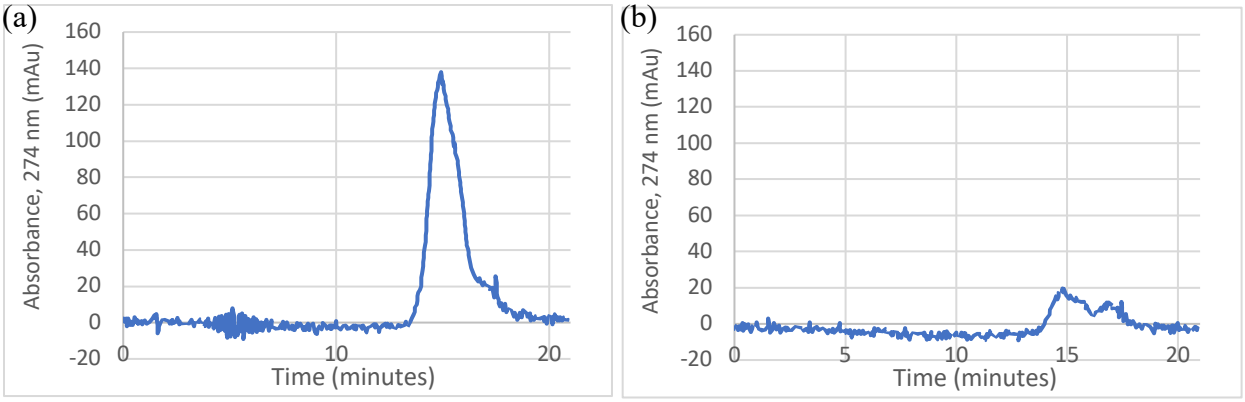

Figure 20: Chromatograms for aqueous polymeric ciprofloxacin run with method NLPCIP12. (a) Aqueous polymeric ciprofloxacin of concentration 100 µg/mL and (b) subsequent blank sample of HPLC grade water.

A polymeric ciprofloxacin standard was then run with method NLPCIP13, which is method NLPCIP11 back-to-back, and a carry over peak at the corresponding elution time was still observed (see Figure 21). The AUC of the second carry over peak was roughly 28% of the AUC seen in first original peak. This suggested that the source of the contamination was the column and not the injection needle.

To confirm the new hypothesis that the column was the source of the contamination, a blank sample was run immediately after a polymeric ciprofloxacin standard using method NLPCIP14, which directs the mobile phase and sample around the column. No carry over peak was seen in the blank run confirming that the column was the source of contamination (see Figure 22).

As a result of this investigation into the source of the carry over signal, future experiments that involved running polymeric ciprofloxacin on the HPLC were done such that the concentration of samples were lower than 25 µg/mL. Additionally, care was taken to include blank samples (HPLC water) at intervals in the LC sequence to both track and minimize carry over.

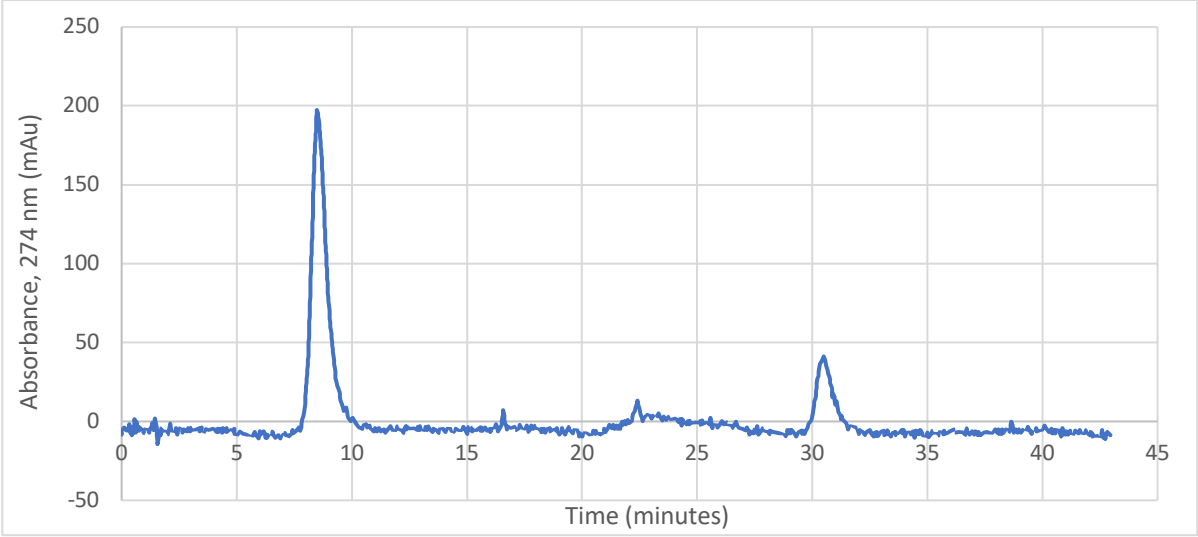

Figure 21: Chromatograms for aqueous polymeric ciprofloxacin of concentration 100 µg/mL run with method NLPCIP13.

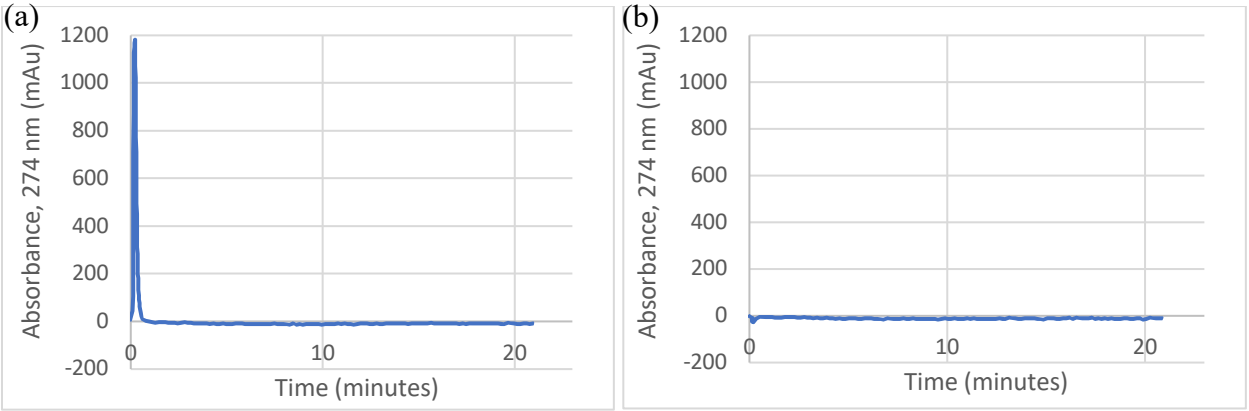

Figure 22: Chromatograms for aqueous polymeric ciprofloxacin were run with method NLPCIP14. (a) Aqueous polymeric ciprofloxacin of concentration 100 µg/mL and (b) subsequent blank sample of HPLC grade water.

Chapter 4. SAMPLE PROCESS ANALYSIS

4.1 INTRODUCTION

Subsequent experiments (described in Chapters 5-7) are performed in human serum. However, unprocessed human serum samples cannot be run on a HPLC instrument. Additionally, pharmacokinetic studies have determined that 20 to 30% of ciprofloxacin is protein bound when administered orally to healthy volunteers [18]. There is therefore a need to develop a sample process method to extract the ciprofloxacin from the human serum for accurate quantification.

There have been previously developed methods used by our lab and our collaborators to extract ciprofloxacin from human serum. One method diluted human serum samples with acetonitrile 5 fold [19]. The samples were centrifuged, and the supernatant was then concentrated 4 times by speedvac before being analyzed. Another method treats human serum samples with 50% acetonitrile (v/v) and centrifuges the samples for 15 minutes [20]. The supernatant was then filtered before being analyzed. A third method dilutes human serum samples 1:1 v/v with 2% aqueous acetic and acetonitrile (84:16). The solution was then diluted again 1:1 with acetonitrile. The samples were then briefly vortexed and then centrifuged for 15 minutes. The supernatant was then filtered before being analyzed. The percent recovery was not reported for these developed methods.

Additional methods of extracting ciprofloxacin have been developed by others outside of our lab. One group developed a methodology for extracting ciprofloxacin from human plasma [21]. 400 µL aliquots of plasma containing ciprofloxacin was diluted with 30 µL of internal standard working solution and one drop of 10 M phosphate buffer. The solution was vortexed before 500 µL of ice-cold acetonitrile was added. The tubes were vortexed again for 5 minutes and then centrifuged. The supernatant was transferred into silanised glass tubes and concentrated with a water bath at 50°C. The samples were resolubilized with 100 µL of the mobile phase (phosphate buffer and acetonitrile 77:23 v/v). The samples were vortexed for 3 minutes and then centrifuged. The supernatant was then run on HPLC for quantification. Using this method, the average percent recovery (n = 5) was 96.90% $\pm$ 1.16% when using silanised tubes and 86.10% $\pm$ 3.59% when using non-silanised tubes.

4.2 Materials And Methods

4.2.1 Initial Sample Process Method

Figure 23: Picture of Frankenstein, the lab made nitrogen drier.

The initial sample process method was based on a method developed by a prior lab member to extract ciprofloxacin from human serum (described above) [19]. To determine the percent recovery of this method, six separate solutions were prepared (see Table 3). Free ciprofloxacin, polymeric ciprofloxacin, or mixed (free and polymeric ciprofloxacin) standard (50 µg/mL, aqueous) was added to either water or human serum (Sigma S7023) (1:1, v/v). 200 µL aliquots of each solution was treated with 800 µL of acetonitrile to promote serum proteins precipitation in human serum samples and to maintain identical sample process conditions for the water samples. Each tube was then briefly vortexed (VWR, Analog Vortex Mixer) and then centrifuged (Beckman Coulter, Microfuge 22R Centrifuge) for 5 minutes at 14,000 rpm. 800 µL of the supernatant was then completely dried down with our lab made nitrogen drier, named "Frankenstein" (see Figure 23). A nitrogen drier was used to concentrate the samples rather than a speedvac (as done by Ida) because previous experiments performed (not shown here) suggested that the speedvac was baking or otherwise contaminating our samples. The dried down samples were then resolubilized in 200 µL of water and briefly vortexed. All samples were then filtered (Millex-GV filter, 0.22 µm pore size) with a syringe (Fisher Scientific, 1 mL plastic sterile

syringe). The resolubilized samples were then transferred into glass LCMS vials (Agilent Technologies, 2 mL vial) with glass inserts (Agilent Technologies, 250 µL pulled point-conical glass inserts) and caps (Agilent Technologies, 9 mm blue screw). The samples were then run with the optimized LC method (NLPCIP11) along with free ciprofloxacin standards ranging from 3.125 µg/mL to 25 µg/mL in concentration. The expected concentration of free and/or polymeric ciprofloxacin samples was 20 µg/mL assuming 100% recovery.

Table 3: Conditions used to determine percent recovery of initial sample process method.

	Matrix	Ciprofloxacin
Condition 1	Water	Free
Condition 2	Water	Mixed: Free and Polymeric
Condition 3	Water	Polymeric
Condition 4	Human serum	Free
Condition 5	Human serum	Mixed: Free and Polymeric
Condition 6	Human serum	Polymeric

4.2.2 Re-solubilization in Acetonitrile

This experiment was performed in an attempt to determine the reason for the lack of polymer signal in the processed samples using the method described in Chapter 4.2.1. Due to the ciprofloxacin's solubility in acetonitrile, we hypothesized that the signal may reappear if the samples were re-solubilized in acetonitrile rather than water. To test this hypothesis, 4 different conditions were tested (see Table 4). Samples under condition 1 were not processed and polymeric ciprofloxacin standards were simply diluted with water to have the same final concentration as the processed samples. For samples under condition 2-4, aqueous polymer standards of concentration 25 µg/mL were prepared. 200 µL aliquots from these samples were then treated with 800 µL of acetonitrile. Each tube was then briefly vortexed (VWR, Analog Vortex Mixer) and then centrifuged (Beckman Coulter, Microfuge 22R Centrifuge) for 5 minutes at 14,000 rpm. 800 µL of the supernatant was then completely dried down with Frankenstein. Samples were resolubilized in 200 µL of water, acetonitrile and water solution (1:1, v/v), or acetonitrile for conditions 2, 3, and 4, respectively. The samples were briefly vortexed and then transferred into glass LCMS vials (Agilent Technologies, 2 mL vial) with glass inserts (Agilent Technologies, 250 µL pulled point-conical glass inserts) and caps (Agilent Technologies, 9 mm

blue screw). The samples were then run with the optimized LC method (NLPCIP11) along with free ciprofloxacin standards ranging from 3.125 µg/mL to 25 µg/mL in concentration. The expected concentration of polymeric ciprofloxacin samples was 20 µg/mL assuming 100% recovery.

Table 4: Conditions used to test effect of different resolubilizing solutions on percent recovery.

	Was the sample processed?	Resolubilized in
Condition 1	No	N/A
Condition 2	Yes	Water
Condition 3	Yes	Acetonitrile, water (1:1, v/v)
Condition 4	Yes	Acetonitrile

4.2.3 Drying Samples in Different Material Tubes

This experiment was performed in an attempt to determine the reason for the lack of polymer signal in the processed samples using the method described in Chapter 4.2.1 and 4.2.2. We hypothesized that the plastic tubes that were being used may be the cause of the lack of polymer signal. This is because potentially the acetonitrile treatment step was causing the loosening and expanding of the plastic polymers and leading to the absorption of the polymer into the tube. To test this hypothesis, 4 different conditions were tested (see Table 5). Samples under condition 1 were not processed and polymeric ciprofloxacin standards were simply diluted with water to have the same final concentration as the processed samples under condition 2, 3, and 4. For conditions 2, 3, and 4, aqueous polymer standards of concentration 25 µg/mL were prepared. 200 µL aliquots from these samples were placed in 2 mL plastic Eppendorf tubes, 1.5 mL plastic Eppendorf tubes, or 0.5 dram glass vials for condition 2, 3 and 4 respectively. These samples were then treated with 800 µL of acetonitrile and each tube was then briefly vortexed (VWR, Analog Vortex Mixer). None of the samples were centrifuged due to the fact that the glass vials could not be centrifuged and to maintain identical sample process methodology. 800 µL of the pseudo-supernatant was then transferred to new tubes and completely dried down with Frankenstein. Samples were resolubilized in 200 µL of water, briefly vortexed, and then transferred into glass LCMS vials (Agilent Technologies, 2 mL vial) with glass inserts (Agilent Technologies, 250 µL pulled point-conical glass inserts) and caps (Agilent Technologies, 9 mm blue screw). The samples were then run with the optimized LC method (NLPCIP11) along with

free ciprofloxacin standards ranging from 3.125 μg/mL to 25 μg/mL in concentration. The expected concentration of polymeric ciprofloxacin samples was 20 μg/mL assuming 100% recovery.

Table 5: Conditions used to test effect of different tube materials on percent recovery.

	Was the sample processed?	Container
Condition 1	No	Plastic 2 mL tube
Condition 2	Yes	Plastic 2 mL tube
Condition 3	Yes	Plastic 1.5 mL tube
Condition 4	Yes	Glass 0.5 dram vial

4.2.4 Percent Recovery in Water

Now focusing on free ciprofloxacin rather than the polymeric ciprofloxacin, we worked to re-evaluate the percent recovery of free ciprofloxacin from water. To test this hypothesis, 4 different conditions were tested (see Table 6). Samples under condition 1 and 3 were not processed and ciprofloxacin standards (free or mixed) were simply diluted with water to have the same final concentration as the processed samples under condition 2, and 4. For condition 2, aqueous 25 μg/mL free ciprofloxacin standards were prepared. For condition 4, a standard with aqueous 25 μg/mL of both free and polymeric ciprofloxacin were prepared. 200 μL aliquots from these samples were then treated with 800 μL of acetonitrile. Each tube was then briefly vortexed (VWR, Analog Vortex Mixer) and then centrifuged (Beckman Coulter, Microfuge 22R Centrifuge) for 5 minutes at 14,000 rpm. 800 μL of the supernatant was then completely dried down with Frankenstein. Samples were resolubilized in 200 μL of water, briefly vortexed, and then transferred into glass LCMS vials (Agilent Technologies, 2 mL vial) with glass inserts (Agilent Technologies, 250 μL pulled point-conical glass inserts) and caps (Agilent Technologies, 9 mm blue screw). The samples were then run with the optimized LC method (NLPCIP11) along with free ciprofloxacin standards ranging from 3.125 μg/mL to 25 μg/mL in concentration. The expected concentration of polymeric ciprofloxacin samples was 20 μg/mL assuming 100% recovery.

Table 6: Conditions used determine percent recovery of ciprofloxacin in water using a method in development.

	Was the sample processed?	Ciprofloxacin
Condition 1	No	Free
Condition 2	Yes	Free
Condition 3	No	Mixed: Free and Polymeric
Condition 4	Yes	Mixed: Free and Polymeric

4.2.5 New Nitrogen Drier

We hypothesized that lower than expected percent recovery when using the methodology described in Chapter 4.2.4 was due to Frankenstein as we occasionally observed some of the sample leaving the tube during the sample drying process due to the difficulty in controlling the pressure. We therefore repeated the experiment described in Chapter 4.2.4 using a new nitrogen dryer (Stuart, Block Heater and Techne, Sample Concentrator) named "Glinda" (see Figure 24).

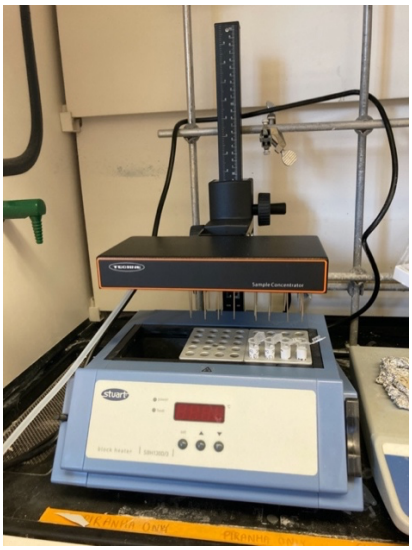

Figure 24: Picture of Glinda, the new nitrogen drier.

4.2.6 Volume and Vortex Time During Re-solubilization

We hypothesized that the percent recovery in water would be increased further by increasing the re-solubilization volume. We also hypothesize that increasing the time the dried down samples were being vortex during the re-solubilization could also increase the precent recovery. To test these hypothesizes, 5 different conditions were tested (see Table 7). Samples under condition 1 were not processed and mixed (free and polymeric) ciprofloxacin were simply diluted with water to have the same final concentration as the processed samples under condition 2 through 5. For

condition 2 through 5, aqueous mixed (free and polymeric) standards of concentration 25 µg/mL were prepared. The mixed standard contained 25 µg/mL of both free and polymeric ciprofloxacin. 200 µL aliquots from these samples were then treated with 800 µL of acetonitrile. Each tube was then briefly vortexed (VWR, Analog Vortex Mixer) and then centrifuged (Beckman Coulter, Microfuge 22R Centrifuge) for 5 minutes at 14,000 rpm. 800 µL of the supernatant was then completely dried down with Glinda. Samples were then resolubilized in 300 µL of water (rather than 200 µL) and vortexed for 30 seconds, 1 minute, 5 minutes, or 20 minutes, for conditions 2, 3, 4, and 5 receptively. Samples were transferred into glass LCMS vials (Agilent Technologies, 2 mL vial) with glass inserts (Agilent Technologies, 250 µL pulled point-conical glass inserts) and caps (Agilent Technologies, 9 mm blue screw). The samples were then run with the optimized LC method (NLPCIP11) along with free ciprofloxacin standards ranging from 3.125 µg/mL to 25 µg/mL in concentration. The expected concentration of polymeric ciprofloxacin samples was 13.33 µg/mL assuming 100% recovery.

Table 7: Conditions used determine percent recovery of ciprofloxacin in water using a method in development.

	Was the sample processed?	Vortex Time
Condition 1	No	N/A
Condition 2	Yes	30 seconds
Condition 3	Yes	1 minute
Condition 4	Yes	5 minutes
Condition 5	Yes	20 minutes

4.2.7 Effect of PBS on Polymer Peak

Experiment described in subsequent chapters diluted the aqueous polymeric ciprofloxacin standards in PBS and human serum incubated multiple days (see Chapter 5 and 7). It was observed that in these samples, there were two peaks. One of the peaks corresponded to free ciprofloxacin and was identified due to the same elution time as the free ciprofloxacin standards. The second peak was thought to be polymer due to the elution time. However, the peak shape was sharp, and was not broad and stout as seen previously (see Figure 13). Another interesting observation was that in the experiments incubating polymeric ciprofloxacin in PBS and human serum for 60 days, the second peak only appear after 24 hours of incubation. In the experiments incubating polymeric ciprofloxacin in PBS and human serum for 2 days in acidic and basic

conditions, the second peak was seen from day 0. To confirm that this second peak did correspond to the polymer, we tested 10 different conditions (see Table 8). Aqueous 37 μg/mL free and polymeric ciprofloxacin standards were prepared. The standards were then diluted 1:1 (v/v) with water or PBS at concentrations ranging from 0.25x to 5x. This range of concentration were chosen because the final concentration of PBS in the acid and base experiments (see Chapter 6 and 7) was 0.25x. We therefore wanted to test concentration of PBS that were less than and significantly greater than 0.25x. Timed aliquots were taken at day 0 and 1 and stored at -80°C. Once all aliquots are taken, the samples were defrosted and transferred into glass LCMS vials (Agilent Technologies, 2 mL vial) with glass inserts (Agilent Technologies, 250 μL pulled point-conical glass inserts) and caps (Agilent Technologies, 9 mm blue screw). The samples were then run with the optimized LC method (NLPCIP11) along with free ciprofloxacin standards ranging from 3.125 μg/mL to 25 μg/mL in concentration. The expected concentration of free and polymeric ciprofloxacin samples was 17 μg/mL assuming 100% recovery.

Table 8: Conditions used determine the effect of PBS on the free and polymeric ciprofloxacin peaks.

	Buffer	Ciprofloxacin
Condition 1	5x PBS	Free
Condition 2	1x PBS	Free
Condition 3	0.5x PBS	Free
Condition 4	0.25x PBS	Free
Condition 5	water	Free
Condition 6	5x PBS	Polymeric
Condition 7	1x PBS	Polymeric
Condition 8	0.5x PBS	Polymeric
Condition 9	0.25x PBS	Polymeric
Condition 10	water	Polymeric

4.2.7 Percent Recovery in Human Serum

The method optimized using aqueous ciprofloxacin was then used on ciprofloxacin in human serum to determine the percent recovery in human serum. First, aqueous 50 μg/mL free ciprofloxacin standards were prepared and diluted 1:1 (v/v) with human serum (Sigma S7023). 200 μL aliquots from the human serum solutions were then treated with 800 μL of acetonitrile.

Each tube was then briefly vortexed (VWR, Analog Vortex Mixer) and then centrifuged (Beckman Coulter, Microfuge 22R Centrifuge) for 5 minutes at 14,000 rpm. 800 µL of the supernatant was then completely dried down with Glinda (Stuart, Block Heater and Techne, Sample Concentrator). Samples were resolubilized in 200 µL of water, briefly vortexed, and then transferred into glass LCMS vials (Agilent Technologies, 2 mL vial) with glass inserts (Agilent Technologies, 250 µL pulled point-conical glass inserts) and caps (Agilent Technologies, 9 mm blue screw). The samples were then run with the optimized LC method (NLPCIP11) along with free ciprofloxacin standards ranging from 3.125 µg/mL to 25 µg/mL in concentration. The expected concentration of polymeric ciprofloxacin samples was 13.33 µg/mL assuming 100% recovery.

4.2.8 Effect of Acetonitrile Ratio and Vortex Time During Protein Precipitation on Percent Recovery, Effect of Acid and Base on Percent Recovery

While a satisfactory percent recovery was achieved with the methodology described in Chapter 4.2.7, we hypothesized the percent recovery could be increased by making the sample process method more favorable for the ciprofloxacin to become disassociated from the serum proteins. This was hypothesized because of the previously mentioned pharmacokinetics property of 20 to 30% protein binding when ciprofloxacin is administered orally [18]. To test this hypothesis, 5 different conditions were tested (see Table 9). First, aqueous 200 µg/mL free ciprofloxacin standards were prepared and diluted 1:1 (v/v) with human serum (Sigma S7023). For conditions 1-3, the samples were then further diluted 1:1 (v/v) with water. For conditions 4 and 5, the samples were diluted 1:1 (v/v) with 10% sulfuric acid or 0.1 N sodium hydroxide, respectively. The effect of acid and base on percent recovery was tested because we predicted that the acid and base would denature or otherwise alter the structure of the serum protein and therefore increase the disassociated of ciprofloxacin from the serum proteins. 127 µL aliquots from the human serum solutions were taken. For conditions 1-3, 73 µL of water was added to the aliquots. For conditions 4 and 5, the aliquots were neutralized with 73 µL of 0.53% sulfuric acid or 2 M sodium hydroxide, respectively. Next, for conditions 1, 3, 4, and 5, 800 µL of acetonitrile was added. For condition 2, 200 µL of acetonitrile was added. This changes the ratio of human serum sample to acetonitrile from 1:4 to 1:1 (v/v). This was tested because of the ratio of human serum to acetonitrile described in the methods described in Chapter 4.1. The samples were then

vortexed. For conditions 1, 2, 4, and 5, the aliquots were vortexed for 10 seconds. For condition 3, the samples were vortexed for 10 minutes. This was tested thinking that the longer vortex time with acetonitrile would increase the disassociation of ciprofloxacin from the serum proteins. All of the samples were then centrifuged (Beckman Coulter, Microfuge 22R Centrifuge) for 5 minutes at 14,000 rpm. For conditions 1, 3, 4, and 5, 800 µL of the supernatant was transferred to a new tube. For condition 2, 200 µL of the supernatant was transferred to a new tube. The samples were then completely dried down with Glinda (Stuart, Block Heater and Techne, Sample Concentrator). All samples were resolubilized in 300 µL of water, briefly vortexed, and then transferred into glass LCMS vials (Agilent Technologies, 2 mL vial) with glass inserts (Agilent Technologies, 250 µL pulled point-conical glass inserts) and caps (Agilent Technologies, 9 mm blue screw). The samples were then run with the optimized LC method (NLPCIP11) along with free ciprofloxacin standards ranging from 3.125 µg/mL to 25 µg/mL in concentration. For conditions 1, 3, 4, and 5, the expected concentration of polymeric ciprofloxacin samples was 16.93 µg/mL assuming 100% recovery. For conditions 2, the expected concentration of polymeric ciprofloxacin samples was 10.58 µg/mL assuming 100% recovery.

Table 9: Conditions used determine the effect acid, base, acetonitrile ratio, and acetonitrile vortex time on percent recovery of free ciprofloxacin in human serum.

	Acid or Base?	Acetonitrile Ratio	Vortex Time
Condition 1	None	1:4	10 seconds
Condition 2	None	1:1	10 seconds
Condition 3	None	1:4	10 minutes
Condition 4	Acid	1:4	10 seconds
Condition 5	Base	1:4	10 seconds

4.3 Results And Discussion

4.3.1 Initial Sample Process Method

The initial sample process method led to lower-than-expected average percent recovery of free ciprofloxacin (see Figure 25). For the free ciprofloxacin diluted in water, the average percent recovery was 23.8% ± 33.1%. For free ciprofloxacin diluted in human serum, the average percent recovery was 51.5% ± 4.5%. For free and polymeric ciprofloxacin diluted in water, the

average percent recovery was 60.7% ± 12.9%. For free and polymeric ciprofloxacin diluted in human serum, the average percent recovery was 49.2% ± 19.3%. The average precent recovery (mean ± standard deviation) was determined based on the theoretical concentration of 20 µg/mL given 100% recovery. No released ciprofloxacin was observed from the polymeric ciprofloxacin samples. These results demonstrate the high variability (high standard deviation) and low percent recovery of the initial sample process method.

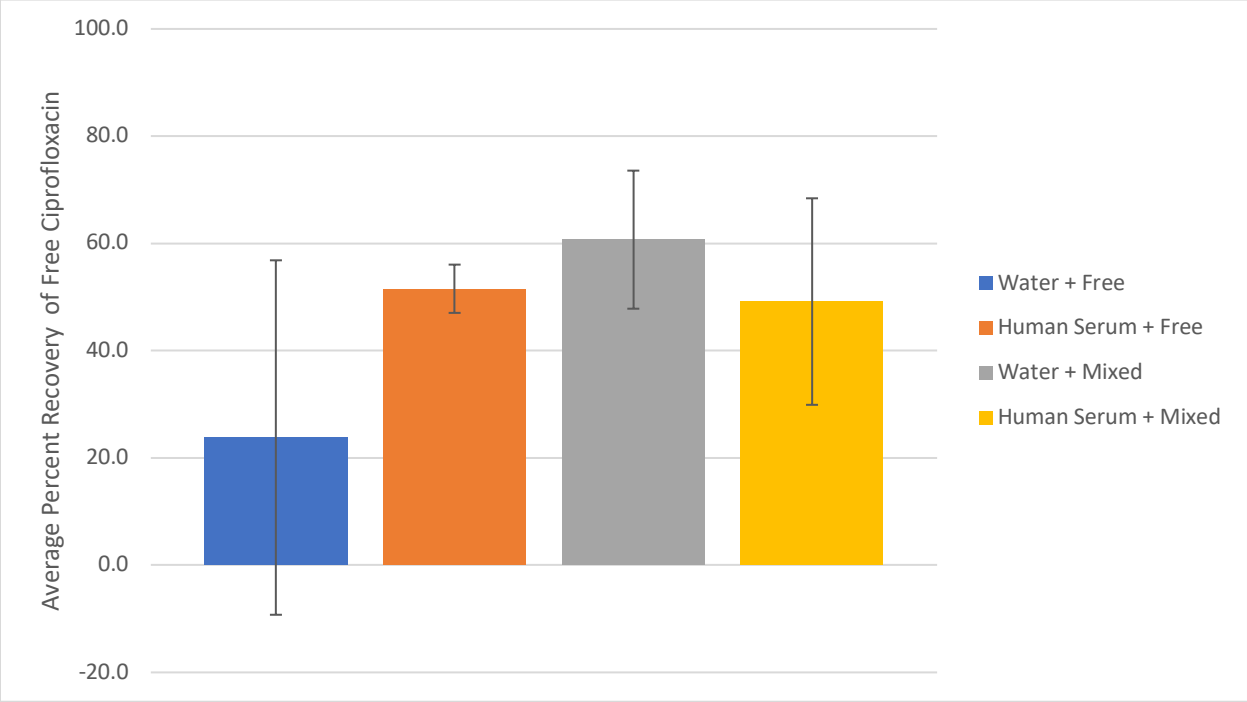

Figure 25: Average percent recovery of free ciprofloxacin using the initial sample process method. Free or mixed (free and polymeric ciprofloxacin) standards diluted with either water or human serum. Error bars represent standard deviation (n = 3).

An intriguing observation was the lack of any polymer signal in the chromatograms for both in the samples created with mixed and polymeric ciprofloxacin standards (see Figure 26 and 27). This phenomenon was observed for both samples diluted with water and human serum. In the chromatograms for the mixed (free and polymeric ciprofloxacin) standards, there was only a peak at roughly 7.8 minutes corresponding to free ciprofloxacin. The chromatogram for the polymeric standards in water look like a blank (water). The chromatogram for the polymeric standards in human serum only contain peaks corresponding to the matrix.

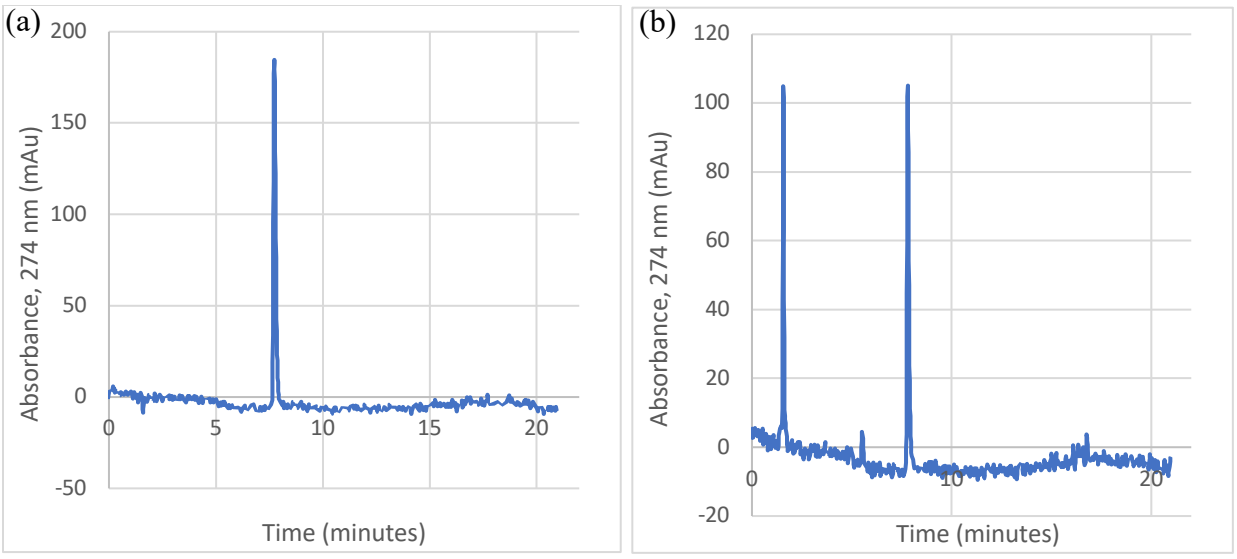

Figure 26: Chromatograms of sample processed and diluted mixed (free and polymeric ciprofloxacin) standards. (a) Mixed (free and polymeric ciprofloxacin) standard diluted with water. (b) Mixed (free and polymeric ciprofloxacin) standard diluted with human serum.

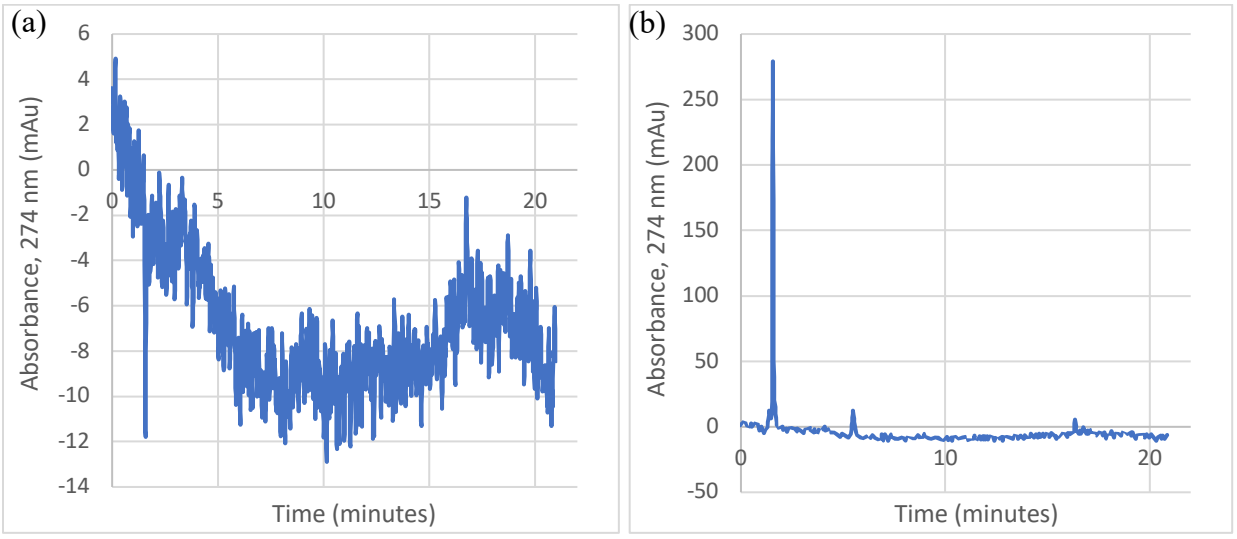

Figure 27: Chromatograms of sample processed and diluted polymeric ciprofloxacin standards. (a) Polymeric ciprofloxacin standard diluted with water. (b) Polymeric ciprofloxacin standard diluted with human serum.

4.3.2 Re-solubilization in Acetonitrile

This experiment was performed in an attempt to determine the reason for the lack of polymer signal in the processed samples using the method described in Chapter 4.2.1. Due to the ciprofloxacin's solubility in acetonitrile, we hypothesized that the signal may reappear if the samples were re-solubilized in acetonitrile rather than water. However, the polymer signal did

not appear when re-solubilized in acetonitrile or a water acetonitrile solution (1:1, v/v). The polymer signal was only observed in the samples that were not sample processed.

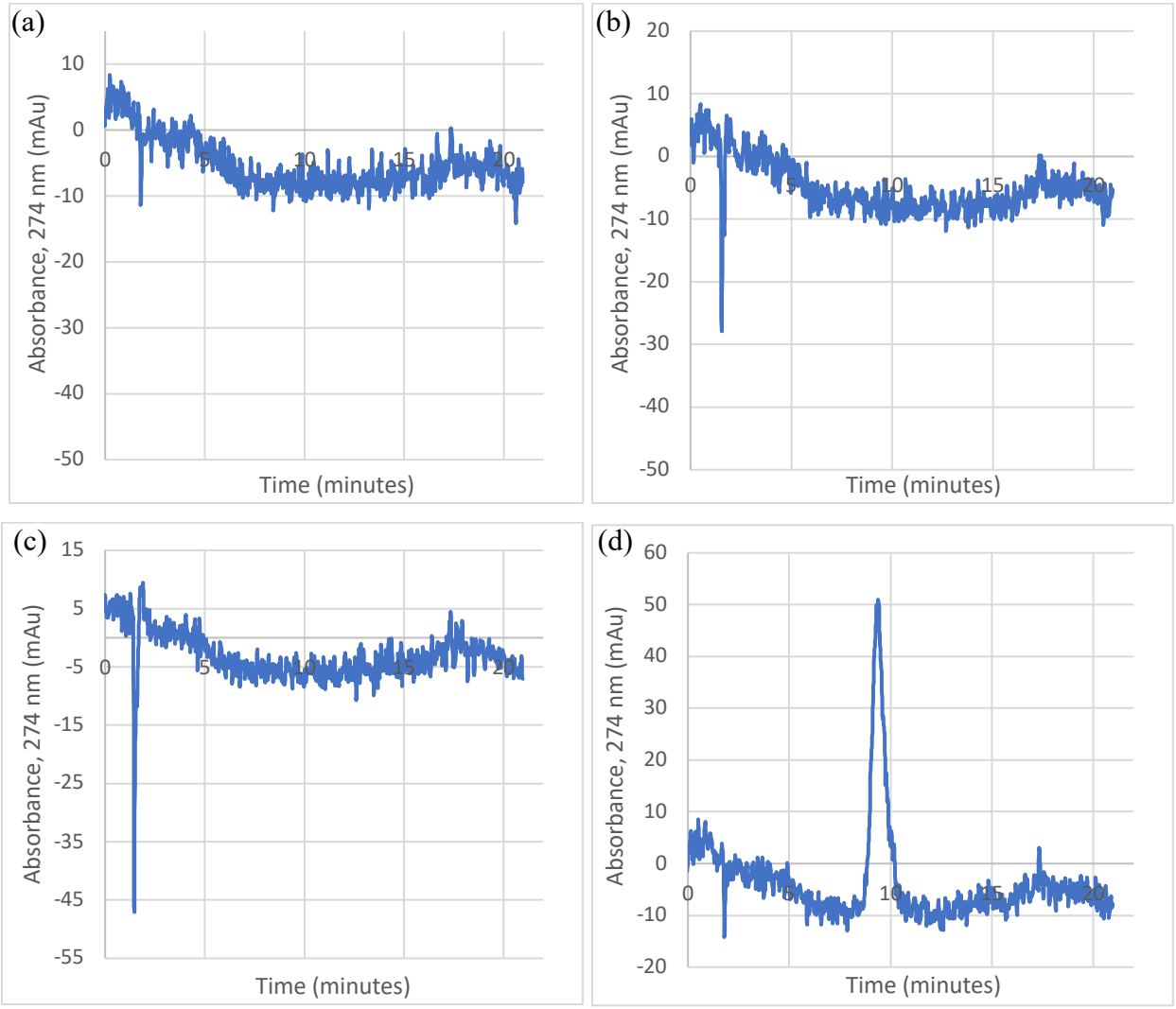

Figure 27: Chromatograms of sample processed polymeric ciprofloxacin standards re-solubilized in various solutions: (a) water, (b) water acetonitrile solution (1:1, v/v), and (c) acetonitrile. (d) Polymeric ciprofloxacin standards that was not sample processed and diluted to the same final concentration as the sample processed samples.

4.3.3 Drying Samples in Different Material Tubes

This experiment was performed in an attempt to determine the reason for the lack of polymer signal in the processed samples using the method described in Chapter 4.2.1 and 4.2.2. We hypothesized that the plastic tubes that were being used may be the cause of the lack of polymer

signal. This is because potentially the acetonitrile treatment step could cause the loosening and expanding of the plastic polymers and leading to the absorption of the polymer into the tube. Unexpectedly, the polymer signal was present in all samples (see Figure 28).

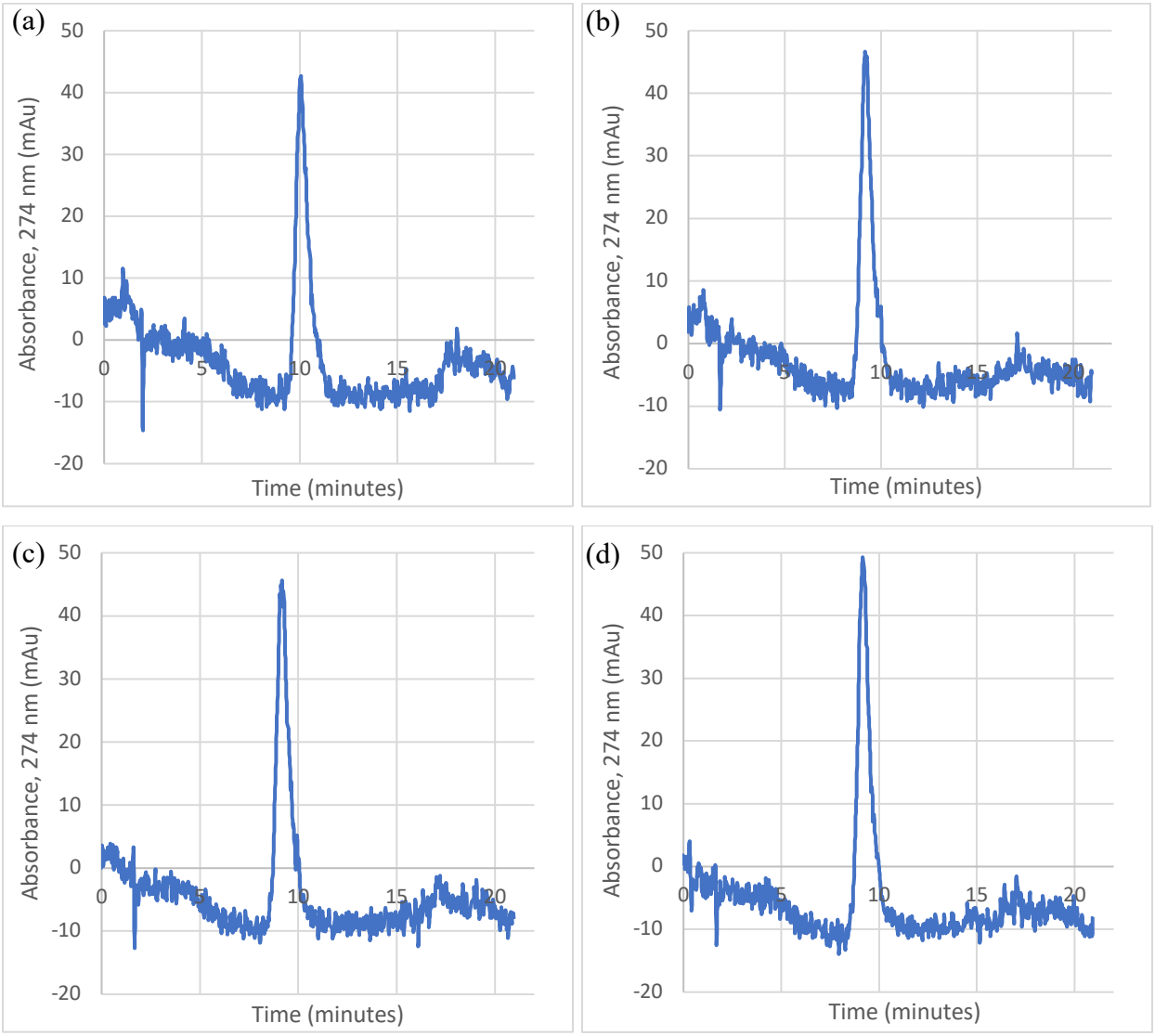

Figure 28: Chromatograms of sample processed polymeric ciprofloxacin standards dried down in various tubes: (a) 2 mL plastic tubes, (b) 1.5 mL plastic tube, or (c) 0.5 dram glass vial. (d) Polymeric ciprofloxacin standards that was not sample processed and diluted to the same final concentration as the sample processed samples.

4.3.4 Percent Recovery in Water

Due to the unreliability of polymer signal and the fact that HPLC is better made for the analysis of smaller molecules, we decided to focus on increasing the percent recovery of free

ciprofloxacin. We therefore worked to re-evaluate the percent recovery of free ciprofloxacin from water (see Figure 29). The average percent recovery for the sample processed free ciprofloxacin standards (condition 2) was 77.7% ± 4.4% (mean ± standard deviation). The average percent recovery for free ciprofloxacin from the mixed standards (condition 4) was 81.2% ± 19.5% (mean ± standard deviation). The average percent recovery of free ciprofloxacin for all of the samples that were not processed (condition 1 and 3) was 102.0% ± 3.9% (mean ± standard deviation). These results are an improvement in percent recovery than the initial method, both in precent recovery and consistency. However, these percent recovery are still not ideal considered the matrix used is water and not human serum.

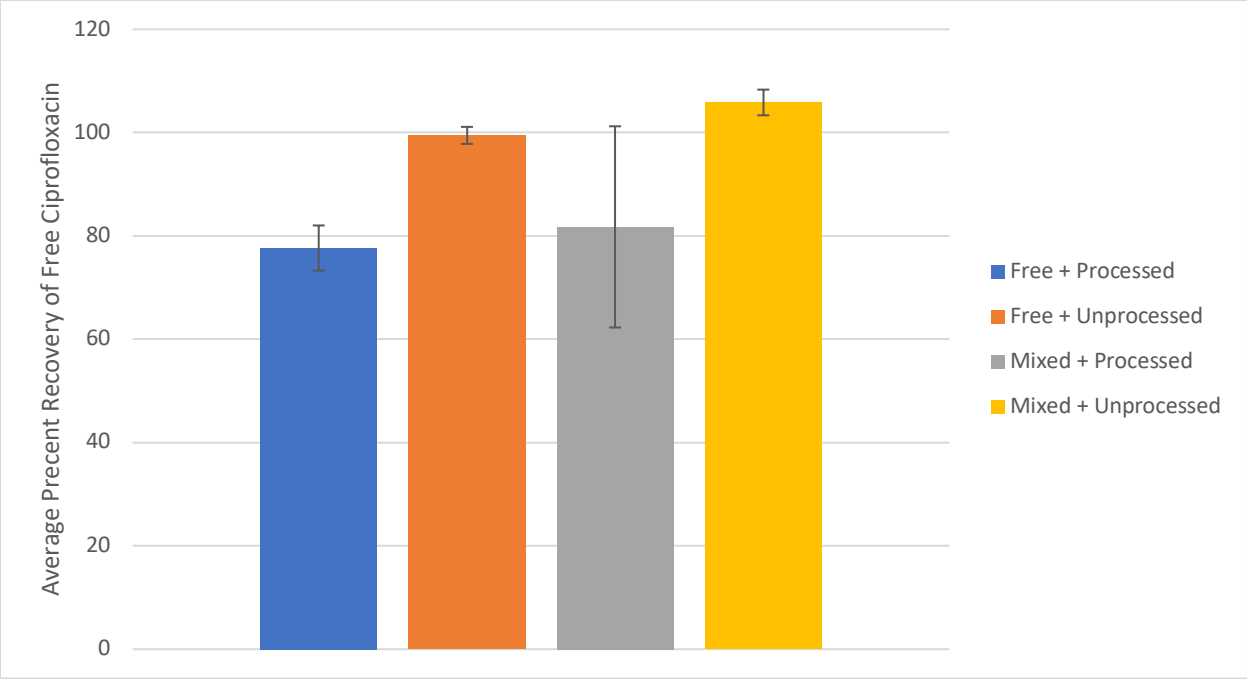

Figure 29: Percent drug recovery of free ciprofloxacin from water when using Frankenstein (n =3).

4.3.5 New Nitrogen Drier

We hypothesized that the average percent recovery could be increased if we used a more reliable nitrogen drier, as while using Frankenstein we occasionally observed some of the sample being splash out of the tube due to the difficulty in adjusting and maintaining a consistent pressure. Using the new nitrogen drier, Glinda, the average percent recovery of free ciprofloxacin in water was re-evaluated (see Figure 30). The average percent recovery for the sample processed free ciprofloxacin standards (condition 2) was 89.2% ± 2.7% (mean ± standard deviation). The

average percent recovery for free ciprofloxacin from the mixed standards (condition 4) was 94.6% ± 10.6% (mean ± standard deviation). The average percent recovery of free ciprofloxacin for all of the samples that were not processed (condition 1 and 3) was 100.5% ± 2.1% (mean ± standard deviation).

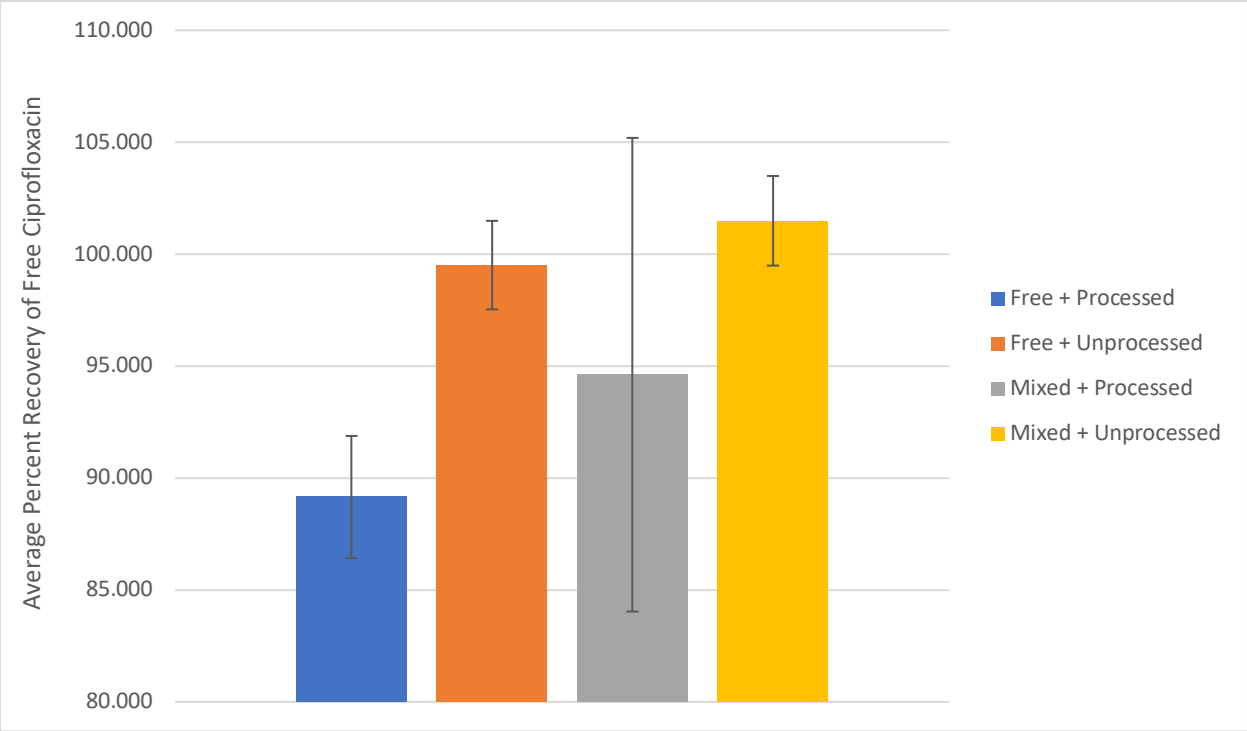

Figure 30: Percent drug recovery of free ciprofloxacin from water when using Glinda (n = 3).

4.3.6 Vortex Time During Re-solubilization

Percent recovery was re-evaluated given the new re-solubilization volume of 300 uL and the effect of vortex time during the re-solubilization step was evaluated (see Figure 31). The average percent recovery (mean ± standard deviation) was 102.4% ± 2.7%, 102.5 ± 4.3%, 99.5% ± 5.7%, and 99.7 ± 3.8% for the samples that were vortexed for 30 seconds, 1 minute, 5 minutes, and 20 minutes, respectively. The average percent recovery (mean ± standard deviation) for the sample that was not sample processed was 98.9 ± 1.7%. These results demonstrate that there is no significant difference in percent recovery based on the vortex time during the re-solubilization step. However, the increase in re-solubilization volume led to a significant increase in percent recovery (comparing results from Chapter 4.3.5 and 4.3.6).

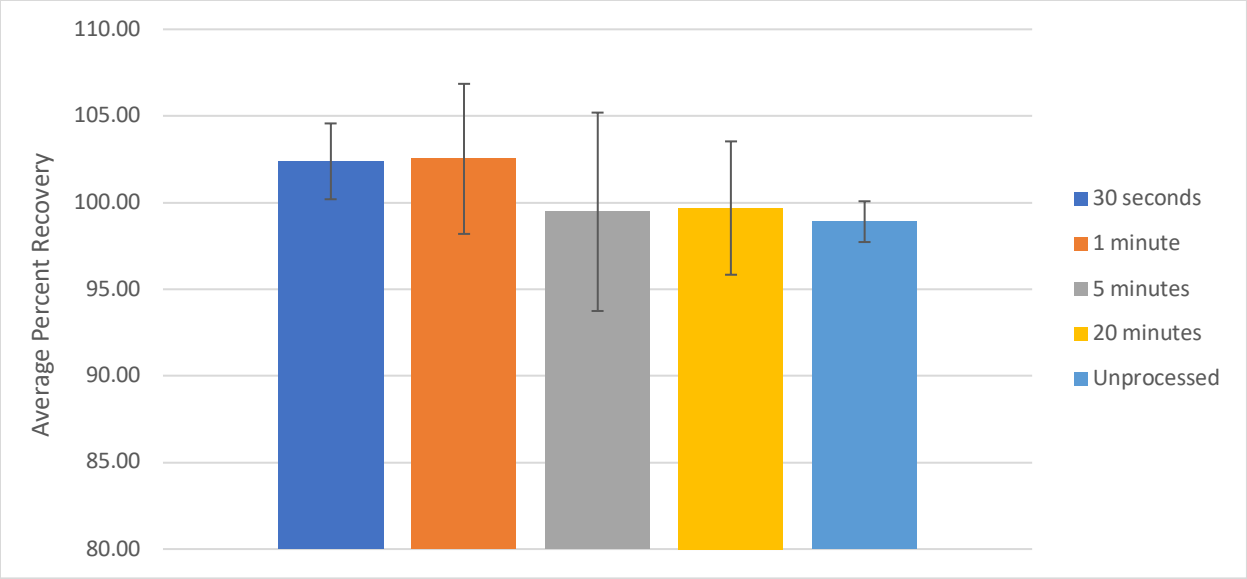

Figure 31: Percent recovery of free ciprofloxacin from water with various vortex times during re-solubilization (n = 3).

4.3.7 Effect of PBS on Polymer Peak

Experiments described in subsequent chapters diluted the aqueous polymeric ciprofloxacin standards in PBS and human serum incubated multiple days (see Chapter 5 and 7). In these experiments, it was observed that were two peaks. One peak corresponded to the release ciprofloxacin, with matching elution time to the free ciprofloxacin standards in the sequence used to create the standard curve. The second peak was thought to be polymer due to the elution time. However, the peak was not broad and stout as seen previously (see Figure 13). Instead, the peak was sharp (see Figure 32).

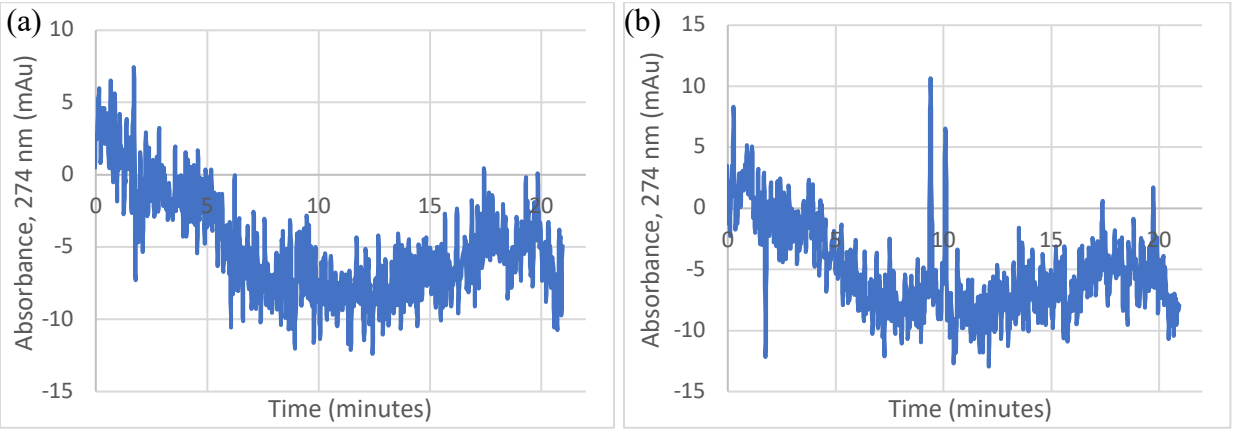

Figure 32: Chromatograms of polymeric ciprofloxacin in PBS incubated for (a) 0 days and (b) 1 day. These chromatograms correspond to samples from the long-term release experiments described in Chapter 5.

Another interesting observation was that in the experiments incubating polymeric ciprofloxacin in PBS and human serum for 60 days, the second peak only appear after 24 hours of incubation. In the experiments incubating polymeric ciprofloxacin in PBS and human serum for 2 days in acidic and basic conditions, the second peak was seen from day 0 (see Figure 52).

We therefore investigated the identity of this second sharp peak. Incubation of free ciprofloxacin in varying concentration of PBS showed no change in the fee ciprofloxacin peak and did not result in a secondary peak (see Figure 33). Incubation of polymeric ciprofloxacin in varying concentration of PBS resulted in the appearance two sharp peaks (see Figure 34). On day 0, only the broad polymer signal and one sharp at roughly 11.3 minutes is observed. On day 1, an additional sharp peak at the same elution time as the broad polymer signal can be observed. We therefore concluded that the secondary sharp peak seen in the experiments described in Chapter 5 and 7 was a product of the polymer interreacting with the matrix (PBS or human serum) and not a complex of the free ciprofloxacin.

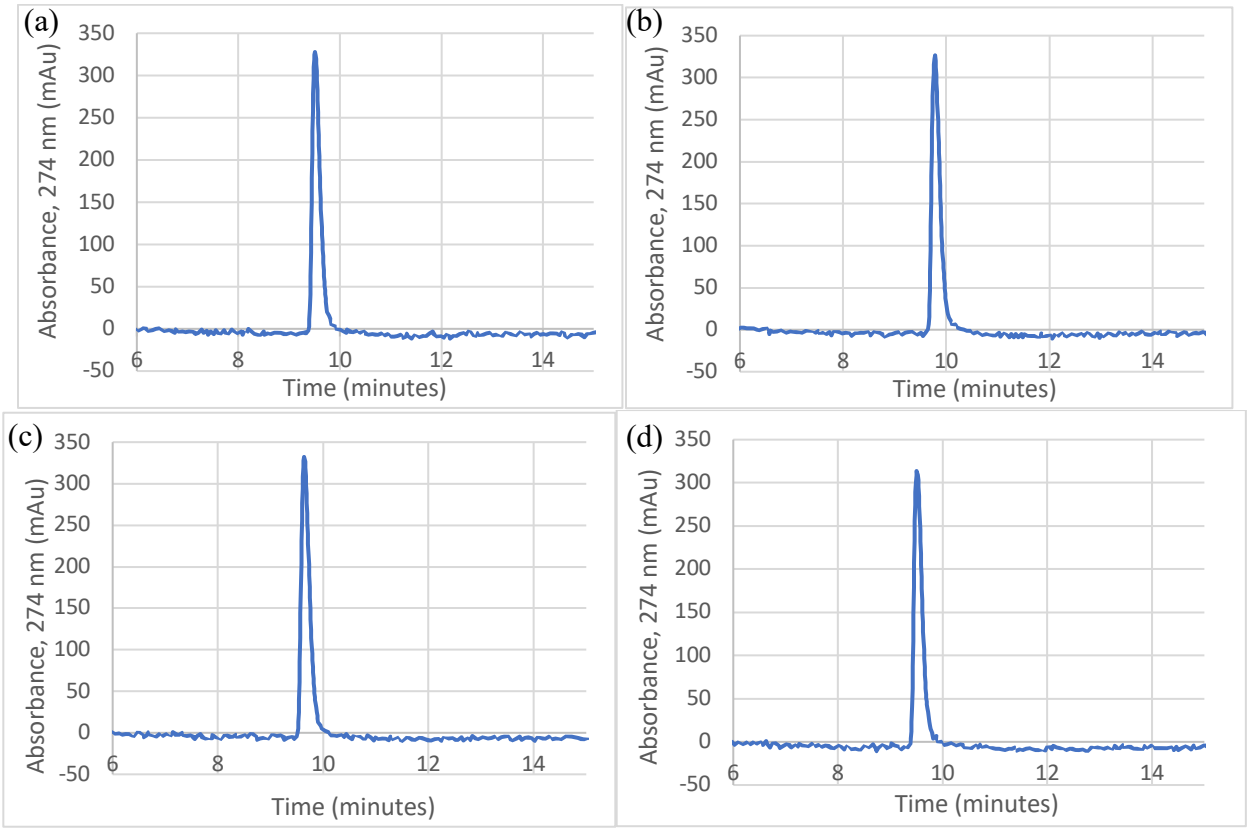

Figure 33: Chromatograms of free ciprofloxacin incubated in various concentration of PBS. Free ciprofloxacin diluted 1:1 with 0.25x PBS on (a) day 0 and (c) day 1. Free ciprofloxacin diluted 1:1 with 5x PBS on (a) day 0 and (c) day 1.

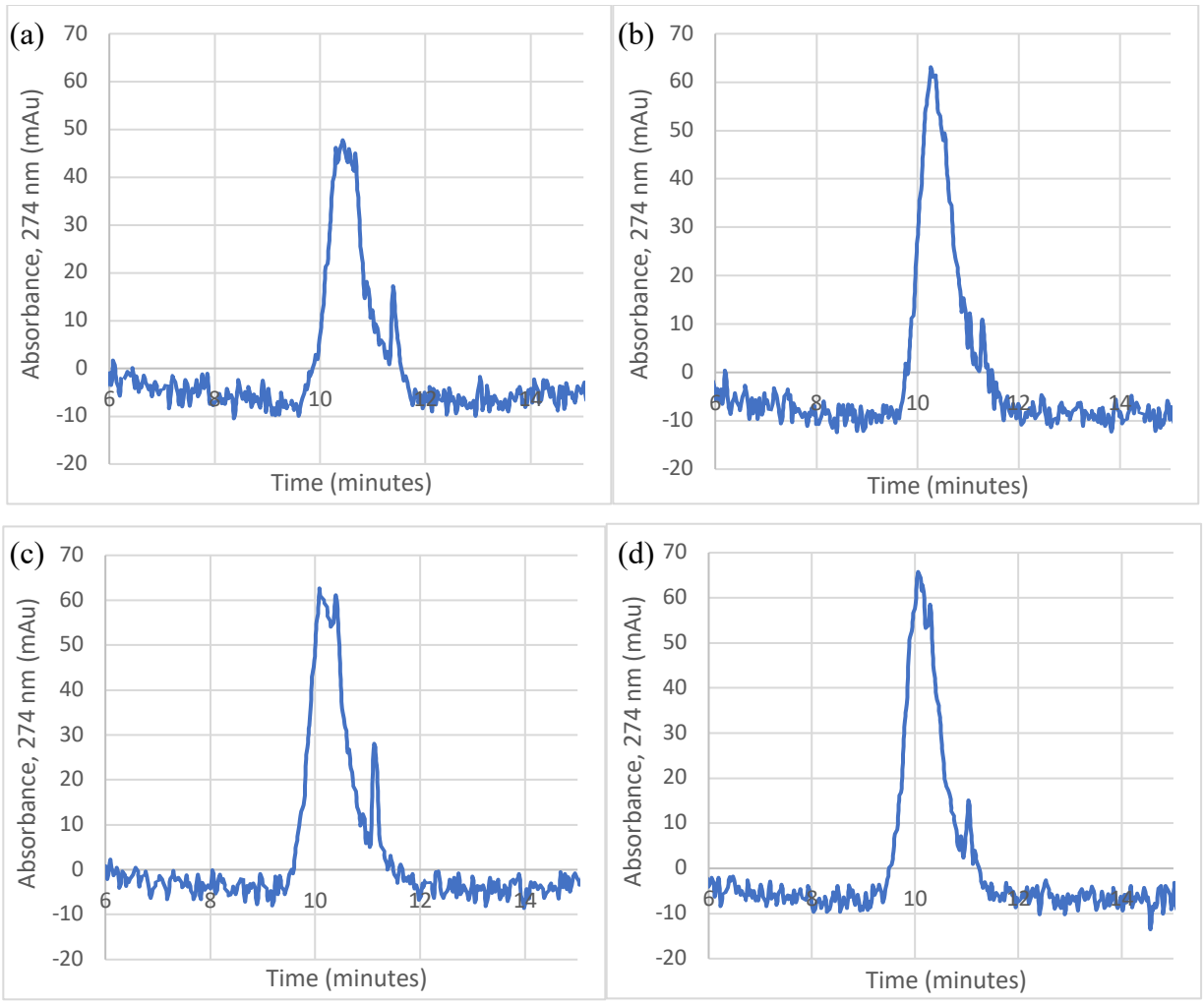

Figure 34: Chromatograms of polymeric ciprofloxacin incubated in various concentration of PBS. Polymeric ciprofloxacin diluted 1:1 with 0.25x PBS on (a) day 0 and (c) day 1. Polymeric ciprofloxacin diluted 1:1 with 5x PBS on (a) day 0 and (c) day 1.

4.3.7 Percent Recovery in Human Serum

The method that was optimized for extracting free ciprofloxacin from water was used on free ciprofloxacin in human serum and the percent recovery was evaluated. The average percent recovery (mean ± standard deviation) was found to be 71.1% ± 1.8% (see Figure 35). This is a significant decrease in precent recovery compared to the average percent recovery of 102.4% ± 2.7% in water. We hypothesized that this was due to the ciprofloxacin binding to the serum proteins and that the percent recovery would increase under conditions favoring the dissociation of ciprofloxacin from the serum proteins.

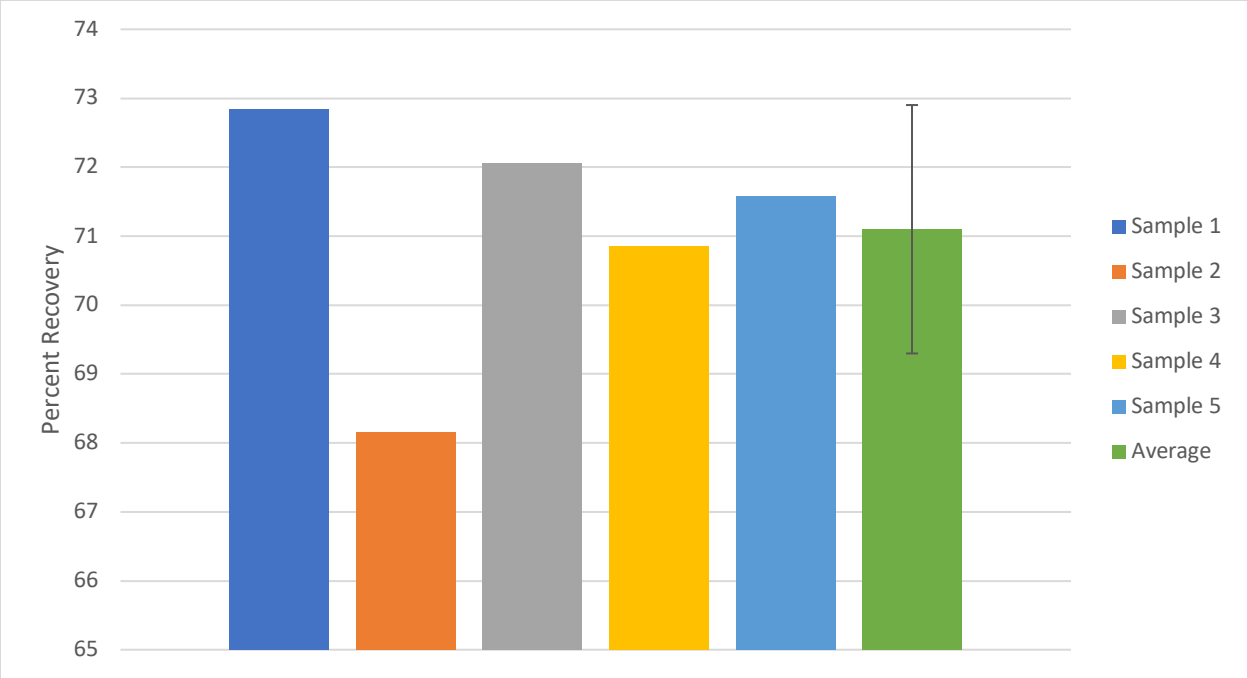

Figure 35: Percent recovery of free ciprofloxacin from human serum (n = 5).

4.3.8 Effect of Acetonitrile Ratio and Vortex Time During Protein Precipitation on Percent Recovery, Effect of Acid and Base on Percent Recovery

To test the hypothesis that conditions favoring the dissociation of ciprofloxacin from the serum proteins would increase the percent recovery, various conditions were tested. For the samples using the optimized method (control, condition 1), the average percent recovery (mean ± standard deviation) was 75.8% ± 2.8%. For the samples that were treated with a lower acetonitrile ratio (condition 2), the average percent recovery was 46.2% ± 31.6%. For the samples that were vortexed with acetonitrile for a longer period of time (condition 3), the average percent recovery was 79.8% ± 3.9%. For the samples that were treated with acid (condition 4), the average percent recovery was 83.6% ± 7.4%. For the samples that were treated with acid (condition 5), the average percent recovery was 82.6% ± 2.7%. As we hypothesized, the conditions that favored increased dissociation of ciprofloxacin from the serum protein (conditions 3-5) led to an increase in average percent recovery. The reason for the average percent recovery decreasing and standard deviation increasing for condition 2 is likely to be because there was not enough acetonitrile to consistently dissociation the ciprofloxacin from the serum protein.

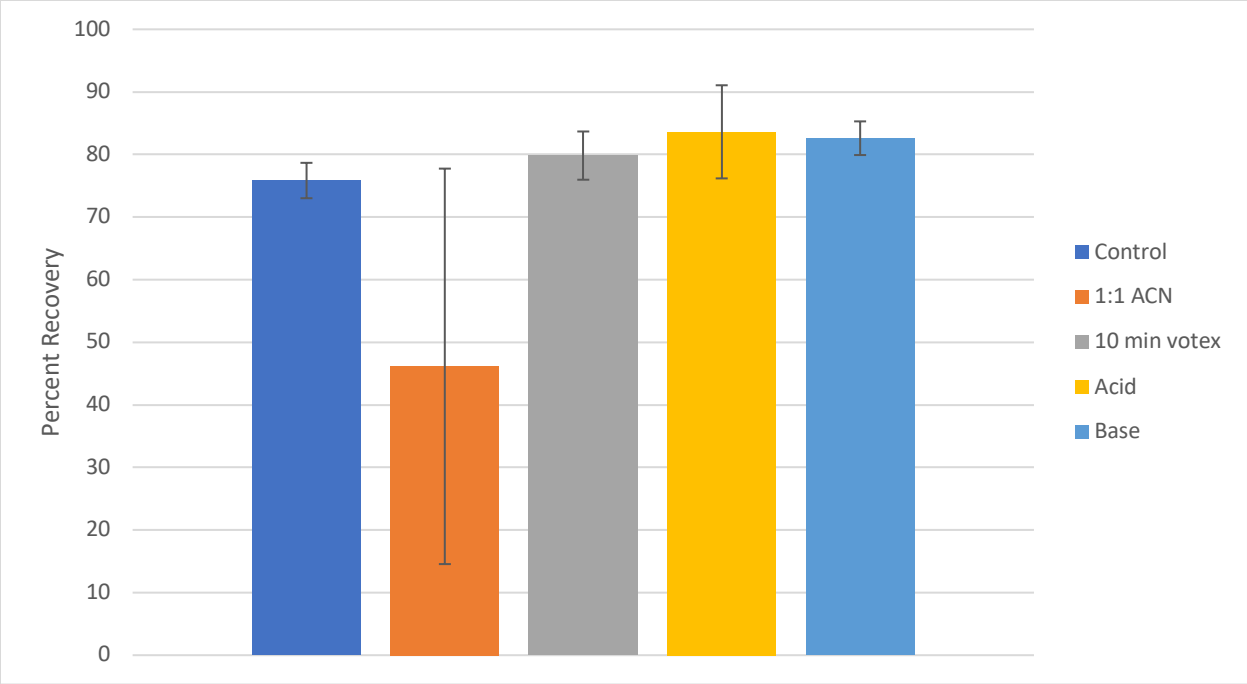

Figure 36: Percent recovery of free ciprofloxacin from human serum under various conditions: control, decrease acetonitril ratio, increased vortex time during acetonitril percipitation, acid, and base (n = 3).

Chapter 5. STABILITY AND RELEASE

5.1 INTRODUCTION

The VC drugamer was designed to be administered as an aerosol to achieve high local concentrations in the lung. However, it was important to investigate the stability of free ciprofloxacin and release of ciprofloxacin from the polymer in human serum, which contains high levels of serum albumin, a protein found to display esterase activity [22]. One reason is because esterases are known to have drug metabolizing activity in lung tissue [23]. This is a point of concern as the VC drugamer contains an ester between the self-immolative spacer and the ciprofloxacin (see Figure 4). As such, the metabolic activity of the esterases could lead to the premature release of the ciprofloxacin in the lung tissue prior to be internalized by the alveolar macrophages. Additionally, subsequent chapters describe experiments on the stability of free ciprofloxacin and release of ciprofloxacin from the polymer in acidic and basic environments. As such, the experiments performed in human serum can help to conclude that any degradation or release observed in subsequent experiments are due to the acidic and basic conditions and not the human serum. Therefore, it is important to investigate the stability of free ciprofloxacin and the release of ciprofloxacin from the polymer when incubated in human serum.

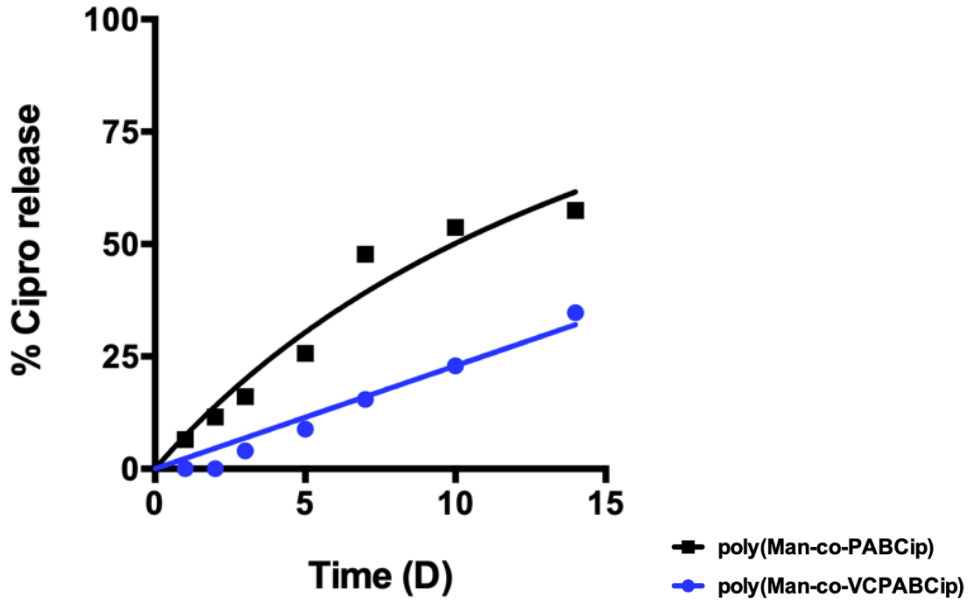

Figure 37: Percent release of ciprofloxacin from the VC drugamer in human serum at 37 °C [19]

Previously, an experiment has been performed characterizing the release of ciprofloxacin from the VC polymer in 90% human serum over 14 days at 37 °C (see Figure 37) [19]. No ciprofloxacin release was observed during the first 2 days. Additionally, the projected half-life was determined to be 21 days. This release profile was compared against a polymer with an ester linker, which was more unstable in human serum and demonstrated a half-life of 8 days.

5.2 Materials And Methods

5.2.1 Long Term Stability of Free Ciprofloxacin, Long Term Release from Polymeric Ciprofloxacin

Six separate solutions were prepared by adding free or polymeric ciprofloxacin standard (100 μg/mL, aqueous) to either water, 1x PBS, or human serum (Sigma S7023) (1:1, v/v). The final concentration of the samples were 50 μg/mL. All samples were then incubated at 37 °C under constant agitation in an orbital shaker (New Brunswick Scientific, innova 4300). One 500 μL timed aliquot was taken from each tube on day 0, 1, 2, 3, 5, 7, 14, 28, 42, and 60. Each aliquot is treated with 1000 μL of acetonitrile to promote serum proteins precipitation in human serum samples and to maintain identical sample process conditions for the water and PBS samples. Each tube was then briefly vortexed (VWR, Analog Vortex Mixer) and then centrifuged (Beckman Coulter, Microfuge 22R Centrifuge) for 5 minutes at 14,000 rpm. 1000 μL of the supernatant was transferred to a fresh tube and stored at -80°C. Once all aliquots are taken, defrost samples were completely dried down with a nitrogen dryer (Stuart, Block Heater and Techne, Sample Concentrator). Samples were then resolubilized in 2000 μL of HPLC grade water. Human serum samples were then filtered (Millex-GV filter, 0.22 um pore size) with a syringe (Fisher Scientific, 1 mL plastic sterile syringe). The resolubilized sample was then transfered into glass LCMS vials (Agilent Technologies, 2 mL vial) with glass inserts (Agilent Technologies, 250 μL pulled point-conical glass inserts) and caps (Agilent Technologies, 9 mm blue screw). The samples were then run with the optimized LC method (NLPCIP11) along with free ciprofloxacin standards ranging from 3.125 μg/mL to 25 μg/mL in concentration. For the free ciprofloxacin samples, the expected concentration of the final samples is 8.33 μg/mL assuming 100% recovery. For the polymeric ciprofloxacin samples, the concentration of the released ciprofloxacin is 8.33 μg/mL assuming 100% recovery and 100% release.

5.3.2 Short Term Stability of Free Ciprofloxacin

Three separate solutions were prepared by adding free ciprofloxacin standard (200 μg/mL, aqueous) to either water, 1x phosphate-buffered solution (PBS), or human serum (Sigma S7023) (1:1, v/v). The final concentration of the samples were 100 μg/mL. All samples were then incubated at 37 °C under constant agitation in an orbital shaker (New Brunswick Scientific, innova 4300). 500 μL timed aliquots was taken from each tube on day 0, 1, 2, 3, 5, and 7. Each aliquot was treated with 1000 μL of acetonitrile to promote serum proteins precipitation in human serum samples and to maintain identical sample process conditions for the water and PBS samples. Each tube was then briefly vortexed (VWR, Analog Vortex Mixer) and then centrifuged (Beckman Coulter, Microfuge 22R Centrifuge) for 5 minutes at 14,000 rpm. 1000 μL of the supernatant was transferred to a fresh tube and stored at -80°C. Once all aliquots were taken, defrosted samples were completely dry down with a nitrogen dryer (Stuart, Block Heater and Techne, Sample Concentrator). Samples were then resolubilized in 2000 μL of HPLC grade water. Human serum samples were then filtered (Millex-GV filter, 0.22 um pore size) with a syringe (Fisher Scientific, 1 mL plastic sterile syringe). The resolubilized sample was then transfered into glass LCMS vials (Agilent Technologies, 2 mL vial) with glass inserts (Agilent Technologies, 250 μL pulled point-conical glass inserts) and caps (Agilent Technologies, 9 mm blue screw). The samples were then run with the optimized LC method (NLPCIP11) along with free ciprofloxacin standards ranging from 3.125 μg/mL to 25 μg/mL in concentration. Assuming 100% recovery, the expected concentration of the final samples is 16.67 μg/mL.

5.3 Results And Discussion

5.3.1 Long Term Stability – in Water

Free ciprofloxacin was incubated in water for 60 days and the concentration of the aliquots taken at various time points was calculated based upon the AUC and the standard curve. The average concentration for each time point remained fairly constant throughout the 60 days (see Figure 38). The average percent expected for all samples was 93.9% ± 6.1% (mean ± standard deviation). Percent expected was calculated based on the theoretical expected concentration of 8.33 μg/mL. This demonstrates the stability of free ciprofloxacin in water for 60 days.

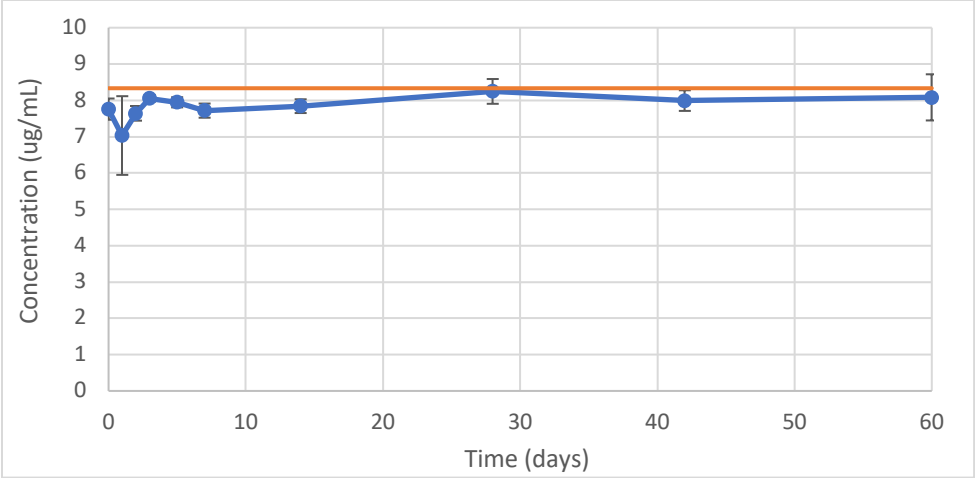

Figure 38: Long term stability of free ciprofloxacin in water. The expected concentration of 8.33 µg/mL is show in orange. The measure concentration of free ciprofloxacin is show in blue (n = 3). The concentration remains fairly stable for the entire 60 day period of incubation. Error bars represent standard deviation.

5.3.2 Long Term Stability – in PBS

Free ciprofloxacin was incubated in PBS for 60 days and the concentration of the aliquots taken at various time points was calculated based upon the AUC and the standard curve. The average concentration for each time point remained fairly constant throughout the 60 days (see Figure 39). The average percent expected for all samples was 104.4% ± 7.4% (mean ± standard deviation). Percent expected was calculated based on the theoretical expected concentration of 8.33 µg/mL. This demonstrates the stability of free ciprofloxacin in PBS for 60 days.

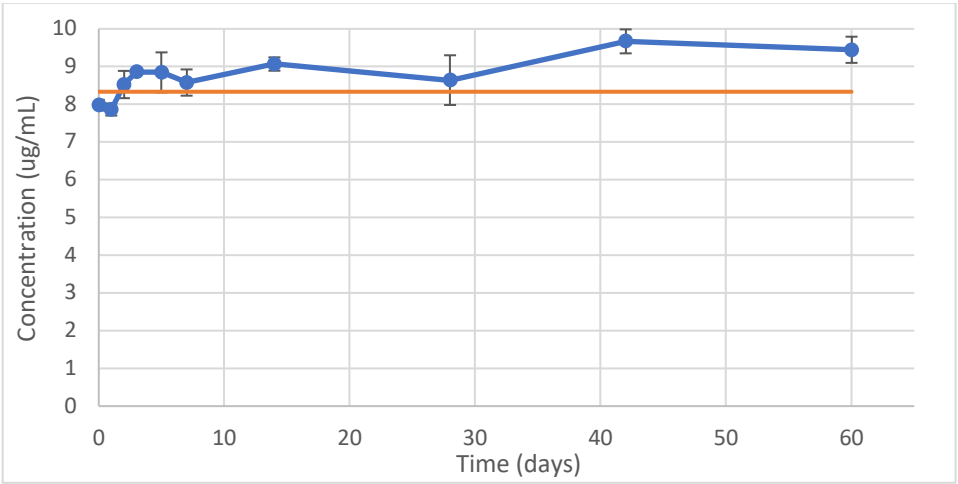

Figure 39: Long term stability of free ciprofloxacin in PBS. The expected concentration of 8.33 µg/mL is show in orange. The measure concentration of free ciprofloxacin is show in blue (n = 3). The concentration remains fairly stable for the entire 60 day period of incubation. Error bars represent standard deviation.

5.3.3 Short Term Stability – in Human Serum

Aliquots of free ciprofloxacin incubated human serum over 60 days were taken. However, some of these samples were spilled during the drying process and therefore were not run on the HPLC for quantification. Due to time constraints, timed aliquots of free ciprofloxacin incubated in human serum were re-taken for 7 days rather than 60 days. Unexpectedly, the average concentration decreased over the 7 days (see Figure 40). The average percent expected was 35%, 49%, 36%, 21%, 26% and 21% for days 0, 1, 2, 3, 5, and 7, respectively. Furthermore, the measured concentrations were much lower than expected with a 32% ± 12% (mean ± standard deviation) average percent expected for all samples. Percent expected was calculated based on the theoretical expected concentration of 16.67 µg/mL. We are uncertain why we saw a low percent expected given the percent recovery demonstrated in Chapter 4. We also do not know why there was a decrease in concentration overtime as there is evidence demonstrating the high stability of ciprofloxacin in human serum up for up to 3 weeks [24] [25].

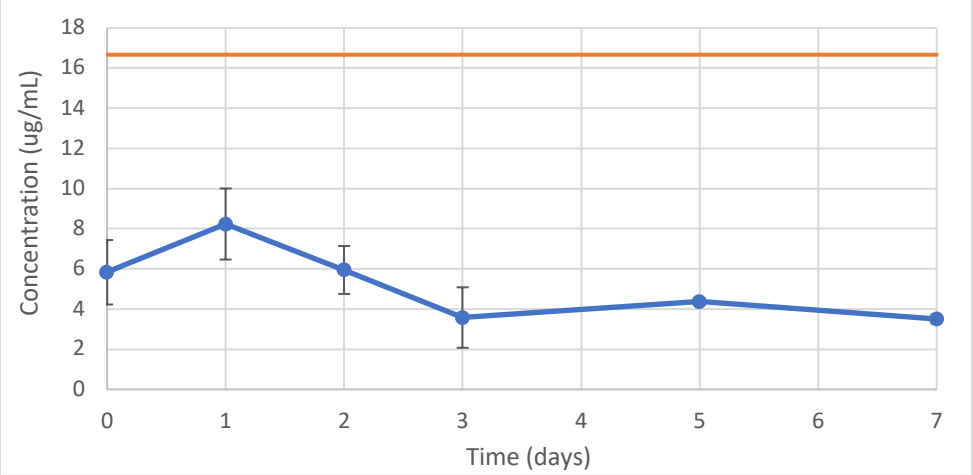

Figure 40: Long term stability of free ciprofloxacin in human serum. The expected concentration of 16.67 µg/mL is show in orange. The measure concentration of free ciprofloxacin is show in blue (n = 3). Error bars represent standard deviation.

5.3.4 Long Term Release – in Water

Polymeric ciprofloxacin was incubated in water for 60 days and the concentration of the released ciprofloxacin was calculated based upon the AUC and the standard curve. No release of ciprofloxacin is observed for the first 14 days of incubation (see Figure 41). The average percent expected was 12%, 17% and 18% on days 28, 42, and 60, respectively. Percent expected was

calculated based on the theoretical expected concentration of 8.33 µg/mL given that all of the ciprofloxacin was released from the polymer and the percent drug weight on the polymer. These results demonstrate the minimal release of ciprofloxacin from the polymer when incubated in water for 60 days.

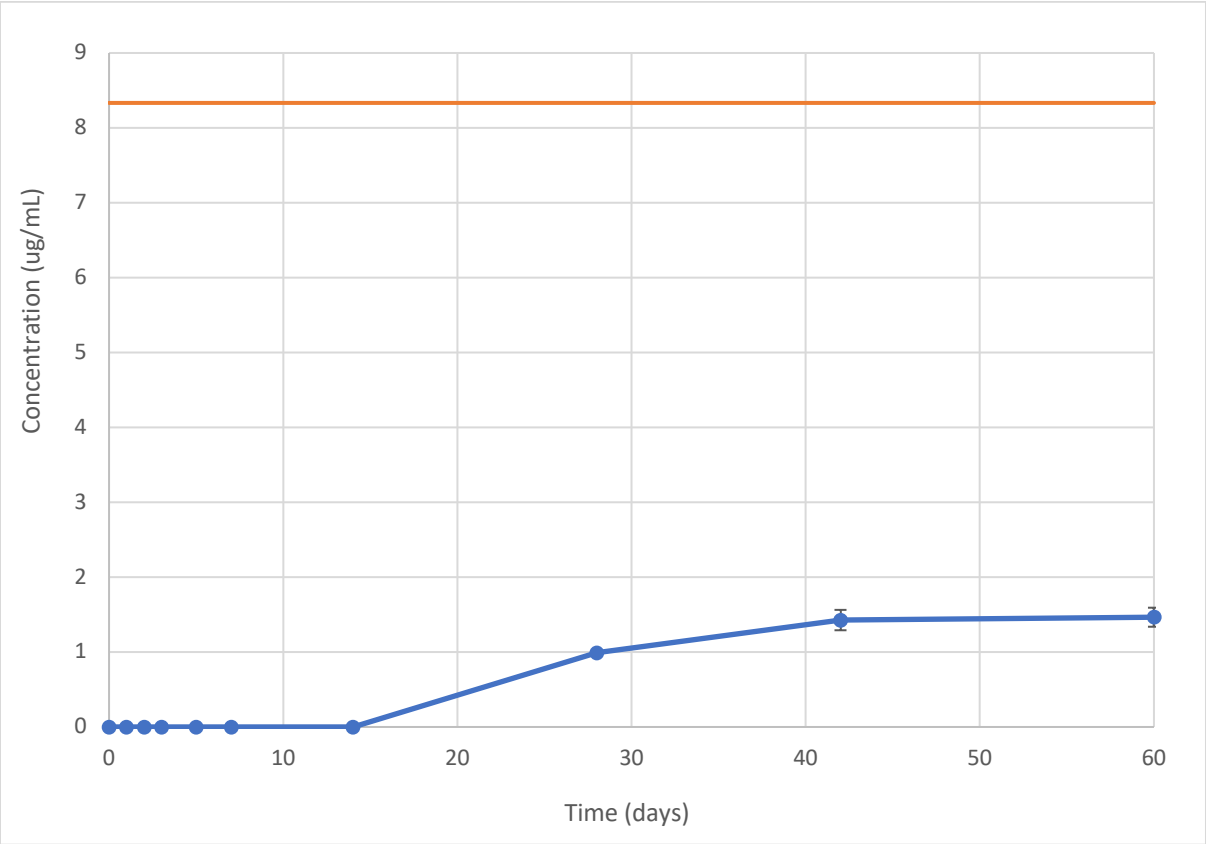

Figure 41: Long term stability of polymeric ciprofloxacin in water. The theoretical complete release concentration of 8.33 µg/mL is show in orange. The measure concentration of released ciprofloxacin is show in blue (n = 3). The percent expected at day 60 is 18%. Error bars represent standard deviation.

5.3.5 Long Term Release – in PBS

Polymeric ciprofloxacin was incubated in PBS for 60 days and the concentration of the released ciprofloxacin was calculated based upon the AUC and the standard curve. No release of ciprofloxacin is observed for the first 5 days of incubation (see Figure 42). The average percent expected was 8%, 13%, 25%, 36%, and 37% on days 7, 14, 28, 42, and 60, respectively. Percent expected was calculated based on the theoretical expected concentration of 8.33 µg/mL given that all of the ciprofloxacin was released from the polymer and the percent drug weight on the polymer. These results demonstrate the stability of the polymer during the first 5 days in PBS.

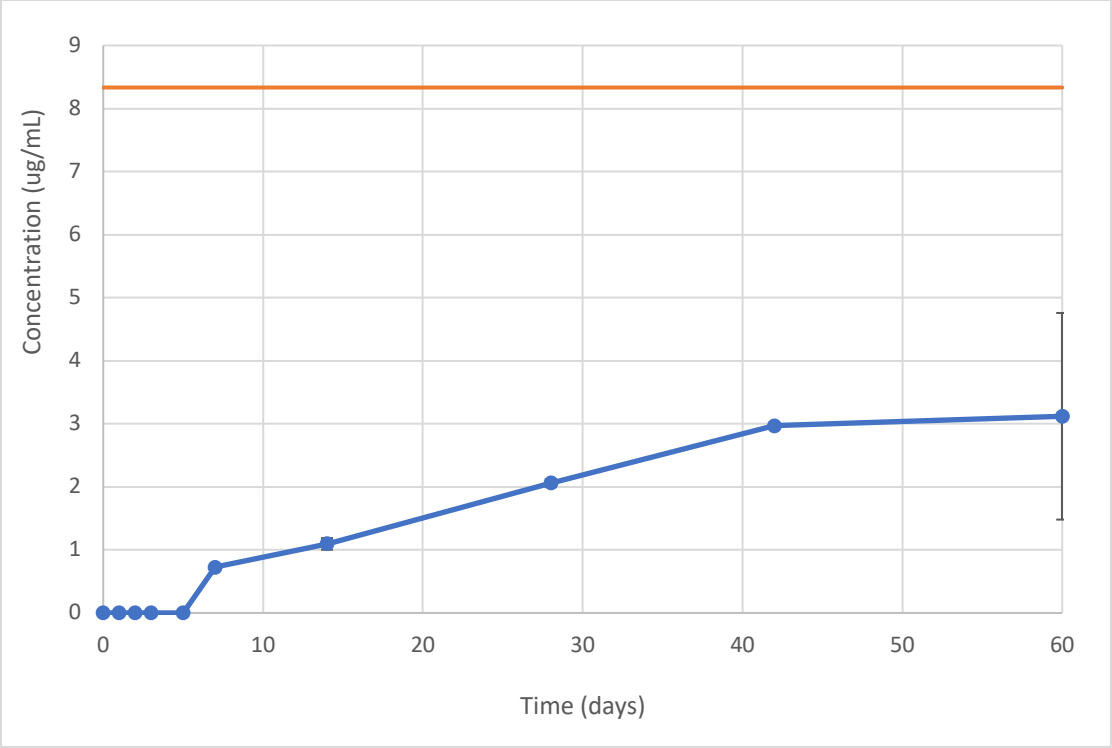

Figure 42: Long term stability of polymeric ciprofloxacin in PBS. The theoretical complete release concentration of 8.33 µg/mL is show in orange. The measure concentration of released ciprofloxacin is show in blue (n = 3). The percent expected at day 60 is 37%. Error bars represent standard deviation.

5.3.6 Long Term Release – in Human Serum

Polymeric ciprofloxacin was incubated in human serum for 60 days and the concentration of the released ciprofloxacin was calculated based upon the AUC and the standard curve. No release of ciprofloxacin is observed until day 2 of incubation (see Figure 43). No substantial release of ciprofloxacin was seen until day 14. The average percent expected was 12%, 17%, 22%, 18%, 59%, 41%, 72%, and 83% on days 2, 3, 5, 7, 14, 28, 42, and 60, respectively. Percent expected was calculated based on the theoretical expected concentration of 8.33 µg/mL given that all of the ciprofloxacin was released from the polymer and the percent drug weight on the polymer. These results demonstrate the minimal non-specific release of ciprofloxacin that occurs during the first week of incubation, and therefore the stability of the polymer in human serum. Based on the minimal or lack of release that occurs during the first 72 hours in water, PBS, human serum, the release observed in subsequent experiments (described in Chapter 6 and 7) can be contributed to the acidic and basic conditions rather than the matrix.

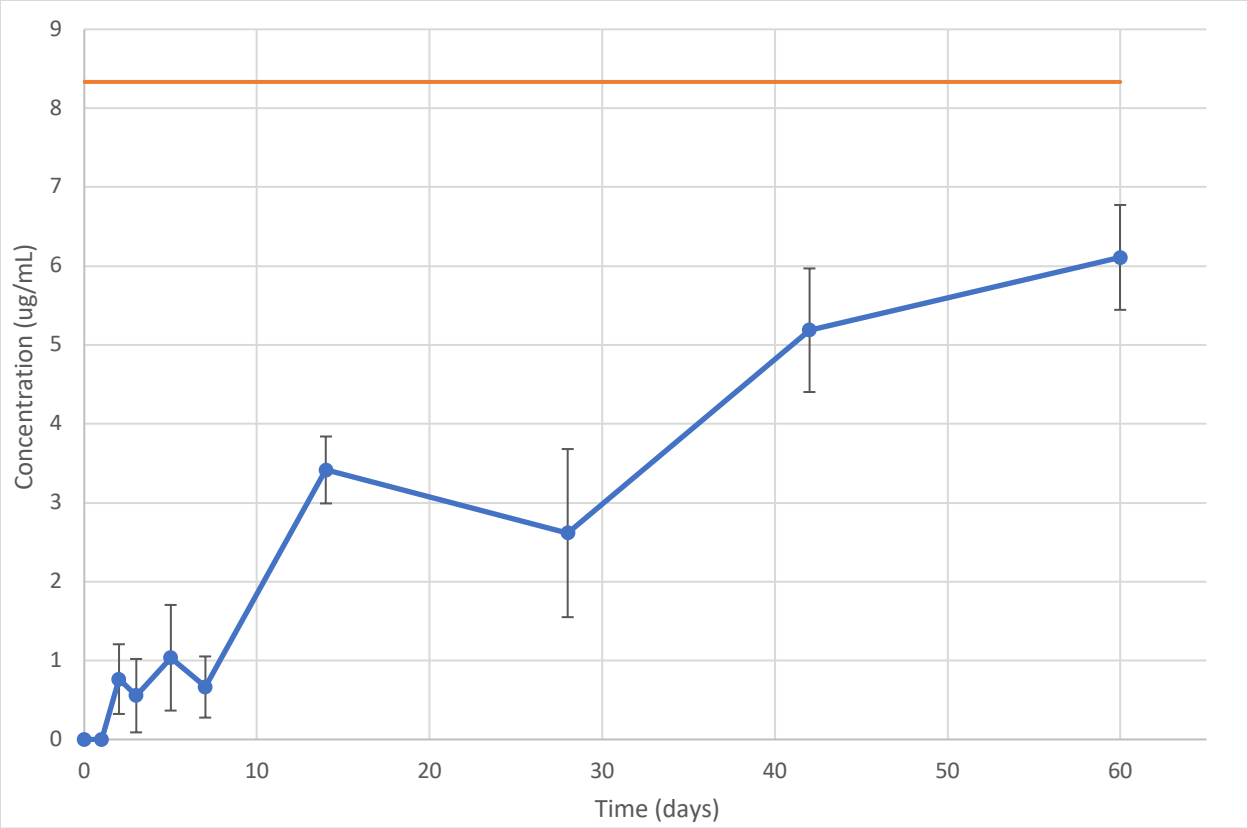

Figure 43: Long term stability of polymeric ciprofloxacin in human serum. The theoretical complete release concentration of 8.33 µg/mL is show in orange. The measure concentration of released ciprofloxacin is show in blue (n = 3). The percent expected at day 60 is 83%. Error bars represent standard deviation.

Chapter 6. ACID AND BASE STABILITY

6.1 INTRODUCTION

As stated above, previously there have been two methods of determining 100% release of ciprofloxacin from the drugamer. The acid hydrolysis method defined 100% release to be 48-hours after incubation in 10% aqueous sulfuric acid [13]. On the other hand, the base hydrolysis method defined 100% release to be 24-hours after incubation in 0.1 N sodium hydroxide [11]. To accurately quantify ciprofloxacin released from the drugamer via acid and base hydrolysis, the stability of free ciprofloxacin in acidic and basic environments first needed to be characterized.

6.2 MATERIALS AND METHODS

Three separate intermediate dilution solutions were prepared by adding free ciprofloxacin standard (200 μg/mL, aqueous) to PBS or human serum (Sigma S7023) (1:1, v/v). Then 10% sulfuric acid, 0.1 N sodium hydroxide, or HPLC grade water (control) was added to the intermediate ciprofloxacin solution (1:1, v/v). This created the final solutions with a ciprofloxacin concentration of 50 μg/mL. The solutions were kept at room temperature and 2 timed aliquots were taken on day 0, 1, and 2 for each condition. 127 μL aliquots were immediately neutralized with 73 μL of either 2 M sodium hydroxide, 0.53% sulfuric acid, or water (control). 800 μL of acetonitrile was then added to promote serum proteins precipitation. Each tube was then briefly vortexed (VWR, Analog Vortex Mixer) and then centrifuged (Beckman Coulter, Microfuge 22R Centrifuge) for 5 minutes at 14,000 rpm. 800 μL of the supernatant was transferred to a fresh tube and stored at -80 °C. Once all aliquots were taken, defrost samples were completely dried down with a nitrogen dryer (Stuart, Block Heater and Techne, Sample Concentrator). Samples were then resolubilized in 300 μL of HPLC grade water. Samples were then filtered (Millex-GV filter, 0.22 um pore size) with a syringe (Fisher Scientific, 1 mL plastic sterile syringe). The resolubilized samples were then transferred into glass LCMS vials (Agilent Technologies, 2 mL vial) with glass inserts (Agilent Technologies, 250 μL pulled point-conical glass inserts) and caps (Agilent Technologies, 9 mm blue screw). The samples were then run with the optimized LC method (NLPCIP11) along with free ciprofloxacin standards ranging from 3.125 μg/mL to 25 μg/mL in concentration. Assuming 100% recovery, the expected concentration of the final samples is 16.933 μg/mL.

6.3 Results and Discussion

6.3.1 Acid and Base Stability in PBS

The aqueous free ciprofloxacin standards were used to create a standard curve, which was then used to determine the concentration of the samples incubated in PBS based upon the measure AUC. In acidic conditions, the percent expected was 95% and 91% at day 0, 88% and 91% at day 1, and 95% and 58% at day 2 (see Figure 44). In basic conditions, the percent expected was 90% and 88% at day 0, 89% and 87% at day 1, and 94% and 91% at day 2 (see Figure 45). In the control, the percent expected was 89% and 90% at day 0, 87% and 91% at day 1, and 88% and 86% at day 2. Percent expected was calculated based on the theoretical expected concentration of 16.933 µg/mL. The minimal change in concentration over the 48-hour period suggest that free ciprofloxacin is stable in acidic and basic environments. This is supported by the chromatograms which consistently show a single sharp peak with an elution time consistent with the free ciprofloxacin standards. Additionally, these percent expected are similar values as the results from Chapter 4.3.8 on percent recovery in samples with acid and base, which is future evidence supporting the stability of ciprofloxacin in acidic and basic conditions. As such, it was concluded that free ciprofloxacin is stable and does not degrade when incubated in PBS with sulfuric acid and sodium hydroxide.

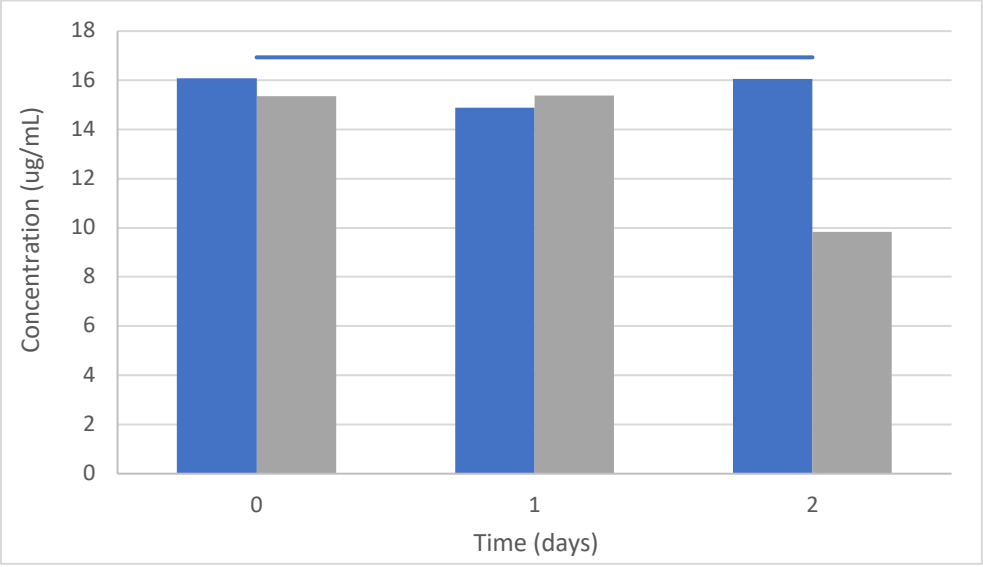

Figure 44: Free ciprofloxacin stability in PBS and an acidic environment. Free ciprofloxacin incubated in PBS with 10% sulfuric acid (n = 2). Based on percent drug weight on the polymer, the expected concentration is 16.933 µg/mL, which is illustrated in the graph as the line above the bars.

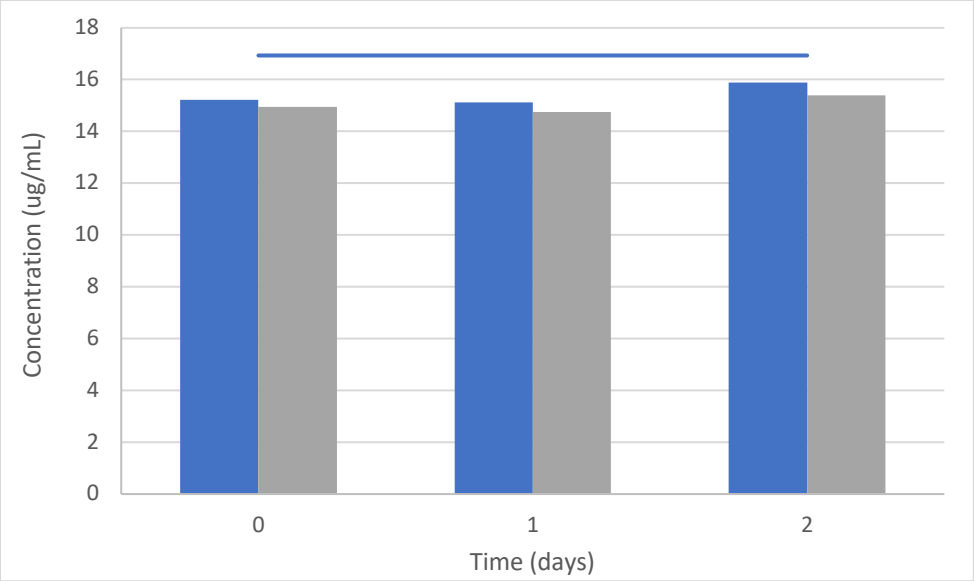

Figure 45: Free ciprofloxacin stability in PBS and a basic environment. Free ciprofloxacin incubated in PBS with 0.1 N sodium hydroxide (n = 2). Based on percent drug weight on the polymer, the expected concentration is 16.933 µg/mL, which is illustrated in the graph as the line above the bars.

6.3.2 Acid and Base Stability in Human Serum

The aqueous free ciprofloxacin standards were used to create a standard curve, which was then used to determine the concentration of the samples incubated in human serum based upon the measure AUC. In acidic conditions, the percent expected was 81% and 92% at day 0, 83% and 93% at day 1, and 83% and 85% at day 2 (see Figure 46). In basic conditions, the percent expected was 90% and 82% at day 0, 82% and 76% at day 1, and 85% and 80% at day 2 (see Figure 47). In the control, the percent expected was 73% and 68% at day 0, 71% and 69% at day 1, and 81% and 81% at day 2. Percent expected was calculated based on the theoretical expected concentration of 16.933 µg/mL. The minimal change in concentration over the 48-hour period suggest that free ciprofloxacin is stable in acidic and basic environments. This is supported by the chromatograms which do not show separating peaks or appearance of new peaks (see Figure 48). Instead, the chromatograms consistently show a single sharp peak with an elution time consistent with the free ciprofloxacin standards. Additionally, these percent expected are similar values as the results from Chapter 4.3.7 and Chapter 4.3.8 on percent recovery in human serum, which is future evidence supporting the stability of ciprofloxacin in acidic and basic conditions. As such, it was concluded that free ciprofloxacin is stable and does not degrade when incubated in human serum with sulfuric acid and sodium hydroxide.

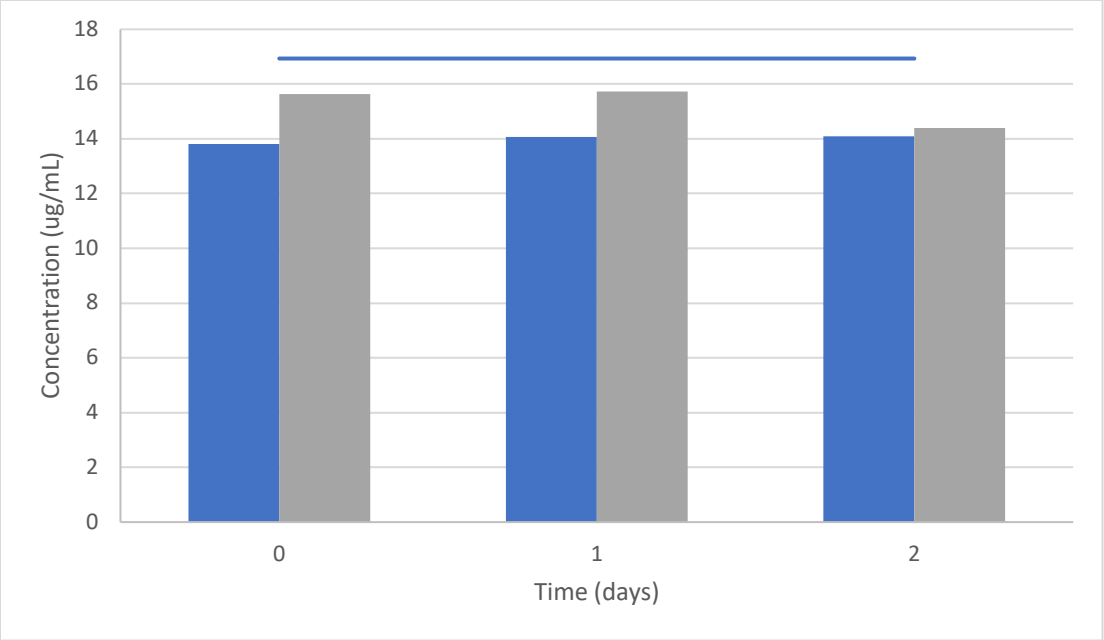

Figure 46: Free ciprofloxacin stability in human serum and an acidic environment. Free ciprofloxacin incubated in human serum with 10% sulfuric acid (n = 2). Based on percent drug weight on the polymer, the expected concentration is 16.933 µg/mL, which is illustrated in the graph as the line above the bars.

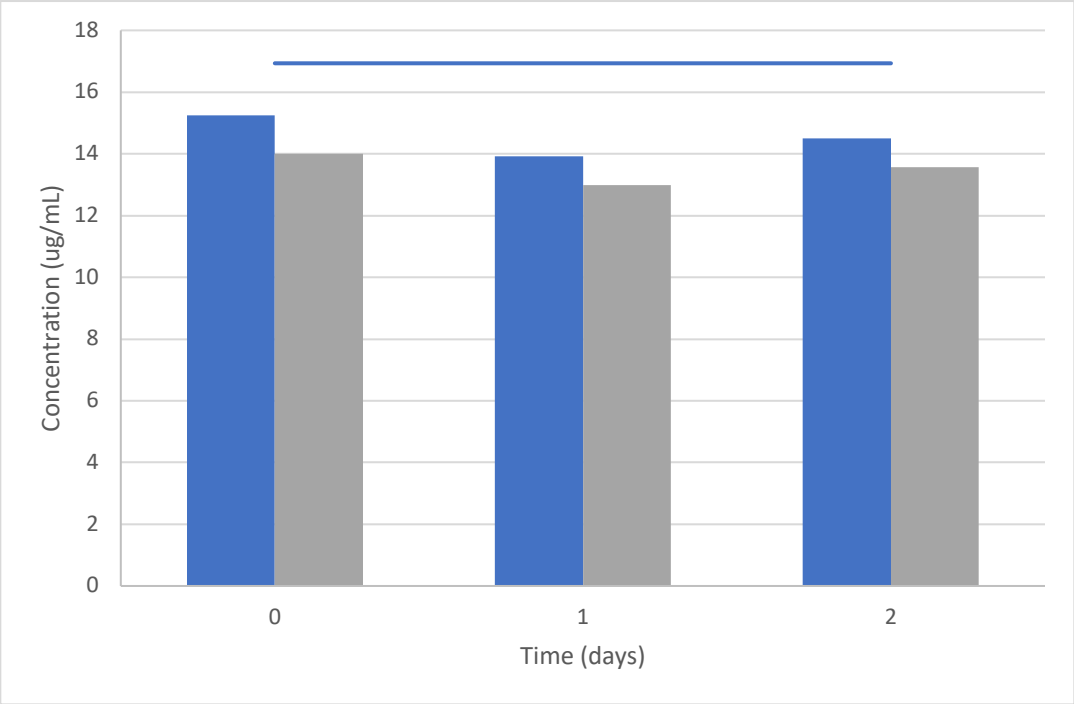

Figure 47: Free ciprofloxacin stability in human serum and a basic environment. Free ciprofloxacin incubated in human serum with 0.1 N sodium hydroxide (n = 2). Based on percent drug weight on the polymer, the expected concentration is 16.933 µg/mL, which is illustrated in the graph as the line above the bars.

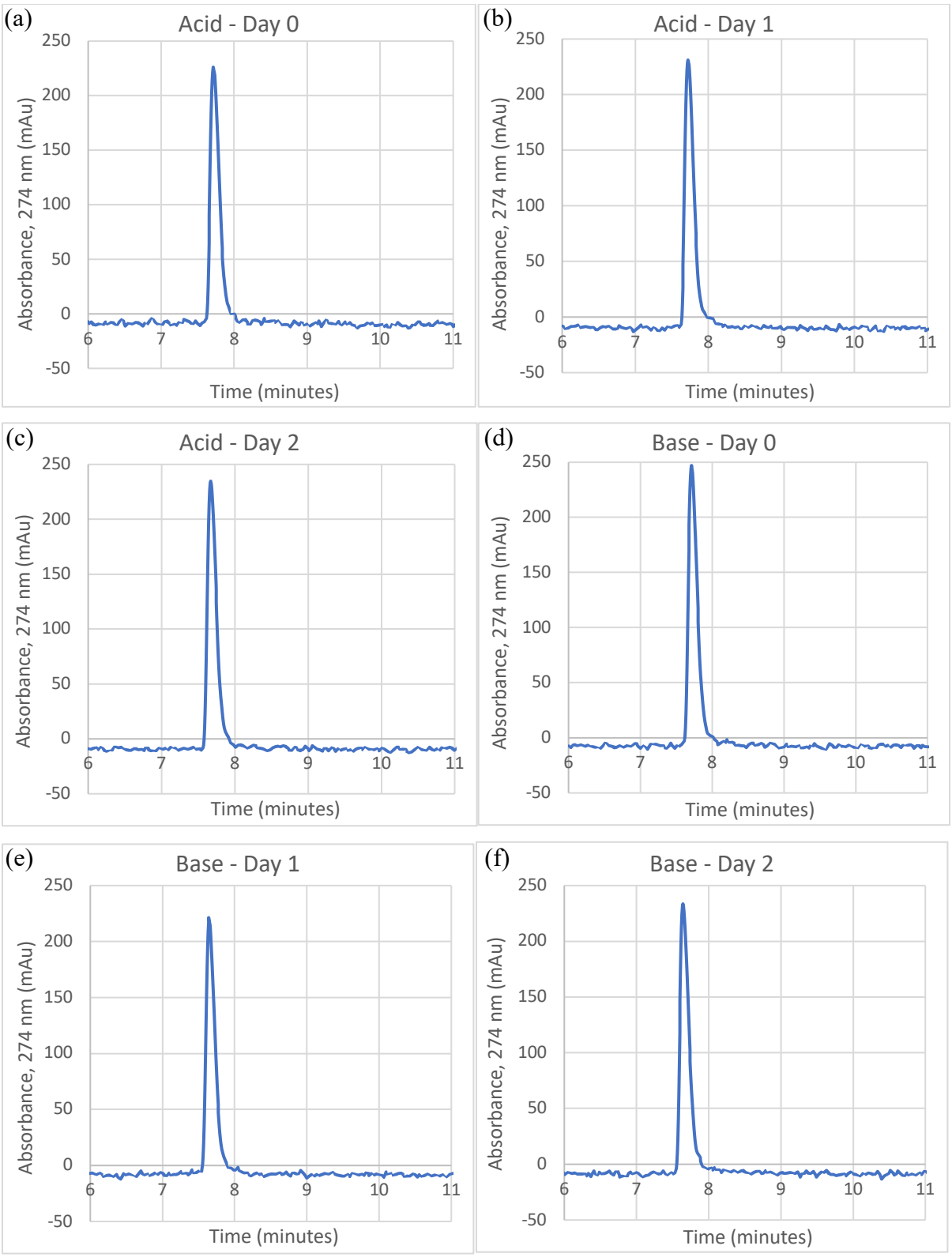

Figure 48: Chromatograms for free ciprofloxacin incubated with human serum under acidic or basic conditions from 6 to 11 minutes. (a, b, c) Chromatograms for sample in acidic conditions at day 0, 1, and 2, respectively. (d, e, f) Chromatograms for samples in basic conditions at 0 hours, 24 hours, and 48 hours respectively.

Chapter 7. ACID AND BASE RELEASE

7.1 INTRODUCTION

As stated above, there have been two approaches for defining 100% release of ciprofloxacin from the drugamer. Acid hydrolysis method involved a 48-hours after incubation in 10% aqueous sulfuric acid [13]. On the other hand, base hydrolysis method involved a 24-hours after incubation in 0.1 N sodium hydroxide [11]. Here these methods will be directly compared to in order to ensure robust methodology and allow for interoperability.

7.2 MATERIALS AND METHODS

7.2.1 Acid and Base Release in PBS

Three separate intermediate dilution solutions were prepared by adding polymeric ciprofloxacin standard (200 μg/mL, aqueous) to PBS (1:1, v/v). Then 10% sulfuric acid, 0.1 N sodium hydroxide, or HPLC grade water (control) was added to the intermediate human serum solution (1:1, v/v). This created the final solutions with a ciprofloxacin concentration of 50 μg/mL. The solutions were kept at room temperature and 2 timed aliquots were taken day 0, 1, 2, and 3 for each condition. 139 μL aliquots were immediately neutralized with 61 μL of either 2 M sodium hydroxide, 0.53% sulfuric acid, or water (control). 800 μL of acetonitrile was then added to promote serum proteins precipitate. Each tube was then briefly vortexed (VWR, Analog Vortex Mixer) and then centrifuged (Beckman Coulter, Microfuge 22R Centrifuge) for 5 minutes at 14,000 rpm. 800 μL of the supernatant was transferred to a fresh tube and stored at -80°C. Once all aliquots are taken, defrost samples were completely dried with a nitrogen dryer (Stuart, Block Heater and Techne, Sample Concentrator). Samples were then resolubilized in 300 μL of HPLC grade water. Samples were then filtered (Millex-GV filter, 0.22 um pore size) with a syringe (Fisher Scientific, 1 mL plastic sterile syringe). The resolubilized sample was then transferred into glass LCMS vials (Agilent Technologies, 2 mL vial) with glass inserts (Agilent Technologies, 250 μL pulled point-conical glass inserts) and caps (Agilent Technologies, 9 mm blue screw). The samples were then run with the optimized LC method (NLPCIP11) along with free ciprofloxacin standards ranging from 3.125 μg/mL to 25 μg/mL in concentration. Assuming

100% recovery and 100% release, the expected concentration of the released ciprofloxacin in the samples is 18.533 μg/mL.

7.2.2 Acid and Base Release in Human Serum

Three separate intermediate dilution solutions were prepared by adding polymeric ciprofloxacin standard (200 μg/mL, aqueous) to human serum (Sigma S7023) (1:1, v/v). Then 10% sulfuric acid, 0.1 N sodium hydroxide, or HPLC grade water (control) was added to the intermediate human serum solution (1:1, v/v). This created the final solutions with a ciprofloxacin concentration of 50 μg/mL. The solutions were kept at room temperature and 2 timed aliquots were taken after day 0,1, and 2 for each condition. 127 μL aliquots were immediately neutralized with 73 μL of either 2 M sodium hydroxide, 0.53% sulfuric acid, or water (control). 800 μL of acetonitrile was then added to promote serum proteins precipitate. Each tube was then briefly vortexed (VWR, Analog Vortex Mixer) and then centrifuged (Beckman Coulter, Microfuge 22R Centrifuge) for 5 minutes at 14,000 rpm. 800 μL of the supernatant was transferred to a fresh tube and stored at -80°C. Once all aliquots are taken, defrost samples were completely dried with a nitrogen dryer (Stuart, Block Heater and Techne, Sample Concentrator). Samples were then resolubilized in 300 μL of HPLC grade water. Samples were then filtered (Millex-GV filter, 0.22 um pore size) with a syringe (Fisher Scientific, 1 mL plastic sterile syringe). The resolubilized sample was then transferred into glass LCMS vials (Agilent Technologies, 2 mL vial) with glass inserts (Agilent Technologies, 250 μL pulled point-conical glass inserts) and caps (Agilent Technologies, 9 mm blue screw). The samples were then run with the optimized LC method (NLPCIP11) along with free ciprofloxacin standards ranging from 3.125 μg/mL to 25 μg/mL in concentration. Assuming 100% recovery and 100% release, the expected concentration of the released ciprofloxacin in the samples is 16.933 μg/mL.

7.3 Results And Discussion

7.3.1 Acid and Base Release in PBS

The aqueous free ciprofloxacin standards were used to create a standard curve, which was then used to determine the concentration of the release ciprofloxacin in the samples incubated in PBS based upon the measure AUC. Percent expected was calculated based on the theoretical expected

concentration of 18.533 µg/mL. In acidic conditions, the percent expected was 16% and 8%, 48% and 30%, 74% and 34%, and 50% and 63% on day 0, 1, 2, and 3, respectively (see Figure 49). In basic conditions, the percent expected was 7% and 6%, 96% and 102%, 94% and 101%, and 94% and 104% on day 0, 1, 2, and 3, respectively (see Figure 50). No release of ciprofloxacin was observed from the polymer incubated in water (control) over the 72 hours. These results suggest that VC-mannose polymer in PBS achieves complete release of the ciprofloxacin when incubated with sodium hydroxide for 24 hours. However, when incubated with sulfuric acid, complete release of the ciprofloxacin was not achieved even after 72 hours. These results are supported by the chromatograms of the samples.

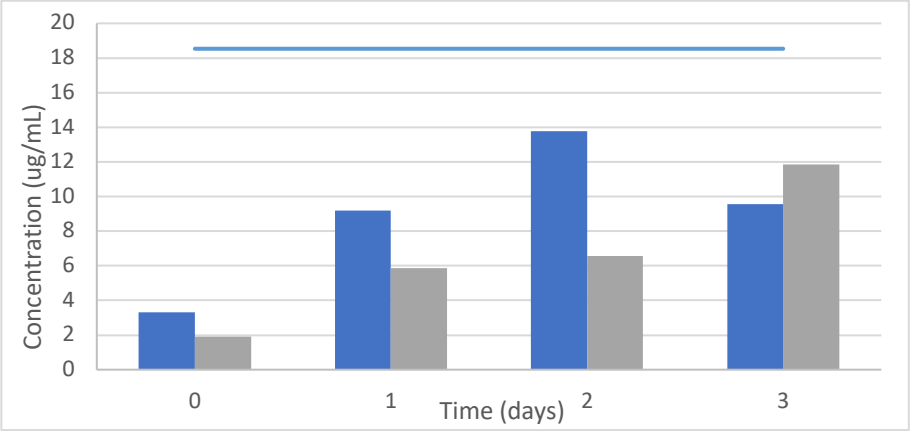

Figure 49: Released ciprofloxacin in PBS and an acidic environment. Polymeric ciprofloxacin incubated in PBS with 10% sulfuric acid (n = 2). Based on percent drug weight on the polymer, the expected concentration is 18.533 µg/mL, which is illustrated in the graph as the line above the bars.

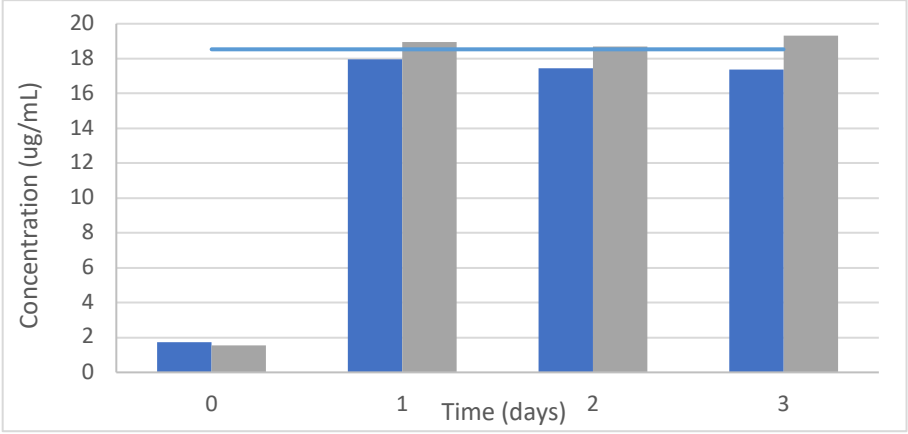

Figure 50: Released ciprofloxacin in PBS and a basic environment. Polymeric ciprofloxacin incubated in PBS with 0.1 N sodium hydroxide (n = 2). Based on percent drug weight on the polymer, the expected concentration is 18.533 µg/mL, which is illustrated in the graph as the line above the bars.

7.3.2 Acid and Base Release in Human Serum

The aqueous free ciprofloxacin standards were used to create a standard curve, which was then used to determine the concentration of the release ciprofloxacin in the samples incubated in human serum based upon the measure AUC (see Figure 51). Percent expected was calculated based on the theoretical expected concentration of 16.933 μg/mL. No released ciprofloxacin was observed in both acidic and basic conditions for day 0. The average concentration (mean ± standard deviation) for the acidic concentration was 4.70 ± 0.06 μg/mL (28% of expected) and 9.47 ± 3.45 μg/mL (56% of expected) on day 1 and 2, respectively. The average concentration for the basic concentration was 18.96 ± 0.60 μg/mL (112% of expected) and 17.00 ± 2.37 μg/mL (100% of expected) on day 1 and 2, respectively. No release of ciprofloxacin was observed from the polymer incubated in water (control) for the entire 48 hours of incubation. Although it was reported that over 100% of the expected released ciprofloxacin was measured after 24 hours, this may be because of an erroneous polymer composition calculation. For example, if the percent drug weight on the polymer was 12% rather than the 11% used to calculation the concentration of ciprofloxacin on the polymer, then the average percent expected for the base condition at 24 hours would be 102.6% ± 3.2%. We therefore believe that it is accurate to conclude that full release was achieved under basic conditions after 24 hours and complete release was not achieved under acidic conditions after 48 hours.

These results are reinforced when observing the chromatograms of the samples (see Figure 52). In the chromatograms of the drugamer incubated under basic conditions, the peak corresponding to the polymer (eluting at roughly 10.3 minutes) is only present at the day 0 time point (see Figure 52 d). Additionally, the peak corresponding to the released ciprofloxacin (eluting at roughly 9 minutes) is present at the day 1 and 2 time points (see Figures 52 e and f). This echoes the results from the calculated concentration that full release is achieved after 24 hours under basic conditions. On the other hand, in the chromatograms of the drugamer incubated under acidic conditions, the peak corresponding to the polymer (eluting at roughly 10.3 minutes) is present at all time points (see Figure 52 a-c). This supports the results from the calculated concentration that complete release of the ciprofloxacin is not achieved even after 48 hours when incubated under acidic conditions.

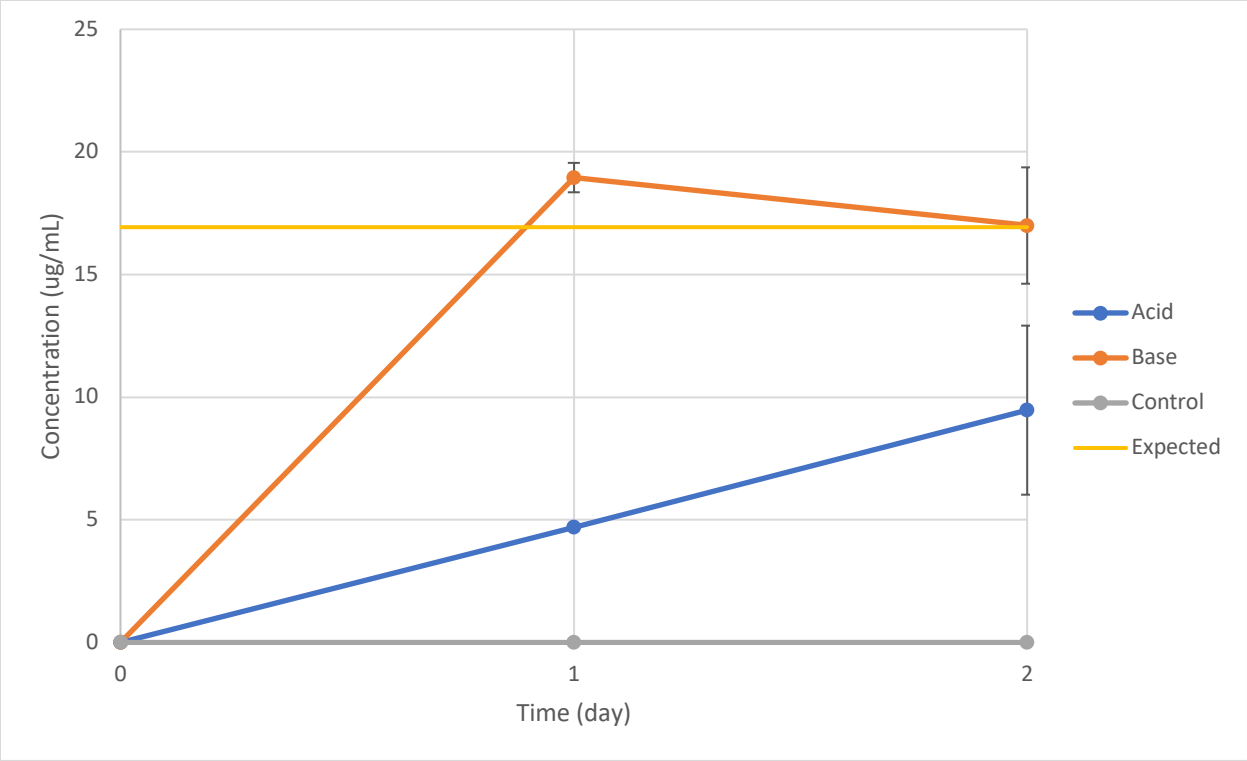

Figure 51: Released ciprofloxacin in human serum and an acidic or basic environment. Polymeric ciprofloxacin incubated in human serum with either 10% sulfuric acid, 0.1 N sodium hydroxide, or water (control). Based on percent drug weight on the polymer, the expected concentration is 16.933 µg/mL. The concentration of the released ciprofloxacin was calculated based on the standard curve and the measured AUC. The error bars represent the standard deviation (n = 3).

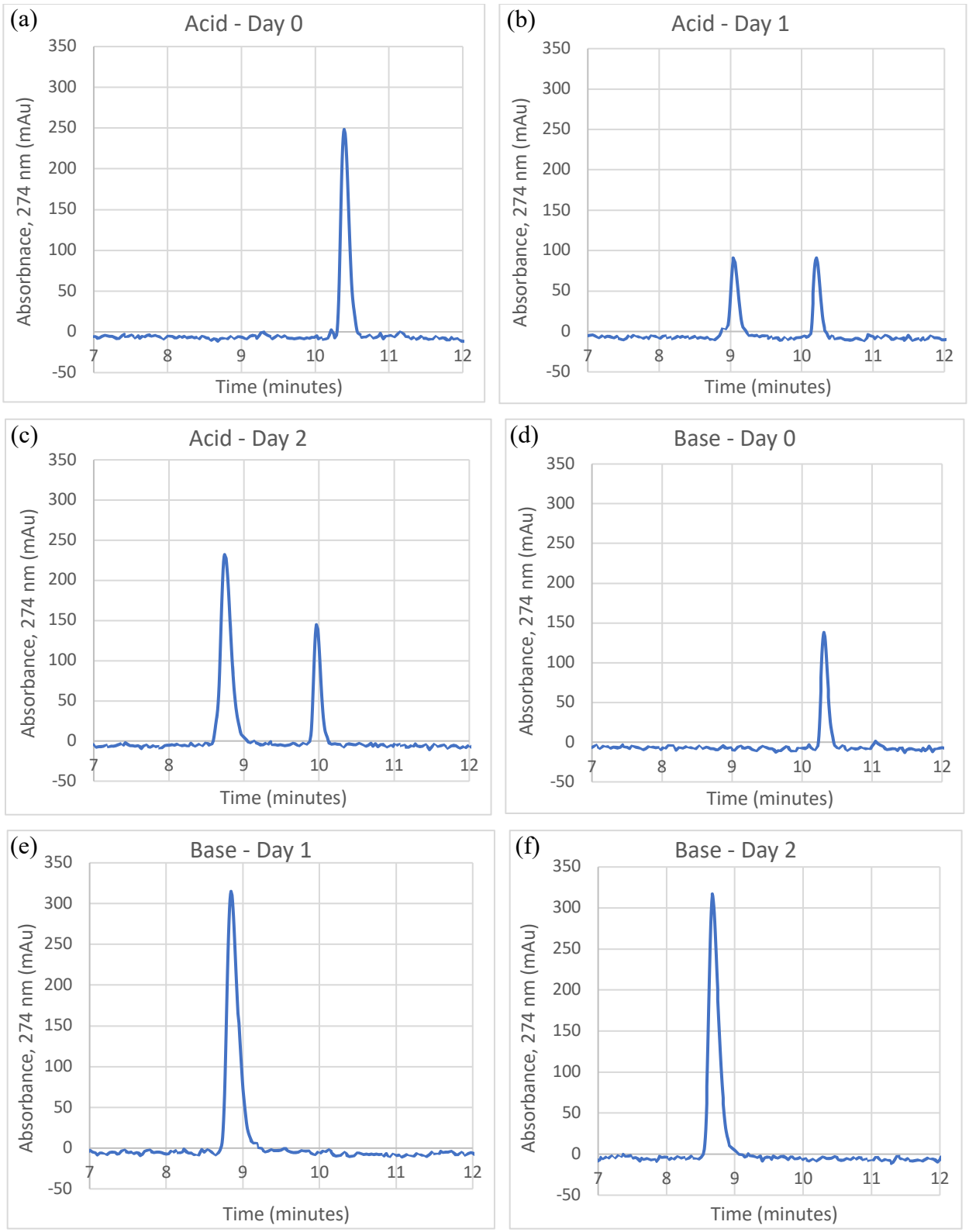

Figure 52: Chromatograms for polymeric ciprofloxacin incubated with human serum under acidic or basic conditions from 7 to 12 minutes. (a, b, c) Chromatograms for acidic conditions at days 0, 1, and 2, respectively. (d, e, f) Chromatograms for basic conditions at days 0, 1, and 2, respectively.

Chapter 8. CONCLUSION

8.1 CORRECTION FACTOR

As mentioned previously, two different methods have been used to define 100% released from the ciprofloxacin drugamer. The acid hydrolysis method defined 100% release through the measured the concentration of free ciprofloxacin after a 48-hour incubation in 10% aqueous sulfuric acid [13]. The base hydrolysis method defined 100% release through the measured concentration of free ciprofloxacin after a 24-hour incubation in 0.1 N sodium hydroxide [11].

Based on the results from the acid and base release and stability experiments (see Chapter 6 and 7), a correction factor that can be applied to results from previously conducted experiments to allow for better interoperability was determined (see Table 10). As no degradation of free ciprofloxacin in acidic and basic environments was found (see Chapter 6), this did not need to be taken into account when analyzing the result from the acid and base release experiments. As it was found that an average of 55.9% $\pm$ 20.4% the free ciprofloxacin was released after 48 hours of incubation in 10% aqueous sulfuric acid (see Chapter 7), the correction factor for the acid hydrolysis method was determined to be 0.56. As it was found that complete release of ciprofloxacin occurred after 24 hours of incubation in 0.1 N sodium hydroxide (see Chapter 7), the correction factor for the base hydrolysis method was determined to be 1.0.

Table 10: Correction factors for the acid and base hydrolysis methods of defining percent release based on the results from the acid and base release experiments.

Method	Used Timed Point	% Expected at 24 hours (Mean $\pm$ SD)	% Expected at 48 hours (Mean $\pm$ SD)	Correction Factor
Acid Hydrolysis	48 hours	27.8% $\pm$ 0.4%	55.9% $\pm$ 20.4%	0.56
Base Hydrolysis	24 hours	111.9% $\pm$ 3.5%	100.4% $\pm$ 14.0%	1.0

8.2 LIMITATIONS OF THE CORRECTION FACTOR

Although correction factors were developed for better interoperability, there are limitations of the developed correction factors. First, the correction factors are dependent on the methodology

used in the acid and base stability and release experiments (see Chapters 6 and 7). The methods described here are not identical to both methods described in the original acid and base hydrolysis methods. For instance, there are difference in the HPLC methods used for analysis, the sample preparation method, and the sample processing methods. Another limitation is the time dependent release of ciprofloxacin from the polymer. As more ciprofloxacin was observed to be released overtime under acidic conditions, any inaccuracy in when the aliquots were taken during experiments (described here and previously conducted experiments using acid hydrolysis to define percent release) would exasperate any inaccuracy in this correction factor. Furthermore, there is potential for an erroneous polymer composition calculation. As full release was not achieved for acid hydrolysis, this could potentially affect the correction factor for the acid hydrolysis method. For example, if the percent drug weight on the polymer was 12% rather than the 11%, then the average percent release would be 51.3% $\pm$ 18.7% at 48 hours. If instead the percent drug weight on the polymer was 10% rather than the 11%, then the average percent release would be 61.5% $\pm$ 22.4%. It should also be noted that there is a high standard deviation for the average percent release for acid hydrolysis at 48 hours. Finally, these correction factors were not made to suggest that the results from previous work are incorrection. Rather, these correction factors were created to allow for more accurate comparisons to be made between experiments using different methods to define percent release.

8.3 Application Of The Correction Factor

The developed correction factors can be applied to the two experiments described in Chapter 1.9. In these two similar experiments, ciprofloxacin polymer with a phenyl ester linker was incubated with model human esterase BChE. However, there were difference in concentration of BChE, the incubation time point used, and method to define percent release (see Table 11). Comparing the reported percent drug released from the phenyl ester polymers, there is a ~10% greater release in the experiment using the acid hydrolysis method despite the shorter time frame and lower concentration of BChE [11, 13]. However, once we have applied the correction factor, in the experiment using acid hydrolysis to define percent release, there was approximately 14% of the ciprofloxacin released after 6 days. Additionally, in the experiment using base hydrolysis to define percent release, there was approximately 15.1% of the ciprofloxacin released after 14

days. These results intuitively make more sense in that more ciprofloxacin was released from the polymer with more time, rather than the opposite occurring.

Table 11: The developed correction factors applied to previously conducted experiments characterizing the released from phenyl ester linker drugamers.

Method	Concentration of BChE	Time Point	% Drug Released	% Drug Released with Correction Factor
Acid Hydrolysis	0.33 µg/mL	6 days	~25%	~14%
Base Hydrolysis	0.5 µg/mL	14 days	~15.1%	~15.1%

8.4 Recommended Method For Defining Percent Drug Release

As mentioned above, the suggested correction factor depends greatly on the utilized method to compare acid and base hydrolysis in Chapter 7. Therefore, based on the results from Chapter 7, here is the recommended methodology for determining percent release in future experiments.

Dilute aqueous 200 µg/mL drugamer standard with human serum (1:1, v/v). Dilute the drugamer solution with 0.1 N sodium hydroxide (1:1, v/v), resulting a solution with a concentration of 50 µg/mL. Incubate the solution for 24 hours at room temperature. Take at least three 127 µL aliquots from the solution and neutralize with 73 µL of 0.53% sulfuric acid. Although this ratio of sample to acid is given, it is recommended to ensure neutralization is occurring by testing the ratio with surrogate aliquots prior to proceeding with this step. To promote protein precipitation, add 800 µL of acetonitrile to each aliquot. Vortex the aliquots and then centrifuge for 5 minutes at 14,000 rpm. Next, transfer 800 µL of each supernatant into new tubes. Completely dry down the supernatants with a nitrogen dryer. Resolubilize the dry samples with 300 uL of water and vortex for 30 seconds. Then pass the resolubilized samples through a filter with 0.22 µm pore size using a sterile syringe into new tubes. Finally, transfer the filter samples into glass LC vials with vial inserts and run on a HPLC with optimized method NLPCIP11 along with free ciprofloxacin standards. The theoretical expected concentration of released ciprofloxacin will be 16.933 µg/mL.

Chapter 9. ACKNOWLEDGEMENTS

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