EVALUATING SOURCES OF PHARMACOKINETIC VARIABILITY USING BREATH TESTING AND MICRODIALYSIS

By

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To my mom, dad, sister, and wife

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ADME	Absorption, Distribution, Metabolism, and Excretion
AUC	Area Under Concentration versus Time Curve
$AUC_{0\text{-}LAST}$	AUC from Zero to Last Time Point
$AUC_{0\text{-}\infty}$	AUC from Zero to Infinity
2-BA	2-Butyl Acetate
BQL	Below Quantification Limit
C_e	Effect Compartment Concentration
C_{MAX}	Maximal Drug Concentration
CRC	Clinical Research Center
CS	Calibration Standard
CV	Coefficient of Variation
DME	Drug Metabolizing Enzymes
E. Coli	*Escherichia coli*
EC_{50}	Concentration at Half-Maximal Effect
EE	Extraction Efficiency
FDA	Food and Drug Administration
HEC	Hydroxyethylcellulose
HIV	Human Immunodeficiency Virus
HQC	High Quality Control concentration
IS	Internal Standard
IOV	Inter-occasion Variability
k_D	Death Rate Constant
k_{MAX}	Maximum Kill-Rate Constant
k_S	Synthesis Rate Constant

k_{SR}	Transfer Rate Constant from Susceptible to Resting Populations
LAMBDA_Z	First-order Elimination Rate Constant
LLOQ	Lower Limit of Quantification
LOD	Limit of Detection
LQC	Low Quality Control concentration
MIC	Minimum Inhibitory Concentration
MQC	Medium Quality Control concentration
MRT	Mean Residence Time
NONMEM®	Nonlinear Mixed Effects Modeling
NSAID	Non-Steroidal Anti-Inflammatory Drug
2-PA	2-Pentyl Acetate
PA	Precision Accuracy batch
PK	Pharmacokinetics
PD	Pharmacodynamics
PPB	Parts per Billion
QC	Quality Control
RPM	Revolutions per Minute
RSE	Relative Standard Error
RT	Retrodialysis
SD	Standard Deviation
SE	Standard Error
T_{MAX}	Time at Maximal Drug Concentration
DWS	Diclofenac Working Stock
WS	Internal Standard Working Solution

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EVALUATING SOURCES OF PHARMACOKINETIC VARIABILITY USING BREATH TESTING AND MICRODIALYSIS

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Pharmacokinetic variability may be caused by genetic and non-genetic factors. Genetic differences can impact drug disposition by affecting the concentrations of drug metabolizing enzymes and/or transporters critical for drug distribution and elimination. Demographic variables, disease states, and drug-drug interactions are known non-genetic causes which may also play a role. Pharmacokinetic variability can lead to differences in drug response. Another important variable leading to variability in drug response is medication adherence. Available methods to measure medication adherence are frequently imprecise or impractical. The availability of a breath test to measure medication adherence in real-time may facilitate medical decision making, improve therapeutic outcomes, and can be used to account for differences in drug response in clinical trials. Pharmacokinetic analyses were performed to evaluate the time-course of breath concentrations for a plethora of volatile markers used to measure definitive adherence. Microdialysis is another sampling technique which uses a semi-permeable membrane to measure free drug concentrations *in vitro* and *in vivo*. *In vitro*, the technique was applied to study the impact of varying albumin concentrations on the protein binding and antimicrobial efficacy of ceftriaxone. *In vivo*, one potential

application of microdialysis is to evaluate topical bioequivalence. With the exception of the vasoconstrictor assay for topical corticosteroids, there is no widely accepted technique which may be used to evaluate bioequivalence for topically applied products. Since microdialysis allows for measurement of free drug concentrations below the skin, it may be a useful technique to compare two products and assess variability in drug absorption. *In vitro* and *in vivo* studies were performed to evaluate the feasibility of using the technique to evaluate bioequivalence using the transdermal patch Flector®.

CHAPTER 1
IMPACT AND SOURCES OF VARIABILITY IN PHARMACOKINETICS

Impact of Variability in Pharmacokinetics

Pharmacokinetics (PK) is a discipline whose aim is to describe the time-course of drug concentrations in the body; in particular, it focuses on absorption, distribution, metabolism and excretion (ADME) processes, all of which play a critical role in dictating how much and how often a drug needs to be administered. PK variability is a common phenomenon which explains, at least in part, why patients respond differently to the same medication. PK variability can be caused by various factors; including differences in genetic and demographic variables (e.g. age, race, and gender), drug-drug interactions with co-administered medications, formulation characteristics, pathological conditions, and circadian rhythms.

The impact of PK variability will depend on the therapeutic index of the drug. For drugs with a narrow therapeutic index, careful dose selection and monitoring is needed. For example, tacrolimus, an immunosuppressive medication with a narrow therapeutic index, can exhibit significant PK variability as a result of differences in the extent of drug absorption and drug metabolism.[1] One study sought to identify sources of PK variability using a population PK analysis conducted with data from 83 adult kidney transplant patients.[2] Using a total of 1,589 trough concentrations, a one-compartment model with first order absorption and elimination was fitted to the data. Two covariates were found to contribute significantly to the variability in tacrolimus's clearance; the number of days post-transplantation and the dosage of a co-administered medication, prednisone. It was noted that clearance values reached 50% of the maximal value after 3.8 (± 0.5) days and a 1.6-fold increase in clearance occurred with prednisone doses greater than

25 milligrams. This example exemplifies how kidney transplantation and co-administration of other medications can affect drug clearance and contribute to variability in drug concentrations.

PK variability observed with medications used in the treatment of human immunodeficiency virus (HIV) has also received significant attention due to the implications that such variability can have on drug efficacy. The importance of identifying sources of variability was shown in a study of 275 HIV-positive patients prescribed nevirapine.[3] Nevirapine is a non-nucleoside reverse transcriptase inhibitor which is administered orally and metabolized by CYP3A4 and CYP2B6. Similar to the tacrolimus example described above, a nonlinear mixed effects modeling approach, using the software NONMEM®, was used to model the population PK of nevirapine. In addition to collecting PK data, the authors genotyped all subjects for genetic variations in *CYP2B6* (516 G>T and 983 T>C); some of which could contribute to variability in the drug's clearance. Indeed, 516T homozygosity and 983C heterozygosity were shown to be important predictors of nevirapine's clearance; in addition to body weight, with a 5% increase in clearance for every 10 kg increase in weight. In this example, data on genetic variants and weight helped explain variability in a nevirapine's PK.

PK variability is known to occur with most drugs; however, determining its impact on drug efficacy will depend on multiple factors. Although not an exhaustive list, here are some factors which one should consider. First, an important variable is a drug's therapeutic index. If at therapeutic concentrations, the risk for toxicity is low and a drug displays a wide therapeutic index, then higher drug concentrations caused by inter- or intra-individual variability will place less of a role. Similarly, if despite significant

variability, drug concentrations remain above some minimal level needed for efficacy, then such variability would be less significant. Second, the drug class and desired efficacy endpoint should be considered. For example, significant PK variability may be more important for an HIV-drug as compared to a medication used in the treatment of nausea; since the risk of adverse effects caused by sub- or supra-therapeutic drug concentrations is greater with the former example. Third, clearly the extent of the variability and the PK properties of the drug are critical factors. For example, for an orally administered drug, inter-individual variability in the extent of absorption is more likely to reach clinical significance for a drug a low oral bioavailability; since small changes can have a profound effect. Last, the time-course of the variability may be important (i.e., short lived vs. constant). If a variable contributing to PK variability is short-lived, then it may be of lesser importance.

Referring back to the tacrolimus and nevirapine examples, significant PK variability can impact drug efficacy. For tacrolimus, one study showed that patients with organ rejection were more likely to have low trough concentrations; with a 55% rejection rate for subjects with trough concentrations between 0 and 10 ng/mL and no rejection episodes observed in patients with a levels of 10-15 ng/mL.[4] For nevirapine, simulations were performed to evaluate the impact of genetic variability in CYP2B6 and weight, on trough concentrations.[3] The simulations showed that individuals with a greater body weight would benefit from a twice daily regimen versus once daily due to a greater risk of sub-therapeutic concentrations with the latter. This increased risk of sub-therapeutic concentrations was negated in subjects who were 516T homozygous or 893C heterozygous due to a lower clearance.

Thus predicting the impact of PK variability is complex as it depends on a multitude of variables. Moreover, when evaluating a patient's response to drug therapy and making dosing recommendations, not only PK, but also sources of pharmacodynamic (PD) variability must be considered.

Important Sources of Pharmacokinetic Variability

Pharmacokinetic Processes

Genetic and non-genetic causes may be responsible for variability in absorption, distribution, metabolism and excretion of drug molecules. The impact of variability in each of these processes will depend on the route of administration and the PK properties of the individual compound.

Absorption

For drugs administered extra-vascularly, variability in the rate and/or extent of absorption can alter the time-course of local and systemic concentrations. Usually a change in the extent of absorption is more likely to reach clinical significance, but this is not necessarily the case when a rapid effect is desired (e.g., analgesics).

Following oral administration, a drug molecule needs to be in solution before it can be absorbed. For passive diffusion to occur, a molecule must also be unionized. As a result, formulation and drug specific-factors which can affect drug dissolution and ionization are important.[5] For example, dipyridamole's absorption has been shown to be erratic; partly due to variability in gastric pH.[6,7] An extended release formulation of dipyridamole and aspirin, uses tartaric acid to provide an acidic local medium and increase the extent of drug absorption. Ketoconazole and itraconazole are also known to exhibit pH-dependent absorption.[8]

Since most drug absorption in the gastrointestinal tract will occur in the small intestine, the rate of gastric emptying, which is altered by food and disease states, will impact the rate of drug absorption. Once at the site of absorption, the physicochemical properties of a drug molecule will dictate its permeability; both across (transcellular) and between (paracellular) cells.[9] Drug transporters (e.g., p-glycoprotein) can contribute to PK variability; often with drugs which exhibit poor permeability across enterocytes. P-glycoprotein is the most widely studied efflux transporter, and genetic variability in its expression has been shown to play an important role in the PK of some drugs (e.g., digoxin).[10–12] In addition to transporters, first-pass metabolism, either in the gut or liver can decrease the extent of drug absorption and thus its oral bioavailability. Such metabolism is also subject to significant inter-individual variability as a result in variable expression of enzymes, demographic characteristics, drug-drug interactions, and disease states.[13]

Distribution

A drug's distribution in the body is dictated by various factors; namely, protein binding, transporter activity, physicochemical properties, and tissue specific properties (e.g., weight, blood flow, composition).[14] Changes in any of these variables can impact tissue distribution and possibly the extent to which a drug molecule reaches its site of action.

The impact of protein binding changes on a drug's PK/PD is a frequent source of debate.[15–24] Its significance will depend on the extent of the binding and a drug's PK properties.[19,24] For drug molecules which bind extensively to plasma and/or tissue proteins, both changes in protein concentrations and binding affinity can result in changes in the unbound drug concentrations. Unbound, free concentrations are

responsible for a drug's pharmacological activity, thus these changes can have a profound impact on drug efficacy. Disease states, including critical illnesses, are known to contribute to variable protein concentrations.[14] For example, renal failure, hepatic insufficiency, and HIV have all been shown to result in changes in albumin or alpha-1 acid glycoprotein concentrations.[25–31] Other factors which may contribute to variable protein concentrations include pregnancy, age, and malnutrition.[32] Alterations in binding affinity may be observed with various disease states, including renal insufficiency, independent of decreases in protein concentrations. This is likely a result of accumulation of various byproducts which are not eliminated properly or a change in the protein structure.[14,32]

Metabolism

Most drugs undergo some degree of biotransformation. Although the liver is the most common site of metabolism, various other organs may be involved (e.g., GI tract, kidneys). In the liver, an alteration in drug metabolism can occur as a result of changes the amount of free drug concentration flowing through the liver, liver perfusion, and/or the concentration of metabolic enzymes responsible for a drug's metabolism. The importance of each of these factors to the hepatic clearance of a drug will depend on the hepatic extraction ratio of the drug. For drugs with a high hepatic extraction ratio (e.g., E_H>0.7), clearance is highly dependent on liver blood flow, and thus changes in protein binding are unlikely to affect hepatic clearance. In contrast, for drugs with a low extraction ratio (e.g., E<0.3), hepatic clearance is dependent on the fraction of total drug which is unbound and the intrinsic clearance of a drug. Thus, low extraction drugs would be susceptible to both protein binding alterations and changes in the intrinsic clearance.

Disease states may be responsible for changes in any of these three factors. For example, hepatic impairment can alter all three variables, while cardiovascular failure could impact drug clearance mostly through a decrease in kidney and liver perfusion.[33] Aside from disease states, drug-drug interactions may also be responsible for changes in intrinsic activity through either inhibition or induction of drug-metabolizing enzymes (DME). Also, inter-individual variability in drug metabolism may be caused by genetic differences in key DME.

Excretion

Frequently drugs undergo biotransformation to a more hydrophilic metabolite, which is then eliminated via the kidneys. Alternatively, a drug can be eliminated unchanged in the urine. For either case, the kidneys are a vital organ involved in drug clearance.

Renal insufficiency can be an important source of PK variability as it can directly impact the elimination of parent compounds and/or their metabolites. Such variability in renal clearance makes dosing of renally eliminated drugs difficult, and increases the risk that a patient will experience adverse effects without proper monitoring.

CHAPTER 2
BREATH TESTING TO ASSESS DEFINITIVE ADHERENCE TO VAGINAL AND ORAL MEDICATIONS

Introduction

Medication adherence refers to the extent to which a patient follows instructions dictated by their physician with regards to a prescribed medication. Many factors can contribute to poor medication adherence; for example, frequency of administration, adverse effects, social status, and poly-pharmacy can all play a role.[34,35] One systematic review of the literature found that patients took between 51 and 71% of their doses; with poorer adherence observed as the total number of daily doses is increased.[36] Another longitudinal study of patients taking antihypertensive medications found that about half of patients had taken a drug holiday (3 or more days off) during the previous year.[37] Moreover, several studies have shown that medication adherence is time dependent, with a longer treatment duration having inferior outcomes.[38,39]

Poor medication adherence can impact the results and conclusions obtained from PK analyses.[40] From a clinical perspective, there are well known consequences of poor medication adherence; which may include poor therapeutic outcomes, increased health care costs, and possibly unwanted side effects. For some conditions (e.g., HIV), near perfect adherence is needed in order to reach favorable outcomes.

Ultimately the impact of poor adherence will depend on the interplay of the PK and PD characteristics of a drug. With regards to the PK, drugs with a longer half-life of elimination will be less impacted by a missed dose as compared to ones with short half-lives.[41] The ratio of a drug's dosing interval and half-life has been referred to as the medication noncompliance impact factor.[41] The smaller the noncompliance impact factor, the less impact a missed dose will have on a drug's PK. Unfortunately, most

drugs have half-lives less than 12 hours.[42] As a result, depending on the dosing frequency, drug concentrations can quickly decrease to sub-therapeutic levels within one day.

The impact of a reduction in drug concentrations will depend on the time-course of drug effects. For some drugs, a drug effect quickly dissipates once drug concentrations fall below some therapeutic range (e.g., analgesics); whereas, for others, drug effects can still be observed despite disappearance of drug concentrations from plasma (e.g., clopidogrel). In the literature, the term "forgiveness" has been used to describe the time with which a drug's effect persists relative to the dosing interval.[42,43] Mathematically this can be described as the difference between the post-dose duration of drug effect (D) and the prescribed dosing interval (I).[42] Thus a long forgiveness can be a result of a long half-life and/or a prolonged PD effect. Ideally the forgiveness is large, reducing the impact of poor adherence. Drugs with a long forgiveness are less susceptible to the adverse effects caused by poor medication compliance.

In practice, obtaining an accurate assessment of medication adherence is difficult and frequently employed measures (e.g., prescription fills, medication diaries) have numerous drawbacks. Methods available to determine *definitive* adherence (e.g., directly observed therapy) are costly and impractical for most disease states. The availability of a breath test to measure medication adherence in real-time, either at home or in a clinic, may facilitate medical decision making and improve therapeutic outcomes. The goal of the analyses described herein was to characterize the PK of two volatile markers and their metabolites. The information gained from these analyses

would aid in the development of a breath test which can be used to assess definitive adherence to oral and vaginal medications.

Methods

Several pilot studies were conducted in order to evaluate the feasibility of using a breath test to assess definitive adherence for vaginal and oral products. In these studies we sought to characterize the PK for two volatile metabolites, 2-pentyl acetate and 2-butyl acetate, as well as their volatile metabolites; namely, 2-pentanol, 2-butanol, 2-pentanone, and 2-butanone. These metabolites are produced in a sequential fashion, where the parent compound is converted to the alcohol metabolite (i.e., 2-pentanol and 2-butanol) and then to ketone metabolite (i.e., 2-pentanone and 2-butanone).

Vaginal Adherence Studies

Two pilot studies were conducted to evaluate the use of a breath test to evaluate adherence to a vaginal product. Study one was conducted at University of Florida and was designed to measure the concentrations of volatile markers following application in a vaginal gel.[44] The ester taggants, 2-pentyl acetate (2-PA) and 2-butyl acetate (2-BA), were formulated in two types of gel, hydroxyethylcellulose (HEC) and tenofovir (TNV) placebo gel. While two additional esters were tested (isopropyl butyrate and 2-pentyl butyrate), these could not be quantified in breath and thus are not described further. The HEC gel was selected because it is commonly used in the formulation of vaginal gels, while the TNV placebo gel was selected to assess adherence for a microbicide formulation containing no active drug. Eight volunteers completed a total of eight 1-hour visits; where 8 separate vaginal formulations (4 esters, 2 formulations) were applied. Following application, breath samples were collected at 0, 1, 2, 3, 4, 5, 7.5, 10, 20, 30, 40, 50, and 60 minutes using a 5 L Tedlar bag and analyzed using a miniature gas

chromatograph and/or gas chromatography-mass spectroscopy. Ester, alcohol, and ketone concentrations were quantified.

Study two was conducted at University of California San Francisco. This study was a double-blind randomized study which enrolled 13 subjects. Again, 2-PA and 2-BA were added to a TNV placebo gel (with no active ingredient) and HEC placebo gel, respectively. The TNV placebo gel was applied to the vagina using a 5 mL syringe applicator. The HEC gel was used as a lubricant on a condom and applied into the vagina with a dildo (15 thrusts). Subjects were randomized to tagged or untagged products (5:1). Each subject came in for two visits (at least 1 day apart); where TNV placebo gel (with or without 2-PA) and HEC gel (with or without 2-BA) were applied, respectively. Sequential breath samples were collected for 75 minutes.

For both studies, analyte breath concentrations reported in parts by billion (ppb) were converted to ng/ml by multiplying each concentration by the molecular weight of the respective molecule. Once all data analysis was completed, results were reported in ppb units. A noncompartmental PK analysis was conducted using WinNonlin (Version 5.2; Pharsight Corporation, St. Louis, MO). Estimates were generated for the following PK parameters: first-order elimination rate constant (Lambda_Z, $minutes^{-1}$), half-life of elimination (Half-life, minutes), maximal drug concentration (C_{MAX}, ppb), time at maximal drug concentration (T_{MAX}, minutes), area under concentration versus time curve from zero to the last time point ($AUC_{0\text{-}LAST}$, min*ppb), area under concentration versus time curve from zero to infinity ($AUC_{0\text{-}\infty}$, min*ppb), percentage of area under concentration versus time curve from zero to infinity which is extrapolated from $AUC_{0\text{-}t}$ (% AUC Extrap) and the mean residence time (MRT, minutes). The area under concentration

versus time curve (AUC) was calculated using the linear trapezoidal rule. All values are reported as mean±SD.

For the first study, statistical analyses were conducted using SAS (Version 9.2; Cary, NC). Since all subjects received each treatment, a paired t-test was used to compare differences between groups. First, we evaluated whether there were any significant differences between the HEC and TNV gel, for each respective molecule. Second, we evaluated whether there were any differences in the PK parameters between the two taggants, for each respective gel type. The latter comparison was conducted in an effort to determine which molecule would be more favorable for use in future studies. An alpha level of 0.05 was used to evaluate statistical significance. All plots were generated using the packages lattice and grid in R (Version 2.12.2).[45]

Oral Adherence Studies

Two studies, denoted as studies three and four, were conducted at University of Florida and sought measure the levels of these flavorants and their metabolites following oral administration. In study three, five fasting, healthy subjects were administered a size zero hard gel capsule (Capsugel, Inc., Greenwood, SC) containing 2-butanol (60 mg), 2-pentanone (60 mg), and L-carvone (30 mg) on six different occasions (i.e., replicates). Subjects directly exhaled into a miniature gas chromatograph (Xhale, Inc., Gainesville, FL), which requires only 10 mL of human breath for analysis. Breath concentrations of 2-butanone and 2-pentanone were determined at 0, 5, 10, 15, 20, 30, 45, and 60 minutes post-ingestion of the capsule.

First, a non-compartmental PK analysis was conducted as previously described. Second, a population pharmacokinetic analysis was conducted to describe the inter- and intra-individual variability, as well as the inter-occasion variability (IOV) using the

software NONMEM® (Version 7.2, Icon Development Solutions, Ellicott City, Maryland). All models were fitted using a first-order conditional estimation method (FOCE) with interaction and the subroutine ADVAN2 TRANS1. Inter-individual variability was incorporated using an exponential function, whereas an additive error model was used for the residual error. It was assumed that the IOV did not vary between visits. During model development, initially no IOV was added, and then it was added to each parameter in a step-wise fashion. The following equation was used to model the inter-individual and IOV for two parameters denoted as K1 and K2:

$$K1 = TVK1 \times EXP(\eta_1 + IOV_{k1})$$

$$K2 = TVK2 \times EXP(\eta_2 + IOV_{k2})$$

η_i, which is used to describe the inter-individual variability, is a normally distributed random variable with mean zero and standard deviation ω^2. Perl-speaks-NONMEM® (PsN, Version 3.4.2) was used for NONMEM® submission, while Wings for NONMEM® (Version 720) facilitated bootstrapping of the final model.[46,47] One thousand bootstrap runs were performed and 95% confidence intervals were calculated using the 2.5th and 97.5th percentile as the lower and upper bound of the bootstrap distribution, respectively. Last, the lattice and Xpose (Version 4.3.3) packages in the software R (Version 2.12.2) were used for generation of graphics and for model diagnostic purposes.[48] All collected data was included during model development. Nested models were compared using the objective function values, goodness of fit plots, and visual predictive checks. Using a chi-square distribution, a decrease in the objective function value of 10.83 ($P<0.001$, 1 degree of freedom) was used to assess statistical significance.

Parameter estimates were obtained for the first-order absorption rate constant (minutes^{-1}), first-order elimination rate constant (minutes^{-1}), and the volume of distribution (L). Although the latter value was estimated, because the concentrations were measured in breath, the parameter estimate has no real physiological meaning. The two first-order rate constants were denoted as K1 and K2 and not assigned to absorption or elimination in the results section because without intravenous data it is difficult to determine whether a “flip-flop” scenario exists. Pentanone was administered and measured in breath directly (i.e., no metabolic conversion is needed); while for butanone, 2-butanol was administered. Since only 2-butanone and 2-pentanone levels were quantified and the conversion to the ketone occurs very quickly (~5 minutes), ketone concentrations were modeled independently. The oral bioavailability for both compounds was assumed to be 100%.

In study four, seven subjects consumed a gelatin capsule (size 0, Capsugel, Inc., Greenwood, SC) containing 2-butanol (40 mg); then breath samples were collected at 0, 0.5, 2, 4, 5, 6, 7, 8, 9, 10, 12.5, 15, 17.5, 20, 25, 30, 45 and 60 minutes following ingestion. Butanone concentrations were quantified using gas chromatography-mass spectrometer. A noncompartmental PK analysis was conducted using WinNonlin (Version 5.2; Pharsight Corporation, St. Louis, MO). Estimates for all relevant parameters were calculated.

Results

Vaginal Adherence Studies

For study one, the PK parameters generated for 2-BA and 2-PA are shown in Table 2-1. When comparing the HEC and TNV gels, for each respective molecule, significant differences were observed in the C_{MAX} for BA and in the elimination rate

constant (Lambda_Z) for 2-PA. When the parameters for 2-BA and 2-PA were compared, within the same gel type, several significant differences were observed. For the HEC containing gels, the elimination rate constant, C_{MAX}, $AUC_{0-\infty}$ and AUC_{0-LAST} were higher for 2-BA, whereas the mean residence time (MRT) were significantly higher for 2-PA. Similar significant differences were also observed with the TNV containing gels, and in addition, the half-life of elimination was significantly longer for 2-PA.

The PK parameters for the alcohol and ketone metabolites of 2-BA and 2-PA are shown in Tables 2-2 and 2-3. When comparing the differences between the HEC and TNV gels for 2-butanol, half-life was significantly greater in the TNV containing gels, whereas C_{MAX} was greater with the HEC gel. For 2-pentanol, AUC_{0-LAST} was significantly higher for the HEC gel. T_{MAX}, the time at maximal concentration, was the only parameter which differed between 2-butanol and 2-pentanol (significantly greater for 2-pentanol in HEC gel). There were no significant differences between 2-butanone and 2-pentanone, regardless of the gel type. For 2-butanone specifically, C_{MAX} and AUC_{0-LAST} were higher for the HEC gel. Similarly, for 2-pentanone, AUC_{0-LAST} was significantly greater for the HEC gel.

For the second study, estimates of the PK parameters for 2-PA, 2-pentanol, and 2-pentanone are shown in Table 2-4. Figures 2-1, 2-2, and 2-3 depict the breath concentration versus time profiles for each of these three molecules, respectively. For 2-PA, in most subjects, breath concentrations reached a maximum within 15 minutes and rapidly declined with a half-life of 25.2 (± 8.9) minutes. In general, the concentrations of the two metabolites, 2-pentanol and 2-pentanone, were lower and persisted for a longer period of time than the parent compound; although the short

sampling time increases the variability in the estimates for these two molecules. PK parameter estimates for 2-BA measured in breath following application through a condom are shown in Table 2-5; while the breath concentration versus time profile is depicted in Figure 2-4. For all subjects, 2-BA was rapidly absorbed and concentrations were detectable in breath within 5 minutes. Upon reaching a maximum, 2-BA concentrations declined rapidly with a half-life of approximately 6 (± 4.7) minutes. Concentrations of 2-butanol and 2-butanone were not detected for any subject following condom application.

Oral Adherence Studies

In study three, all five subjects successfully completed a total of six independent studies (i.e., replicates). For all 30 visits, 2-butanone and 2-pentanone could be quantified in breath using the miniature gas chromatograph. The concentration-versus-time plots for each respective molecule stratified by subject are depicted in Figure 2-5.

The PK parameter estimates from the non-compartmental analysis for 2-butanone and 2-pentanone are shown in Tables 2-6 and 2-7, respectively. In most cases, 2-butanone and 2-pentanone concentrations could be quantified in breath within 5 minutes post-ingestion and were still detectable at 60 minutes. When the data for all 30 studies is averaged, the elimination half-life for 2-butanone and 2-pentanone is 21.6 (±8) and 23.7 (±9.1) minutes, respectively. Average C_{MAX} and T_{MAX} were 1376 (± 819.8) ppb and 17.8 (± 8.8) minutes for 2-butanone; and similar values were observed for 2-petanone (1,424.2 (± 741.3) ppb and 14.8 (± 8) minutes). Within each subject, when the six replicates were averaged, 2-butanone $AUC_{0\text{-}LAST}$ varied between 9,582.4 and 68,147.9 min*ppb. Similar variability in drug exposure ($AUC_{0\text{-}LAST}$) was observed for 2-pentanone (13,782.3 – 68,007.7 min*ppb).

In the population PK analysis, a one-compartment body model with first-order absorption described the PK of both 2-butanone and 2-pentanone. The goodness-of-fit plots showing the individual and population predicted values showed no obvious trend for either molecule (Figure 2-6). The conditional weighted residuals were within an acceptable range (-2 to 2) for 95% of the points for both molecules (Figure 2-7). Individual plots stratified by both subject and replicate are shown in Figures 2-8 and 2-9. Visual predictive checks for both molecules helped verify that a one-compartmental body model described the data reasonably well (Figures 2-10 and 2-11).

For 2-butanone specifically, the typical model estimates for the two first-order rate constants (K1 and K2) were 0.129 and 0.034 $minutes^{-1}$, respectively (Table 2-8). Inter-individual variability was moderate and more pronounced for K1 (73.7% K1 and 39% K2); whereas the opposite was true for the IOV (27.7% K1 and 62.2% K2). For 2-pentanone, the typical model estimates for K1 and K2 were 0.078 and 0.061 $minutes^{-1}$, respectively. A similar pattern was observed with the inter-individual and IOV. Inter-individual variability was more pronounced for K1 (56.5% K1 and 38.7% K2), but the opposite was true for the inter-occasion variability (36.3% K1 and 85.6% K2).

For the second oral adherence study, PK parameter estimates for 2-butanone are shown in Table 2-9. Breath concentrations of 2-butanone were detectable at 5 minutes for most subjects and the time at maximal concentration (T_{MAX}) was 6.57 (1.51) minutes. The half-life of the molecule is 10.9 (9.61) minutes; thus demonstrating a rapid elimination from breath, with undetectable levels observed at 60 minutes in four subjects. The mean residence time for a 2-butanone molecule is estimated to be 12.89 (5.95) minutes.

Discussion

The results of these pilot studies demonstrate that 2-butyl acetate, 2-pentyl acetate, and their metabolites can be measured in breath following vaginal and oral administration. In most cases, detectable concentrations were observed as early as 5 minutes and up to 60 minutes. The availability of a validated method which uses a portable device for detection of these volatile markers can allow for a real-time assessment of medication adherence.

Considerable PK variability was observed during each study, which could be attributed to various factors. For example, there is inherent variability in PK processes between individuals. In this study, variability can result from differences in drug absorption, distribution to the lungs, and elimination from the gas phase of exhaled breath. Second, there can be variability caused by the technique itself. Last, assay variability can be another source of variability in the concentrations measured.

In the study conducted in five subjects with six replicates each (study three), for the non-compartmental analysis, the observed variability in drug exposure is likely a result of differences in both clearance and absorption. When comparing the results from the noncompartmental and population PK analyses for 2-butanone, one could conclude that the estimate for K1 likely represents first-order absorption rate constant, whereas K2 is the first-order elimination rate constant. If this is true, then for 2-butanone, the results suggest that a larger portion of the inter-individual variability is caused by differences in absorption, whereas there is greater IOV in the elimination. The extent of the variability was similar for 2-pentanone, although estimates for the two first-order rate constants were almost the same.

Despite the observed PK variability, in all 30 visits, 2-pentanone and 2-butanone concentrations could be quantified. For the development of a medication adherence system, documenting the presence of one of these two exogenous molecules would suffice to document definitive adherence to a medication.

Table 2-1. Pharmacokinetic parameters for the parent taggants (2-butyl acetate or 2-pentyl acetate) in human breath (n=8) following vaginal administration. The taggants were formulated in either hydroxyethylcellulose (HEC) or tenofovir placebo (Placebo) gels.

	2-butyl acetate		2-pentyl acetate	
Parameter	HEC	Placebo	HEC	Placebo
LAMBDA Z ($minutes^{-1}$)	0.05 (0.03)[b]	0.04 (0.01)[b]	0.02 (0.01)	0.02 (0.01)[a]
Half-life (minutes)	18.0 (7.1)	19.4 (4.5)	61.8 (50.3)	33.7 (15.5)[b]
C_{MAX} (ppb)	590.3 (261.2)[a,b]	381.6 (105.0)[b]	100.4(49.6)	94.23 (39.5)
T_{MAX} (minutes)	8.1 (2.6)	8.4 (4.8)	9.4 (4.2)	8.8 (4.4)
$AUC_{0\text{-}LAST}$ (minutes*ppb)	15011.0 (7540.9)[b]	10782.8 (2821.8)[b]	3540.8 (2053.4)	3220.8 (1954.5)
$AUC_{0\text{-}\infty}$ (minutes*ppb)	17033.3 (8229.3)[b]	12420.7 (3071.0)[b]	7216.3 (5595.0)	4968.5 (4010.7)
% AUC Extrap	11.4 (7.4)	13.4 (7.2)	45.0 (19.3)[a,b]	28.3 (12.5)[b]
MRT (minutes)	20.5 (4.0)	22.6 (1.7)	24.8 (2.5)[b]	25.3 (3.2)[b]

*The data is reported as the mean with standard deviation in parentheses. $P<0.05$ was used to evaluate statistical significance.

[a]HEC compared to placebo gel.

[b]2-butyl acetate and 2-pentyl acetate for a respective gel.

Table 2-2. Pharmacokinetic parameters for the alcohol metabolites (2-butanol or 2-pentanol) in human breath (n=8) following vaginal administration. The taggants were formulated in either hydroxyethylcellulose (HEC) or tenofovir placebo (Placebo) gels.

	2-butanol		2-pentanol	
Parameter	HEC	Placebo	HEC	Placebo
LAMBDA Z ($minutes^{-1}$)	0.03 (0.03)	0.02 (0.01)	0.02 (0.018)	0.02 (0.02)
Half-life (minutes)	29.4 (12.9)	54.1 (23.8)[a]	61.9 (36.4)	57.0 (35.4)
C_{MAX} (ppb)	42.3 (18.5)[a]	21.8 (12.7)	36.5 (22.9)	27.3 (29.0)
T_{MAX} (minutes)	9.4 (3.2)	24.4 (21.9)	19.4 (12.1)[b]	21.4 (18.4)
$AUC_{0\text{-}LAST}$ (minutes*ppb)	1279.7 (878.8)	804.3 (431.0)	1460.9 (799.9)[a]	954.4 (742.7)
$AUC_{0\text{-}\infty}$ (minutes*ppb)	2052.7 (1557.2)	1858.1 (974.6)	3788.8 (3106.0)	3623.9 (4223.1)
% AUC Extrap	35.9 (11.6)	53.5 (15.4)[a]	52.0 (21.0)	46.8 (28.2)
MRT (minutes)	23.7 (6.2)	28.4 (5.9)	29.5 (3.1)	29.9 (4.7)

*The data is reported as the mean with standard deviation in parentheses. $P<0.05$ was used to evaluate statistical significance.

[a]HEC compared to placebo gel.

[b]2-butanol and 2-pentanol for a respective gel type.

Table 2-3. Pharmacokinetic parameters for the ketone metabolites (2-butanone and 2-pentanone) in human breath (n=8) following vaginal administration. The taggants were formulated in either hydroxyethylcellulose (HEC) or tenofovir placebo (Placebo) gels.

	2-butanone		2-pentanone	
Parameter	HEC	Placebo	HEC	Placebo
LAMBDA Z ($minutes^{-1}$)	0.02 (0.02)	0.01 (0.01)	0.01 (0.01)	0.02 (0.01)
Half-life (minutes)	56.9 (33.8)	64.5 (27.6)	328.9 (466.8)	68.9 (50.1)
C_{MAX} (ppb)	52.1 (21.1)[a]	28.1 (15.8)	48.6 (35.5)	33.4 (30.8)
T_{MAX} (minutes)	18.8 (16.9)	31.3 (24.9)	26.3 (6.9)	38.8 (22.0)
$AUC_{0\text{-}LAST}$ (minutes*ppb)	2204.4 (1108.6)[a]	1165.6 (631.9)	2111.7 (1511.5)[a]	1271.9 (1048.6)
$AUC_{0\text{-}\infty}$ (minutes*ppb)	4916.7 (3231.5)	3419.9 (2590.4)	16920.0 (19512.2)	2442.7 (1879.2)
% AUC Extrap	44.5 (20.6)	57.1 (11.5)	72.1 (20.4)	50.6 (21.8)
MRT (minutes)	28.6 (4.0)	31.4 (2.6)	32.4 (2.8)	33.6 (4.3)

*The data is reported as the mean with standard deviation in parentheses. P<0.05 was used to evaluate statistical significance.
[a]HEC compared to placebo gel.

Table 2-4. Pharmacokinetic parameters for the appearance 2-pentyl acetate, 2-pentanol, and 2-pentanone in human breath (n=13) following vaginal administration. The taggant was formulated in tenofovir placebo (Placebo) gel.

	2-pentyl acetate	2-pentanol	2-pentanone
LAMBDA_Z ($minutes^{-1}$)	0.031 (0.012)	0.008 (0.006)	0.009 (0.007)
Half-life (minutes)	25.2 (8.9)	195.2 (254.9)	155.9 (159.2)
C_{MAX} (ppb)	270.4 (218.2)	56.7 (28.4)	66.6 (41.8)
T_{MAX} (minutes)	14.4 (12.1)	23.3 (7.9)	46.7 (15.8)
$AUC_{0\text{-}LAST}$ (minutes*ppb)	7881.2 (4757.1)	3140.2 (1494.1)	3673.5 (2239.6)
$AUC_{0\text{-}\infty}$ (minutes*ppb)	10047.3 (6558.4)	15927.8 (23291.5)	18669.5 (17266.9)
% AUC Extrap	18.6 (8.9)	61.2 (23.4)	63.4 (23.6)
$MRT_{0\text{-}last}$ (minutes)	30.1 (4.3)	37.0 (2.0)	42.4 (2.7)

*The data is reported as the mean with standard deviation in parentheses.

Table 2-5. Pharmacokinetic parameters for the appearance 2-butyl aceate in human breath (n=13) following condom application. The taggant was formulated in a hydroxyethylcellulose (HEC) gel.

	2-Butyl Acetate
LAMBDA_Z ($minutes^{-1}$)	0.156 (0.079)
Half-life (minutes)	6.0 (4.7)
C_{MAX} (ppb)	49.9 (49.9)
T_{MAX} (minutes)	6.4 (3.2)
$AUC_{0\text{-}LAST}$ (minutes*ppb)	412.4 (445.1)
$AUC_{0\text{-}\infty}$ (minutes*ppb)	499.3 (523.4)
% AUC Extrap	19.1 (15.1)
$MRT_{0\text{-}last}$ (minutes)	8.0 (2.3)

*The data is reported as the mean with standard deviation in parentheses.

Table 2-6. Pharmacokinetic parameters for 2-butanone following oral administration (n=5, 6 replicates each).

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5
LAMBDA_Z (minutes^{-1})	0.033 (0.008)	0.034 (0.005)	0.024(0.008)	0.043 (0.010)	0.046 (0.017)
Half-life (minutes)	22.6 (6.1)	20.6 (3.2)	30.7 (9.0)	16.4 (3.4)	17.5 (8.9)
C_{MAX} (ppb)	1296.6 (450.7)	1926.2 (239.9)	1020.7 (710.0)	2238.2 (708.6)	398.0 (257.9)
T_{MAX} (minutes)	21.7 (10.3)	20.8 (4.9)	11.7 (10.8)	18.3 (8.2)	16.7 (7.5)
$AUC_{0\text{-}LAST}$ (minutes*ppb)	45297.5 (16577.2)	66465.5 (5168.5)	19607.5 (11598.9)	68147.3 (18757.5)	9581.2 (7641.1)
$AUC_{0\text{-}\infty}$ (minutes*ppb)	59105.5 (19880.7)	82627.9 (10044.4)	25623.4 (15158.8)	77592.6 (19556.2)	10998.5 (8575.8)
% AUC Extrap	22.6 (11.0)	19.2 (3.5)	23.2 (4.7)	12.58 (3.3)	12 (10.5)
$MRT_{0\text{-}last}$ (minutes)	29.3 (5.9)	27.7 (1.6)	22.7 (5.5)	26.2 (3.2)	22.9 (3.6)

*The data is reported as the mean with standard deviation in parentheses.

Table 2-7. Pharmacokinetic parameters for 2-pentanone following oral administration (n=5, 6 replicates each).

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5
LAMBDA_Z (minutes^{-1})	0.029 (0.007)	0.030 (0.004)	0.023 (0.009)	0.040 (0.08)	0.042 (0.015)
Half-life (minutes)	24.8 (5.6)	23.2 (2.6)	34.6 (13.3)	17.9 (3.5)	17.8 (5.2)
C_{MAX} (ppb)	1328.2 (395.9)	1849.5 (180.0)	1334.0 (859.2)	2072.4 (696.6)	536.4 (290.3)
T_{MAX} (minutes)	20.0 (9.5)	15.8 (4.9)	10.0 (10.0)	15.0 (7.7)	13.3 (6.1)
$AUC_{0\text{-}LAST}$ (minutes*ppb)	49094.4 (17665.2)	68007.7 (6248.7)	31020.6 (18913.3)	66163.9 (17201.9)	13782.7 (9108.3)
$AUC_{0\text{-}\infty}$ (minutes*ppb)	65626.4 (21988.9)	87240.2 (9621.5)	45946.8 (34359.7)	76449.6 (19188.4)	15806.3 (10707.1)
% AUC Extrap	24.5 (9.7)	21.9 (2.6)	27.1 (9.1)	13.6 (2.7)	11.1 (5.5)
$MRT_{0\text{-}last}$ (minutes)	28.4 (4.9)	27.3 (1.4)	23.7 (4.6)	25.6 (2.6)	22.2 (3.4)

*The data is reported as the mean with standard deviation in parentheses.

Table 2-8. Population parameter estimates from the final model and bootstrap analysis.

	2-Butanone		2-Pentanone	
Parameter And Model	Estimate (SD Scale) ±SE From Final Model	Bootstrap Estimate (95% CI)[a]	Estimate (SD Scale) ±SE From Final Model	Bootstrap Estimate (95% CI)[b]
Structural Model				
K1 ($minutes^{-1}$)	0.13 ± 0.04	0.13 (0.07-0.25)	0.08 ± 0.02	0.08 (0.05-0.11)
K2 ($minutes^{-1}$)	0.04 ± 0.01	0.04 (0.01-0.06)	0.06 ± 0.02	0.07 (0.04-0.11)
V (L)[c]	0.15 ± 0.02	0.18 (0.12-0.33)	0.19 ± 0.04	0.20 (0.16-0.27)
Variance Model				
Ω^2 (K1)	0.54 (73.7%) ± 0.31	0.55 (0-0.89)	0.32 (56.5%) ± 0.24	0.48 (0-0.70)
Ω^2 (K2)	0.15 (39.0%) ± 0.14	0.46 (0-1.63)	0.15 (38.7%) ± 0.12	0.27 (0-0.60)
IOV (K1)	0.08 (27.7%) ± 0.64	0.28 (0.11-0.55)	0.13 (36.3%) ± 0.10	0.37 (0.20-0.54)
IOV (K2)	0.39 (62.2%) ± 0.20	0.71 (0.39-1.44)	0.73 (85.6%) ± 0.29	0.86 (0.40-1.53)
Residual Error (ppb)	264.87 ± 61.02	256.55 (147.0 – 368.88)	222.92 ± 35.06	218.27 (146.29 – 282.13)

[a]2-Butanone: 1,000 runs; 98.8% of runs with successful minimization; 53.5% of runs with successful covariance step.
[b]2-Pentanone: 1,000 runs; 98.3% of runs with successful minimization; 64.3% of runs with successful covariance step
[c]No physiological meaning as drug concentrations were measured in breath.

Table 2-9. Pharmacokinetic parameters for exhaled 2-butanone from subjects (n=7) after orally consuming 2-butanol (40 mg).

Parameter	Value
LAMBDA_Z ($minutes^{-1}$)	0.21(± 0.26)
Half-life (minutes)	10.9 (± 9.6)
C_{MAX} (ppb)	548 (± 235)
T_{MAX} (minutes)	6.6 (± 1.5)
$AUC_{0\text{-}LAST}$ (minutes*ppb)	5227 (± 3858)
$AUC_{0\text{-}\infty}$ (minutes*ppb)	5585 (± 3976)
% AUC Extrap	6.69 (± 9.18)
$MRT_{0\text{-}last}$ (minutes)	12.9 (± 6.0)

The data is reported as the mean with standard deviation in parentheses.

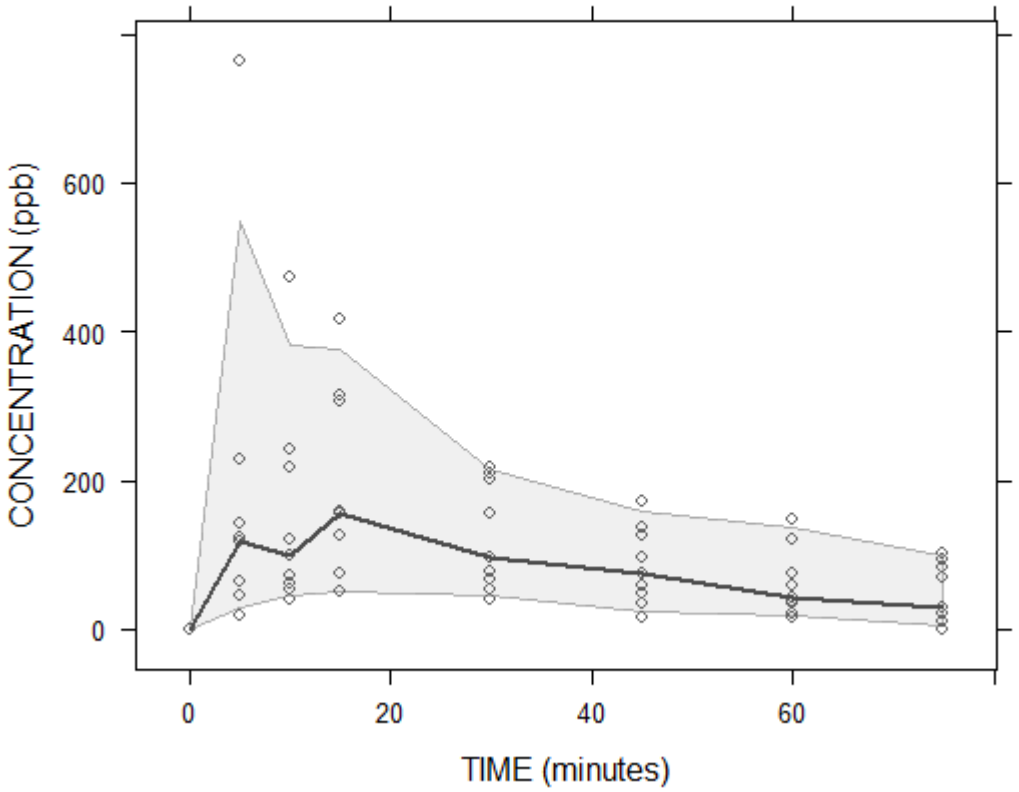

Figure 2-1. Concentration versus time plot for 2-pentyl acetate following vaginal administration (n=13). The taggant was formulated in tenofovir placebo (Placebo) gel. The dark line signifies median values for each time point, while the shaded region represents the 5th and 95th percentiles.

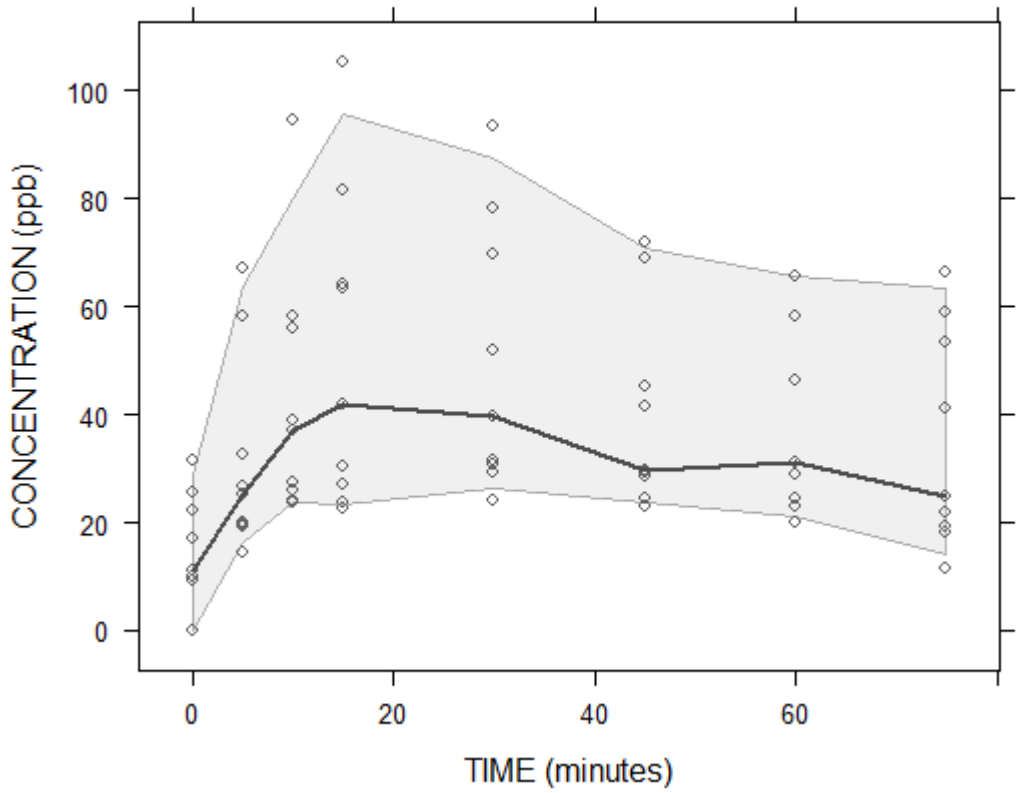

Figure 2-2. Concentration versus time plot for 2-pentanol following vaginal administration (n=13). The taggant was formulated in tenofovir placebo (Placebo) gel. The line signifies median values for each time point, while the shaded region represents the 5th and 95th percentiles.

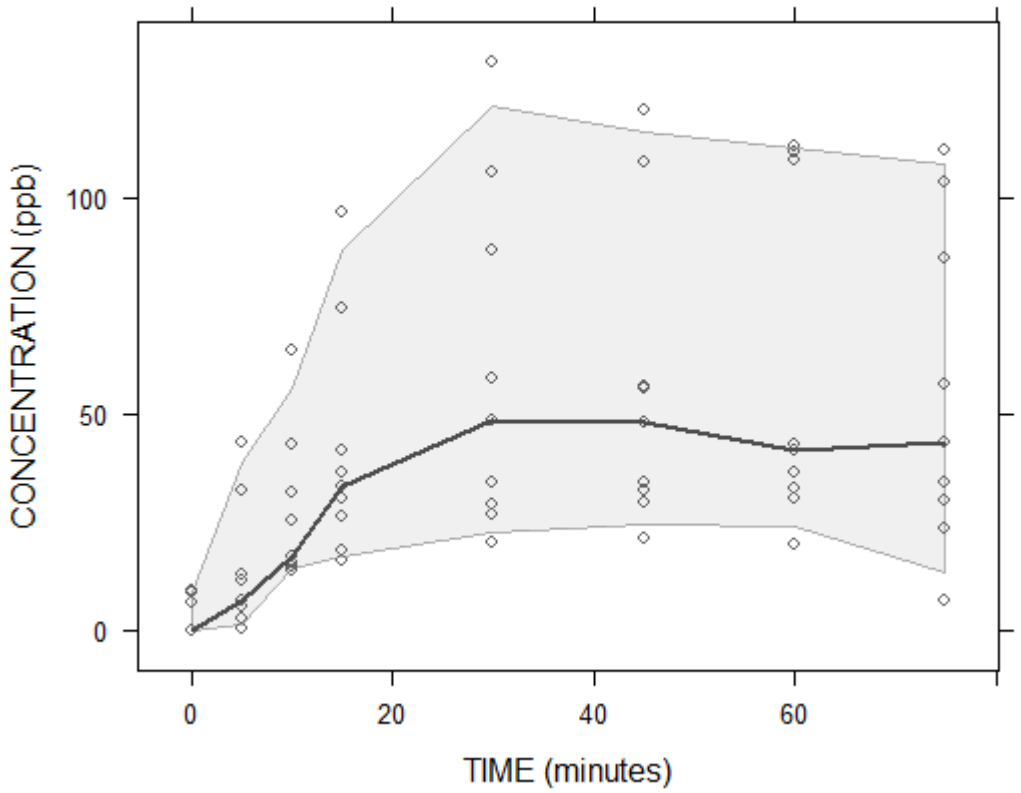

Figure 2-3. Concentration versus time plot for 2-pentanone following vaginal administration (n=13). The taggant was formulated in tenofovir placebo (Placebo) gel. The line signifies median values for each time point, while the shaded region represents the 5th and 95th percentiles.

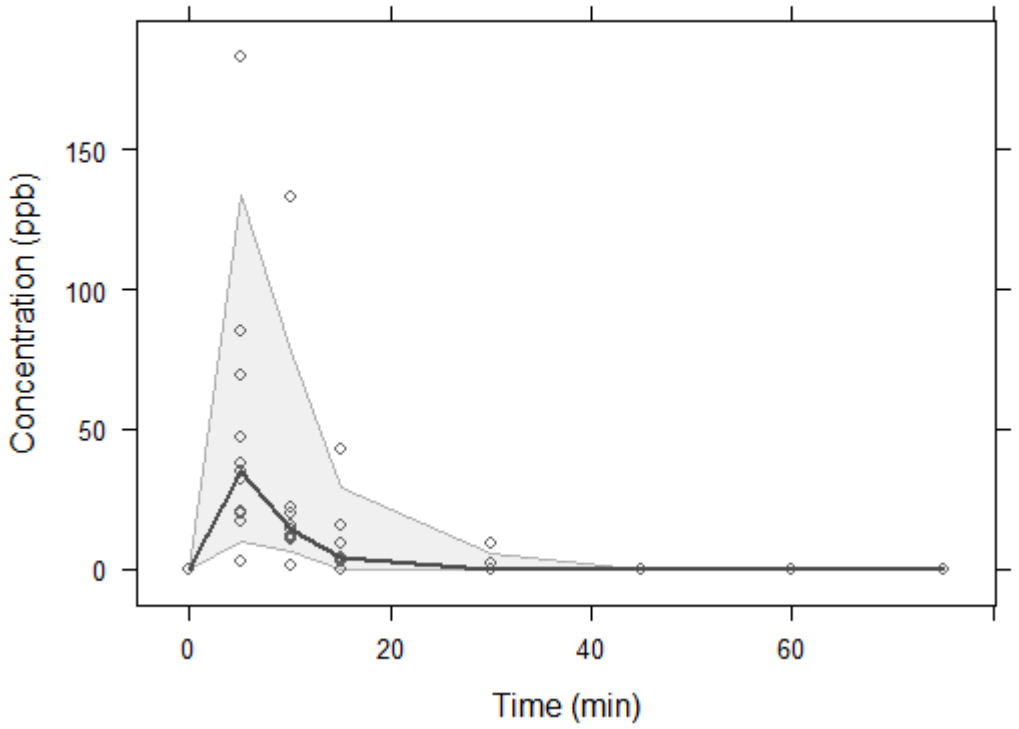

Figure 2-4. Concentration versus time plot for 2-butyl acetate following application using a condom (n=13). The taggant was formulated in hydroxyethylcellulose (HEC) gel. The line signifies median values for each time point, while the shaded region represents the 5th and 95th percentiles.

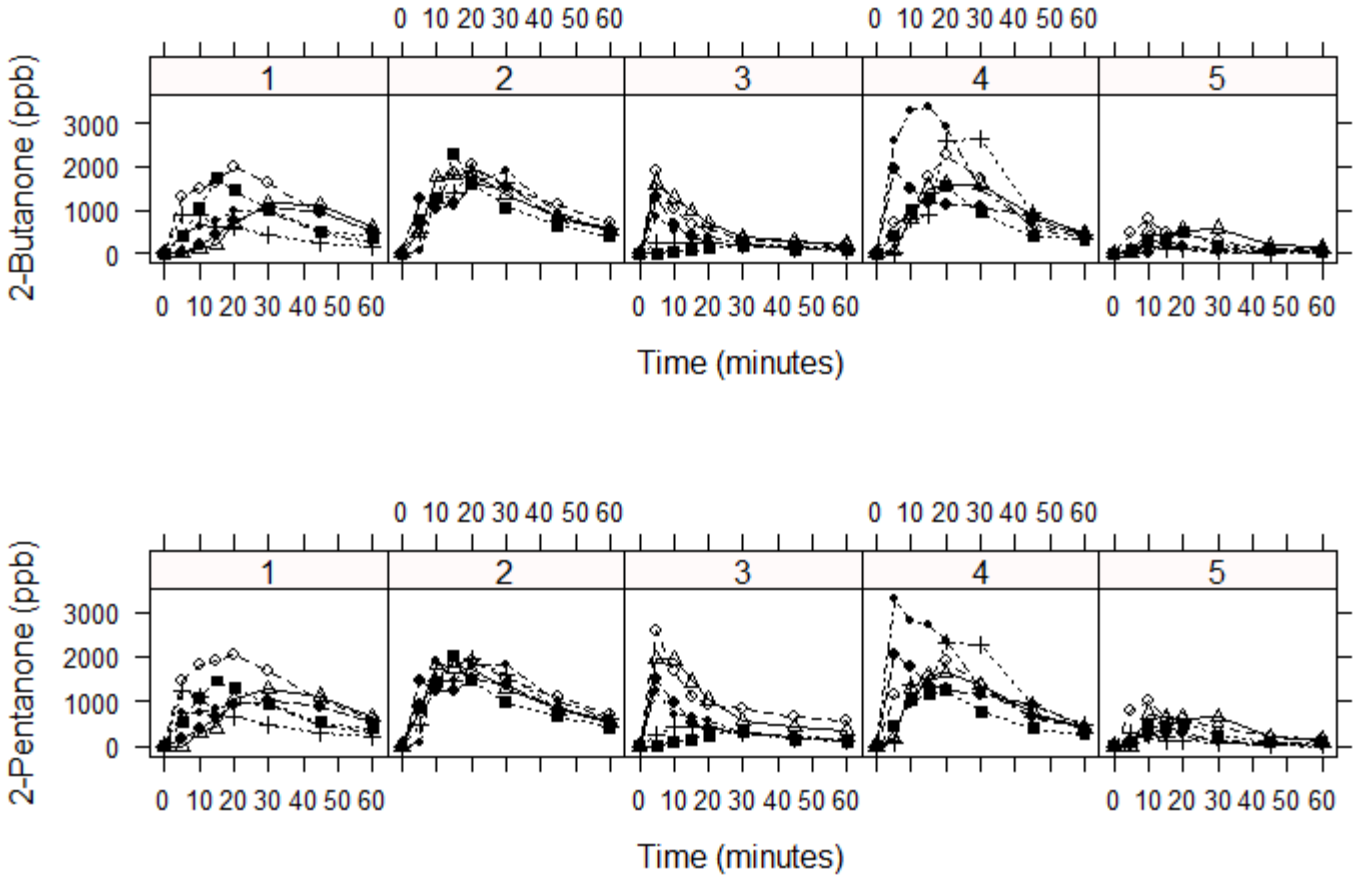

Figure 2-5. Mean concentration (µg/mL) versus time (minutes) profile by subject for 2-butanone (top) and 2-pentanone (bottom). Individual replicates (n=6) are plotted for each subject (Replicate 1: open circles; Replicate 2: open triangles; Replicate 3: crosses; Replicate 4: closed squares; Replicate 5: closed circles; Replicate 6: closed triangles).

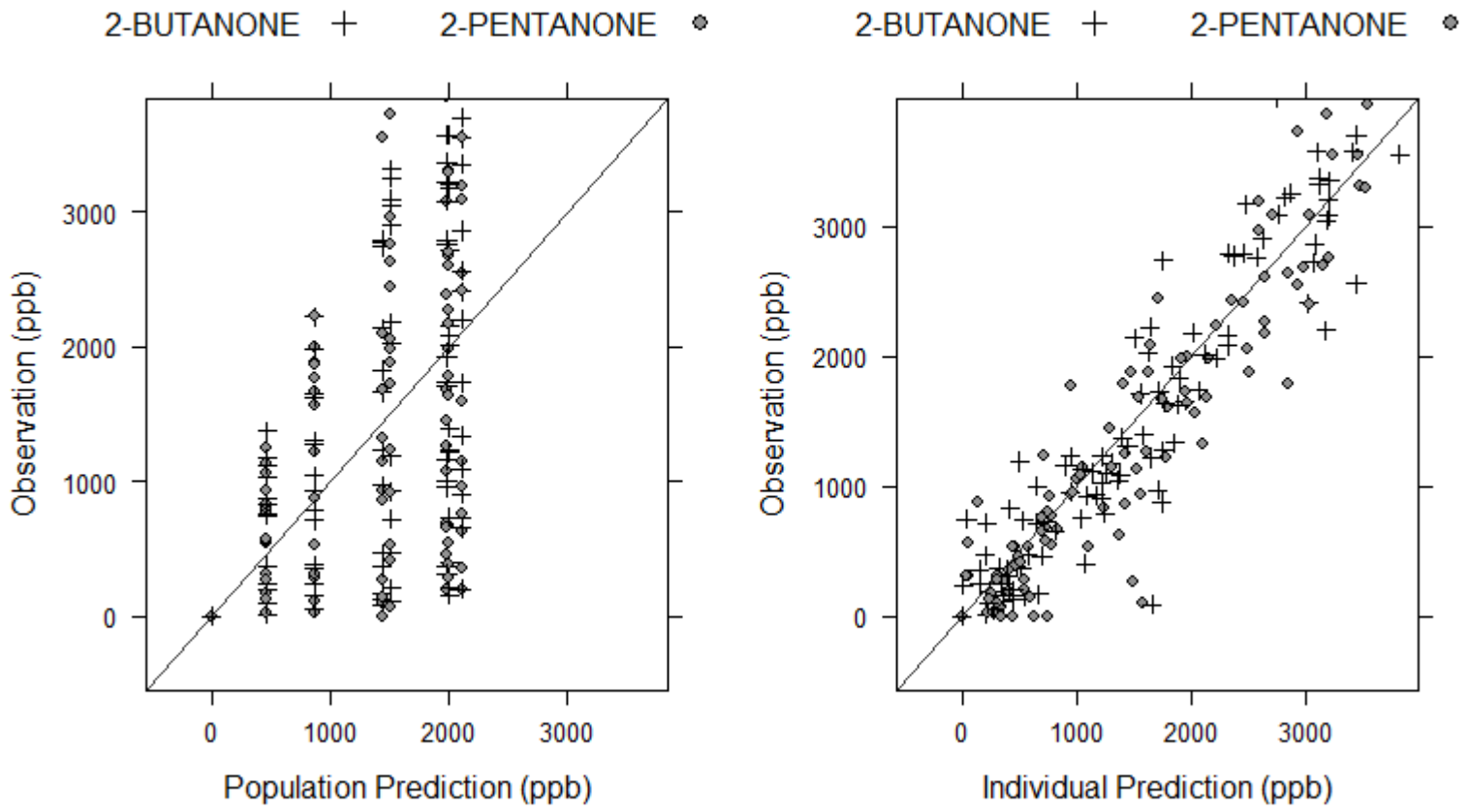

Figure 2-6. Goodness-of-fit plots for 2-butanone and 2-pentanone concentrations.

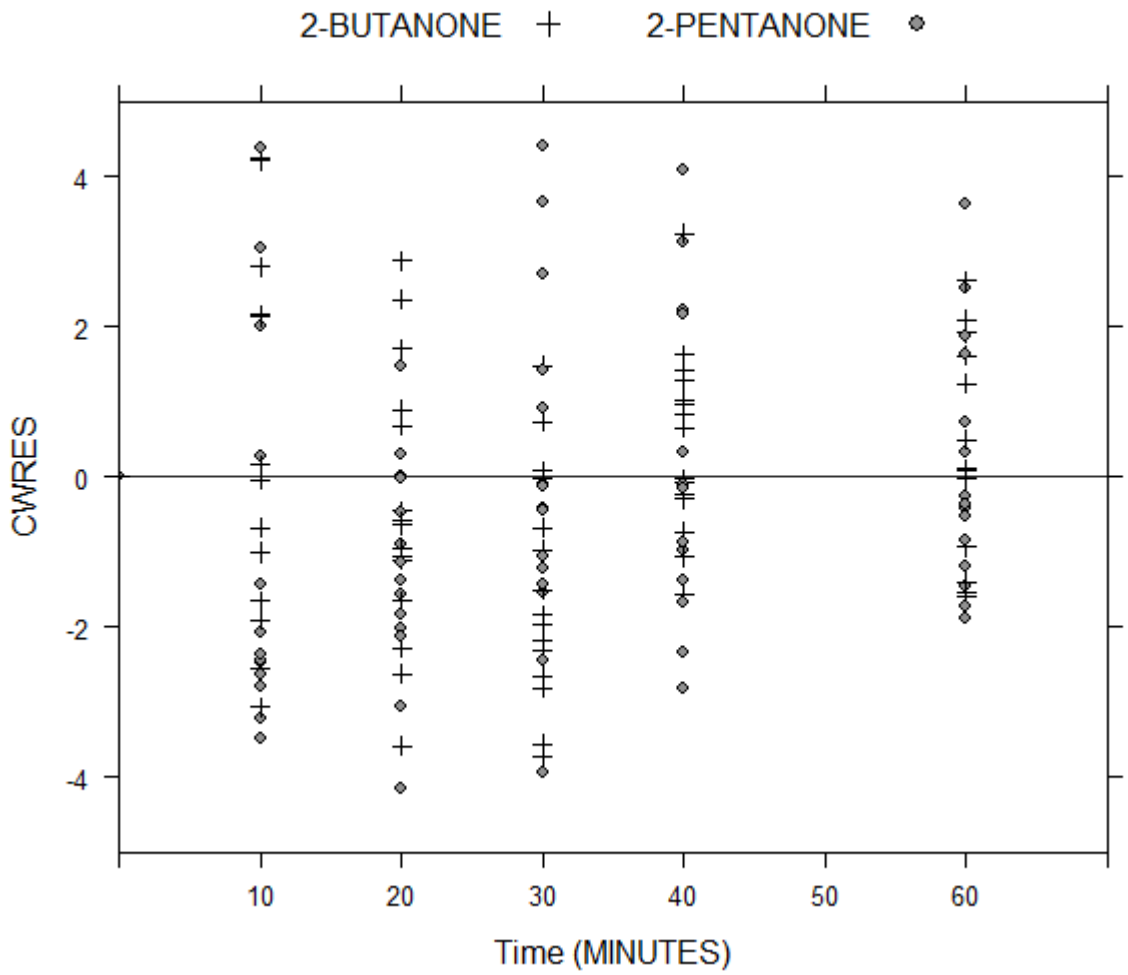

Figure 2-7. Conditional weighted residuals (CWRES) versus time.

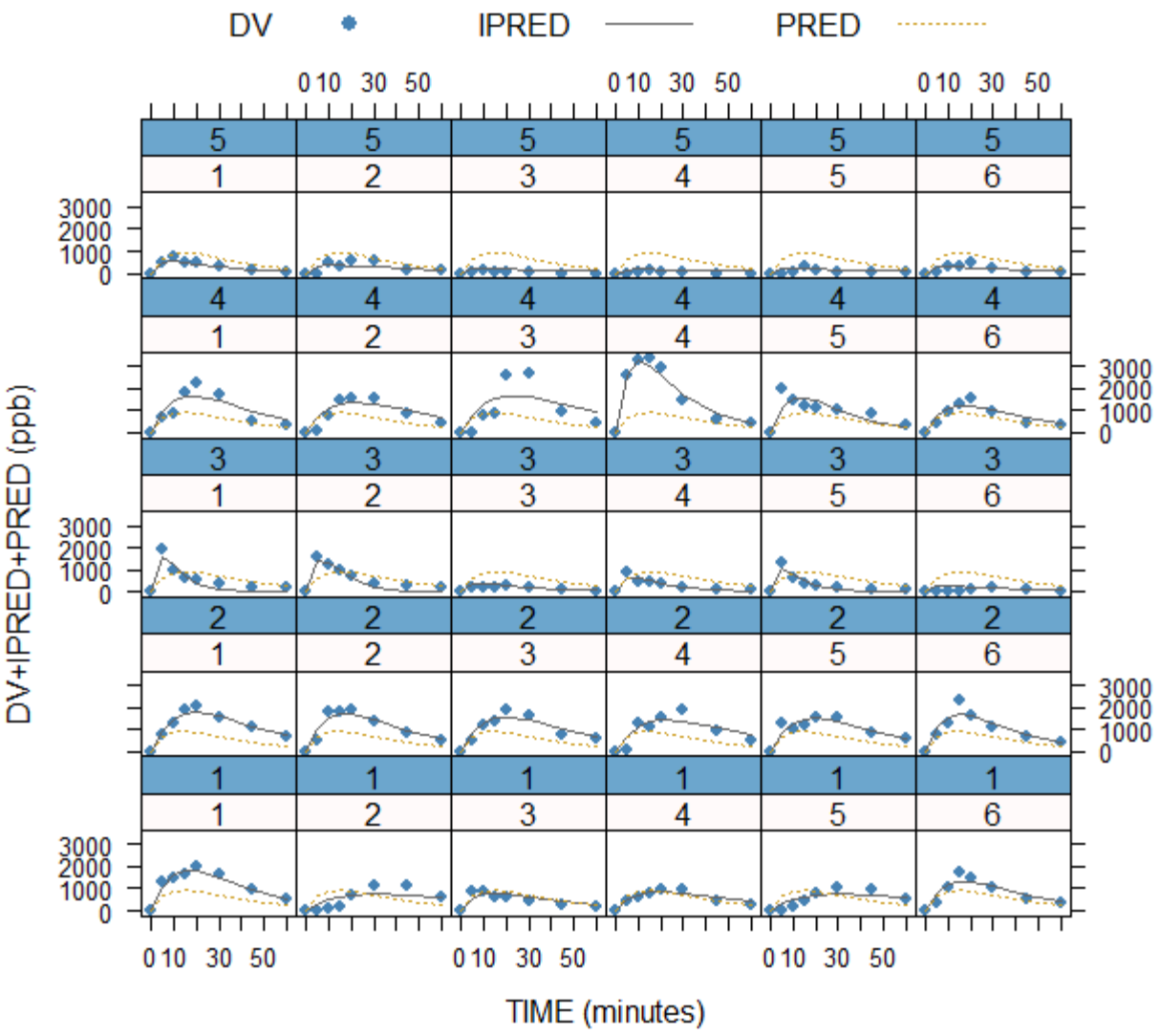

Figure 2-8. Individual concentration-versus-time plots for 2-butanone (5 subjects with 6 replicates each).

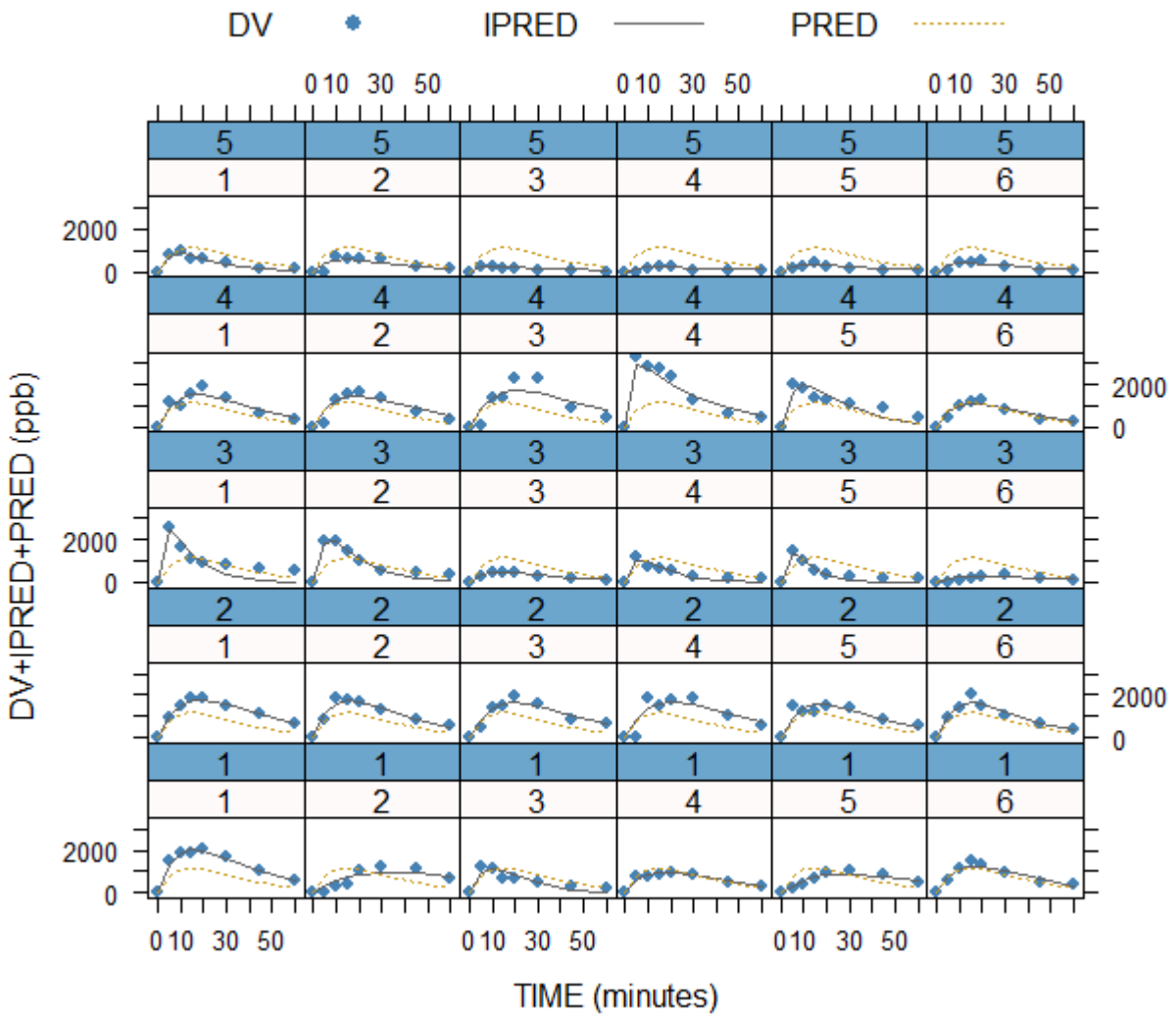

Figure 2-9. Individual concentration-versus-time plots for 2-pentanone (5 subjects with 6 replicates each).

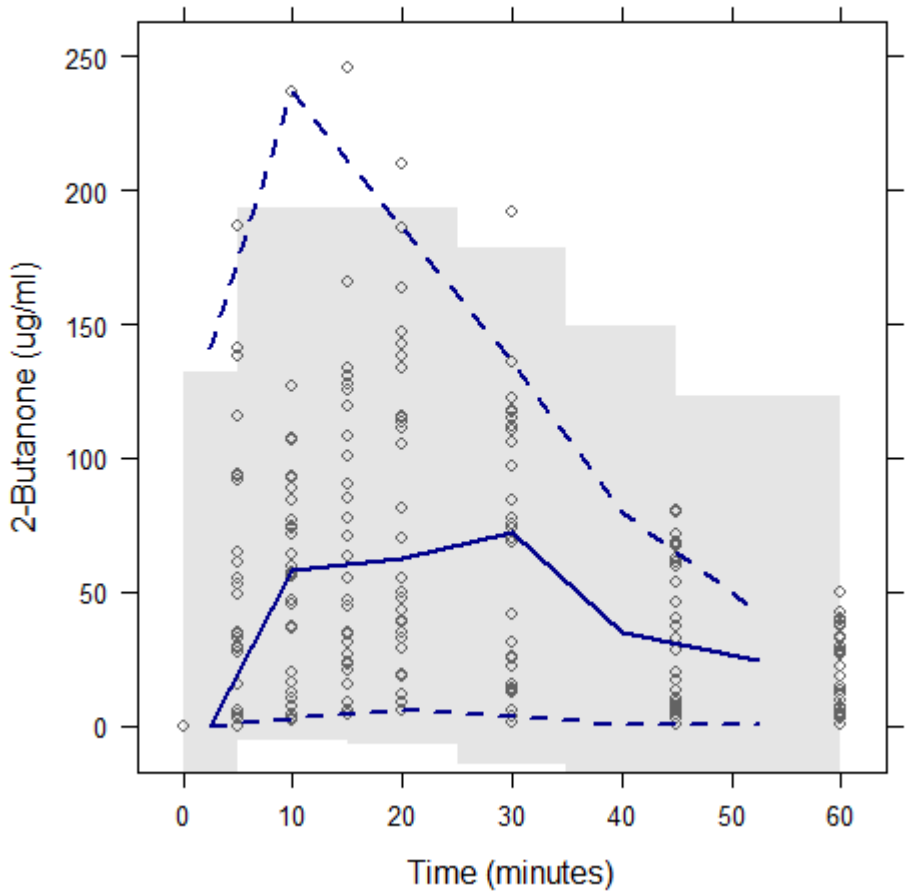

Figure 2-10. Visual predictive check for 2-butanone. The shaded area represents the prediction interval based on 1,000 simulations; whereas the dashed and solid lines represent the 2.5-, 50-, and 97.5-th percentiles for the observed data.

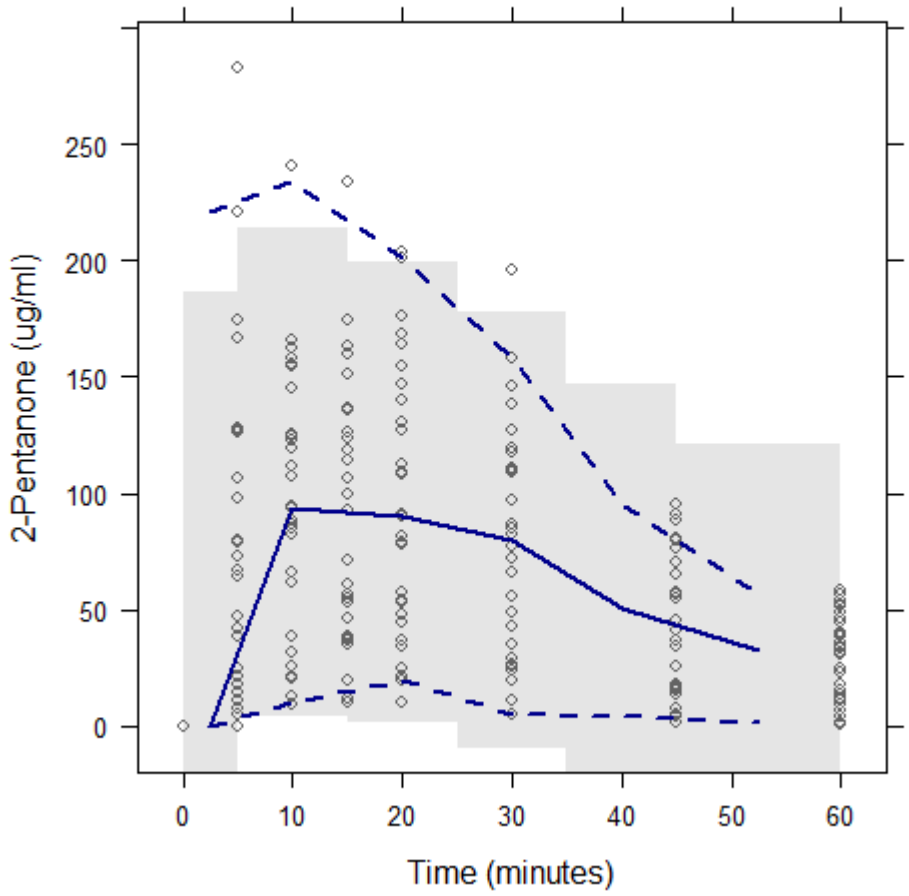

Figure 2-11. Visual predictive check for 2-pentanone.The shaded area represents the prediction interval based on 1,000 simulations; whereas the dashed and solid lines represent the 2.5-, 50-, and 97.5-th percentiles for the observed data.

CHAPTER 3
INFLUENCE OF VARYING PROTEIN CONCENTRATIONS ON THE ANTIMICROBIAL EFFICACY OF CEFTRIAXONE

Introduction

Ceftriaxone is a third-generation β-lactam antibiotic which displays broad bactericidal activity against gram-negative bacteria, such as *Haemophilus influenza* or *Neisseria meningitides*, as well as coverage against gram-positive pathogens such as *Streptococcus pneumonia.* Due to its high plasma protein binding (up to 98%), ceftriaxone displays a significantly longer half-life (~ 7-8 hours) than other *beta*-lactam-antibiotics.[49] Since only the unbound drug is responsible for the antibacterial activity, protein binding must be accounted for when determining an appropriate dosing scheme. As a result, investigators often add a protein supplement or plasma *in vitro* in an effort to elucidate the role of protein binding on antibacterial activity.

There are several factors which must be considered when studying the impact of protein binding in an *in vitro* setting. These include the type of protein supplement used, the technique used to measure the degree of protein binding, and the interpretation of the results.[23] One study reported that available protein supplements may differ in their impact on protein binding and antimicrobial efficacy.[50] Aside from the type of protein supplement utilized, the protein concentration used may also be important. Frequently a protein concentration of 4 g/dl is used, although this may not be appropriate for all drugs. We sought to evaluate the impact of adding varying protein concentrations on the protein binding and antimicrobial efficacy of ceftriaxone using microdialysis and bacterial time-kill curve experiments, respectively.

Microdialysis is a versatile tool which may be used to measure free drug concentrations both in an *in vitro* and *in vivo* setting. With microdialysis, a probe is

placed into the tissue of interest (when used *in vivo*) and perfused with a physiological solution commonly referred to as the "perfusate." Due to diffusion processes, drug flows down its concentration gradient and is taken up into the perfusate. The collected sample (i.e., dialysate) contains a fraction of the drug which is in the surrounding medium. Equilibrium between the surrounding medium and perfusate is not established, and thus the fraction of drug diffusing through the membrane has to be determined using recovery experiments (e.g., extraction efficiency, retrodialysis). Since only the free unbound drug can pass through the membrane of the probe, microdialysis can be used *in vitro* to evaluate the binding of drugs to plasma proteins.

Methods

Protein Binding Studies

Chemicals and equipment

A Metler balance (AB104) was used to weigh all chemicals. Vortexing and pH measurement was done using a Kraft Apparatus (model PV-5) and a Corning pH meter (model 430), respectively. An Agilent 1100 series HPLC and Restek C-18 column (Pinnacle DB 5 µm) was used for analysis of the collected samples. A Harvard apparatus 22 syringe pump was used for infusion of the drug solution, while the CMA 60 microdialysis probe was used to measure free drug concentrations in all studies. Ceftriaxone (Sigma C-5793), hexadecyl trimethyl-ammonium bromide (HDTA, Fisher Scientific 03042), human serum albumin (HSA, Calbiochem CAS 70024-90-7), and lactated ringer's solution (Baxter) were used for reagent and sample preparation.

Reagent preparation

Ceftriaxone calibration samples (31.25, 62.5, 125, 250, 500, and 1000 ng/mL) were prepared in lactated ringer's solution. The mobile phase was prepared by mixing

0.2 liters of 7.5 mM KH_2PO_4 (MW 136.09 g/mol) dissolved in water (204 mg KH_2PO_4) with 0.8 liters of HPLC-grade methanol. Then the ion pairing agent, HDTA (MW 364.46 g/mol), was added at a concentration of 1.2 mM (455 mg HDTA). The pH of the mobile phase was adjusted to 8.8. The mobile phase was filtered and sonicated for 20 minutes.

Sample preparation

Experiments were conducted to evaluate ceftriaxone's recovery using the CMA 60 probe (M Dialysis, Solna, Sweden). For the extraction efficiency (EE) experiments, where the drug is diluted in the surrounding media, ceftriaxone's recovery was tested using three concentrations (50, 100, and 150 µg/mL) prepared in lactated ringer's solution. For the retrodialysis (RT) experiments, where the drug is diluted in the perfusate, similarly three concentrations were prepared (50, 100, and 150 µg/mL) by dissolving the drug in lactated ringer's solution. During preparation of drug solutions, exposure to light was minimized.

Using the same drug concentrations tested in the recovery experiments, protein binding experiments were conducted using varying concentrations of human serum albumin. On the first day, 50 µg/mL drug solutions containing varying concentrations of human serum albumin (no protein, 0.5, 1.5, 2.5, 3.5, 4.5, or 5.5 g/dl) were prepared in triplicate. On the second and third day, the same protein concentrations were tested using the 100 and 150 µg/mL drug concentrations, respectively.

Ceftriaxone's protein binding was also measured using undiluted and diluted pooled human plasma in an effort to evaluate the impact of varying protein concentrations in plasma. For the experiments with diluted plasma, undiluted plasma was diluted by one-half and one-fourth using lactated ringer's solution. Actual protein

concentrations were measured using an albumin assay kit (BioChain® BCG Albumin Assay Kit, Hayward, CA).

Recovery experiments

Before any protein binding experiments could be conducted, the recovery of the drug was determined. EE and RT experiments were conducted on separate days. For the EE experiments, a 5 mL syringe containing lactated ringer's solution was used as the perfusate. The probe was placed in a drug solution containing 50, 100, or 150 µg/mL of ceftriaxone. Lactated ringer's solution was infused through the probe at flow rate of 1.5 µl/min for a period of 15 minutes (equilibration period). Then dialysate samples were collected for a period of 20 minutes each (collection period). A total of three separate samples were collected; each twenty minutes apart. The recovery (R %) was calculated according to Equation 3-1; using the measured concentration in the dialysate ($C_{dialysate}$) and the known concentration in the perfusate ($C_{perfusate}$).

$$R\ \% = 100 \times \frac{C_{dialysate}}{C_{perfusate}} \qquad 3\text{-}1$$

In the RT experiments, the drug is infused in the perfusate (i.e., lactated ringer's solution). Syringes containing 50, 100, or 150 µg/mL of ceftriaxone were fastened to the syringe pump. First, the drug solution was allowed to equilibrate through the probe for a period of 15 minutes. Next there was a 20 minute sample collection period. A total of three separate samples were collected; each twenty minutes apart. The recovery in the RT experiments was calculated using equation 3-2.

$$R\ \% = 100 - \left(100 \times \frac{C_{dialysate}}{C_{perfusate}} \right) \qquad 3\text{-}2$$

Microdialysis experiments

Using microdialysis, the protein binding of ceftriaxone (50, 100, and 150 μg/mL) was tested using varying concentrations of human serum albumin (no protein, 0.5, 1.5, 2.5, 3.5, 4.5, or 5.5 g/dl). During the protein binding experiments, first the lowest protein concentration (i.e., no protein) was tested and in a stepwise fashion increasing protein concentrations were evaluated. Briefly, the probe was placed in a drug solution and then there was a 15 minute equilibration period where lactated ringer's solution was perfused through the probe. Once the equilibration period was complete, there was a 20 minute sample collection period. This process was repeated for each protein concentration. Samples were analyzed immediately upon collection. All experiments were conducted in triplicate.

A similar procedure was followed for protein binding experiments conducted using undiluted and diluted plasma. On three separate days, the protein binding for one ceftriaxone concentration (50, 100, or 150 μg/mL) was measured in undiluted plasma as well as plasma diluted by one-half and one-fourth using lactated ringer's solution. In each case there was a 30 minute equilibration period followed by a 20 minute sample collection period. In addition, there was a 15 minute "flushing" period in between each run where the probe was placed in lactated ringer's solution. Samples were analyzed immediately upon collection. All experiments were conducted in triplicate.

Microbiological Experiments

Chemicals and equipment

A Metler balance (AB104) was used to weigh all chemicals. The A-JUST (Abbott Lab) Turbidmeter and McFarland Equivalence Turbidity Standards were used to prepare bacterial dispersions. A Harvard apparatus 22 syringe pump was used for

infusion of the drug solution during microdialysis experiments, while the CMA 20 and 60 microdialysis probes were utilized for determination of free drug concentrations during the bacterial time-kill curves. Ceftriaxone (Sigma C-5793), hexadecyl trimethyl-ammonium bromide (HDTA, Fisher Scientific 03042), human serum albumin (Calbiochem CAS 70024-90-7), and lactated ringer's solution (Baxter) were used for reagent and sample preparation. All microdialysis samples were analyzed using a PE200 series pump and autosampler (Perkin Elmer, Norwalk, CT, USA); as well as a triple quadruple API 4000 mass spectrometer (Applied Biosystems, Carlsbad, CA, USA) equipped with an electrospray ion source. The column of choice was a Symmetry® C-18 column (4.6 x 500 mm, 3.5 µm particle size; Waters, Dublin, Ireland). Corning 24- and 96-well plates (#3524), 50 mL tissue culture flasks, and Remel 5% sheep blood agar plates were used during bacterial time-kill curves.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration of ceftriaxone against *E. coli* (ATCC 25922) was determined in the presence and absence of human serum albumin. For the former case, first, the broth used in the experiments was prepared. For determination in triplicate, 20 mL of Mueller-Hinton-broth (Becton Dickenson, BBL 211443) was prepared by suspending 440 mg of the powder in 20 mL of purified water. The broth was mixed thoroughly, heated with frequent agitation, and allowed to boil for 1 minute to completely dissolve the powder. The broth was autoclaved at 121°C for 15 minutes. Last, 500 µL of the prepared broth was added into each of the 24 wells on the cell culture plate. Second, primary and secondary stock solutions (2 and 0.1 mg/mL) of ceftriaxone were prepared. Then 10 µl of the secondary stock was added to the first well and serial dilutions were performed to obtain the following concentrations: 0.0078, 0.0157, 0.0313,

0.0625, 0.125, 0.25, 0.5, and 1 µg/mL. Third, a dispersion of bacteria containing 1.5 x 10^8 CFU/mL was prepared. Of this bacterial dispersion, 10 µL was added to each well.

Negative (containing no drug or bacteria) and positive (containing no drug but bacteria) control samples were prepared. For the negative control, 500 µL of broth was dispensed into each well; and no bacteria were added. For the positive controls, 500 µL of broth and 10 µL of 1.5 x 10^8 CFU/mL *E. coli* dispersion were added into each well. All well plates were incubated at 37°C for 20 hours. The minimum inhibitory concentration was determined as the ceftriaxone concentration where no visible growth was observed.

The minimum inhibitory concentration was then determined in the presence of human serum albumin. Broth was prepared in a similar fashion as described above except that albumin was added. To prepare broth containing 2 and 4 g/dl of human serum albumin, 0.4 and 0.8 grams of human serum albumin was each added to 20 mL of broth. All other procedures were performed in a similar fashion. These experiments were performed on two separate dates.

Bioanalytical method development and validation

Following completion of the minimum inhibitory concentration experiments, static bacterial time-kill curve experiments were performed. During these experiments, one objective was to evaluate the feasibility of using microdialysis to quantify free drug concentrations directly in the culture flasks containing the bacteria, broth, and drug solution. Before this objective could be tested, development and validation of a method to detect ceftriaxone in broth samples was completed.

For preparation of calibration standards and quality controls (QC), a CMA 20 probe was placed in a broth solution and dialysate was collected for a period of 12

hours. The dialysate was then mixed one-to-one with a cefazolin solution (2000 ng/mL, internal standard); centrifuged at 10,000 rpm for 5 minutes; and the supernatant was collected. This supernatant will be referred to as the “working solution.” Using the working solution, calibration standards were prepared ranging from 7.81-1000 ng/mL. In addition, QC samples (8, 20, 100, and 800 ng/mL) were also prepared with the working solution.

During analysis, the precursor and product ion transitions monitored were m/z 555.2/396.2 and m/z 555.2/167.0 for ceftriaxone and 455.1/323.0 for cefazolin. The mobile phase consisted of methanol (75%) and 10 mM ammonium acetate in water (pH 7.2). A flow rate and injection volume of 0.45 mL/min and 10 µL was used, respectively. The method was validated according to the guidelines set forth by the United States Food and Drug Administration (FDA).

Static bacterial time-kill curves

Bacterial time-kill curves were conducted to evaluate the effect of varying protein concentrations on the antimicrobial efficacy of ceftriaxone against *E. coli* (ATCC 25922). To achieve this goal, three scenarios were evaluated. First, the time-kill profile of ceftriaxone was evaluated in the absence of human serum albumin. Then it was evaluated in the presence of 2 and 4 g/dl of human serum albumin separately. Each scenario was conducted in triplicate. In each case, seven different ceftriaxone concentrations were evaluated, each representing multiples of the MIC (0.25*MIC, 0.5*MIC, MIC, 2*MIC, 4*MIC, 8*MIC,16*MIC); where the MIC was determined to be 0.06 µg/mL in the previously described experiments. In addition to these seven concentrations, negative and growth controls were also included.

For each scenario, nine 50 mL culture flasks were filled with 29.6 mL of Mueller Hinton broth. Then, with the exception of the negative control, 100 µL of a bacteria dispersion containing 1.5 x 10^8 CFU/mL was added. Flasks were incubated for 2 hours at 37°C. A primary stock solution (4.8 mg/mL) was prepared by adding 48 mg of ceftriaxone to 10 mL of triple deionized water. Then a secondary stock was prepared (480 µg/mL), and serially diluted, to obtain concentrations between 0.015-0.96 µg/mL; each concentration corresponding to an MIC multiple.

After two hours of incubation, flasks were taken out of the incubator and a 20 µL aliquot was removed from each flask for spot inoculation. Before being returned to incubator, 300 µL of a drug solution was added to each flask but the positive and negative controls. For the spot inoculation, a 10-fold serial dilution scheme was performed. Briefly, 180 µL of sterile saline was added to each well and a 20 µl sample was taken out of each flask. Serial dilutions were performed by taking a 20 µl from the previous well. Last, 10 µL was spotted five times per dilution on one- fourth of each 5% sheep blood agar plate. Plates were incubated for 20 to 24 hours at 37°C and then viable counts were determined.

Upon completion of the three scenarios described above (i.e., no protein, 2 g/dl, and 4 g/dl), additional bacterial time-kill curves were performed to study the bacterial regrowth observed when no human serum albumin was added. In these experiments, 0.5*MIC, MIC, 1.5*MIC, 2*MIC, 2.5*MIC, 3*MIC, and 3.5*MIC were studied. In addition, negative and growth controls were performed.

Microdialysis experiments

During bacterial time-kill curve experiments, a 2.5 mL sample of culture media was drawn out of each flask at 2, 6, 8, 10, 14, 18, and 24 hours for measurement of free drug concentrations. One of three probes was placed in a 5mL glass tube containing 2.5 mL of the removed aliquot. After a 15 minute equilibration period, a dialysate sample was collected for 20 minutes. In between each time point there was at least 5 minutes of flushing to prevent potential clogging of the microdialysis probe. Each sample was stored at -70°C until the time of analysis. Additional experiments were performed to evaluate ceftriaxone's recovery in broth using EE and RT. During the follow-up experiments to study bacterial regrowth, microdialysis experiments were not performed.

PK/PD modeling

The data from the static time-kill curves was analyzed in NONMEM® version 7.2 using the first order conditional estimation method algorithm with ADVAN9. Perl-speaks-NONMEM® was used for generation of visual predictive checks (PsN, Version 3.4.2), while the xpose4 and lattice packages in the software R were used for data visualization.[46–48] NONMEM® workflow was managed using Piraña.[51]

A semimechanistic PK/PD model previously developed and validated by Neilsen et al. was fitted to the data.[52,53] In this model, the total bacterial population is divided into two subpopulations; one which is growing (S) and another which is in a resting phase (R). Movement from the former to the latter is dictated by a rate constant, k_{SR}, which is dependent on the total bacterial concentration (i.e., S+R) as shown in Equation 3-3. The parameters k_S and k_D represent bacterial synthesis and death rate constants.

$$k_{SR} = \frac{(k_S - k_D)}{N_{MAX}} * (S + R) \quad \text{3-3}$$

For the PK model, drug concentrations measured using microdialysis were included in the data analysis. For time points where free concentrations were not measured, the average of the previous and later time points was used. To describe a delay in drug effect, an effect compartment (C_e) was added to the PK model, where movement between compartments is dictated by the rate constant k_{e0}. The drug effect was modeled using a sigmoidal E_{MAX} model (Equation 3-4) where parameters representing the maximal drug effect (k_{MAX}) and the concentration at which half-maximal effect is observed (EC_{50}) are estimated. To account for the observed bacterial regrowth, an adaption function was included to account for a change in the EC_{50} with time.[54,55] As shown in equation 3-5, this adaptation function is a function of a maximal adaption (ß) and a rate of adaptation constant (Ƭ).

$$\text{EFFECT} = \frac{k_{MAX} * C_e^{\gamma}}{C_e^{\gamma} + \alpha * EC_{50}^{\gamma}} \quad \text{3-4}$$

$$\alpha = 1 + \beta * (1 - e^{-\tau * t \circ conc}) \quad \text{3-5}$$

Thus, the bacterial concentration in the growing and resting subpopulations is dictated by equations 3-6 and 3-7.

$$\frac{dS}{dt} = k_S * (1 - DRUG) * S - k_D * S - k_{SR} * S \quad \text{3-6}$$

$$\frac{dR}{dt} = k_{SR} * S - k_D * R \quad \text{3-7}$$

Since experiments were performed in triplicate, three data points were available for each time point. The lower limit of detection (LOD) for the bacterial counts was set to

20 CFU/ML. For bacterial counts below the LOD, 10 CFU/ML was entered as first value in consecutive series while all other LOD data was omitted.[56]

Results

Protein Binding Studies

Ceftriaxone's recovery using EE and RT was determined to be 75.2% and 82.1%, respectively. The results of the protein binding studies are shown in Figure 3-1. In both the experiments with human serum albumin and pooled human plasma, as the protein concentration is increased, ceftriaxone's protein binding increases from 0 to 90%. Slightly greater binding is observed with pooled human plasma as compared to human serum albumin. With human plasma, a slight increase in the fraction unbound is observed as the drug concentration is increased.

Microbiological Experiments

When determining the minimum inhibitory concentration, addition of human serum albumin resulted in a higher drug concentration needed to inhibit visible growth (Table 3-1). No difference was noted when comparing 2 and 4 g/dl of human serum albumin.

In the bacterial time-kill curves, in the absence of human serum albumin, 4*MIC, 8*MIC, and 16*MIC resulted in no visible growth after 8-10 hours (Figure 3-2). For 2*MIC and MIC, regrowth was observed after 10 and 6 hours, respectively. With all other MIC multiples, bacterial growth was observed immediately (i.e., 2 hours). In the presence of 2 g/dl of human serum albumin, due to lower free concentrations, only the 16*MIC multiple resulted in no observable growth after 10 hours (Figure 3-3). For 8*MIC, regrowth occurred after 6 hours of sampling. For all other multiples, bacterial growth occurred immediately. When 4 g/dl of human serum albumin was added, only

16*MIC prevented regrowth; while regrowth was observed after 4 hours for the 8*MIC concentration (Figure 3-4). For all other MIC multiples, regrowth occurred after 2 hours.

In the follow-up experiments to study bacterial regrowth, only the highest drug concentration (i.e., 3.5*MIC) resulted in visible regrowth (Figure 3-5). Although the time to regrowth varied (3*MIC 20 hours, 2.5*MIC 10 hours, 2*MIC 8 hours, 1.5*MIC 8 hours, MIC 6 hours, 0.5*MIC 2 hours), for all other MIC multiples bacterial regrowth was observed.

As expected the addition of HSA impacted the antimicrobial efficacy of ceftriaxone. This reduced efficacy is to a decreased amount of free drug available in each flask. Mean free drug concentrations observed for the six highest drug concentrations are shown in Figures 3-6, 3-7, and 3-8. As the protein concentration is increased, there is an observable reduction in free ceftriaxone concentrations. Ceftriaxone's recovery in broth, determined using EE and RT, was 87.2 and 55.9, respectively.

Parameter estimates for the PK/PD model describing the bacterial time-kill curve data are shown in Table 3-2. Visual predictive checks for each scenario are shown in Figures 3-9 – 3-12. With the exception of the highest drug concentration in the presence of 4 g/dl HSA; it appears that the model results in a reasonable fit of the observed data.

Discussion

The goal of the described experiments was to evaluate the role of variable protein binding on the antimicrobial efficacy of ceftriaxone using microdialysis. In the protein binding experiments, as the human serum albumin concentration was increased, an increase in the protein binding of ceftriaxone was observed. When pooled human plasma was diluted, a similar relationship was observed; with slightly greater binding

observed when compared to the human serum albumin experiments. This difference can be explained by the availability of additional proteins in plasma which can interact with and bind to ceftriaxone.

The observed decrease in free drug concentrations as protein concentrations were increased resulted in an observed change in ceftriaxone's efficacy. Addition of protein resulted in a 2-4 fold increase in the MIC of the drug. No difference in the MIC was observed when comparing the 2 and 4 g/dl experiments. A lack of a difference may be attributed to the experimental setup. Although the MIC provides a rough estimate of the concentration needed to inhibit visible growth, it may be difficult to observe the effect of relatively small differences in free drug concentration. Moreover, the technique itself is subject to investigator bias as determination of this concentration is subjective.

Bacterial time-kill curves were performed to account for changes in growth with time. Addition of HSA to the culture flasks resulted in an immediate growth or regrowth for all drug concentrations minus the highest MIC multiple (i.e., 16*MIC). For the 2 and 4 g/dl scenarios, the major difference was faster regrowth in the 8*MIC flasks. Additional follow-up experiments were then performed to evaluate the range of drug concentrations which resulted in bacterial regrowth (0.5*MIC-3.5*MIC). Regrowth was observed at all concentrations except 3.5*MIC. As the drug concentration was increased, the time-to-regrowth was prolonged. The observed regrowth is likely a result of resistance development to ceftriaxone; although this was not tested directly.

An additional goal of these studies was to evaluate the feasibility of using microdialysis to measure free drug concentrations in culture flasks during bacterial time-kill curve experiments. Microdialysis allowed for a measurement of free drug

concentrations at the site of action; data which could then be correlated with the PD data using PK/PD modeling. A noticeable decease in free drug concentrations was observed as the protein concentration was increased. Significant variability was observed in the 4 g/dl scenario and it was difficult to differentiate culture flasks on the basis of free drug concentrations. In addition, when protein was added to the flasks, a noticeable increase in free drug concentrations occurred at approximately 14 hours. Although follow-up experiments have not been performed to study the mechanism of this change; one potential mechanism may be a conformational change in the structure of the protein which occurs halfway through the experiments.

Table 3-1. Effect of human serum albumin on the minimum inhibitory concentration (MIC) of ceftriaxone against *E. coli* (ATCC 25922).

		Human Serum Albumin Concentration	
Replicate	No Protein	2 g/dL	4 g/dL
Trial 1 (01/21/2010)			
MIC (mg/mL)	0.125	0.25	0.25
MIC (mg/mL)	0.125	0.5	0.25
MIC (mg/mL)	0.125	0.25	0.25
Mode	0.125	0.25	0.25
Trial 2 (07/30/2010)			
MIC (mg/mL)	0.0625	0.25	0.5
MIC (mg/mL)	0.125	0.5	0.25
MIC (mg/mL)	0.0625	0.25	0.25
Mode	0.0625	0.25	0.25

Table 3-2. Parameter and relative standard error estimates for an effect compartment model developed to describe bacterial time-kill curve data.

Parameter	Estimate	RSE (%)
k_{max} ($hours^{-1}$)	1.19	14.6
EC_{50} (µg/mL)	0.04	31.6
k_S ($hours^{-1}$)	4.38	6.8
N_{MAX} (cfu/mL)	5.2×10^8	19.5
K_d ($hours^{-1}$)	1.63	4.1
ϒ	0.91	26.8
K_{e0} ($hours^{-1}$)	100 FIX	-
ß	17.5	14.3
Ƭ(L*µg/mL)	0.08	20.9
Residual Error (LN cfu/mL)	2.54	7.3

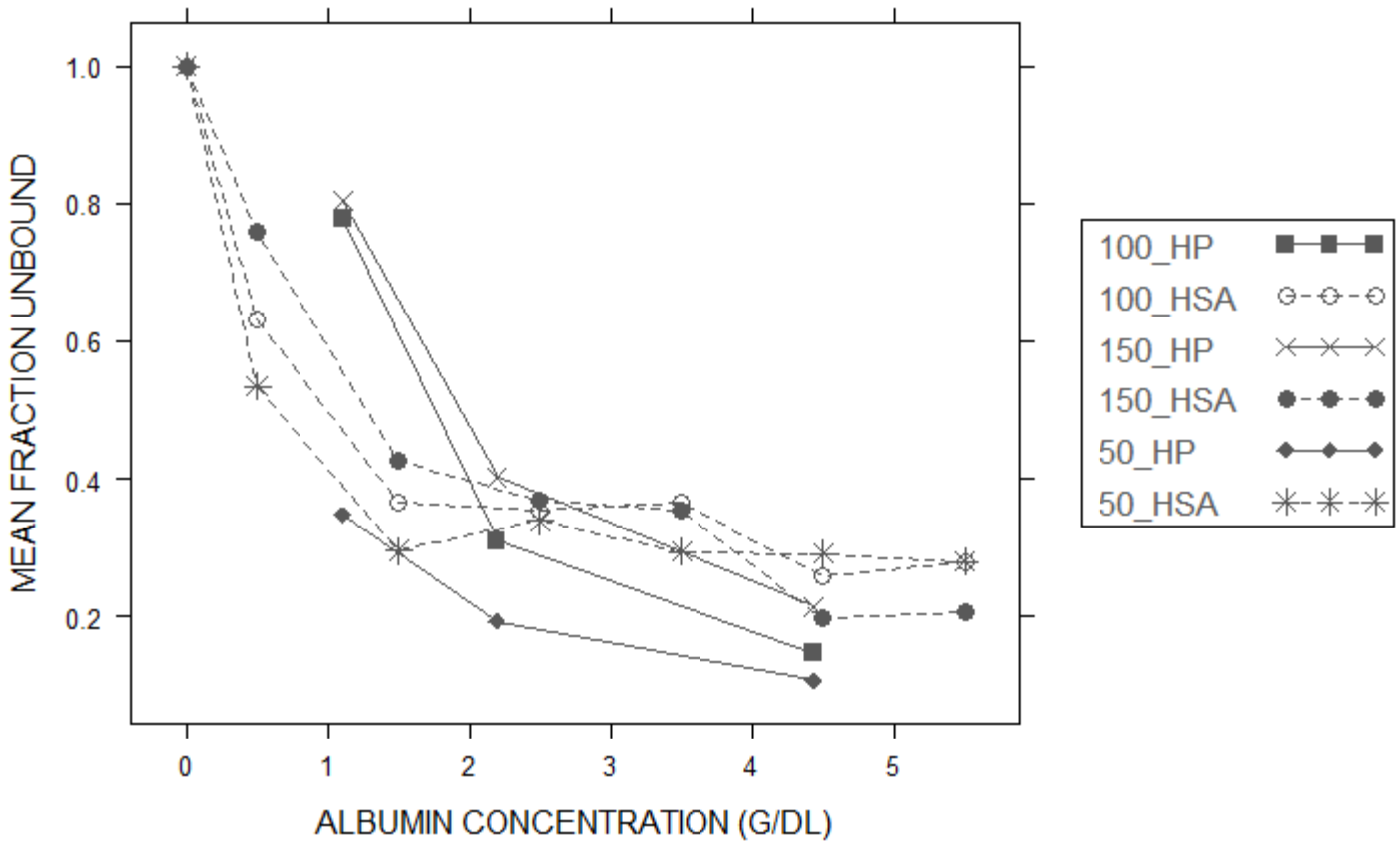

Figure 3-1. Protein binding experiments using human serum albumin and pooled human plasma. Mean fraction unbound is reported (n=3).

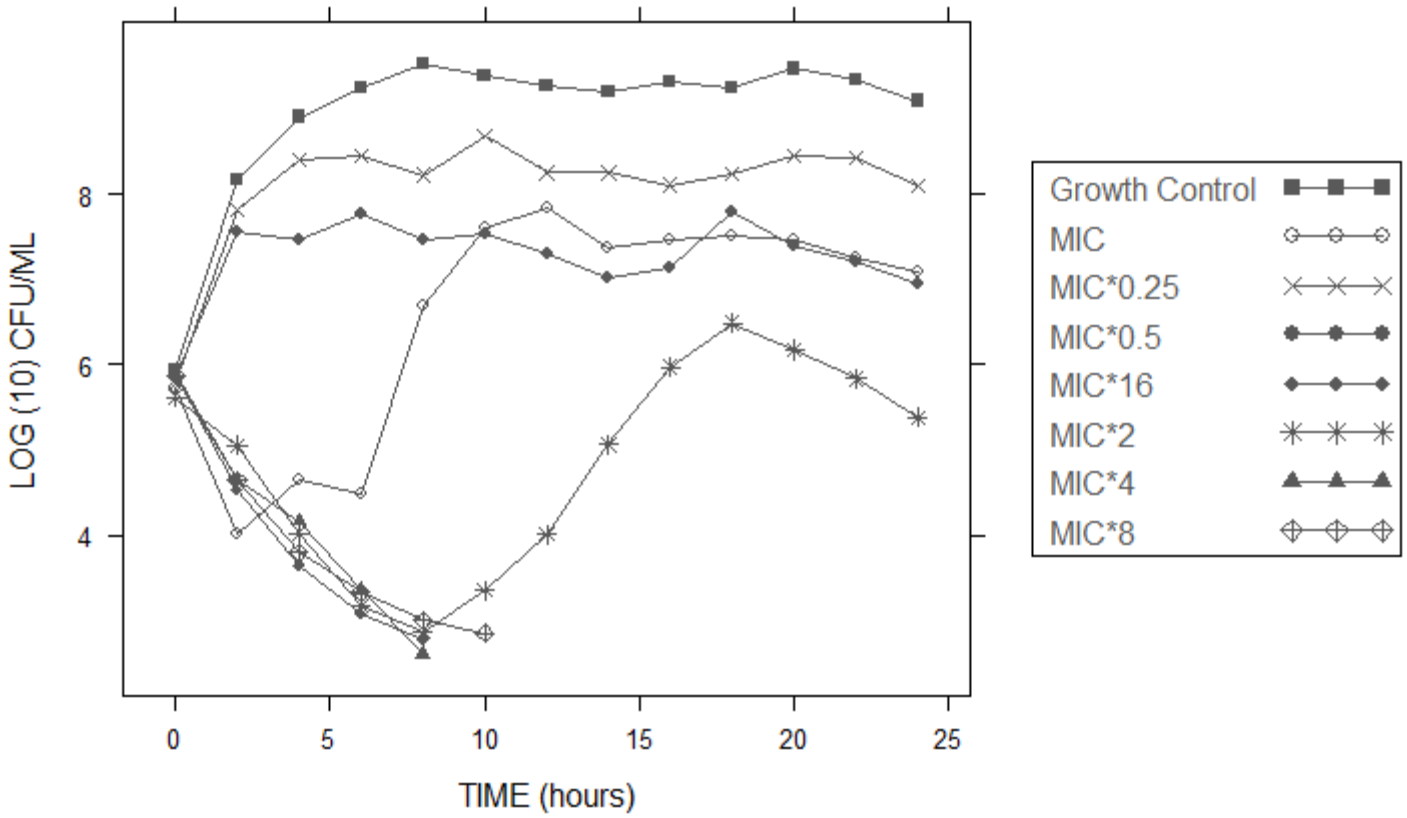

Figure 3-2. Bacterial time curves in the absence of human serum albumin. The data plotted is the mean of experiments performed in triplicate.

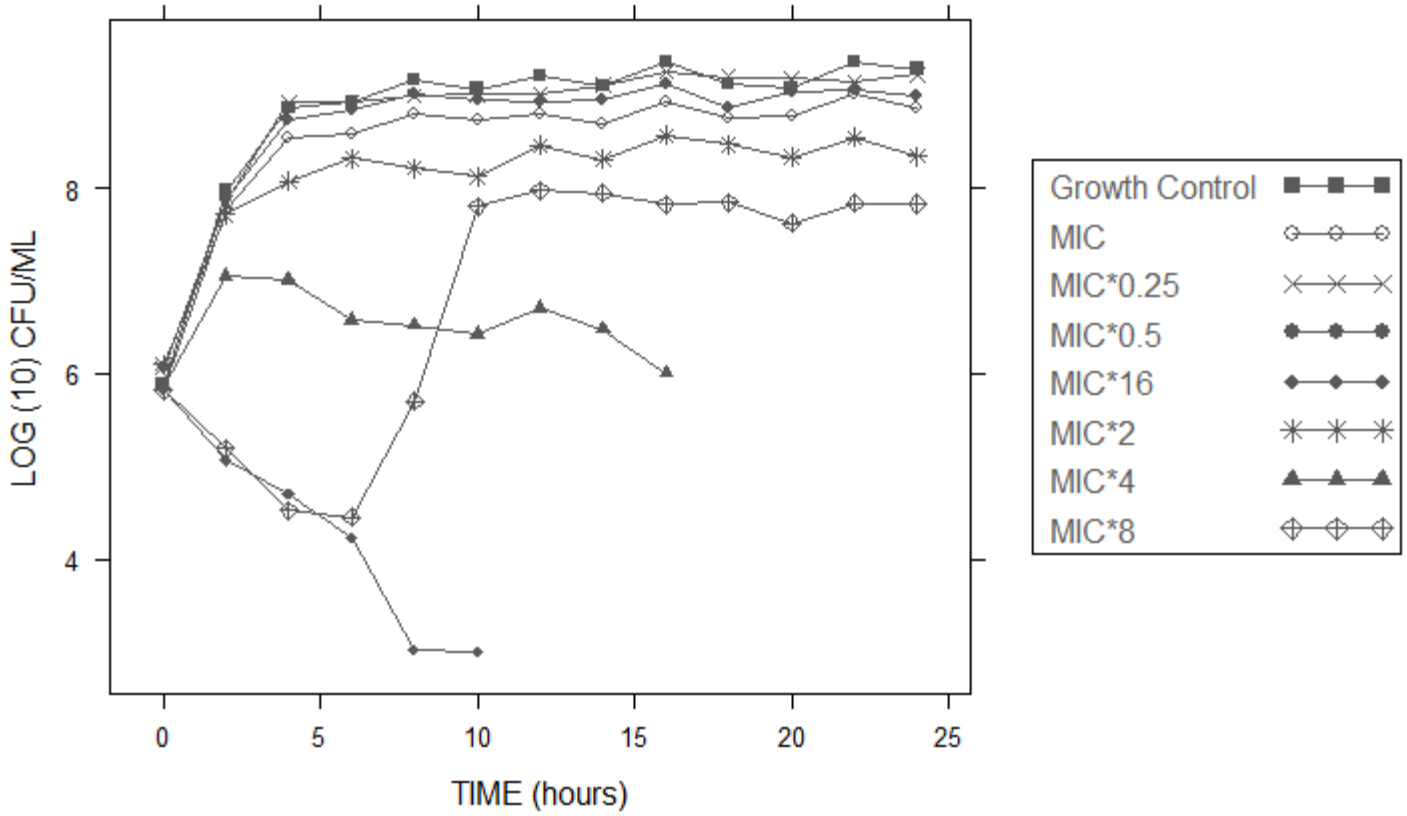

Figure 3-3. Bacterial time curves in the presence of 2 g/dL of human serum albumin. The data plotted is the mean of experiments performed in triplicate.

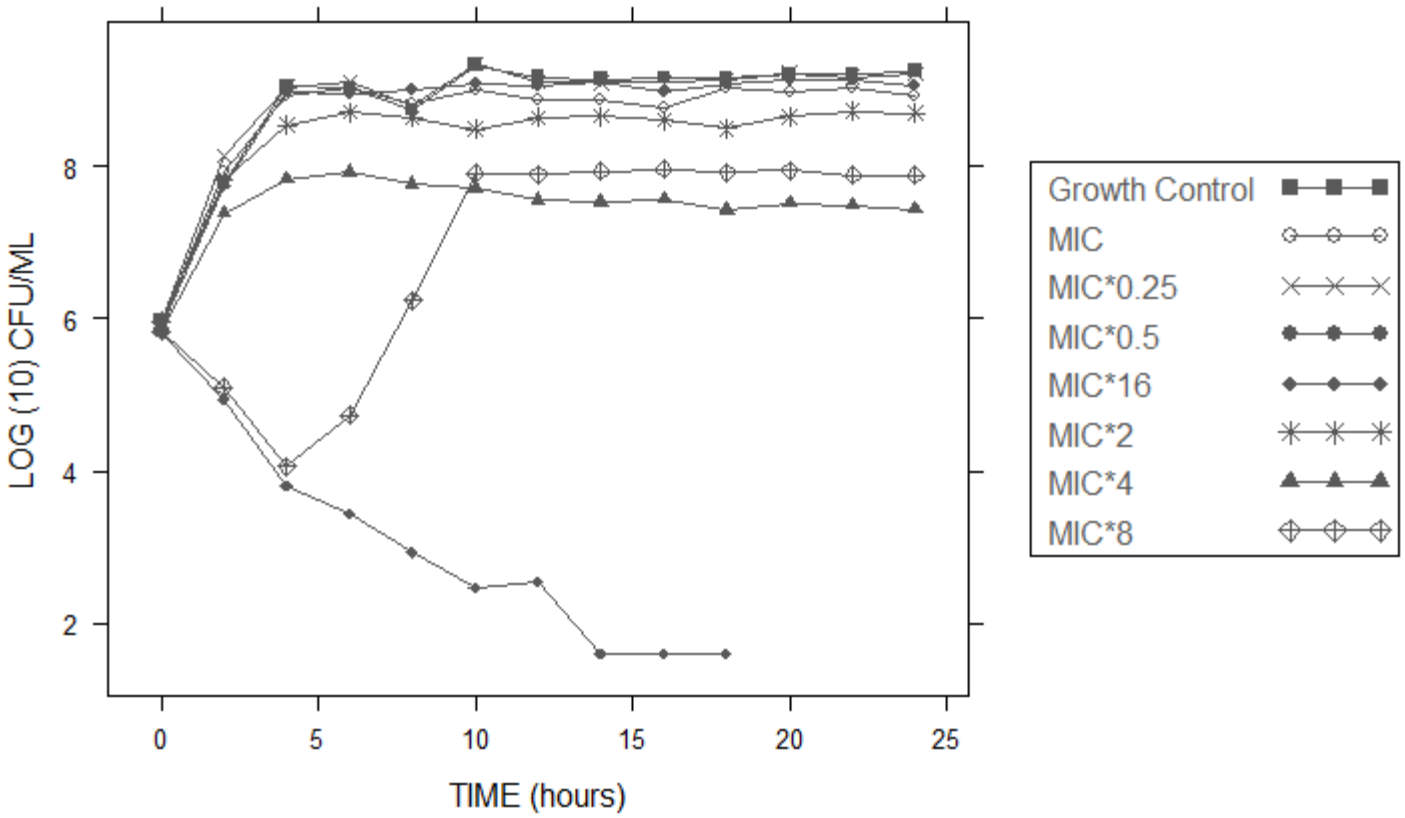

Figure 3-4. Bacterial time curves in the presence of 4 g/dL of human serum albumin. The data plotted is the mean of experiments performed in triplicate.

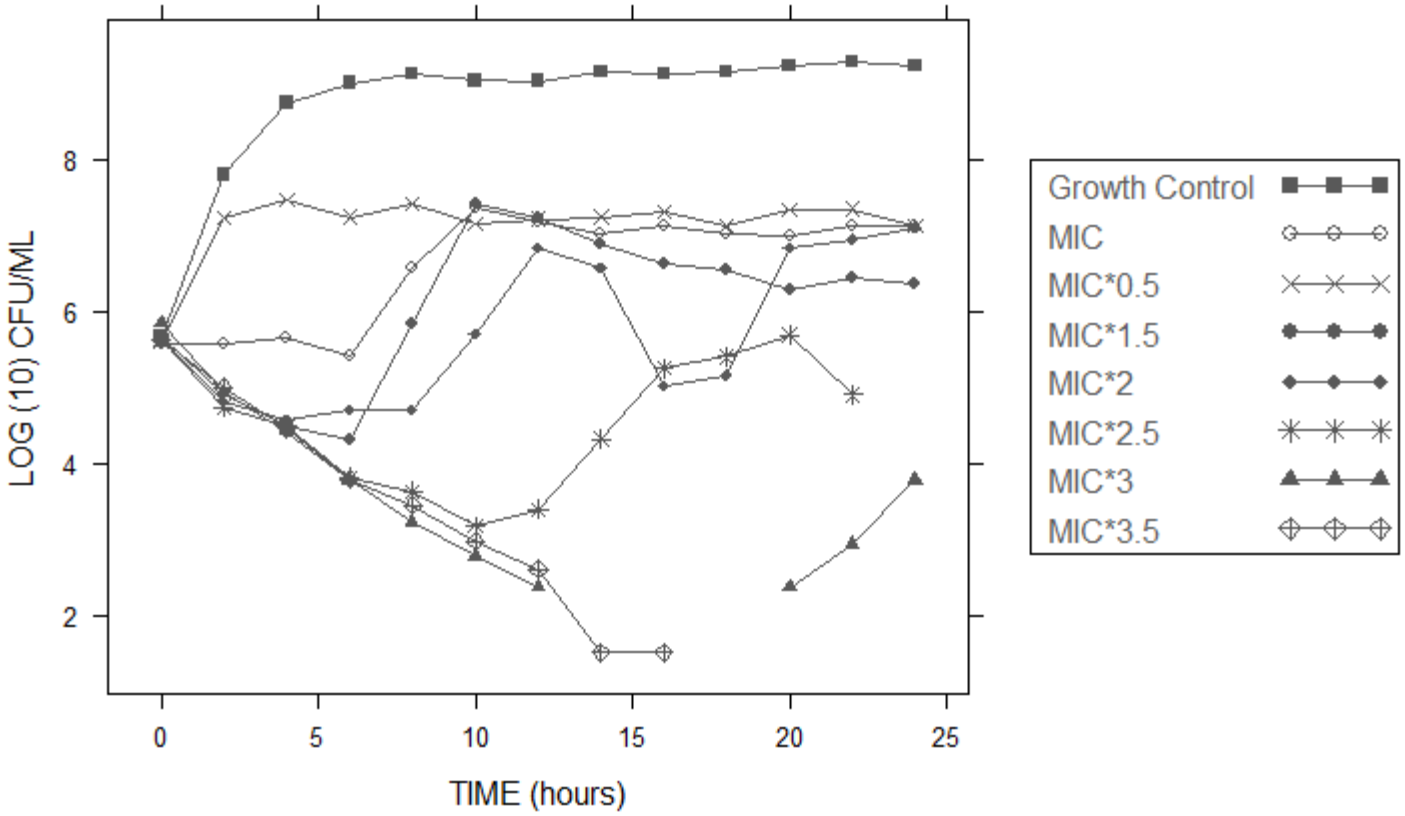

Figure 3-5. Follow-up bacterial time curves performed to study the development of drug resistance to ceftriaxone in the absence of human serum albumin. The data plotted is the mean of experiments performed in triplicate.

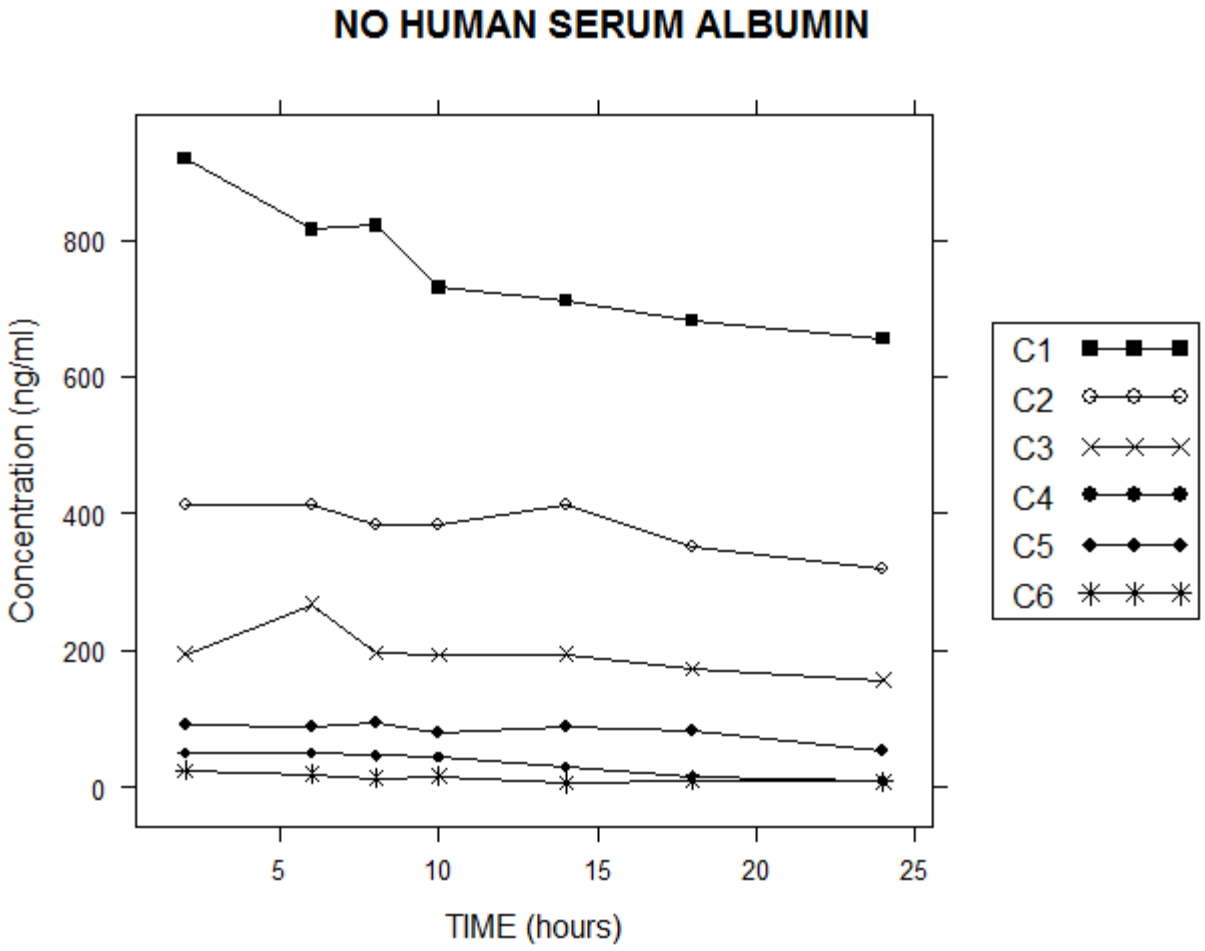

Figure 3-6. Free ceftriaxone concentrations in the absence of human serum albumin and measured using microdialysis. The data plotted is the mean of experiments performed in triplicate. Expected concentrations for each flask were: C1 960 ng/mL; C2 480 ng/mL; C3 240 ng/mL; C4 120 ng/mL; C5 60 ng/mL; C6 30 ng/mL.

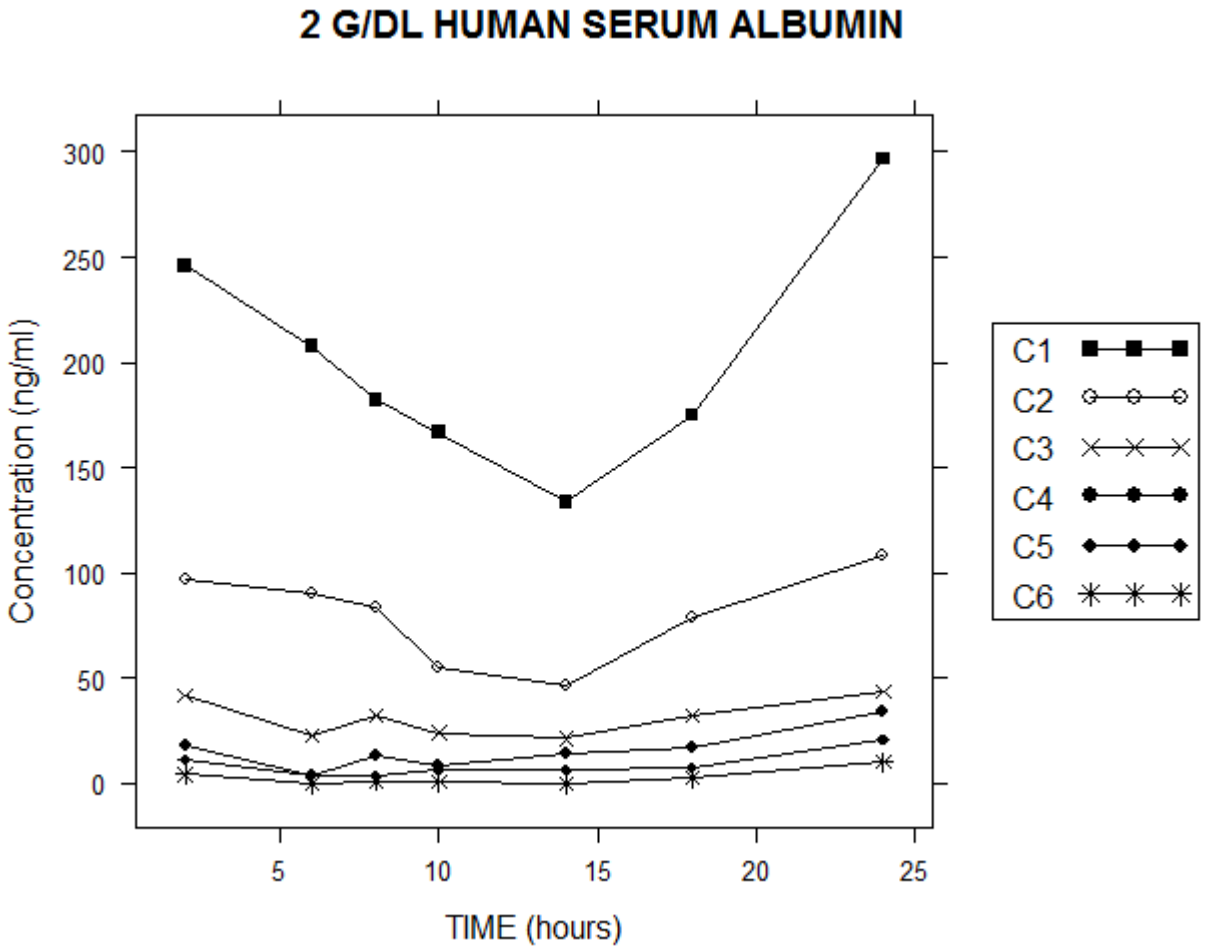

Figure 3-7. Free ceftriaxone concentrations in the presence of 2 g/dL of human serum albumin and measured using microdialysis. The data plotted is the mean of experiments performed in triplicate.

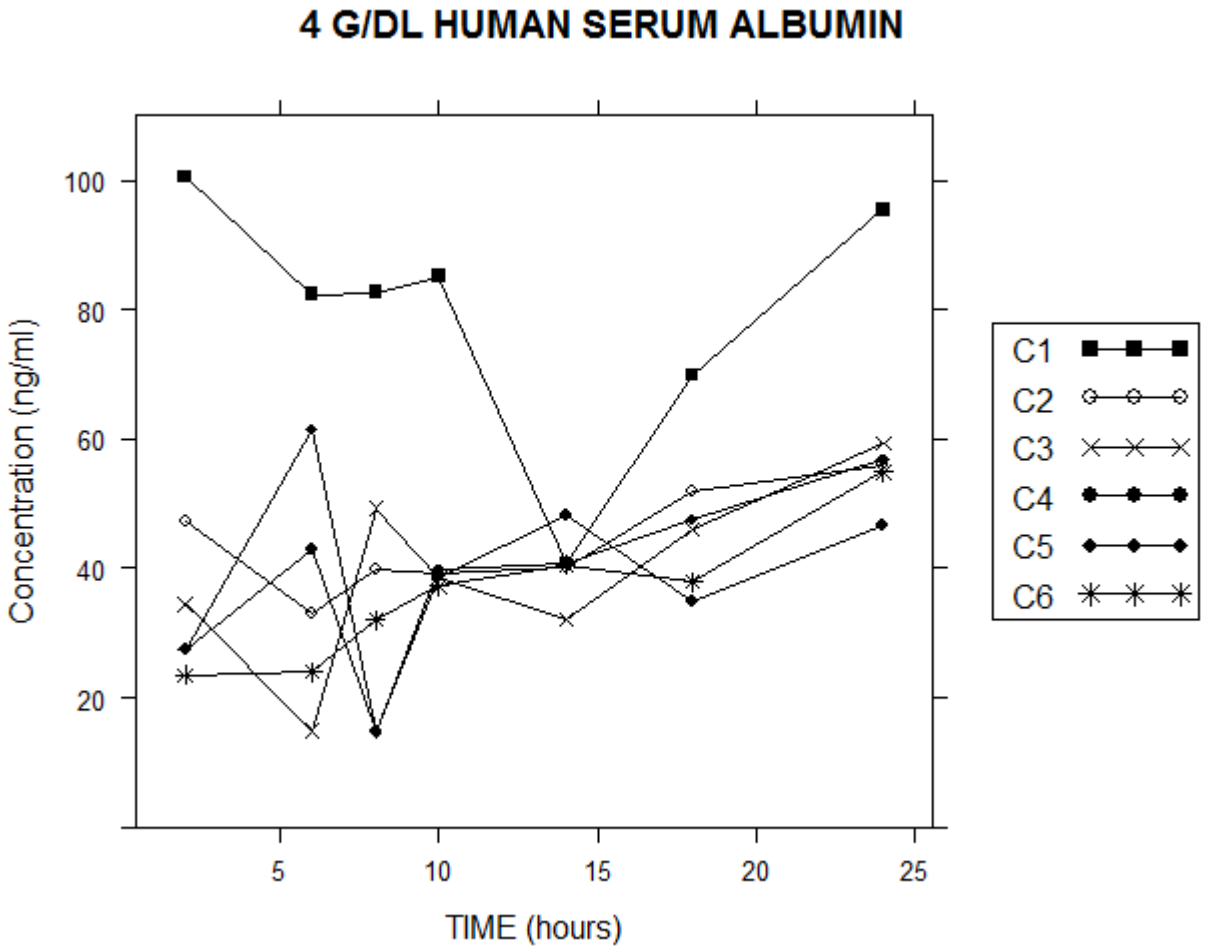

Figure 3-8. Free ceftriaxone concentrations in the presence of 4 g/dL of human serum albumin and measured using microdialysis. The data plotted is the mean of experiments performed in triplicate.

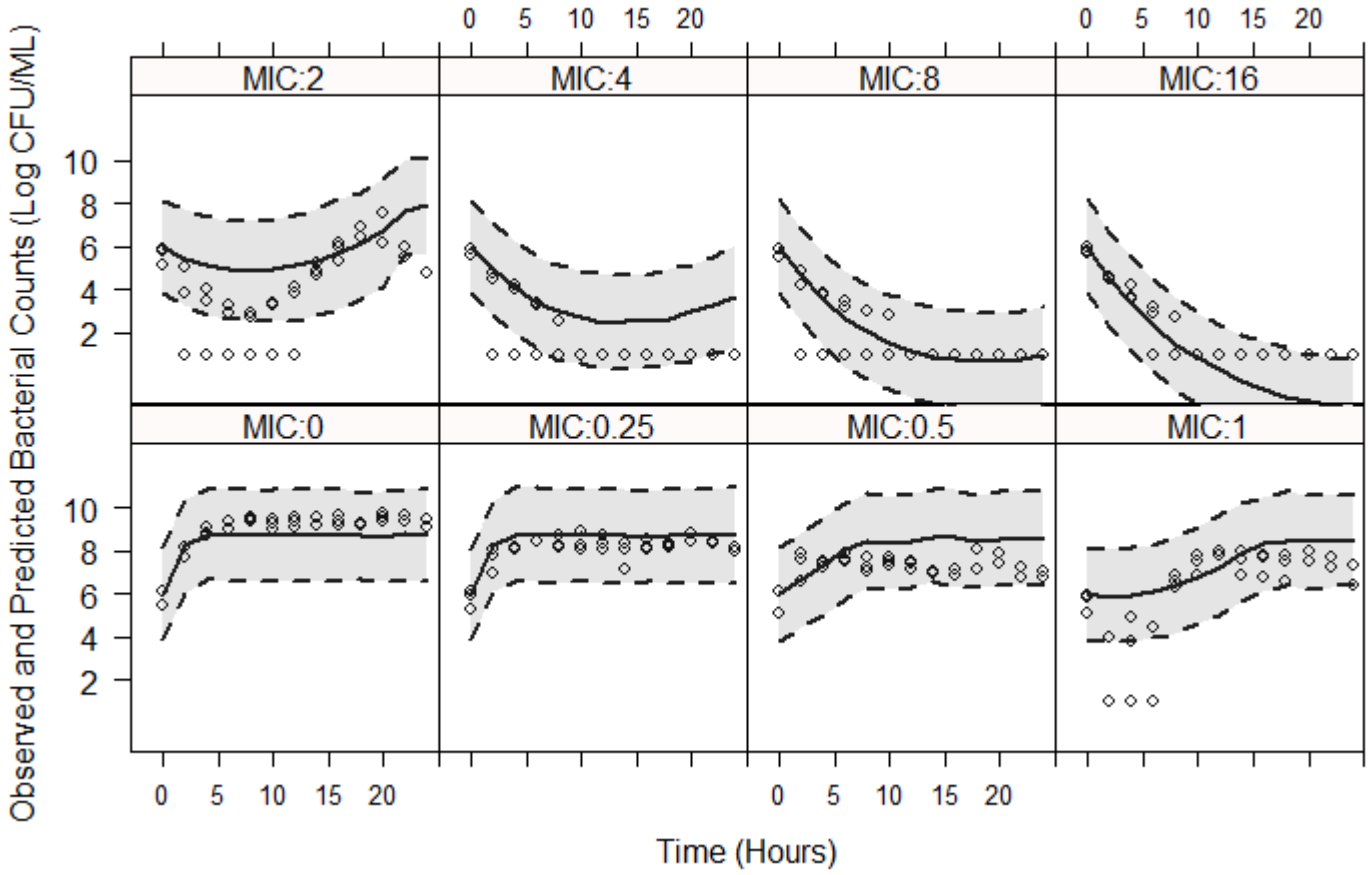

Figure 3-9. Observed data (open circles) and 95% prediction intervals (shaded area) in the absence of human serum albumin. Individual plots represent the various MIC multiples.

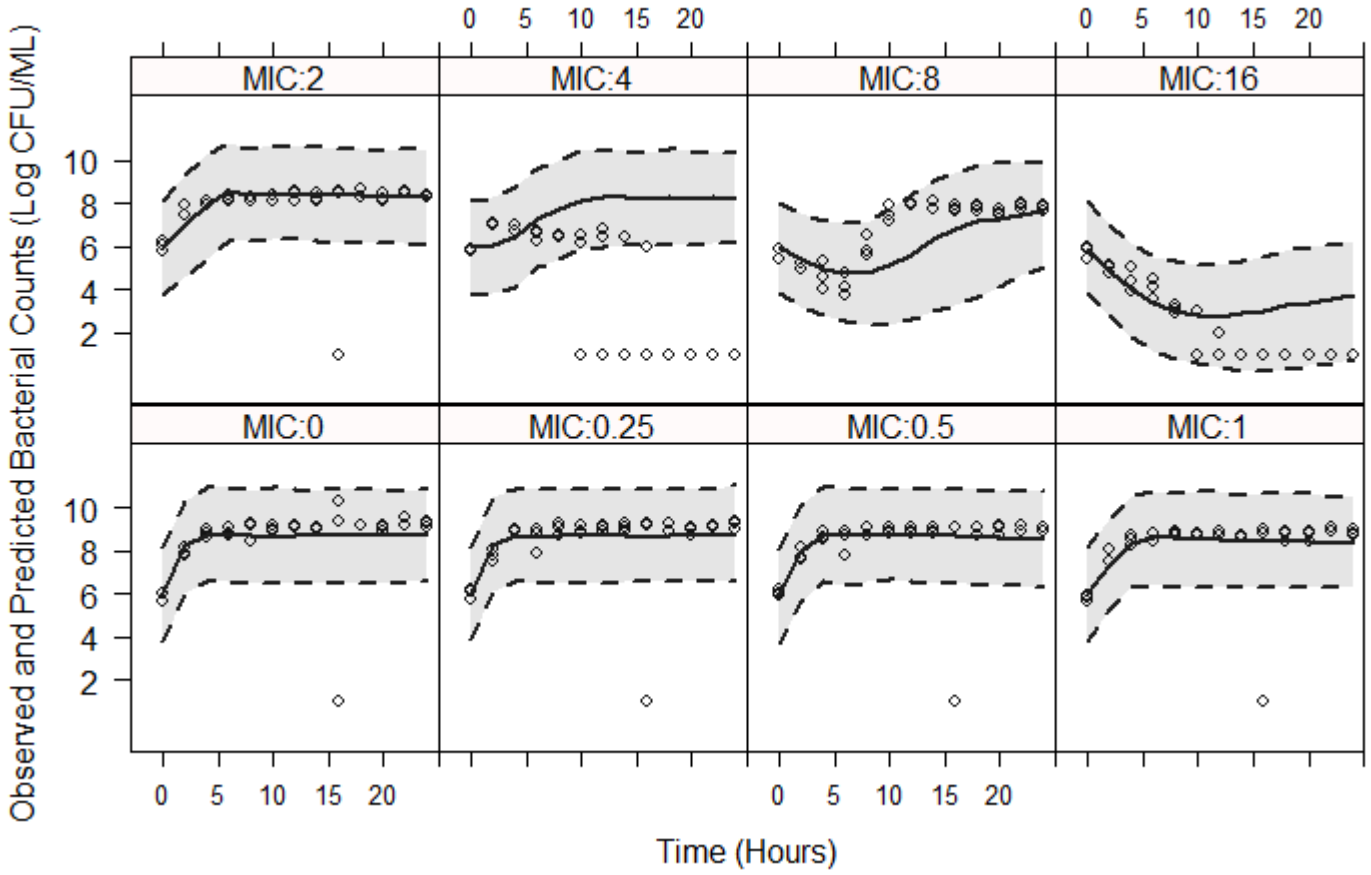

Figure 3-10. Observed data (open circles) and 95% prediction intervals (shaded area) in the presence of 2 g/dL human serum albumin. Individual plots represent the various MIC multiples.

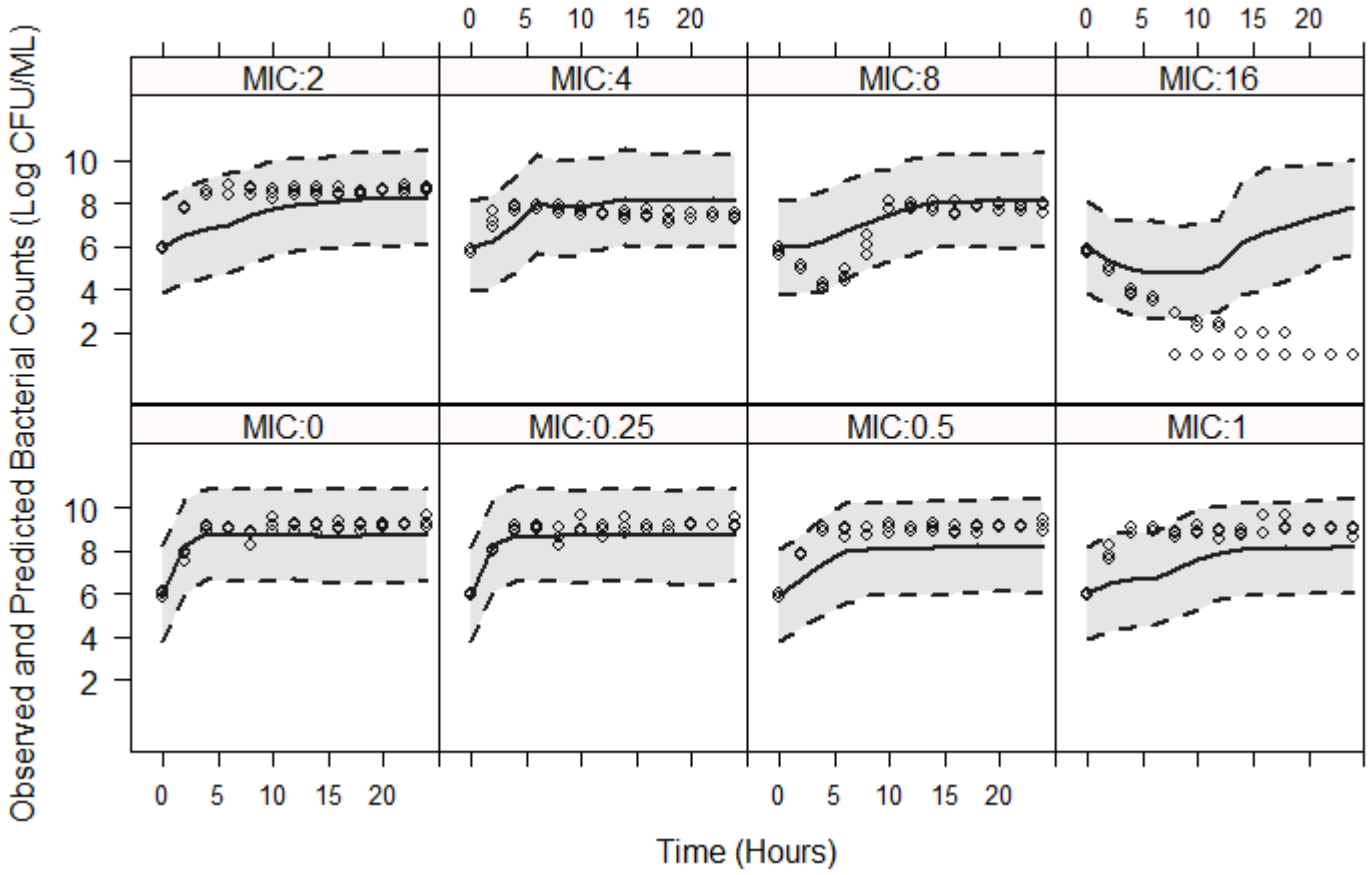

Figure 3-11. Observed data (open circles) and 95% prediction intervals (shaded area) in the presence of 4 g/dL human serum albumin. Individual plots represent the various MIC multiples.

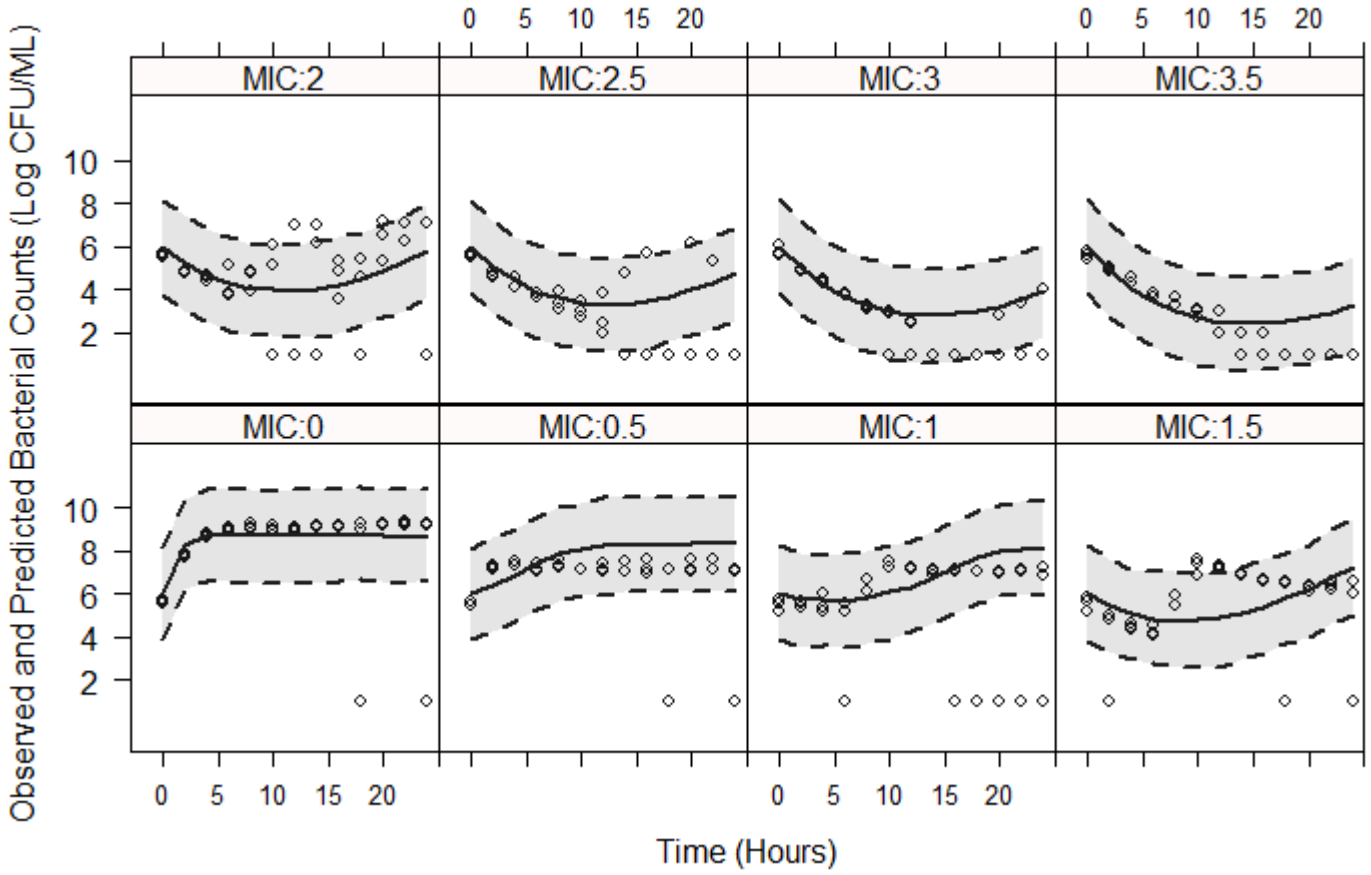

Figure 3-12. Observed data (open circles) and 95% prediction intervals (shaded area) for bacterial time-kill curves performed to evaluate resistance development.

CHAPTER 4
DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS TO QUANTIFY DICLOFENAC CONCENTRATIONS IN MICRODIALYSIS AND PLASMA SAMPLES

Introduction

Diclofenac sodium (2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid) is a nonsteroidal anti-inflammatory drug (NSAID) administered either locally or systemically for its analgesic and anti-inflammatory properties. It is commercially available as a sodium and potassium salt. Diclofenac is a weak acid with pKa of 4 and a partition coefficient in n-octanol/water of 13.4.[57] Following a 50-mg oral dose of diclofenac, a maximal concentration of 7.8 µg/mL were observed; whereas much lower peak plasma concentrations (0.7- 6 ng/mL) were observed following administration as a transdermal patch (Flector®).[58] Following oral administration, diclofenac has a half-life of elimination and an oral bioavailability of approximately 1.8 hours and 50%, respectively.[58] The low oral bioavailability is largely a result of significant first-pass metabolism.

Several publications have described the development of bioanalytical methods to quantify diclofenac concentrations in plasma, urine, and microdialysis samples using high performance liquid chromatography (HPLC) and liquid chromatography mass spectroscopy (LC/MS).[59–67] One study compared the use of an HPLC-UV method and tandem mass spectrometry methods for detection of diclofenac in microdialysis samples.[60] A lower limit of quantification was observed with the LC/MS method (10 vs. 1 ng/mL). In addition, a higher number of false positives and negative values were observed with the HPLC-UV assay when analyzing biological samples collected in a clinical microdialysis study performed using Voltaren® Emulgel®.

The major objectives of the studies described herein was to develop and validate two analytical methods in accordance to published guidelines set forth by the U.S.

FDA.[68] These methods would be used to quantify diclofenac concentrations in microdialysis and plasma samples collected from a clinical study in which a single-dose of Flector® is applied.

Methods

Chemicals and Equipment

The following chemicals and reagents were used:

- Water, double distilled, Corning AG-3
- Methanol, Fisher, #A452-4
- Hexane, Acros, #26836-0025
- 0.9% Sodium Chloride Injection USP (Saline), Premixed Bag, 500 mL, Exp. January 2012, Baxter LOT C817619
- Ammonium acetate, Fisher, #A639500
- Acetic Acid, Glacial, Fisher, #A-38
- O-phosphoric acid 85%, Fisher, #A260-500
- Diclofenac sodium salt (Purity ≥ 99%), Sigma-Aldrich® D6899- 10G, 127K1220
- Indomethacin (Purity ≥ 99%), Sigma-Aldrich® 17378- 5G, 088K0666

The following equipment was used:

- Eppendorf Research® (adjustable) pipettes, capacity of 20, 200 & 1000 µl, Eppendorf
- Eppendorf Centrifuge, 5810R, Eppendorf
- Jouan Centrifuge Evaporator RC10-10 and Edwards RV5 Pump
- epT.I.P.S., 200 & 1000 µl tips for Eppendorf Research® pipettes, Eppendorf
- Vortex Maxi Mix II, Thermo Scientific
- Microcentrifuge Tubes with Flat Top Cap, 1.5 mL, Fisher LOT 10430508
- Centrifuge Tubes, 15mL, Corning 430055

- Centrifuge Tubes, 50mL, Corning 430828
- Borosilicate Culture Tubes, 13 x 100 mm, Fisher
- Vial, Crimp/Snap, 12x32, 300µL, PP, Concial, Sun Sri$_{TM}$ 500 102, LOT 00117973
- Seal, AL Crimp, 11mm, TFE/Red Rubber, Silver, Sun Sri$_{TM}$ 200 100, LOT 00118189
- PE200-series pump
- Autosampler, Perkin Elmer, Norwalk, CT, USA
- Symmetry® C-18 column (4.6 x 50 mm, 3.5 µm particle size), Waters, Dublin, Ireland, Part No. WAT200625, Lot No. 0196302231
- 0.2 µm filter, Millipore, Cork, Ireland
- Triple- quadrupole API 4000 mass spectrometer, Applied Biosystems, Carlsbad, CA, USA
- Leybold TW 700 turbo molecular pump, Oerlikon- Leybold, Cologne, Germany

Microdialysis Samples

Reagent preparation

A primary stock diclofenac solution (1 mg/mL) was prepared by dissolving 10 mg of diclofenac sodium in 10 mL of a one-to-one mixture containing methanol and deionized water. Similarly, a primary stock solution of indomethacin (1 mg/mL), an internal standard in our method, was prepared by mixing 10 mg of indomethacin with 10 mL of methanol. Then a secondary indomethacin stock solution was prepared by mixing 100 µL of the primary stock solution with 9.9 mL of a one-to-one mixture containing methanol and sodium chloride 0.9%. Last, a “working solution” (WS) of indomethacin was prepared by adding 200 µL of the secondary stock with 39.8 mL of a one-to-one mixture containing methanol and 0.9% sodium chloride.

This WS containing indomethacin was then used to dilute the primary diclofenac solution. A secondary diclofenac study was prepared by adding 10 µL of diclofenac primary stock with 990 µL of WS. Then a third diclofenac stock was prepared by adding 100 µL of the secondary diclofenac stock to 990 µL of WS. Calibration and QC samples were prepared as described below.

Double Blank: 100 µl MeOH: Saline (1:1) (double blank)
Blank: 100 µl working solution (blank)
Standard 1 (100 ng/mL): Third Stock (100 µL) is added to 900 µL WS (C1)
Standard 2 (50 ng/mL): Standard 1 (500 µL) is added to 500 µL WS (C2)
Standard 3 (25 ng/mL): Standard 2 (500 µL) is added to 500 µL WS (C3)
Standard 4 (12.5 ng/mL): Standard 3 (500 µL) is added to 500 µL WS (C4)
Standard 5 (6.25 ng/mL): Standard 4 (500 µL) is added to 500 µL WS (C5)
Standard 6 (3.13 ng/mL): Standard 5 (500 µL) is added to 500 µL WS (C6)
Standard 7 (1.56 ng/mL): Standard 6 (500 µL) is added to 500 µL WS (C7)
Standard 8 (0.78 ng/mL): Standard 7 (500 µL) is added to 500 µL WS (C8)
Standard 9 (0.39 ng/mL): Standard 8 (500 µL) is added to 500 µL WS (C9)
QC1 (80 ng/mL): Third Stock (80 µL) is added to 920 µL of WS (HQC)
QC2 (10 ng/mL): Quality Control 1 (125 µL) is added to 875 µL of WS (MQC1)
QC3 (4 ng/mL): Quality Control 2 (400 µL) is added to 600 µL of WS (MQCC2)
QC4 (1 ng/mL): Quality Control 2 (100 µL) is added to 900 µL of WS (LQC)
QC5 (0.39 ng/mL): Standard 8 (100 µL) is added to 100 µL of WS (LLOQ)

During the method validation process, at least one calibration curve and three sets of quality control samples were prepared and assessed for accuracy and precision. Following preparation in micro-centrifuge tubes, 100 µL of each sample was transferred to 300 µL vials.

Method conditions

As the mobile phase, HPLC-grade methanol and 10 mM ammonium acetate buffer adjusted to pH 4.2 was used at a constant ratio of 75:25, respectively. The ammonium acetate buffer was prepared by weighing 385.5 mg of HPLC-grade ammonium acetate and dissolving it in 500 mL of deionized water. The pH was adjusted to 4.2 using acetic

acid. The buffer was filtered and sonicated for 15 minutes. A flow rate of 0.45 mL/min was used.

All samples were analyzed using a triple-quadrupole API 4000 mass spectrometer. The system was operated in multiple reaction monitoring (MRM) mode and was operated using the Analyst software version 1.4.2 (MDS Sciex, Toronto, Canada). Precursor and product ions were monitored for a dwell time of 250 ms at m/z 296.1/215, m/z 296.1/249.9, and m/z 358.2/139 in positive ion mode. For each of these precursor and product ion combinations, the declustering potential (DP) was set to 35, 35, and 65 volts; while the collision energy was 29, 19, and 25 volts. A run time and injection volume of 6 minutes and 10 µL was used, respectively.

Stability studies

Stability samples were conducted to evaluate the stability of diclofenac in a working solution containing a one-to-one mixture of methanol and 0.9% sodium chloride. Short- and long-term, freeze-thaw, and primary stock stability studies were conducted. For the short term stability studies, stability was evaluated following 24 hours of storage in the auto-sampler. Long term stability was assessed following 30 days of storage at -70°C. Samples were exposed to three freeze-thaw cycles were they were frozen at -70°C, then thawed and analyzed after three cycles. Primary stock stability was assessed by diluting the primary stock to 100 ng/mL and then comparing the observed concentrations on day 1 and day 8. Except for the primary stock stability, all stability studies were also studied with addition of dextran 3%. Dextran may be added to the perfusate in clinical microdialysis studies in order to prevent ultrafiltration.

Plasma Samples

Reagent preparation

The mobile phase consisted of HPLC-grade methanol and 10 mM ammonium acetate adjusted to pH 4.2. Preparation of the ammonium acetate buffer was previously described. Indomethacin was again used as the internal standard in this method. First, “IS Stock 1” (1 mg/mL) was prepared by dissolving 10 mg of indomethacin in 10 mL of methanol. Then “IS Stock 2” (10 µg/mL) was prepared by adding 100 µL of IS Stock 1 to 9.9 mL methanol. Last, “IS working solution” (750 ng/mL) was prepared using 150 µL of IS Stock 2 and 1.85 mL of cold methanol.

Diclofenac stock solutions were prepared. A diluent of deionized water and methanol in a one-to-one mixture was prepared. Then primary and secondary drug stocks were prepared similar to indomethacin using a one-to-one mixture of deionized water and methanol. A third drug stock (1000 ng/mL) was prepared using 1 mL of the secondary stock and 9 mL of diluent. Next, diclofenac working stocks (DWS) were prepared as shown below.

DWS1 (1000 ng/mL): 4 mL of third stock
DWS2 (500 ng/mL): 2 mL WS1 plus 2 mL diluent
DWS3 (250 ng/mL): 2 mL WS2 plus 2 mL diluent
DWS4 (125 ng/mL): 2 mL WS3 plus 2 mL diluent
DWS5 (62.5 ng/mL): 2 mL WS4 pus 2 mL diluent
DWS6 (31.25 ng/mL): 2 mL WS5 plus 2 mL diluent
DWS7 (15.63 ng/mL): 2 mL WS6 plus 2 mL diluent

Using these working stocks, calibration standards and QC samples were prepared as follows:

Double Blank: 200 µl plasma
Blank: 200 µl plasma is added to 10 µl IS WS
Standard 1 (100 ng/mL): 20 µL DWS1 added to 180 µL plasma and 10 µL IS WS
Standard 2 (50 ng/mL): 20 µL DWS2 added to 180 µL plasma and 10 µL IS WS
Standard 3 (25 ng/mL): 20 µL DWS3 added to 180 µL plasma and 10 µL IS WS
Standard 4 (12.5 ng/mL): 20 µL DWS4 added to 180 µL plasma and 10 µL IS WS
Standard 5 (6.25 ng/mL): 20 µL DWS5 added to 180 µL plasma and 10 µL IS WS
Standard 6 (3.13 ng/mL): 20 µL DWS6 added to 180 µL plasma and 10 µL IS WS
Standard 7 (1.56 ng/mL): 20 µL DWS7 added to 180 µL plasma and 10 µL IS WS
HQC (80 ng/mL): 16 µL DWS1 is added to 184 µL plasma and 10 µL IS WS
MQC (50 ng/mL): 20 µL DWS2 is added to 180 µL plasma and 10 µL IS WS
LQC (5 ng/mL): 20 µL QC2 is added to 180 µL plasma and 10 µL IS WS
LLOQ (1.56 ng/mL): 20 µL DWS7 is added to 180 µL plasma and 10 µL IS WS

QC samples were prepared in bulk. Then precision accuracy batches (PA) were analyzed on three separate days. For each PA batch, QC samples were prepared six times using the extraction process detailed below (steps 1-17). Similarly, for preparation of calibration standards (CS), Steps 3-17 were followed.

- Step 1: Remove 200 µL out of each bulk six times (HQC, MQC, LQC, LLOQ).
- Step 2: Add 10 µL of IS (35 ng/mL) to each sample.
- Step 3: Vortex for 10 seconds.
- Step 4: Add 50 µL of 1:1 dilution of 85% o-phosphoric Acid.
- Step 5: Vortex each tube for 30 seconds.
- Step 6: Add 2 mL of hexane.
- Step 7: Vortex for 2 minutes.
- Step 8: Centrifuge at 2000xg for 10 minutes.
- Step 9: Separate all supernatant with glass Pasteur pipettes.
- Step 10: Evaporate all samples to dryness under vacuum.
- Step 11: Add 2 mL of hexane.
- Step 12: Vortex for 2 minutes.
- Step 13: Centrifuge at 2000xg for 10 minutes.
- Step 14: Separate all supernatant with glass Pasteur pipettes.
- Step 15: Evaporate all samples to dryness under vacuum.
- Step 16: Reconstitute in 100 µl of diluent (TDW: MeOH, 50:50).
- Step 17: Place 100 µl in a vial for analysis.

The impact of the matrix was evaluated by preparing QC samples in diluent and spiking each QC in blank plasma processed as shown above. Matrix effect QC samples were prepared as follows:

HQC (80 ng/mL): 16 µl WS1 is added to 74 µL diluent and 10 µL IS WS
MQC (50 ng/mL): 20 µl WS2 is added to 70 µL diluent and 10 µL IS WS
LQC (5 ng/mL): 20 µl MQC is added to 70 µL diluents and 10 µL IS WS
LLOQ (1.56 ng/mL): 20 µl WS7 is added to 70 µL diluent and 10 µL IS WS

These samples were then added to plasma processed as follows:

- Step 1: 200 µL of blank human plasma is placed in 24 glass tubes.
- Step 2: Add 50 µl of 1:1 dilution of 85% o-phosphoric Acid.
- Step 3: Vortex for 30 seconds.
- Step 4: Add 2 mL of hexane.
- Step 5: Vortex for 2 minutes.
- Step 6: Centrifuge at 2000xg for 10 minutes.
- Step 7: Separate all supernatant with glass Pasteur pipettes.
- Step 8: Evaporate all samples to dryness under vacuum.
- Step 9: Add 2 mL of hexane.
- Step 10: Vortex for 2 minutes.
- Step 11: Centrifuge at 2000xg for 10 minutes.
- Step 12: Separate all supernatant with glass Pasteur pipettes.
- Step 13: Evaporate all samples to dryness under vacuum.
- Step 14: Reconstitute in 100 µL of each QC sample.
- Step 15: Place 100 µl of each sample in a vial for injection.

Last, to calculate the recovery of the drug following this extraction process, quality control samples were prepared in diluents as described above and injected directly.

Stability studies

Studies were conducted to evaluate the short term and freeze-thaw stability of diclofenac in plasma samples. For the short term stability studies, stability was evaluated following 24 hours of storage in the auto-sampler. Samples were exposed to three freeze-thaw cycles were they were frozen at -70°C, then thawed and analyzed after three cycles.

Results

During validation of an analytical method to quantify diclofenac in microdialysis samples, accuracy and precision were assessed. All calibration standards were within 15% accuracy on three separate days (Table 4-1). Three sets of quality controls were analyzed on each validation day. Of these samples, no more than one quality control sample per day failed to meet accuracy standards (Tables 4-2 and 4-3). Inter-day precision was less than 15% for all quality controls (Table 4-4). Short- and long-term, freeze-thaw, and primary stock stability studies resulted in acceptable accuracy and precision values (Tables 4-5 - 4-8).

An analytical method was also developed to quantify diclofenac concentrations in plasma samples. Again accuracy and precision values for the calibration standards and quality controls met acceptability criteria set forth by the U.S. FDA (Tables 4-9, 4-10, and 4-11). Diclofenac appeared stable in plasma following 24 hours of storage in an autosampler and 3 freeze-thaw cycles (Tables 4-12 and 4-13). The average recovery of the analyte was approximately 62%; an average matrix enhacement of 39% was observed (Figure 4-1). Greater matrix enhancement was observed at lower diclofenac concentrations.

Discussion

The two developed analytical methods met acceptable standards during three days of validation. Although the recovery values were relatively low, they were reproducible. Significant matrix enhancement was observed; although it was more pronounced at low diclofenac concentrations.

Table 4-1. Calibration curves for microdialysis samples analyzed validation Days 1-3.

Sample Name	Sample Type	Calculated Concentration (ng/mL)	Accuracy (%)
Day 1			
Double Blank	Double Blank	N/A	N/A
Blank	Blank	N/A	N/A
0.390	Standard	0.387	99.3
0.781	Standard	0.817	105.
1.562	Standard	1.55	99.0
3.125	Standard	2.81	89.9
6.25	Standard	6.12	98.0
12.5	Standard	12.7	101.
25	Standard	25.9	104.
50	Standard	50.2	100.
100	Standard	104.	104.
DAY 2			
Double Blank	Double Blank	N/A	N/A
Blank	Blank	N/A	N/A
0.390	Standard	0.411	105.
0.781	Standard	0.739	94.6
1.562	Standard	1.47	94.3
3.125	Standard	3.03	96.9
6.25	Standard	6.19	99.1
12.5	Standard	12.5	100.
25	Standard	25.3	101.
50	Standard	50.8	102.
100	Standard	103.	103.
DAY 3			
Double Blank	Double Blank	N/A	N/A
Blank	Blank	N/A	N/A
0.390	Standard	0.339	87.0
0.781	Standard	0.684	87.6
1.562	Standard	1.61	103.
3.125	Standard	2.84	90.7
6.25	Standard	5.84	93.4
12.5	Standard	12.0	96.2
25	Standard	24.1	96.3
50	Standard	46.6	93.2
100	Standard	96.5	96.5

Table 4-2. Quality control samples analyzed on validation Days 1 and 2.

Sample Name	Sample Type	Calculated Concentration (ng/mL)	Accuracy (%)
Day 1			
LLOQ	Quality Control	0.401	103.
LQC	Quality Control	1.33	133.
MQC2	Quality Control	4.29	107.
MQC1	Quality Control	10.8	108.
HQC	Quality Control	88.6	111.
LLOQ	Quality Control	0.416	107.
LQC	Quality Control	1.03	103.
MQC2	Quality Control	4.24	106.
MQC1	Quality Control	10.8	108.
HQC	Quality Control	90.2	113.
LLOQ	Quality Control	0.433	111.
LQC	Quality Control	1.13	113.
MQC2	Quality Control	4.29	107.
MQC1	Quality Control	11.4	114.
HQC	Quality Control	86.0	108.
Day 2			
LLOQ	Quality Control	0.408	105.
LQC	Quality Control	0.958	95.8
MQC2	Quality Control	3.76	94.1
MQC1	Quality Control	10.1	101.
HQC	Quality Control	78.7	98.4
LLOQ	Quality Control	0.397	102.
LQC	Quality Control	0.982	98.2
MQC2	Quality Control	4.07	102.
MQC1	Quality Control	10.3	103.
HQC	Quality Control	81.3	102.
LLOQ	Quality Control	0.390	100
LQC	Quality Control	1.01	101
MQC2	Quality Control	3.25	81.3
MQC1	Quality Control	9.07	90.7
HQC	Quality Control	86.1	108

Table 4-3. Quality control samples analyzed on validation Day 3.

Sample Name	Sample Type	Calculated Concentration (ng/mL)	Accuracy (%)
Day 3			
LLOQ	Quality Control	0.444	114.
LQC	Quality Control	0.931	93.1
MQC2	Quality Control	3.83	95.6
MQC1	Quality Control	9.74	97.4
HQC	Quality Control	79.9	99.9
LLOQ	Quality Control	0.436	112.
LQC	Quality Control	1.03	103.
MQC2	Quality Control	3.98	99.4
MQC1	Quality Control	9.63	96.3
HQC	Quality Control	80.9	101.
LLOQ	Quality Control	0.372	95.3
LQC	Quality Control	1.01	101.
MQC2	Quality Control	3.96	98.9
MQC1	Quality Control	9.68	96.8
HQC	Quality Control	81.1	101.

Table 4-4. Inter-day precision for microdialysis samples analyzed validation Days 1-3.

	Coefficient of Variation [%]		
	Day 1	Day 2	Day 3
LLOQ	3.84	8.54	10.11
LQC	13.13	5.53	7.48
MQC2	0.68	9.11	3.22
MQC1	3.15	5.65	2.56
HQC	2.40	3.32	5.59

Table 4-5. Short term stability studies of diclofenac in microdialysis samples stored in an auto-sampler for 24 hours. Each concentration was prepared in triplicate.

Sample Name	Observed Concentration (0 hours)	Observed Concentration (24 hours)
No Dextran		
ST LQC a	0.851	0.948
ST LQC b	0.894	1.02
ST LQC c	0.813	1.07
ST HQC a	76.3	75.5
ST HQC b	77.9	76.6
ST HQC c	78.8	79.0
Mean L	0.85	1.01
SD	0.04	0.06
Precision	4.75 %	6.06 %
Accuracy	85.27 %	101.27 %
Mean H	77.67	77.03
SD	1.27	1.79
Precision	1.63 %	2.32 %
Accuracy	97.08 %	96.29 %
Dextran Added		
ST LQC a D	0.95	0.826
ST LQC b D	0.999	0.96
ST LQC c D	1.09	0.832
ST HQC a D	78.0	82.7
ST HQC b D	85.9	89.1
ST HQC c D	81.6	87.2
Mean L	1.01	0.87
SD	0.07	0.08
Precision	7.01 %	8.67 %
Accuracy	101.30 %	87.27 %
Mean H	81.83	86.33
SD	3.96	3.29
Precision	4.83 %	3.81 %
Accuracy	102.29 %	107.92 %

Table 4-6. Long term stability studies of diclofenac in microdialysis samples stored at -70°C for 30 days. Each concentration prepared in triplicate.

Sample Name	Observed Concentration (0 hours)	Observed Concentration (24 hours)
No Dextran		
LT LQC a	1.03	0.88
LT LQC b	1.03	0.871
LT LQC c	1.12	0.973
LT HQC a	77.3	75.9
LT HQC b	77.0	76.7
LT HQC c	79.0	75.1
Mean L	1.06	0.91
SD	0.05	0.06
Precision	4.90 %	6.22 %
Accuracy	106.00 %	90.80 %
Mean H	77.77	75.90
SD	1.08	0.80
Precision	1.39 %	1.05 %
Accuracy	97.21 %	94.88 %
Dextran Added		
LT LQC a D	0.943	1.07
LT LQC b D	0.857	1
LT LQC c D	0.722	0.808
LT HQC a D	81	87.2
LT HQC b D	84	94.1
LT HQC c D	86.6	97.5
Mean L	0.90	0.96
SD	0.06	0.14
Precision	6.76 %	14.14 %
Accuracy	90.00 %	95.93 %
Mean H	83.87	92.93
SD	2.80	5.25
Precision	3.34 %	5.65 %
Accuracy	104.83 %	116.17 %

Table 4-7. Freeze-thaw stability studies of diclofenac in microdialysis samples frozen at -70°C.

Sample Name	Observed Concentration (0 hours)	Observed Concentration (cycle 3)
No Dextran		
FT LQC a	1.16	0.883
FT LQC b	0.997	0.971
FT LQC c	0.942	0.783
FT HQC a	81.4	73.1
FT HQC b	86.0	75.5
FT HQC c	80.7	76.9
Mean L	1.03	0.88
SD	0.11	0.09
Precision	10.97 %	10.70 %
Accuracy	103.30 %	87.90 %
Mean H	82.70	75.17
SD	2.88	1.92
Precision	3.48 %	2.56 %
Accuracy	103.38 %	93.96 %
Dextran Added		
FT LQC a D	1.25	1.44
FT LQC b D	1.13	1.02
FT LQC c D	1.01	0.907
FT HQC a D	79.1	67.4
FT HQC b D	81.1	68.3
FT HQC c D	81.9	70.2
Mean L	1.13	1.12
SD	0.12	0.28
Precision	10.62 %	25.02 %
Accuracy	113.00 %	112.23 %
Mean H	80.70	68.63
SD	1.44	1.43
Precision	1.79 %	2.08 %
Accuracy	100.88 %	85.79 %

Table 4-8. Primary stock stability studies of diclofenac in microdialysis samples stored in at 2 to 8°C for 7 days.

Sample Name	Observed Concentration (0 hours)	Observed Concentration (24 hours)
PS a	112.00	104.00
PS b	112.00	108.00
PS c	115.00	113.00
Mean	113.00	108.33
SD	1.73	4.51
Precision	1.53 %	4.16 %
Accuracy	113.00 %	108.33 %

Table 4-9. Calibration curves for plasma samples analyzed on validation Days 1-3.

Sample Name	Sample Type	Calculated Concentration (ng/mL)	Accuracy (%)
Day 1			
Double Blank	Double Blank	N/A	N/A
Blank	Blank	N/A	N/A
1.562	Standard	1.62	104
3.125	Standard	2.98	95.4
6.25	Standard	5.56	89
12.5	Standard	14.1	113
25	Standard	24.1	96.6
50	Standard	47.9	95.8
100	Standard	126	126
DAY 2			
Double Blank	Double Blank	N/A	N/A
Blank	Blank	N/A	N/A
1.562	Standard	1.41	90.5
3.125	Standard	3.6	115
6.25	Standard	6.98	112
12.5	Standard	11.7	93.9
25	Standard	24	95.9
50	Standard	33	66.1
100	Standard	97	97
DAY 3			
Double Blank	Double Blank	N/A	N/A
Blank	Blank	N/A	N/A
1.562	Standard	1.71	110
3.125	Standard	3.21	103
6.25	Standard	6.27	100
12.5	Standard	11.9	95
25	Standard	25.3	101
50	Standard	54.3	109
100	Standard	107	107

Table 4-10. Precision accuracy (PA) batches for plasma samples analyzed on validation Days 1-3.

	LLOQ 1.56		LQC 5		MQC 50		HQC 80	
	[DRUG]	ACCURACY (%)	[DRUG]	ACCURACY (%)	[DRUG]	ACCURACY (%)	[DRUG]	ACCURACY (%)
PA-1	1.85	118.60	5.12	102.47	42.22	84.44	69.01	86.26
	2.40	153.84	3.51	70.22	40.00	80.01	65.76	82.20
	1.60	102.25	4.36	87.20	40.40	80.81	66.27	82.84
	1.81	116.27	4.25	85.04	42.59	85.17	66.07	82.59
	1.74	111.50	4.54	90.71	55.18	110.37	81.37	101.71
	1.50	96.20	5.16	103.22	55.69	111.39	78.65	98.31
	1.12	71.75	5.77	115.50	46.24	92.48	80.23	100.28
PA-2	1.42	91.01	3.64	72.78	50.55	101.09	83.99	104.99
	1.33	85.36	4.49	89.75	39.86	79.71	99.52	124.40
	1.30	83.56	4.29	85.77	44.09	88.17	80.21	100.26
	1.37	87.99	4.60	91.95	42.16	84.33	81.50	101.87
	1.59	101.88	4.34	86.82	37.58	75.16	78.51	98.14
	1.64	105.43	4.52	90.30	51.47	102.93	69.43	86.78
	1.67	107.20	4.90	97.90	40.92	81.84	69.60	87.00
PA-3	2.64	169.30	4.10	82.02	50.01	100.02	69.12	86.41
	1.56	100.28	5.09	101.74	49.68	99.35	68.00	85.00
	1.58	101.10	4.70	93.91	45.93	91.85	71.77	89.71
	1.80	115.36	3.89	77.71	51.01	102.02	NA	NA
N	18.00	18.00	18.00	18.00	18.00	18.00	17.00	17.00
MEAN (ng/mL)	1.66	106.60	4.51	90.28	45.87	91.73	75.24	94.04
SD (ng/mL)	0.37	23.65	0.56	11.28	5.58	11.16	8.93	11.16
CV (%)	22.18	22.18	12.49	12.49	12.17	12.17	11.87	11.87

Table 4-11. Inter-day precision for plasma samples analyzed on validation Days 1-3.

	Coefficient of Variation [%]		
	Day 1	Day 2	Day 3
LLOQ	17.34	11.34	22.71
LQC	13.68	15.46	10.22
MQC	16.02	1.69	8.42
HQC	9.81	9.31	1.97

Table 4-12. Short term stability studies of diclofenac in plasma samples stored in an auto-sampler for 24 hours. Each concentration prepared in triplicate.

Sample Name	Observed Concentration (0 hours)	Observed Concentration (24 hours)	Sample Name	Observed Concentration (0 hours)	Observed Concentration (24 hours)
LLOQ 1	1.85	1.69	MQC 1	42.20	43.80
LLOQ 2	2.40	2.24	MQC 2	39.90	42.60
LLOQ 3	1.59	1.48	MQC 3	40.50	38.40
LLOQ 4	1.81	1.83	MQC 4	42.50	42.70
LLOQ 5	1.74	1.73	MQC 5	55.20	51.60
LLOQ 6	1.50	1.59	MQC 6	55.60	55.80
Mean (ng/mL)	1.82	1.76	Mean (ng/mL)	45.98	45.82
SD (ng/mL)	0.32	0.26	SD (ng/mL)	7.36	6.52
CV (%)	17.40	15.00	CV (%)	16.01	14.22
Accuracy (%)	116.35	112.82	Accuracy (%)	91.97	91.63
LQC 1	5.11	4.59	HQC 1	68.90	61.90
LQC 2	3.51	4.23	HQC 2	65.60	64.00
LQC 3	4.35	4.36	HQC 3	66.10	61.00
LQC 4	4.24	4.48	HQC 4	66.10	69.70
LQC 5	4.53	3.96	HQC 5	81.50	78.00
LQC 6	5.16	5.08	HQC 6	78.50	78.80
Mean (ng/mL)	4.48	4.45	Mean (ng/mL)	71.12	68.90
SD (ng/mL)	0.61	0.38	SD (ng/mL)	7.04	7.96
CV (%)	13.67	8.49	CV (%)	9.90	11.55
Accuracy (%)	89.67	89.00	Accuracy (%)	88.90	86.13

Table 4-13. Freeze-thaw studies of diclofenac in plasma samples.

Sample Name	Observed Concentration for PA-1 (ng/mL)	Observed Concentration After 3 F/T Cycles (ng/mL)	Sample Name	Observed Concentration for PA-1 (ng/mL)	Observed Concentration After 3 F/T Cycles (ng/mL)
LQC 1 (F/T)	5.12	5.21	HQC 1 (F/T)	69.01	82.50
LQC 2 (F/T)	3.51	5.36	HQC 2 (F/T)	65.76	92.40
LQC 3 (F/T)	4.36	5.87	HQC 3 (F/T)	66.27	75.80
LQC 4 (F/T)	4.25	5.69	HQC 4 (F/T)	66.07	90.90
LQC 5 (F/T)	4.54	4.77	HQC 5 (F/T)	81.37	62.60
LQC 6 (F/T)	5.16	5.55	HQC 6 (F/T)	78.65	71.80
Mean (ng/mL)	4.49	5.41	Mean (ng/mL)	71.19	79.33
SD (ng/mL)	0.61	0.39	SD (ng/mL)	6.99	11.52
CV (%)	13.68	7.22	CV (%)	9.81	14.52
Accuracy (%)	89.81	108.17	Accuracy (%)	88.99	99.17
MQC 1 (F/T)	42.22	43.10			
MQC 2 (F/T)	40.00	48.60			
MQC 3 (F/T)	40.40	62.60			
MQC 4 (F/T)	42.59	47.40			
MQC 5 (F/T)	55.18	46.00			
MQC 6 (F/T)	55.69	55.70			
Mean (ng/mL)	46.02	50.57			
SD (ng/mL)	7.37	7.23			
CV (%)	16.02	14.31			
Accuracy (%)	92.03	101.13			

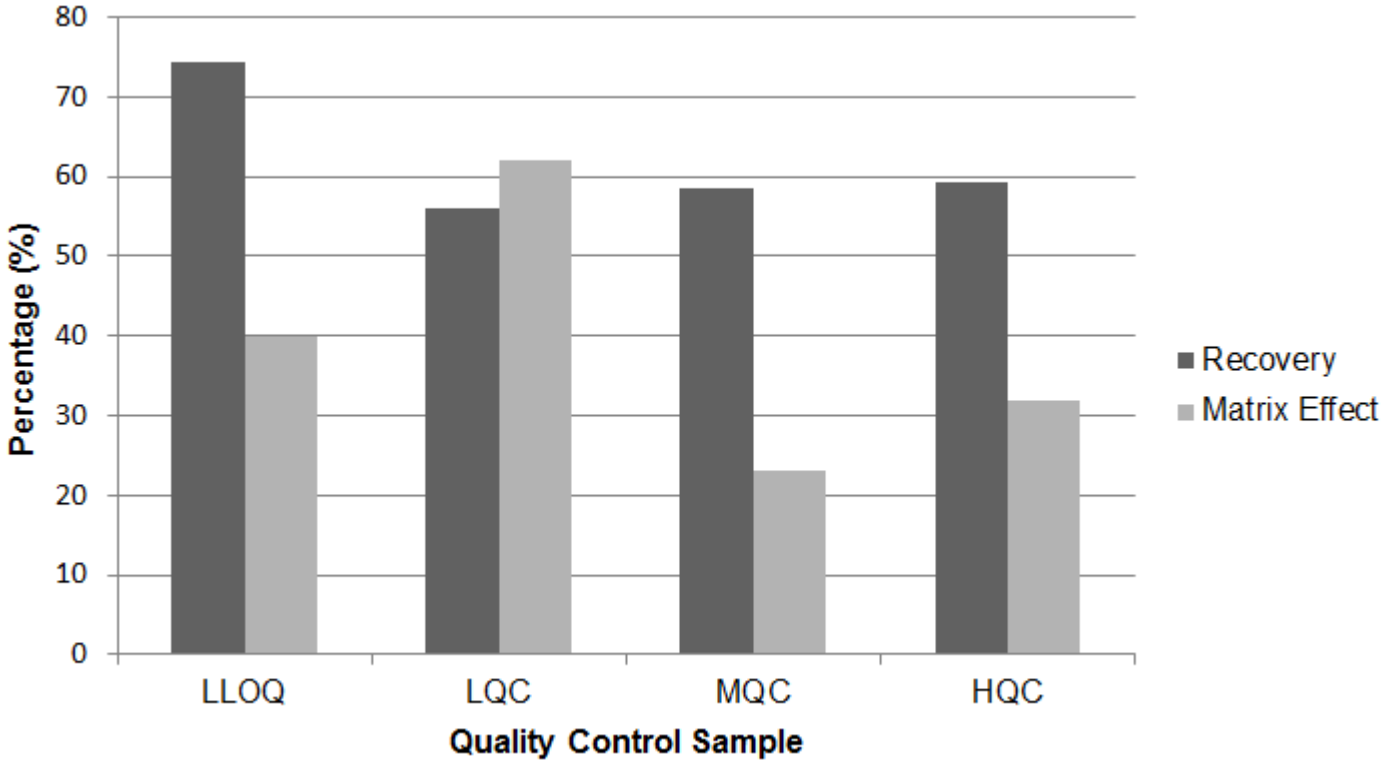

Figure 4-1. Percentage recovery and matrix effect following diclofenac extraction from plasma.

CHAPTER 5
IN VITRO STUDIES TO EVALUATE THE FEASIBILITY OF USING FLECTOR® IN A CLINICAL MICRODIALYSIS STUDY TO EVALUATE TOPICAL BIOEQUIVALENCE

Introduction

Microdialysis is a technique used to quantify free drug concentrations in both *in vitro* and *in vivo* settings.[69–73] When used *in vivo*, concentrations of endogenous and exogenous compounds can be quantified in most tissues.[74–79] Thus the technique is commonly used for assessment of drug delivery, distribution kinetics and determination of drug concentrations in tissue fluids. Microdialysis may be applied in drug discovery, preclinical, and clinical stages of drug development.

In an *in vivo* microdialysis study, a probe with a semi-permeable membrane is placed in the tissue. A syringe pump delivers the perfusate to the probe at a constant flow rate, where it enters through an inlet and travels down the probe, allowing for passive diffusion to occur. Dialysate samples are collected at pre-defined intervals. During passive diffusion, an exchange between the peri-probe fluid (fluid immediately surrounding the probe) and perfusate occurs. However, at commonly used flow rates (~1.5 µL/min) and membrane lengths (3-10 nm), an equilibrium between the extracellular fluid and the perfusate does not occur. In other words, the analyte concentration inside and outside the probe will not be the same in most cases. The concept of recovery is used to relate these two concentrations and determine the “true” tissue concentration.

In vitro microdialysis experiments are usually done beforehand in order to determine the feasibility of performing the experiments *in vivo*. These experiments are especially important when you are dealing with a novel compound whose recovery and binding characteristics with a microdialysis probe are unknown. Also, since the analyte

concentration in the “bulk” solution is known, the impact of various factors on the recovery may be studied. This includes the effects that flow rate, perfusate, drug, and membrane characteristics may have on relative recovery. RT and EE are frequently used to determine *in vitro* recovery.

During *in vitro* EE experiments, the drug of interest is dissolved in a physiological media which surrounds the probe. The goal is these experiments is to mimic what occurs *in vivo*, where a microdialysis probe is placed in a tissue containing an analyte of interest. The percent recovery (R %) is calculated as the ratio between the drug concentration measured in the dialysate and the known concentration in the physiological medium surrounding the probe (Equation 5-1).

$$R\,\% = 100 * \frac{C_{dialysate}}{C_{media}} \qquad 5\text{-}1$$

For RT experiments, drug is dissolved in the perfusate and infused through the probe at a constant flow rate. This method is typically used *in vivo* to calculate recovery. The percent recovery (R %) is calculated as the ratio of drug concentration in the dialysate and the known concentration in the perfusate (Equation 5-2).

$$R\,\% = 100 - \left(100 * \frac{C_{dialysate}}{C_{perfusate}} \right) \qquad 5\text{-}2$$

The goal of the studies described herein was to study the *in vitro* recovery and dissolution profile of dicofenac sodium, a non-steriodal anti-inflammatory drug (NSAID), prior to conducting a clinical microdialysis study using the product Flector®. This product is a transdermal patch containing 180 mg of diclofenac epolamine and is indicated for the treatment of minor strains, sprains, and contusions.[80] In addition to

studying the dissolution profile of a Flector® patch, a method was developed to quantify the residual content in used Flector® patches.

Methods

In Vitro Microdialysis Studies

Six rounds of recovery experiments were performed. For each round, the recovery of diclofenac using a CMA 66 microdialysis probe was determined in triplicate using both EE and RT experiments. Dextran was added in varying concentration to prevent ultrafiltration across the membrane.

Reagent preparation

For the first three rounds, EE and RT experiments were performed using diclofenac solutions containing 5, 10, and 25 ng/mL. These concentrations were attained by preparing a 1 mg/mL drug solution in methanol and water mixture (50:50); and 10 µg/mL and 1 µg/mL drug solutions in sodium chloride 0.9%. For the EE experiments, the perfusate was made up of dextran (3%, low fraction) solution in sodium chloride 0.9%. For the RT experiments, the three desired drug concentrations were prepared by diluting the 1 µg/mL drug solution with a solution of dextran 3% in sodium chloride 0.9%.

Rounds four through six were performed with diclofenac concentrations of 2, 15, and 40 ng/mL. Dextran concentrations were also varied between 1-3%. The desired drug concentrations were prepared by diluting a 1 µg/mL diclofenac drug solution in sodium chloride 0.9% for the EE experiments and a dextran-containing sodium chloride 0.9% solution for RT.

Study protocol

For EE evaluation, the probe is placed in a solution containing diclofenac dissolved in sodium chloride 0.9%; while a perfusate (dextran in sodium chloride 0.9%) is infused at 1.5 µl/min. In round 1, an equilibration period of 15 minutes was performed, while 30 minutes was used for all other rounds. Following the equilibration period, two dialysate samples were collected, each 20 minutes apart. Once sample collection is complete, the probe is transferred to the next highest drug concentration, and another 30 minute equilibration period is performed before beginning sample collection. Once all three concentrations have been evaluated, the experiments are repeated two additional times. In RT scenario, the same procedure was followed, except that drug syringes were switched for each drug concentration. In addition, the probe was placed in a "fresh" sodium chloride 0.9% solution following completion of each concentration.

Dissolution Studies

Chemicals and equipment

The following chemicals and reagents were used:

- Water, double distilled, Corning AG-3
- Methanol, Fisher, #A452-4
- Ammonium acetate, Fisher, #A639500
- Diclofenac sodium salt (Purity ≥ 99%), Sigma-Aldrich® D6899- 10G, 127K1220
- Indomethacin (Purity ≥ 99%), Sigma-Aldrich® 17378- 5G, 088K0666
- 7 Flector® patches, King Pharmaceuticals®, Inc

The following equipment was used:

- Vanderkamp® 600, six- spindle dissolution tester, QC-0372
- Sonicater Fisher Scientific, FS 110H
- 3 White Back Red Spirit Lab Thermometers, Fisherbrand 305 mm
- Bulb pipette 10 mL, 20 mL, 25 mL
- Microcentrifuge Tubes with Flat Top Cab, 1.5 mL, Fisher
- Centrifuge Tubes 15 mL, Corning 430055
- Research® adjustable pipettes, capacity of 20,200 & 1000 µl, Eppendorf

- Redi-Tip™ general purpose pipet tips 200 &1000µl, Fisher Scientific
- Vortex Maxi Mix II, Thermo Scientific
- Vial, Crimp/Snap 12x32, 300µl,PP ,Concial, Sun Sri™ 500-102
- Seal, AL Crimp, 11 mm, TFE/ Red Rubber, Silver Sri™ 200-100
- Aluminum screening
- Plastic fishing line, diameter 0.11 mm
- 35 Blue Vinyl Electrical Tape (19 mm)

Experimental setup

Each of the six vessels in the Vanderkamp® 600 was filled with 1 L of heated double distilled water following placement of a modified patch holder. A constant temperature of 32°C was maintained in each vessel. A modified patch holder was created in order to accommodate the size of each vessel. Briefly, a rectangular piece of aluminum window screen (18x65 cm) was cut and sown together in a circular fashion using plastic finishing line. For each vessel, a Flector® patch was opened and the drug-containing side of the patch was affixed to the circular aluminum mesh using electrical tape. The modified patch holder assembly was then placed in the vessel.

Study protocol

Once the patch holder was in place and the vessel filled with water, the power was switched on; the paddle speed was adjusted to 50 rpm; and the paddle lowered so that it is approximately halfway down the patch's height. The timer was started and 1 mL aliquots were taken at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, and 24 hours. The samples were frozen and analyzed at a later date using the previously described LC/MS method. Before analysis, the samples were diluted in an effort to obtain drug concentrations in the range of the validated method. The results were plotted using the lattice and ggplot 2 packages in the statistical software R.[45,81]

Residual Content Studies

The goal of these studies was to develop a method to quantify diclofenac concentrations in unused and used Flector® patches. Four separate methods were identified and tested.[82–85] These methods differed in the extracting agent and extraction time used. Method A used 0.1 M hydrochloric acid in methanol and a 2 hour extraction time; Methods B and C both used methanol and 1 and 2 hour extraction times, respectively; Method D used 2-propanol and an extraction time of 16 hours.

Three patches were cut into 16 pieces and all four methods were tested. The method tested on each piece was pre-defined in an effort to test each method on different corners of the patch. Each piece was placed in a 50 mL centrifuge tube; 30 mL of extracting agent was added; and each tube was placed in a shaker (Eberbach Corporation, Ann Arbor, Michigan) for the pre-defined extraction time. When complete, a 1 mL aliquot was taken from each tube and centrifuged (Marathon 16KM, Fisher Scientific) at 12,000 rpm for 10 minutes. Samples were then frozen until the time of analysis. Before analyzing, samples were diluted so that the analyzed concentrations were within the limits of the validated method.

Based on preliminary studies, method C (methanol, 2 hour extraction time) was chosen for further development and validation. In the validation studies, three unused patches were processed and 10 mL of extracting agent was used. In an effort to study the extraction process using “used” patches, four patches were applied to chicken breast skin for 12 hours, and then the extraction process using methanol and a 2 hour extraction time was performed.

Results

Recovery experiments were performed *in vitro* to evaluate the feasibility of using diclofenac as a model drug in a clinical microdialysis study. These experiments were performed in six rounds, each in triplicate, where the drug and dextran concentrations were varied in an effort to evaluate their impact on the recovery of the study drug (Table 5-1) using EE and RT experiments. In all scenarios studied, the recovery of the study drug exceeded 75%; ranging between 77.6-99.6%. When comparing the results of the EE and RT experiments within a round, a slightly higher recovery was observed with RT. High recovery values were obtained across a range of diclofenac concentrations (2-40 ng/mL). Dextran concentration did not appear to have a large impact on the observed recovery value.

Studies were performed to study the rate of dissolution of the product Flector®. After a period of 24 hours, the average amount of diclofenac released was 110.1 (± 10.8) mg of diclofenac was released (Figure 5-1). An increase in variability around the mean was observed at the last three time points (i.e., 10, 12, and 24 hours).

A method was developed and validated to quantify the amount of diclofenac in new and used Flector® patches. Four methods which differed in the solvent and extraction time were tested. Of these four methods, the use of methanol with a 2 hours extraction time, proved to be most reproducible. Validation results for this method are shown in Figure 5-2. On average, three unused Flector® patches (n=3) cut into 16 pieces each resulted in diclofenac concentrations reasonably close to expected values (i.e., 40.5 ng/mL per piece). The average diclofenac content for an entire Flector® patch was 123 mg. When four unused patches were applied to chicken breasts for a period of

12 hours, the mean residual content was 82.4 mg; corresponding to 36.6% of drug content released.

Discussion

Diclofenac's recovery value determined using EE and RT exceeded 85% when dextran (3%) was added to the perfusate. As expected, only minor differences were observed in the measured recovery when comparing the values observed using EE and RT. The probe manufacturer recommends that dextran 3% be added to the perfusate to prevent ultrafiltration across the membrane; with results of these experiments supporting use of this agent. The high *in vitro* recovery values observed using a linear microdialysis probe supported further study in humans.

The dissolution experiments performed helped to describe the rate at which diclofenac is released from the product Flector® *in vitro*. The expected amount of pure diclofenac in an unused Flector® patch is 129.6 mg. After a period of 24 hours, an average of 110.1 (± 10.8) mg was released. The greatest variability was observed with later time points (i.e., 10, 12, and 24 hours); possibly due to the patches swelling to a greater extent over time.

In vitro experiments were performed to develop and validate a method which can be used to quantify residual diclofenac content in used Flector® patches. First, we tested various methods which varied in the extracting agent and the extraction time used. Second, the selected method was validated by processing three unused Flector® patches. Last, four patches were applied to chicken breast purchased at a local supermarket. After 12 hours of application, the patches were processed according to the validated method and percentage of diclofenac released from each patch was calculated. Of the four methods tested, methanol and a 2 hour extraction time resulted

in the most reproducible results. Upon validation with three patches, a mean of 123 mg was extracted. This value is reasonably close to the expected 129.6 mg of pure diclofenac one would expect. After application to chicken skin, an average of 36.6% of the patch content was released. When comparing the amount of diclofenac released after 12 hours for the dissolution and chicken breast experiments; 79.7 mg and 47.2 mg were observed. This difference in the amount released is not surprising since greater release was observed when the patch was placed in water; whereas with the latter scenario the patch was applied directly on chicken breast.

Table 5-1. Recovery results for extraction efficiency and retrodialysis experiments. Each round was performed in triplicate.

Round	Dextran Concenration	Diclofenac Concentration	EE Mean (SD)	RT Mean (SD)
1	3%	5, 10 and 25 ng/ml	85.7 (18.2)	97.2 (3.7)
2	3%	5, 10 and 25 ng/ml	88.7 (10.8)	96.1 (6.6)
3	3%	5, 10 and 25 ng/ml	98.2 (11.7)	99.6 (0.7)
4	3%	2, 15 and 40 ng/ml	87.7 (16.1)	95.3 (5.3)
5	1%	2, 15 and 40 ng/ml	94.1 (12.0)	93.8 (7.8)
6	2%	5, 15 and 40 ng/ml	77.6 (11.4)	87.4 (12.9)

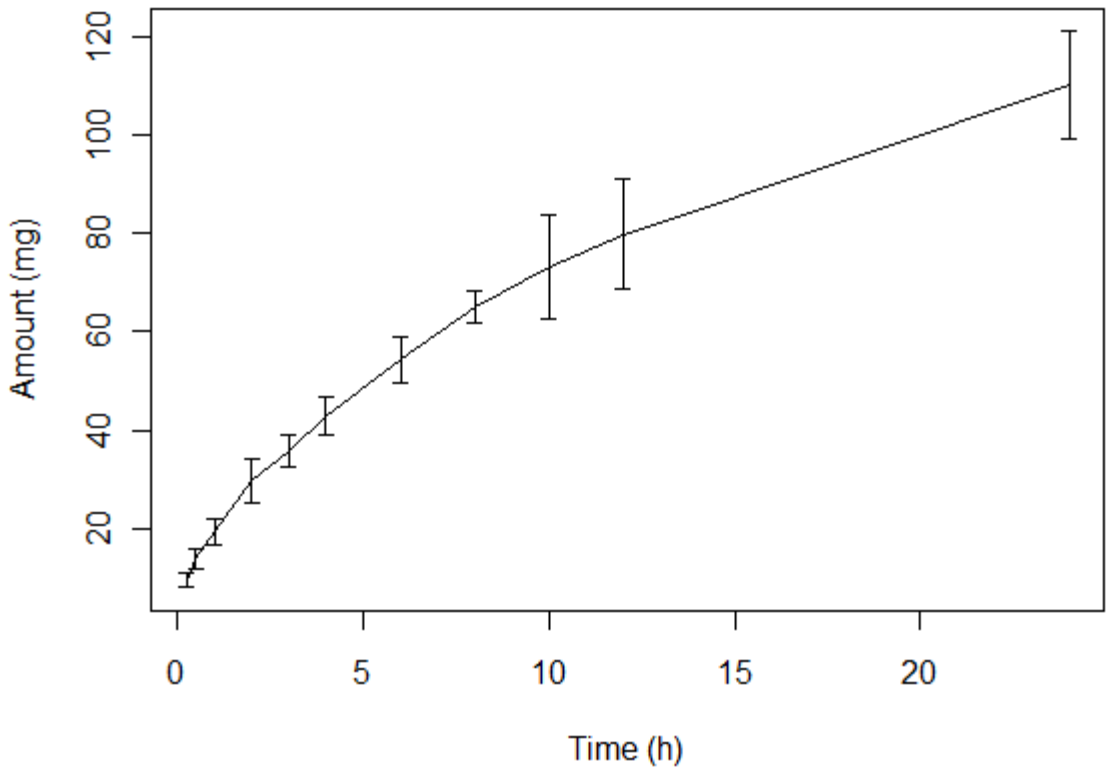

Figure 5-1. Mean dissolution profile for experiments performed using six Flector® patches. Error bars represent the standard deviation.

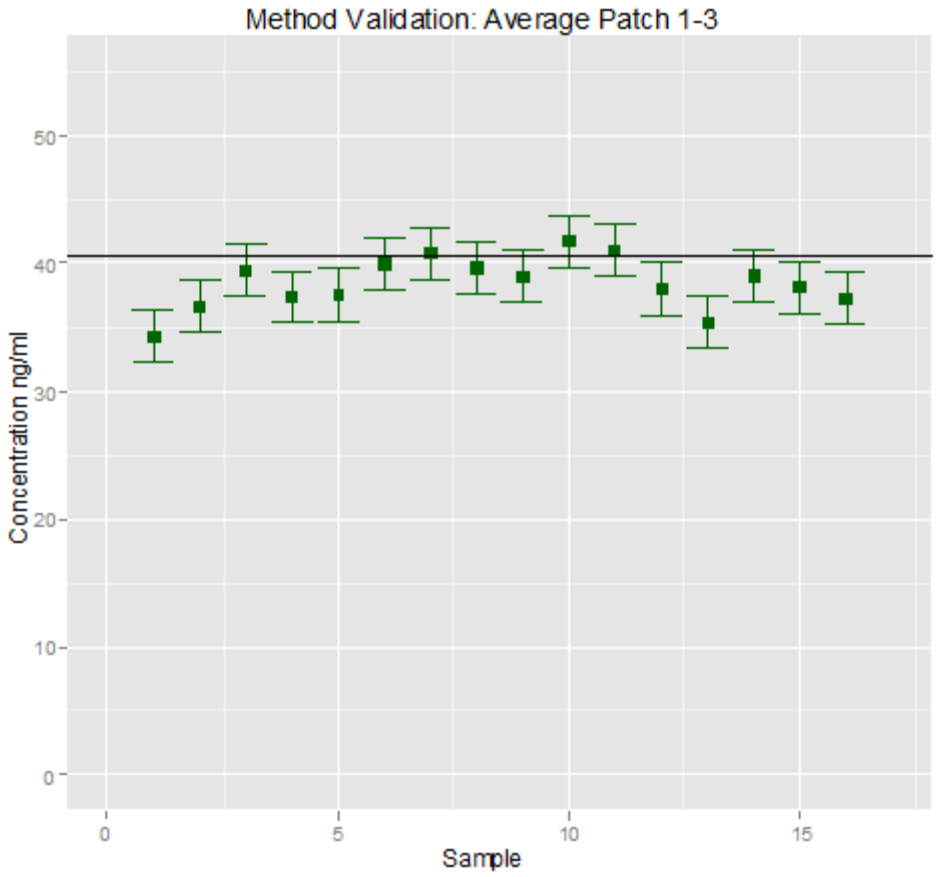

Figure 5-2. Mean results obtained after cutting a Flector® patch into sixteen pieces and extracting the drug content using methanol. The solid line represents the expected concentration for each piece.

CHAPTER 6
USE OF MICRODIALYSIS TO EVALUATE THE EFFECT OF SKIN PROPERTIES AND APPLICATION SITE ON THE TOPICAL BIOEQUIVALENCE OF DICLOFENAC: A FEASIBILITY PILOT STUDY

Introduction

Evaluating the recovery of a study drug *in vivo* is a key objective of all clinical microdialysis studies. The recovery value should be determined for each subject and microdialysis probe. Several microdialysis studies have been performed to evaluate the tissue distribution of diclofenac *in vivo*.[59,86–90] In one study, the diclofenac's recovery was reported to be 66%±12% (mean ± SE) for superficial layers of the skin; whereas a slightly lower value was reported for deep layers (63%±15%).[59] Another study reported an *in vivo* recovery value of 70.5%±22.9% (mean ± SD) for diclofenac.[88]

We sought to conduct a pilot study to aid in preparation for a larger pivotal study. The objectives of the pilot study were to evaluate the feasibility of using a linear microdialysis probe; evaluate diclofenac's recovery *in vivo*; and determine the length of time needed for all diclofenac to be washed out following infusion of a low concentration administered for recovery determination. The methods and results described herein pertain to a pilot study conducted in three healthy subjects.

Methods

Volunteers

Three healthy male or female subjects between 18 and 55 years old (inclusive) provided written informed consent and underwent a screening examination prior to study enrollment. The study was approved by the Institutional Review Boards at the University of Florida and FDA.

Inclusion criteria

- Body mass index (BMI) of 18.5 and 32 kg/m^2 (inclusive).
- Non-smoker status for at least 12 months prior to study entry.
- Healthy on the basis of physical examination, medical history, and vital signs.
- Normal results for clinical laboratory tests performed at screening.
- Female subjects must be postmenopausal, on proper contraception, or abstinent.
- Alcohol may not be consumed 72 hours prior to study admission.
- Diclofenac-containing medications will be avoided while enrolled in the study.
- Subject must adhere to prohibitions and restrictions detailed in the protocol.
- Subjects must provide written informed consent.

Exclusion criteria

- Clinically significant abnormal values in the performed laboratory tests.
- Significant medical illness precluding participation in this clinical study.
- History of atopic eczema, dry skin or ichtyosis.
- Excessive hair at the site of application.
- Known allergies or hypersensitivity to diclofenac-containing products.
- Abnormal physical examination of vital signs.
- Subject administered an investigational drug within 60 days.
- Pregnant or breast-feeding.
- Recent history of surgery (within 3 months prior to screening).
- Clinically significant acute illness within 7 days prior to study drug administration.
- Recent acute blood loss or donation of blood.
- Employees of the investigator or study center.
- Over-the-counter or prescription use of other NSAID products.

Prohibitions and restrictions

- Subjects will avoid strenuous exercise for 48 hours prior to the study visit.
- There will be no alcohol consumption for at least 72 hours prior to the study visit.
- Smoking is prohibited during study participation.
- Blood and/or plasma donation is prohibited during study participation.
- Subjects will inform the study team if they become pregnant during study participation.
- Subjects will refrain from using topical moisturizers for 48 hours prior to the study participation.

Screening

The following screening procedures were performed for all subjects prior to study entry: 1) medical history was evaluated and a physical exam was performed; 2) inclusion and exclusion criteria were verified; 3) height, body weight and body mass index (BMI) were determined; 4) vital signs (systolic/diastolic blood pressure, pulse rate and temperature) following 5 minutes in the supine position were recorded; 5) clinical laboratory testing was performed (comprehensive metabolic panel and complete blood count); 6) a serum pregnancy test was performed during the initial screening procedure. 7) a urine pregnancy test was performed upon admission to the Clinical Research Center (CRC).

Recovery assessment

During microdialysis studies, recovery determination is a critical study component in order to correctly calculate tissue concentrations of a desired analyte. The RT method was used to evaluate probe recovery. With the RT method, a known drug concentration is infused through the probe prior to administration of the study drug. The change in analyte concentration in the perfusate is then compared with the concentration in the dialysate and the recovery of the probe is calculated using the equation shown below.

$$R\,(\%) = 100 - \left(100 \times \frac{\text{drug A}_{\text{dialysate}}}{\text{drug A}_{\text{perfusate}}} \right) \qquad \text{6-1}$$

In this pilot study, a diclofenac concentration of 25 ng/mL was selected for recovery determination using the RT method. This concentration was selected based on several factors. First, published microdialysis studies evaluating the local penetration of diclofenac (other formulations) were considered.[59,86,88] In these studies, mean maximum concentrations of diclofenac in subcutaneous tissue varied between 13.1 ng/mL and 5

μg/mL following administration of diclofenac as a spray gel (4%) and Voltaren Emulgel (6% and 300 mg/100 cm^2). Second, we evaluated data reported in the NDA package for Flector® in order to determine what range of tissue concentrations would be likely for this product. Although local concentrations of Flector® have not been studied in humans, based on plasma concentrations (0.7 – 6 ng/mL 10-20 hours after single application), we felt that a concentration of 25 ng/mL was reasonable.[80] Third, we considered the total dose of diclofenac delivered using this concentration. For a 25 ng/mL concentration infused for 1.5 hours at a rate of 1.5 μL/minute, a total of 3.375 ng of diclofenac would be delivered. We felt that this was reasonable given what is considered to be a therapeutic dose. Last, we considered what concentration would be appropriate given the lower limit of quantification for our analytical method. We feel confident that we will be able to quantify diclofenac concentrations in the dialysate using a concentration of 25 ng/mL.

Study protocol

In the feasibility study, each subject visited the CRC on two separate outpatient visits; the first for screening purposes and the second for determination of probe recovery. Once a subject provided written informed consent, he/she was screened by a study physician to verify that all inclusion criteria were met. Any subjects meeting any of the exclusion criteria were not allowed to participate. Once a subject was enrolled in the study, they were asked to visit the clinical research center (CRC) between 8-10 AM on Day 1.

On Day 1, upon admission to the CRC, a study physician inserted three microdialysis probes (CMA 66, M Dialysis, Solna, Sweden) into subcutaneous tissue in the abdomen and the recovery of the probe was determined using the RT method

(Table 6-1). Lidocaine 1% was used during probe insertion to minimize the discomfort to the subject. Next, there was a 30-minute equilibration period where sodium chloride 0.9% solution (containing dextran 3%) was infused at a rate of 1.5 µL/minute. Probe recovery was then determined using the RT method. A drug solution containing 25 ng/mL was infused through each probe at a constant rate of 1.5 µL/minute for 60 minutes in order to reach a steady state with the tissue. Then a dialysate sample was collected for 30 minutes. Post-retrodialysis, sodium chloride 0.9% (containing dextran 3%) was infused through the probes, and dialysate samples were collected every 30 minutes for 3.5 hours in order to determine the length of time needed until disappearance of the drug in the dialysate. The recovery experiments lasted approximately 6 hours.

A follow-up phone call will be performed within 72 hours of the subject being discharged. Subjects who participated in the feasibility study were not required to participate in the main study (the two studies will be treated separately).

Drug Analysis

The LC/MS method described in the previous chapter was used for quantification of diclofenac in microdialysis samples.

Results

No adverse events occurred during the three study visits. Subjects reported minimal discomfort during probe insertion. During the follow-up phone call, two subjects reported minor bruising around the needle insertion site, but stated that there was no pain or tenderness. No other complaints were reported.

The demographic characteristics of all three subjects are shown in Table 6-2. For each subject, measurements of probe depth were obtained for each probe (Table 6-3).

There was some inter-individual variability in the degree of probe depth, likely a result of differences in body weight. Within each individual, there were only small differences in the probe depth.

For all three subjects the calculated recovery by loss was approximately 100% (Tables 6-4 – 6-6). For Subject 3, one probe did not function properly, as noted by the lack of dialysate during collection. A major objective of this feasibility was to evaluate the time necessary for complete washout of diclofenac following recovery determination. For Subject 1, diclofenac concentrations were below the limit of quantification (BQL) 30 minutes after recovery determination. For Subjects 2 and 3, diclofenac concentrations could still be quantified 3.5 and 3 hours following infusion of diclofenac for recovery determination (Tables 6-7 – 6-9).

Discussion

In each of the three subjects, three microdialysis probes were inserted, an ultrasound measurement of probe depth was obtained, and the probe recovery was determined. No major adverse events related to probe insertion or removal were reported. With the exception of one probe in Subject 3, all probes worked properly and dialysate samples could be collected. It is not clear what resulted in the poor functionality of this single probe. Recovery by loss was approximately 100% in all subjects. The time-to-washout for diclofenac differed between the three subjects; varying between 0.5-3.5 hours. This variability in the necessary washout period may be explained (at least in part) due to differences in body weight. The results of this study aided in the planning of the pivotal study and demonstrated that diclofenac's high recovery would not be a limiting factor *in vivo*.

Table 6-1. Time and events table for the pilot study.

Phase	Screening	Recovery Experiments (N=3)		
Study Day	Prior to Study Entry	Day 1		
Time		0-0.5 h	0.5–1.5 h	1.5-5.5 h
Residence in Clinic			Outpatient Visit	
Medical history	X			
Inclusion/Exclusion Criteria	X			
Physical examination	X			
Weight, height and BMI	X			
Vital signs/oral temperature	X	X		
Clinical Lab Testing	X			
Serum Pregnancy Test	X			
Urine Pregnancy Test		X		
Diclofenac probe calibration (3.5 hours)		EQUILI-BRATION (Saline)	EQUILI-BRATION (Drug Solution)	Sample Collection (every 30 minutes)
AE Reporting			Continuous	

Table 6-2. Demographic characteristics for subjects participating in the pilot study.

Variable	Subject 1	Subject 2	Subject 3
Age (years)	28	45	20
Height (cm)	182.7	169	172.7
Weight (kg)	101.1	90.5	56.7
BMI	29	31.7	19

Table 6-3. Measurement of probe depth in three healthy subjects.

Variable	Subject 1	Subject 2	Subject 3
Probe 1	8 mm	10 mm	5 mm
Probe 2	9 mm	11 mm	5 mm
Probe 3	10 mm	12 mm	5 mm

Table 6-4. Recovery determination for Subject 01.

Sample Type	Perfusate	Probe Number	Diclofenac Concentration (ng/mL)
Sample From Diclofenac Syringe 1	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	1	17.18
Sample From Diclofenac Syringe 2	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	2	17.36
Sample From Diclofenac Syringe 3	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	3	14.92
Sample From Placebo Syringe 1	0.9% Saline With 3% Dextran 40	1	BQL
Sample From Placebo Syringe 2	0.9% Saline With 3% Dextran 40	2	BQL
Sample From Placebo Syringe 3	0.9% Saline With 3% Dextran 40	3	BQL
Retrodialysis (1.5 – 2 Hours)	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	1	BQL
Retrodialysis (1.5 – 2 Hours)	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	2	BQL
Retrodialysis (1.5 – 2 Hours)	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	3	BQL
		1	100%
		2	100%
Relative Recovery		3	100%

*BQL = Below Quantification Limit (0.39 ng/mL)

Table 6-5. Recovery determination for Subject 02.

Sample Type	Perfusate	Probe Number	Diclofenac Concentration (ng/mL)
Sample From Diclofenac Syringe 1	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	1	17.52
Sample From Diclofenac Syringe 2	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	2	18.78
Sample From Diclofenac Syringe 3	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	3	13.58
Sample From Placebo Syringe 1	0.9% Saline With 3% Dextran 40	1	BQL
Sample From Placebo Syringe 2	0.9% Saline With 3% Dextran 40	2	BQL
Sample From Placebo Syringe 3	0.9% Saline With 3% Dextran 40	3	BQL
Retrodialysis (1.5 – 2 Hours)	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	1	BQL
Retrodialysis (1.5 – 2 Hours)	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	2	BQL
Retrodialysis (1.5 – 2 Hours)	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	3	BQL
Relative Recovery		1	100%
		2	100%
		3	100%

*BQL = Below Quantification Limit (0.39 ng/mL)

Table 6-6. Recovery determination for Subject 03.

Sample Type	Perfusate	Probe Number	Diclofenac Concentration (ng/mL)
Sample From Diclofenac Syringe 1	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	1	17.56
Sample From Diclofenac Syringe 2	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	2	19.48
Sample From Diclofenac Syringe 3	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	3	19.56
Sample From Placebo Syringe 1	0.9% Saline With 3% Dextran 40	1	BQL
Sample From Placebo Syringe 2	0.9% Saline With 3% Dextran 40	2	BQL
Sample From Placebo Syringe 3	0.9% Saline With 3% Dextran 40	3	BQL
Retrodialysis (1.5 – 2 Hours)	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	1	BQL
Retrodialysis (1.5 – 2 Hours)	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	2	NA[a]
Retrodialysis (1.5 – 2 Hours)	Diclofenac 25 ng/mL In 0.9% Saline With 3% Dextran 40	3	BQL
		1	100%
		2	NA[a]
Relative Recovery		3	100%

*BQL = below quantification limit (0.39 ng/mL)
[a]No dialysate obtained through this probe.

Table 6-7. Diclofenac washout period in Subject 01.

Sample Type	Perfusate	Probe Number	Diclofenac Concentration (ng/mL)
Post-RT (2 – 2.5 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (2 – 2.5 Hours)	0.9% Saline With 3% Dextran 40	2	BQL
Post-RT (2 – 2.5 Hours)	0.9% Saline With 3% Dextran 40	3	16.78
Post-RT (2.5 – 3 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (2.5 – 3 Hours)	0.9% Saline With 3% Dextran 40	2	BQL
Post-RT (2.5 – 3 Hours)	0.9% Saline With 3% Dextran 40	3	BQL
Post-RT (3 – 3.5 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (3 – 3.5 Hours)	0.9% Saline With 3% Dextran 40	2	BQL
Post-RT (3 – 3.5 Hours)	0.9% Saline With 3% Dextran 40	3	BQL
Post-RT (3.5 – 4 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (3.5 – 4 Hours)	0.9% Saline With 3% Dextran 40	2	BQL
Post-RT (3.5 – 4 Hours)	0.9% Saline With 3% Dextran 40	3	BQL
Post-RT (4 – 4.5 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (4 – 4.5 Hours)	0.9% Saline With 3% Dextran 40	2	BQL
Post-RT (4 – 4.5 Hours)	0.9% Saline With 3% Dextran 40	3	BQL
Post-RT (4.5 – 5 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (4.5 – 5 Hours)	0.9% Saline With 3% Dextran 40	2	BQL
Post-RT (4.5 – 5 Hours)	0.9% Saline With 3% Dextran 40	3	BQL
Post-RT (5 – 5.5 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (5 – 5.5 Hours)	0.9% Saline With 3% Dextran 40	2	BQL
Post-RT (5 – 5.5 Hours)	0.9% Saline With 3% Dextran 40	3	BQL

*BQL = Below Quantification Limit (0.39 ng/mL)

Table 6-8. Diclofenac washout period in Subject 02.

Sample Type	Perfusate	Probe Number	Diclofenac Concentration (ng/mL)
Post-RT (2 – 2.5 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (2 – 2.5 Hours)	0.9% Saline With 3% Dextran 40	2	BQL
Post-RT (2 – 2.5 Hours)	0.9% Saline With 3% Dextran 40	3	BQL
Post-RT (2.5 – 3 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (2.5 – 3 Hours)	0.9% Saline With 3% Dextran 40	2	BQL
Post-RT (2.5 – 3 Hours)	0.9% Saline With 3% Dextran 40	3	BQL
Post-RT (3 – 3.5 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (3 – 3.5 Hours)	0.9% Saline With 3% Dextran 40	2	BQL
Post-RT (3 – 3.5 Hours)	0.9% Saline With 3% Dextran 40	3	BQL
Post-RT (3.5 – 4 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (3.5 – 4 Hours)	0.9% Saline With 3% Dextran 40	2	1.894
Post-RT (3.5 – 4 Hours)	0.9% Saline With 3% Dextran 40	3	1.938
Post-RT (4 – 4.5 Hours)	0.9% Saline With 3% Dextran 40	1	5.1
Post-RT (4 – 4.5 Hours)	0.9% Saline With 3% Dextran 40	2	1.888
Post-RT (4 – 4.5 Hours)	0.9% Saline With 3% Dextran 40	3	BQL
Post-RT (4.5 – 5 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (4.5 – 5 Hours)	0.9% Saline With 3% Dextran 40	2	BQL
Post-RT (4.5 – 5 Hours)	0.9% Saline With 3% Dextran 40	3	2.9
Post-RT (5 – 5.5 Hours)	0.9% Saline With 3% Dextran 40	1	8.76
Post-RT (5 – 5.5 Hours)	0.9% Saline With 3% Dextran 40	2	BQL
Post-RT (5 – 5.5 Hours)	0.9% Saline With 3% Dextran 40	3	2.96

*BQL = Below Quantification Limit (0.39 ng/mL)

Table 6-9. Diclofenac washout period in Subject 03.

Sample Type	Perfusate	Probe Number	Diclofenac Concentration (ng/mL)
Post-RT (2 – 2.5 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (2 – 2.5 Hours)	0.9% Saline With 3% Dextran 40	2	NA
Post-RT (2 – 2.5 Hours)	0.9% Saline With 3% Dextran 40	3	BQL
Post-RT (2.5 – 3 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (2.5 – 3 Hours)	0.9% Saline With 3% Dextran 40	2	NA
Post-RT (2.5 – 3 Hours)	0.9% Saline With 3% Dextran 40	3	0.894
Post-RT (3 – 3.5 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (3 – 3.5 Hours)	0.9% Saline With 3% Dextran 40	2	NA
Post-RT (3 – 3.5 Hours)	0.9% Saline With 3% Dextran 40	3	0.85
Post-RT (3.5 – 4 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (3.5 – 4 Hours)	0.9% Saline With 3% Dextran 40	2	NA
Post-RT (3.5 – 4 Hours)	0.9% Saline With 3% Dextran 40	3	BQL
Post-RT (4 – 4.5 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (4 – 4.5 Hours)	0.9% Saline With 3% Dextran 40	2	NA
Post-RT (4 – 4.5 Hours)	0.9% Saline With 3% Dextran 40	3	BQL
Post-RT (4.5 – 5 Hours)	0.9% Saline With 3% Dextran 40	1	1.66
Post-RT (4.5 – 5 Hours)	0.9% Saline With 3% Dextran 40	2	NA
Post-RT (4.5 – 5 Hours)	0.9% Saline With 3% Dextran 40	3	BQL
Post-RT (5 – 5.5 Hours)	0.9% Saline With 3% Dextran 40	1	BQL
Post-RT (5 – 5.5 Hours)	0.9% Saline With 3% Dextran 40	2	NA
Post-RT (5 – 5.5 Hours)	0.9% Saline With 3% Dextran 40	3	BQL

*BQL = Below Quantification Limit (0.39 ng/mL)

CHAPTER 7
USE OF MICRODIALYSIS TO EVALUATE THE EFFECT OF SKIN PROPERTIES AND APPLICATION SITE ON THE TOPICAL BIOEQUIVALENCE OF DICLOFENAC: THE MAIN STUDY

Introduction

For most topically applied products, clinical end-point studies must be conducted to establish bioequivalence between two products.[91] The need for these trials may increase drug development costs and limit the availability of cheaper generic drugs which are applied topically. The only widely accepted method by the U.S. FDA is the vasoconstrictor assay used to establish topical bioequivalence with topical glucocorticoids.[92] In 2007, the FDA acknowledged the need for the need for further research to identify techniques to evaluate bioequivalence for topical dermatological products. [93,94] In this report, four techniques were acknowledged; pharmacokinetic studies, skin stripping, microdialysis, and near infrared spectroscopy.

Each of these techniques has advantages and disadvantages which could potentially limit their use. *In vitro* tests are frequently performed using excised human/animal skin, although the lack of live tissue and circulation, among other factors, has limited its applicability to evaluating scale-up or post-approval changes for a product.[91] Pharmacokinetic studies are of limited usefulness unless concentrations are sufficiently high in the systemic circulation to allow detection and if they reflect delivery to the site of action.[94] Skin stripping is a technique by which drug content is quantified in the stratum corneum. This technique may be particularly useful for drugs whose site of action is the stratum corneum.[91,95–97] For other topical drugs, the measured amount is assumed to provide a reflection of what is occurring in lower layers of the skin. Although significant research has shown the value of the technique for bioequivalence

determination, one of its limitations is the lack of standardization in the study design. Near infrared spectroscopy is an imaging technique which may be useful for drug molecules with spectral characteristics necessary for detection.[91,98,99]

Dermal microdialysis is a technique under investigation for use in the evaluation of bioequivalence for topically applied products. Microdialysis allows for the continuous monitoring of drug concentrations in the desired tissue layer; an advantage it provides over other available techniques. One paper sought to compare microdialysis and the tape-stripping method in healthy volunteers.[100] When comparing lidocaine cream and ointment products, both methods reached the same conclusion; 3-5 fold greater penetration was obtained with the cream formulation. In this study, four microdialysis probes were inserted in two penetration areas. The authors reported a 19% intrasubject variability between probes and 20% for the two penetration areas.

The current study described herein sought to evaluate the use of microdialysis for the evaluation of topical bioequivalence using the transdermal patch Flector®. The main study will enroll a sufficient number of subjects in order to complete all treatments in six healthy subjects. In an effort to evaluate the sensitivity of the microdialysis technique, two different batches of Flector® will be evaluated. Moreover, to evaluate the impact of administration site, both test and reference products will be compared after a single dose is applied in the abdomen and thigh. These sites were selected because they are anatomically distinct and they’re practical sites which allow subjects to remain relatively mobile throughout the study. The study design is described below.

Methods

Volunteers

Six healthy male or female subjects between 18 and 55 years old (inclusive) will provide written informed consent and undergo a screening examination prior to enrollment. Subjects who participated in the pilot study could participate in the main study, but were not obliged to do so. The study was approved by the Institutional Review Boards at the University of Florida and FDA.

Inclusion criteria

- Body mass index (BMI) of 18.5 and 32 kg/m^2 (inclusive).
- Non-smoker status for at least 12 months prior to study entry.
- Healthy on the basis of physical examination, medical history, and vital signs.
- Normal results for clinical laboratory tests performed at screening.
- Female subjects must be postmenopausal, use proper contraception, or practice abstinence.
- Alcohol may not be consumed 72 hours prior to study admission.
- Diclofenac-containing medications will be avoided while enrolled in the study.
- Willing to adhere to the prohibitions and restrictions specified in this protocol.
- Subjects must provide written informed consent.
- Subjects should be willing avoid the use of body oils, creams, lotions, or powders to the test areas for a period of 48 hours before the application of patches.

Exclusion criteria

- Clinically significant abnormal values in the clinical laboratory tests.
- Significant medical illness precluding participation in this clinical study.
- History of atopic eczema, dry skin or ichtyosis.
- Excessive hair at the site of application.
- Known allergy to Flector® Patch and/or diclofenac-containing products.
- Abnormal physical examination of vital signs.

- Subject administered an investigational drug within 60 days.
- Pregnant or breast-feeding.
- Recent history of surgery (within 3 months prior to screening).
- Clinically significant acute illness within 7 days prior to study drug administration.
- Recent acute blood loss or donation of blood.
- Employees of the investigator or study center.
- Over-the-counter or prescription use of other NSAID products.

Prohibitions and restrictions

- Subjects will avoid strenuous exercise for 48 hours prior to the study visit.
- There will be no alcohol consumption for at least 72 hours prior to the study visit.
- Smoking is prohibited during study participation.
- Blood and/or plasma donation is prohibited during study participation.
- Subjects will inform the study team if they become pregnant during study participation.
- Subjects will refrain from using topical moisturizers for 48 hours prior to the study participation.

Screening

The following screening procedures are performed for all subjects prior to study entry: 1) medical history is evaluated and a physical exam performed; 2) inclusion and exclusion criteria are verified; 3) height, body weight and body mass index (BMI) are determined; 4) vital signs (systolic/diastolic blood pressure, pulse rate and temperature) following 5 minutes in the supine position are recorded; 5) clinical laboratory tests are performed (comprehensive metabolic panel and complete blood count); 6) a serum pregnancy test is performed during the initial screening procedure. 7) a urine pregnancy test is performed upon admission to the CRC; 8) evaluation of skin type using the Fitzpatrick skin type scale and a measurement of the transepidermal water loss (TEWL).

Recovery Assessment

Similar the pilot study, recovery is determined using RT with a diclofenac 25 ng/mL drug solution prepared in 0.9% sodium chloride containing dextran 3%. The recovery will be calculated using Equation 7-1.

$$R\,(\%) = 100 - \left(100 \times \frac{\text{drug A}_{\text{dialysate}}}{\text{drug A}_{\text{perfusate}}} \right) \qquad 7\text{-}1$$

Study Protocol

Upon providing informed consent, enrolled subjects undergo an outpatient screening at the CRC. This screening will be performed by a study physician prior to study entry. For the first treatment phase (only), an evaluation of the subject's skin type is performed. The evaluation of the skin type includes use of the Fitzpatrick skin type scale and a measurement of the transepidermal water loss (TEWL); this information will not be used for screening purposes, but these measures may be included as covariates during data analysis.

On the evening of "Day -1" (1 day before probe placement), the subject is admitted to the CRC, where he/she will stays overnight. Upon admission, vital signs, skin and oral temperature are recorded, a urine pregnancy test is completed, and the subject is offered a dinner meal.

On the morning of "Day 1," subjects receive a standardized breakfast 30 minutes prior to dosing. Between 8:00 – 10:00 AM, a study physician inserts three microdialysis probes (CMA 66, CMA Microdialysis, Inc.) into the subcutaneous tissue of the abdomen or thigh based on a predetermined randomization scheme. Lidocaine 1% is used during probe insertion to minimize discomfort to the study subject.

Prior to the start of the main study the recovery of the probe is determined. First, there is a 30-minute equilibration period where sodium chloride 0.9% (containing dextran 3%) is infused through each probe at a rate of 1.5 µL/minute. Dextran is added to the sodium chloride 0.9% to prevent ultra-filtration over the membrane; a technique which is recommended by the manufacturer. Second, probe recovery is determined using the RT method; where a drug solution containing 25 ng/mL (and 3% Dextran) is infused through each probe at a constant rate of 1.5 µL/minute for 60 minutes in order to reach a steady state with the tissue. Then a dialysate sample is collected for 30 minutes. Before the patch is applied, there is a 4 hour washout period where sodium chloride 0.9% (containing dextran 3%) is infused through the probe.

Then the study patch is applied and sample collection begins. During the sample collection period, sodium chloride 0.9% (containing dextran 3%) is infused continuously at a rate of 1.5 µL/min. For all treatments, the room temperature is maintained at 70-75°F. The patch is removed 12 hours following application. No other patches are applied (single dose only). In total, sample collection continues for a total of 24 hours. Blood samples (about 3 mL each) for analysis of diclofenac plasma concentrations are collected immediately before application of the study patch (i.e., 0 hours) and at 0.5, 1, 1.5, 2, 4, 6, 8, 10, and 12 hours following application. The patch is removed at 12 hours. Blood samples are collected at 14, 16, and 24 hours; all time points occurring after patch removal. Additional blood samples (5 mL each) are collected at 2 and 12 hours to quantify diclofenac's protein binding. Microdialysis samples for analysis of diclofenac concentrations are collected at 20-minute intervals for 8 hours.

The subject remains one additional night at the CRC and is discharged the next morning following probe removal. There is a one-week washout period (at least), and then subject will return for the reference (or test) product. This next study section is done in the same administration site, but the contralateral side. Then there is another one-week washout period (at least) before the next administration site is evaluated. The same flow of events (minus the screening and skin evaluation) described above is repeated for the remaining two treatment phases.

Drug Analysis

Plasma and microdialysis samples are analyzed using an LC-MS assay which was validated according to FDA guidelines and described previously.

CHAPTER 8
DISCUSSION

PK variability may increase the risk of experiencing a sub- or supra-therapeutic drug response. This is particularly problematic for drugs with a narrow therapeutic index. For example, inter-individual variability in drug exposure to cytotoxic drugs can vary between 2-10 fold.[101–104] Inter- and intra-individual variability may be caused by differences or alterations in drug absorption, distribution, metabolism, and excretion of drug molecules. In addition to variability in PK processes, poor medication adherence can impact the results obtained in PK analyses; as well as contribute to differences in drug response.

Currently available direct and indirect methods of adherence measurement are either imprecise or impractical in most settings. Examples of direct measurement include biological assays and directly observed therapy; whereas, pill counts, self-reporting, and electronic monitors indirectly document adherence.[105] Breath testing may provide another objective method to document adherence. With breath testing, volatile drug molecules may be measured directly. Another option would be to measure volatile markers which are safe and have no impact on a dosage form or the pharmacological response obtained from an active ingredient.

Pentyl acetate and butyl acetate are two volatile markers, which are metabolized to alcohol and ketone metabolites. Breath concentrations of the parent compounds and/or their metabolites could be measured in breath within minutes of administration via the vaginal or oral route. When evaluating the inter-individual and inter-occasion variability for these markers, PK variability was observed in both absorption and elimination processes. Despite these results, use of volatile metabolites in various

dosage forms may provide a valuable technique to assess medication adherence in clinical trials and practice.

Microdialysis is a versatile technique which may aid in a direct assessment of PK variability by measurement free drug concentrations. *In vitro* the technique was used to assess differences in the protein binding and antimicrobial efficacy of ceftriaxone. When used to measure free drug concentrations in culture flasks, differences in drug concentrations occurring over time were observed. Measured concentrations could then be correlated directly with drug effect using a PK/PD modeling approach.

The sampling technique may also be used to measure differences in skin absorption and may provide a method to evaluate bioequivalence for topically applied products. The results of the preliminary studies described herein provide a framework to conduct a clinical microdialysis study using the product Flector®. Sensitive analytical methods were developed to quantify diclofenac concentrations in microdialysis and plasma samples. A method was developed and validated which could be used to quantify residual drug content in Flector® patches. Data obtained from measurement of diclofenac in the subcutaneous layers of the skin, plasma samples, and used patches, may be combined to evaluate the feasibility of using the technique to evaluate topical bioequivalence for the product Flector®.

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BIOGRAPHICAL SKETCH

Daniel Gonzalez was born in Miami, FL. He has one sibling, Danisa Borges, and is married to Kady Rae Gonzalez. Daniel graduated from Southwest Miami Senior High School in 2002; completed two years of pre-pharmacy studies at University of Florida; and 4 years towards his professional degree. In May 2008, he was awarded the Doctor of Pharmacy degree by the University of Florida, and had the highest grade point average in his graduating class. In August 2008, he began his graduate studies at University of Florida; working under the direction of Professor Hartmut Derendorf in the Department of Pharmaceutics. While in graduate school, Daniel was an active member of the American College of Clinical Pharmacology, American College of Clinical Pharmacy, American Society for Clinical Pharmacology and Therapeutics, American Association of Pharmaceutical Scientists, and the American Society of Pharmacometrics. He received his Ph.D. from the University of Florida in the summer of 2012.