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Title : In Vitro and In Silico Approaches to Evaluate the Pharmacokinetics and Pharmacodynamics of Combination Antibiotic Therapy Against Drug-resistant Bacteria

By

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Abstract

This thesis focuses on combating antibacterial resistance by developing novel in vitro and in silico techniques. In vitro techniques such as in vitro pharmacodynamic (IVPD) modeling are powerful tools for investigating pharmacokinetic and pharmacodynamic response of antibiotics against bacteria. The standard IVPD model in the literature works for simulating monotherapy and combination therapy of drugs having similar half live. But it does not work for combination therapy of drugs having different half live. The method present in the literature for combination therapy of drugs with different half live was described by Blaser. By utilizing Blaser's method, it was observed that the concentration of drug having a longer half-life could not be achieved as expected in vivo. Therefore, it was essential to develop a novel in vitro pharmacodynamic model to address this limitation. The novel IVPD model in this thesis has overcome this issue by varying the infusion rate at which the drug with longer half-life was being supplemented to the central vessel. The change in infusion rate was calculated to mimic the in vivo plasma concentration of the longer half-life drug. The novel IVPD model was verified by running a 48 hour experiment where the concentration of drug with longer half-life (ceftriaxone) was monitored.

Another aspect of this research was dedicated to developing a physiologically based pharmacokinetic and pharmacodynamic (PBPK-PD) model for combination therapy of amicrobial medications acting synergistically (ampicillin and ceftriaxone). PBPK modeling is a dynamic method that predicts in vivo systemic drug exposure in humans based on the compound's physicochemical properties and absorption, distribution, metabolism and excretion (ADME) characteristics. Interlinking it with the pharmacodynamic model would help to understand the change in pharmacodynamic response caused due to alterations in the pharmacokinetics of drug that impact systemic exposure. An advantage of developing PBPK-PD model for combination

therapy is it can act as a predictive tool to optimize dosing regimen and understand the pharmacodynamic response in special populations (renal impaired patients, pediatrics, pregnant women, etc.).

To develop the PBPK-PD model, substrate profiles for ampicillin and ceftriaxone were first created and verified in healthy volunteers against published literature. Verification was performed by visual predictive check and by calculating the fold error for maximum concentration (Cmax) and area under the curve (AUC). A custom PD model was developed using lua script code which can simulate a pharmacodynamic response for drugs acting synergistically. The PBPK model was interlinked with the PD model. The PBPK-PD model was verified against in vitro results published in the literature. The PD end point was the observed decrease in bacterial count over a period of 72 hours. A dosing regimen of ampicillin 2g q 4 hours and ceftriaxone 2g q 12 hours was simulated using the PBPK-PD model. It was observed that the PBPK-PD model developed in this research could capture the in vitro pharmacodynamic experiment data.

Once verified, the PBPK-PD model was extended to a population of severe renal impaired patients. PBPK-PD model was used to justify the change in dose frequency of ampicillin when given in combination with ceftriaxone in severe renal impaired patients' population. Two dosing regimens were simulated in severe renal impaired patients: 1) ampicillin 2g q 8 hours and ceftriaxone 2g q 12 hours, and 2) ampicillin 2g q 6 hours and ceftriaxone 2g q 12 hours.

In a patient population with renal impairment a regimen comprised of ampicillin IV 2000 mg every q-6 hours and ceftriaxone IV 2000 mg q-12 hours achieved complete eradication of bacteria. The novel PBPK-PD model created in this dissertation research is of clinical significance as an in silico approach can be used to optimizing dosing regimens in special patient populations being treated with a combination of antimicrobial drugs acting synergistically.

CHAPTER 1
INTRODUCTION

1.1 Antibiotic Resistance in *Enterococcus faecalis*

Enterococcus faecalis is a gram-positive facultative bacterium that is normally found in the gastrointestinal fluid of human and animals [1, 2]. *E. faecalis* can cause diseases such as urinary tract infection, meningitis, bacteraemia and infective endocarditis. Infective endocarditis caused by this organism was first described in 1899 [1]. Infective endocarditis is a disease that affects the endocardium surface of the heart which results in the formation of vegetation on the inner lining of heart valves due to the interaction between bacteria and the immune system [3]. The outcome of infective endocarditis is cardiac failure. The patient demographic that is at risk to develop infective endocarditis due to *E. faecalis* are individuals with underlying heart complications such as damaged cardiac valve. Pathogenesis of infective endocarditis caused by *E. faecalis* can be explained in two steps: first bacteria adhere to the tissue via cell surface adhesions, and second the bacteria develop a biofilm in the cardiac valve tissue[1].

Bactericidal therapy is required to achieve clinical cure in infective endocarditis caused by enterococci. Enterococcal bacterial growth depends upon penicillin binding protein (PBP) enzymes that link a precursor pentapeptide molecule to the peptidoglycan cell wall. β-lactams are structural analogs of precursor pentapeptide molecules and bind to PBP, which leads to the inhibition of cell wall growth due to the production of reactive oxygen species. However, β-lactam tolerance has been observed due to an increase in low affinity PBPs (PBP4 in *E. faecalis*) which weakly binds to β-lactam due to abolition of reactive oxygen species by enzyme superoxide dismutase [2]. The low affinity of PBP-4 is caused due to mutation in pbp-4 gene A617T that facilitates the binding of β-lactam binding [70]. Thus, monotherapy with β-lactams such as ampicillin is associated with poor bactericidal effect, but combination therapy with other antimicrobials can achieve bactericidal activity.

Combination therapy of ampicillin plus aminoglycosides such as gentamicin was first considered as a treatment towards elimination of *E. faecalis*. However, the utility of this combination therapy is limited by the development of high resistance towards aminoglycosides, ranging from 23%-73% in *E. faecalis* [1]. The reason behind acquired resistance to aminoglycosides is the obtainment of a bifunctional gene coding for APH(2'')-Ia-AAC(6')-Ie. This enzyme inhibits bactericidal activity of gentamicin by phosphorylation at the 2' hydroxy position, which prevents antibiotic binding to the 30s ribosomal unit, averting the inhibition of protein synthesis leading to loss of bactericidal activity [2].

An alternative combination against *E. faecalis* is the combination of ampicillin plus ceftriaxone. Ampicillin binds to PBP4 whereas ceftriaxone binds to PBP 2 and PBP 3 leading to the inability of pentapeptide to link with the peptidoglycan cell wall which causes inhibition of cell wall growth leading to bacterial death [4]. An open label, non-randomized multicentre study for the treatment of infective endocarditis caused by *E. faecalis* was performed using the combination therapy of ceftriaxone (2g IV every 12 hours) and ampicillin (2g IV every 4 hours). This study showed that the combination was effective in 71.4% of patients having the high resistant aminoglycoside strain and 72.7% of patients having non-high resistant aminoglycoside strain of *E. faecalis* [1].

1.2 In Vitro Pharmacodynamic Model

In vitro pharmacodynamic (IVPD) models are used to quantify the pharmacodynamics of an antibiotic against a strain of bacteria, by simulating the in vivo systemic exposure of the antibiotic over time. IVPD is extensively used as a preclinical modelling tool to optimize drug dosing regimens against various strains of bacteria, including multidrug resistant organisms [5, 6]. Based on working principles, IVPD models are of two kinds: a static model and a dynamic model. In the static model, bacteria are exposed to a constant concentration of antibiotic in a culture vessel. The bacterial count is then observed over time. In the dynamic model, bacteria are exposed to changes in drug concentration over time. In vitro dynamic models are intended to mimic the systemic profile of a drug in vivo after dosing. This is achieved by simulating the expected maximum plasma concentration (Cmax) and elimination half-life of the drug.

The basic set up of the one compartment in vitro dynamic model is illustrated in Figure 1. The model consists of a central vessel which has three ports, a reservoir, a waste container, and a pump. The reservoir supplements fresh medium at a predefined flow rate (controlled by the pump) to the central vessel. The central vessel contains bacteria suspended in a predefined amount of medium. Drug is administered as a bolus dose through the sampling port into the central vessel. Over time, drug is eliminated from the central vessel (due to the system flow rate) into the waste container. Samples are collected at predetermined times via the sampling port [5,71]

Mathematical background for IVPD model: The change in drug concentration in the central vessel as a function of time can be explained by the following equation:

$$c = c_0 * e^{-kt}$$ equation 1

c is the concentration c_0 is the concentration at time zero, t is time and k is elimination rate constant. When drug is introduced to the system as a constant rate infusion, the following equation applied:

$$c = \frac{k_0}{Cl} * (1 - e^{-kt}) \qquad \text{equation 2}$$

where Cl is clearance (exit flow rate) and k_0 is the infusion rate. The dose is calculated to reflect the unbound concentration of drug in-vivo. The volume of the central vessel is 250mL and the dose is calculated based on the volume of the central vessel and the initial concentration of the drug to be achieved.

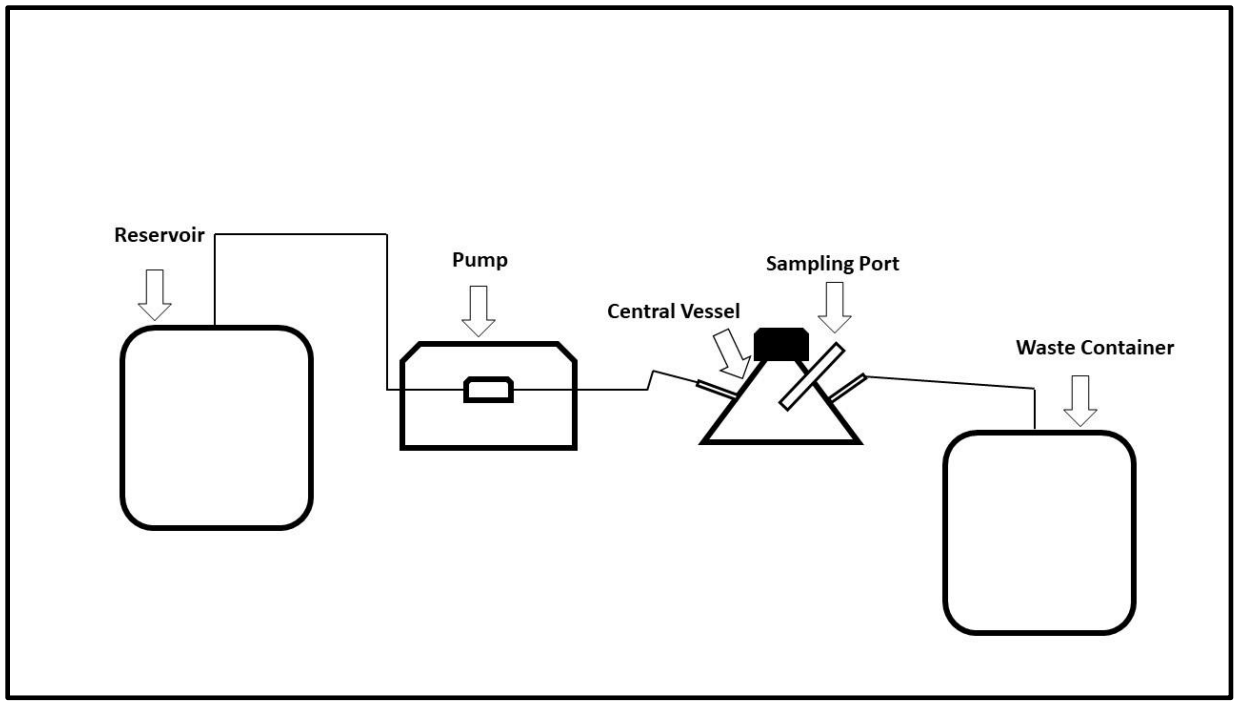


Figure 1. System for in vitro pharmacodynamic modeling of antibiotics for studying drug exposure over time against bacteria

In Vitro Pharmacodynamic Model for Combination Therapy

Blaser described an IVPD model for combination therapy of drugs having two different half lives. This model is widely used by researchers in the field. The model consists of two vessels: a supplement vessel and a central vessel. The kinetics of the system can be defined by a one compartment model. For combination therapy of two drugs having different half lives, the clearance (i.e., the flow rate) of the in vitro system must be set up based on the drug having a shorter half-life (faster clearance). This results in faster elimination of the other drug having longer half-life (slow clearance). To compensate for this loss, the drug with the slower clearance (longer half-life) must be supplemented to the central vessel. Blaser describes the rate at which the drug (having longer half-life) is to be supplemented to the central vessel as the difference between the clearance of the drug with the shorter half-life and the drug with the longer half-life. The following equations describe Blaser's methodology to estimate the concentration of drug with longer half-life in the central and supplement vessel [32]:

$$Cs = \frac{D \times Vs}{\Delta t \times Vc \times (Cl - Cl_1)}$$ (In supplement vessel) equation 3

$$Cc = \frac{D}{(\Delta t \times Cl)}$$ (In central vessel) equation 4

Cs and Cc is the concentration of drug having longer half-life in supplement vessel and central vessel, respectively, and $D/\Delta t$ is the dose per time interval. Vs and Vc are the volumes in supplement vessel and central vessel, and Cl and Cl_1 is the clearance of drug having longer half-life and drug having shorter half-life, respectively [32]. However, we have discovered that this method is inadequate as the drug with longer half-life is being supplemented at a higher constant flow rate leading to faster elimination of the drug from the system. As a result, the model fails to

capture the in vivo systemic profile of the medication. Therefore, an alternative approach is needed to create a reliable and representative IVPD model for combination therapy of drugs having different half live

1.3 Physiologically Based Pharmacokinetic Modeling and Simulation

Although in vitro pharmacodynamic models can predict bacterial growth in vivo and allow for optimization of antibiotic dosing, they do not consider immunological factors such as host defense mechanisms and uptake of pathogens. Additionally, in some cases bacterial growth in vitro is faster than that expected in vivo [64]. Therefore, there is a need for a model that can effectively translate in vitro data into in vivo predictions [2].

Simcyp is a physiologically based pharmacokinetic (PBPK) modelling platform that simulates systemic drug exposure across a range of virtual patient populations. PBPK modeling was introduced by Torell in 1937. [8] Over the years there has been a significant growth in the field due to computational advancement and evolution of advances in preclinical testing methodologies. PBPK modeling is a dynamic method that predicts in vivo systemic exposure based on physicochemical properties and in vitro or preclinical absorption, distribution, metabolism, and excretion (ADME) data of an investigational drug or test compound [9]. In contrast to static methods, PBPK modeling simulates the plasma-time profile curve based, among other things, on changing enzyme kinetics and transporters. Applications of PBPK modeling include drug-drug interaction (DDI) assessment, which aids in dosing regimen selection of at victim drug, prediction of sampling points for a specialized population, understanding effect of inter-subject variability on PK parameters within a population, in vitro in vivo extrapolation and predicting the endpoint pharmacodynamics. [10]

The importance of PBPK modelling is reflected in the recent regulatory guidance that have been developed to help make decisions for clinical studies and labelling of new drug entities using PBPK modelling and simulation. Between 2008 and 2017, the FDA received 130 investigational new drug applications and 94 new drug applications containing PBPK analyses [11]. This signifies the increase application PBPK modeling in drug development. Presently, its primary role is in DDI assessment, but over time the application of PBPK modelling and simulation data in regulatory submissions is expected to grow as pharmaceutical scientists and regulators gain confidence in the approach.

Components of PBPK model

PBPK modeling is typically a bottom-up approach, which simulates clinical drug behaviour based on information gathered during preclinical evaluation [8]. However, the PBPK approach is also used in the early stages of clinical development to assess disposition of a compound in various scenarios such as inter-ethnic variability, special populations, and drug interaction potential.

A basic PBPK model, the minimal PBPK model, considers body as the central compartment which is connected to several peripheral compartments and is used to measure pharmacokinetic parameters such as clearance and volume of distribution [12]. A more complex PBPK model, the full PBPK model, includes compartments for organs such as brain, heart, lung, kidney, gonads, thymus, and muscle, responsible for drugs pharmacological response [9] Each organ is defined by its tissue volume and the blood flow rate. To measure PK parameters in this type of PBPK model, two approaches have been defined: perfusion rate-limited kinetics for small lipophilic molecules and permeability rate-limited kinetics for hydrophilic molecules. Perfusion rate limited kinetics explains the uptake of the lipophilic molecule where the blood flow to tissue the rate is limiting

step and for hydrophilic molecule the rate limiting step is transport across the cell membrane, explaining the permeability limited kinetics [8]. Figure 2 provides a schematic diagram of the whole PBPK model [9]. In this research, Simcyp was used to develop a PBPK model for ceftriaxone in healthy volunteers and explore the potential synergistic effect of ceftriaxone ampicillin on bactericidal pharmacodynamics. Further the PBPK model was integrated with a PD model for optimizing dosing regimen of ampicillin in severe renal impaired patients.

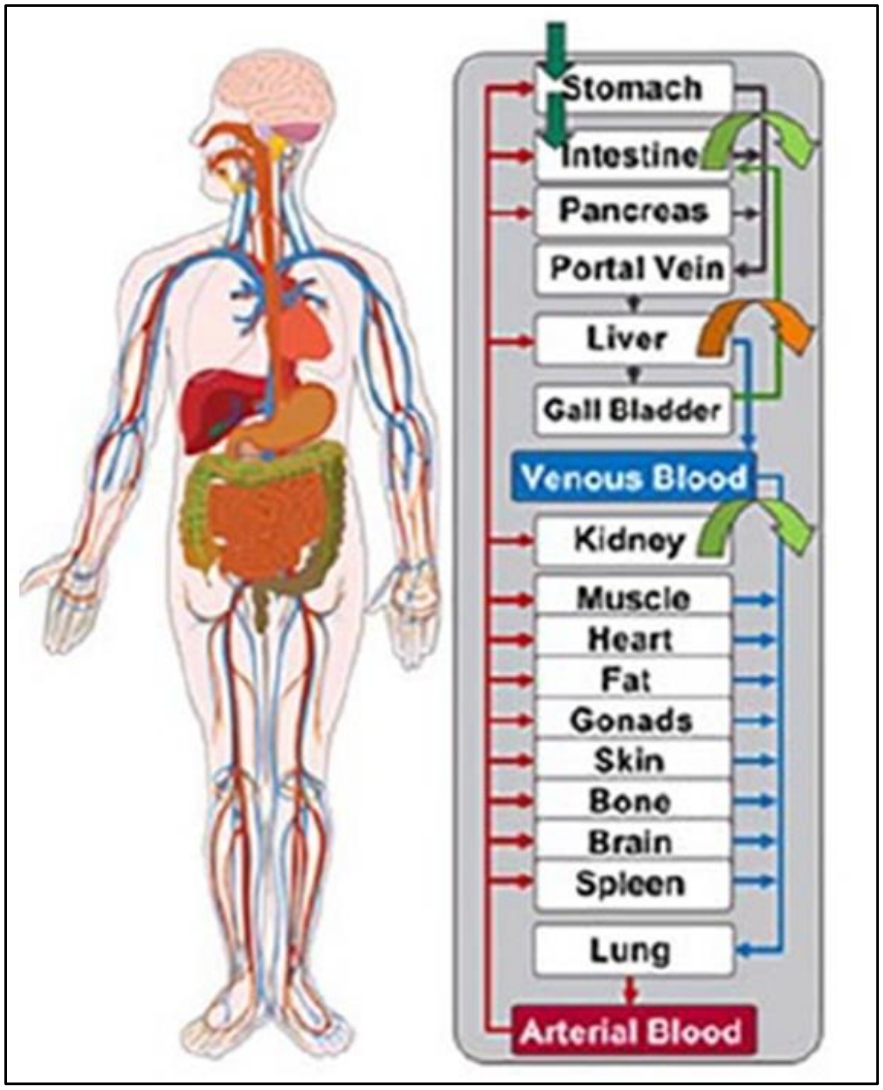


Figure 2. Schematic representation of whole PBPK model [8]

There are three main components of the Simcyp simulator: the population, the substrate profile, and the trial design. Researchers, depending upon their specific aims, can utilize either Simcyp's library of in-built profile or modify individual components according to their needs.

Simcyp Populations

The Simcyp simulator has in built populations that are based on real life demographic data from subjects who have taken part in clinical studies and available public health records. The predefined populations in Simcyp simulator include Healthy Volunteers, and special patient groups such as cancer, cirrhosis, rheumatoid arthritis, obese, paediatric, pregnancy, psoriasis , preterm, geriatric and chronic renal failure. A custom population can also be developed by modifying underlying physiology of individual organs [13].

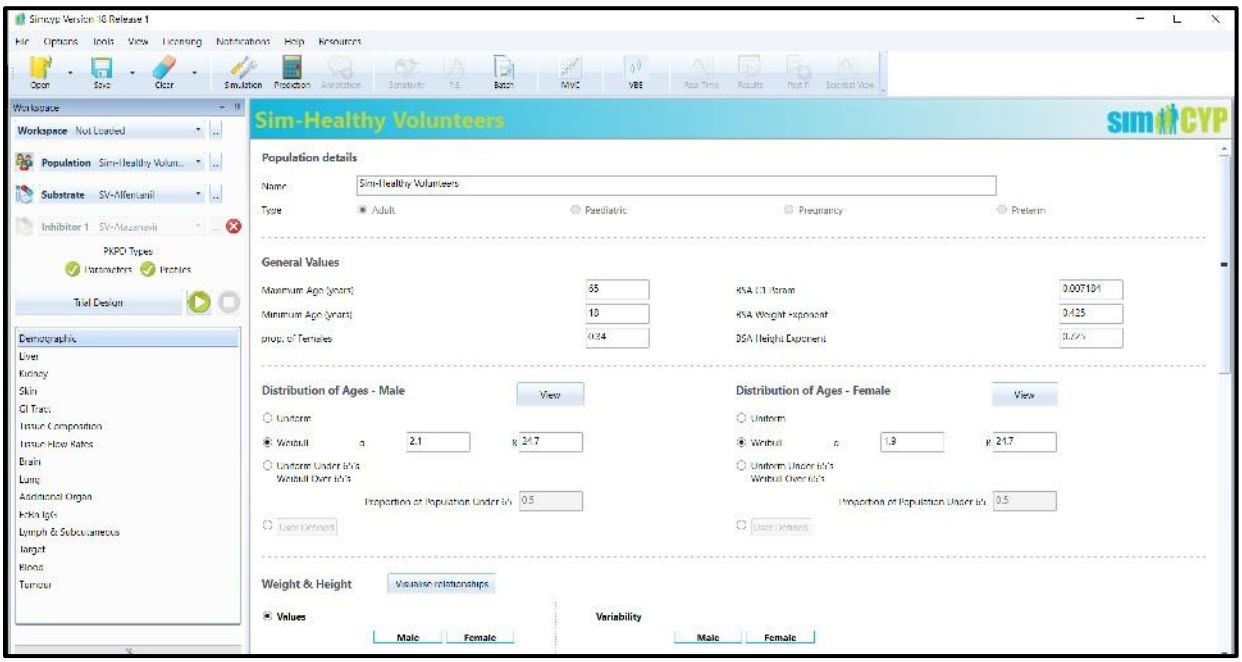


Figure 3. Screenshot of population page adapted from Simcyp version 18

Simcyp Substrate

The substrate screen enables the user to view the drug-specific data associated with a particular compound that will be used in a Simcyp simulation. The following parameters are described in the Simcyp substrate screen: Physicochemical and Blood Binding, Absorption, Distribution, Elimination, Interactions (competitive and mechanism-based inhibition and induction) and Transport (efflux and uptake in both the gut and liver).

Besides a basic 1st order absorption model, Simcyp has two inbuilt absorption model namely advanced dissolution absorption and metabolism (ADAM) and Multi-layer gut wall with ADAM (M-ADAM) model. The ADAM model includes various parameters that affect the rate and extent of oral absorption. Parameters include surface area of the GI tract, luminal pH effect on the ionisation of the compound, transporter density, fed vs fasted state, solubility, dissolution, permeability, and precipitation.

The Distribution component of Simcyp offers two distribution models: minimal PBPK model and a full PBPK model. These models are used to predict the distribution of drug in the body. The Minimal PBPK model predicts concentration of drug in four compartments. These four compartments are systemic, liver, portal vein and the single adjusting compartment (SAC) The SAC is a non-physiological compartment which permits adjustment to the drug concentration profile in the Systemic compartment. The minimal PBPK model describes drug distribution using the parameter volume of distribution at steady state (Vss). The full PBPK distribution model makes use of a number of time-based differential equations in order to simulate the concentrations in various organ compartments: the blood (plasma), adipose, bone, brain, gut, heart, kidney, liver, lung, muscle, pancreas, skin and spleen. Organ and tissue accumulation is based on tissue: plasma

partitioning (Kp). Inter-individual variability is introduced through tissue volume prediction taking account of age, sex, weight and height. [33]

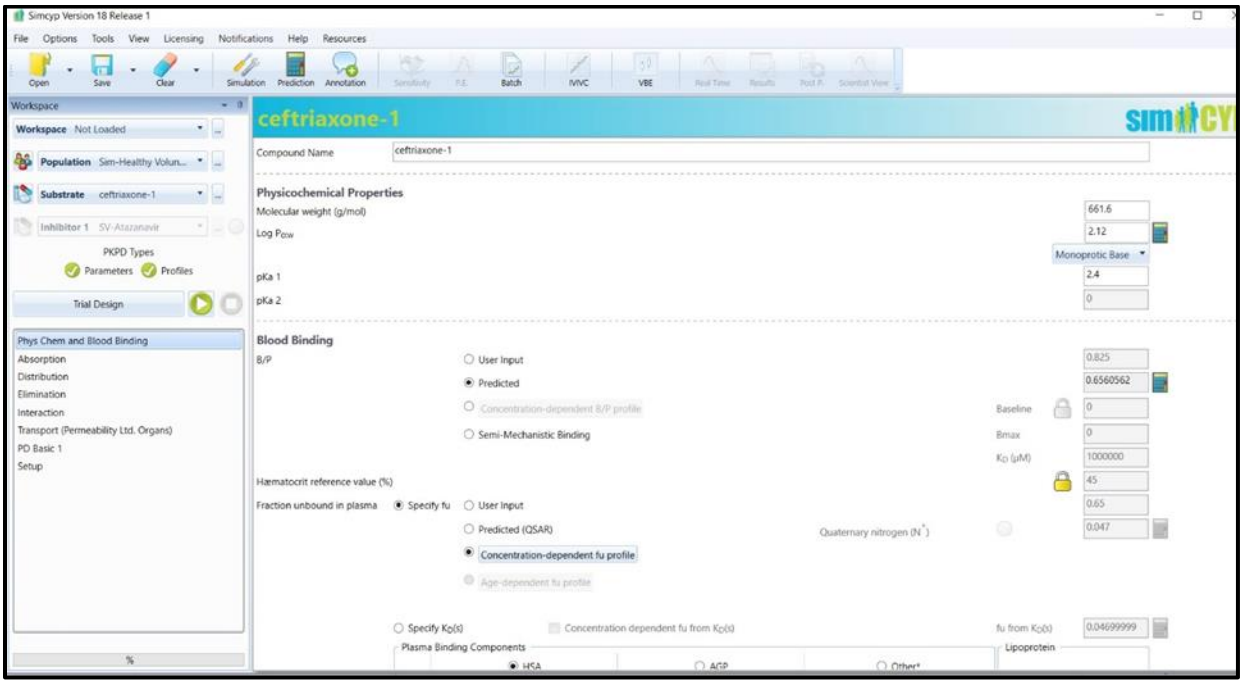


Figure 4. Screenshot of substrate profile adapted from Simcyp version 18

Simcyp Trial Design

The trial design option is where the user can define the dosing regimen, trial size, demographics, study duration and fasted/fed status.

Trials: The user needs to define the population for the study, number of trials to be run, the duration of study and to select the gender ratio followed by the age range. Users can select from virtual population, representative population and multiple populations.

Dosing regimen: The user needs to input dosing related parameters such as route of administration for substrate, dose of the substrate, whether it is a single dose or multiple dose and has to select the condition which is fed or fasted state.

Sampling plan: The sampling site option allows the definition of a peripheral sampling site. This option allows the correction of early distribution differences between the sampling site venous compartment and the central venous compartment (often reported by PBPK models) which are observed following intravenous administration. The sampling time allows the user to customize the sampling schedule in the output to mimic the output from clinical studies and/or to accommodate identification of time-sensitive pharmacokinetic parameters such as $T_{max.}$

Analytical Error: Analytical error features provide an additional flexibility in defining the design of clinical studies and to mirror experimental data. When the user selects the 'Analytical Error' tab from the Trial Design, a screen for the analytical error appears showing both analytical error and Lower Limit of Quantification selections.

Data Analysis: This screen allows the calculation of additional estimates of AUC and the calculation of power for comparisons between populations. [33]

1.4 Pharmacokinetic/Pharmacodynamic (PK/PD) Modeling for Antibacterial Therapy

An increased presence of multidrug resistant bacteria poses a major threat to infected patients due to an increased likelihood of treatment failure [16]. As the development of new antibiotics is limited, the efficacy of combination therapy using available antibiotics against these pathogens must be evaluated. Combination therapy of antibiotics produces either a synergistic effect or an additive effect [73]. A synergistic effect is achieved when one drug by itself shows no or minimal antibacterial effect, but when given in combination with another drug enhances the overall effect produced [73]. An example of synergistic combination is ampicillin and ceftriaxone against *E. faecalis* [84]. Additive effects occur when two drugs individually have effect against the bacteria and when combined the effect is equal to the sum of the effect produced individually by the drugs

[73]. An example of additive effect is rosuvastatin and cefixime against *Klebsiella pneumoniae* [74]. In order for combination therapy to have a successful outcome it is important to understand the pharmacokinetic (PK) and pharmacodynamic (PD) properties of the antibiotics [75].

Establishing the PK/PD relationship is an essential tool at the early stage of drug development to optimize dosing regimen for antibiotics and understanding emergence of resistance. PK/PD indices for explaining antibiotic effect are AUC/MIC (the ratio of area under the concentration time-curve over the period of 24 hours and the minimum inhibitory concentration), T>MIC (time above the MIC during a dosing interval) and Cmax/MIC (maximum concentration/MIC) [15]. PK/PD indices can be predicted by using in vitro pharmacodynamic models and animal models. Although in vitro and animal models are good quantifying tools it is difficult to translate the results into clinical trials for diseased populations [76].

This challenge can be overcome by integrating the results from in vitro and animal models to physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) models. PBPK models help to understand change in PK of drugs caused due to intrinsic and extrinsic factors in humans [76]. Simulating PBPK/PD model for antibiotics can help evaluate dosing regimens not previously tested in clinical trials, and to understand the bacterial killing over time in diseased population [76]. PBPK/PD models thus provide a clinical trial scenario in diseased population and potentially save time and cost [76].

Simcyp is a PBPK software which has multiple inbuilt populations. It has the capability of integrating PBPK with PD models [17]. The PD response model in Simcyp can be set up for compounds, metabolites, and inhibitors. The user has the option of creating a PD response model by linking up to three PD 'response units' in a linear fashion, with links representing (generalized) transduction. Thus, each response unit can be regarded as a building block for constructing more

complex response models. Simcyp has inbuilt basic PD response models such as Hill or Sigmoid E_{max} models, mechanism-based receptor binding-activation models, and stimulus-response-models. Additionally, Simcyp provides the user with various PK input parameter options to apply to the PD model. These input parameters include total dose, average dose rate, plasma, red blood cells, generic oral absorption compartment, and single adjusting compartment. The output of the PD model can be converted to an Excel file or used as an input for the second PD response. The user can build a custom PD response model using the PD custom option. In PD custom model, the user writes equations with the help of lua scripting to develop a novel in vitro mathematical model. The input for the PD model can be selected from the input PK parameters mentioned [17].

In this research, a PBPK-PD model was developed for a synergistically acting drug combination of ampicillin and ceftriaxone against *E. faecalis*. There are no published PBPK-PD models for simulating synergistic antimicrobial therapy using Simcyp. Therefore, a customized PD model using lua scripting was created that models the systemic exposure of ampicillin and ceftriaxone linked to a synergistic response against bacteria. This PBPK-PD model was then utilized to optimize the dose frequency of ampicillin when given in combination with ceftriaxone in severe renal impaired patients (GFR<30mL/min).

1.5 Ceftriaxone

Ceftriaxone(6*R*,7*R*)-7-[[(2*Z*)-2-(2-amino-1,3-thiazol-4-yl)-2-methoxyiminoacetyl]amino]-3-[(2-methyl-5,6-dioxo-1*H*-1,2,4-triazin-3-yl)sulfanylmethyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid is a third generation cephalosporin. It is active against gram-positive and gram-negative bacteria such as *haemophilius influenzae, Neisseria gonorrhoea,* and *Neisseria meningitidis.* Ceftriaxone is not active against enterococcus when administered as a monotherapy. Ceftriaxone when given in combination with ampicillin binds to the active site of penicillin-binding protein (PBP-2 and PBP-3) which results in inhibition of cell wall synthesis leading to bacterial cell death. [18] The FDA approved ceftriaxone dosing regimen approved in adults and children is 1g to 2g once daily or in equally divided doses twice a day. [19]

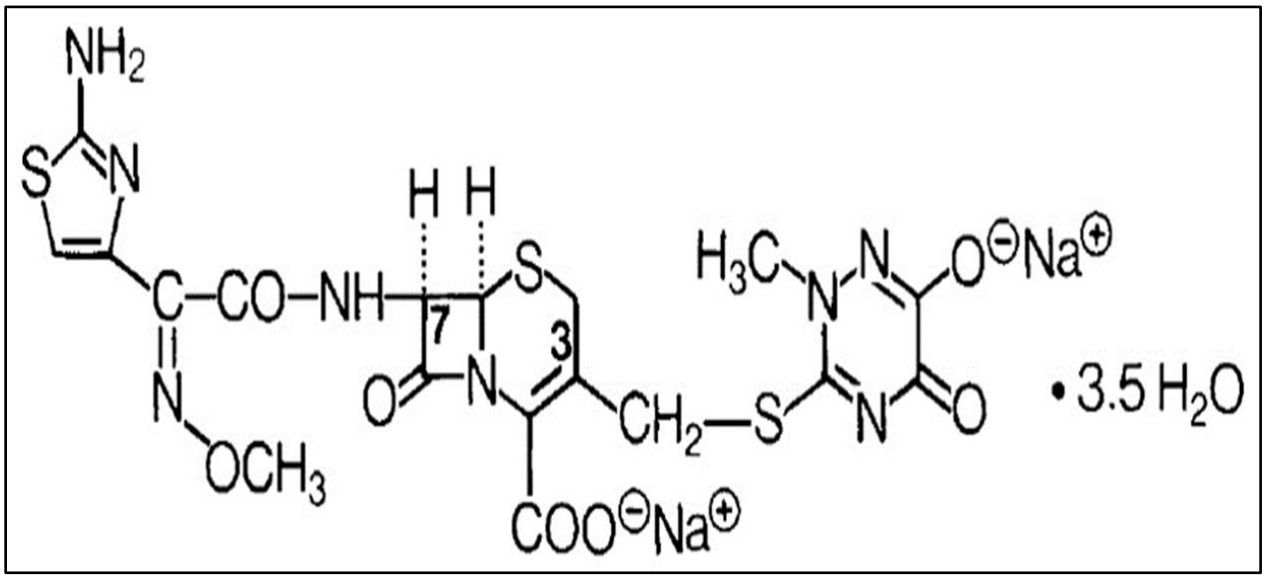


Figure 5. Ceftriaxone structure [20]

Ceftriaxone Pharmacokinetics

A clinical study demonstrated that ceftriaxone plasma Cmax and area under the curve (AUC) increased proportional to dose in healthy volunteers, indicating that ceftriaxone follows linear pharmacokinetics [20]. Compared to other cephalosporins, ceftriaxone has a longer elimination half-life (~6.6-7 hour) which is of clinical importance as it reduces dosing frequency needed to achieve therapeutic effect [22].

Absorption

Ceftriaxone is not absorbed orally due to part to its chemical instability in gastrointestinal fluids. Because of its poor oral bioavailability, ceftriaxone is administered intravenously [23]. Ceftriaxone is a hydrophilic compound and is not a substrate for PEPT1, an intestine transporter that mediated drug absorption into the blood stream.

Distribution and Clearance

Ceftriaxone has a reported volume of distribution of 10L, indicating that its distribution is confined primarily to the blood and plasma, consistent with the compound's hydrophilic nature. In terms of clearance, 30-60% of the drug is eliminated unchanged via the biliary route and the rest is eliminated via renal route. Therefore, there is no dose adjustment needed in patients suffering from renal dysfunction.

1.6 Ampicillin

Ampicillin ((2*S*, 5R, 6R)-6-[[(2*R*)-2-amino-2-phenylacetyl] amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid) is a β-lactam antibiotic. It is active against both gram-positive as well as gram-negative bacteria. Ampicillin is used as a monotherapy for the treatment of respiratory tract infections, urinary tract infections, skin and soft tissue infections. Ampicillin binds to penicillin binding protein (PBP-4) which inhibits the transpeptidation reaction followed by blocking peptidoglycan synthesis which abolishes the cell wall synthesis process leading to cell death [24]. However, monotherapy of ampicillin causes a bacteriostatic effect as there is increase in low affinity PBP-4 and overexpression of PBP-2 and PBP-3. Hence, ampicillin plus ceftriaxone has been considered as an option for the treatment of infective endocarditis caused by *E. faecalis* [25]. This study will focus on the bactericidal effect of ampicillin plus ceftriaxone against *E. faecalis*.

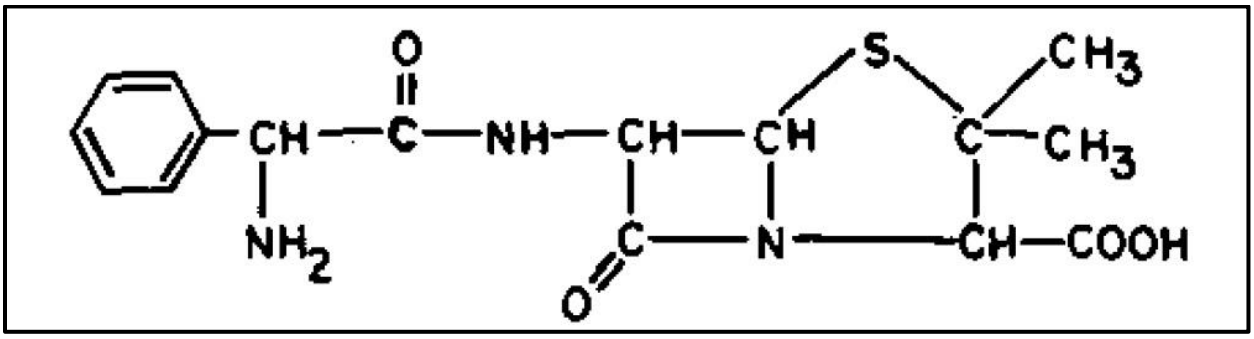


Figure 6. Ampicillin structure [24]

Ampicillin Pharmacokinetics

Jusko et al. [3] performed clinical studies in normal and anephoric subjects to understand the pharmacokinetics of ampicillin. It was observed that following intravenous administration to

healthy volunteers, the plasma concentration declined in a bi-exponential manner over time. No significant difference was observed in clearance and volume of distribution of ampicillin as the dose increases, which indicates that ampicillin follows linear kinetics [26]. The elimination half-life of ampicillin is observed to be 1.3 hours [25].

Absorption

Ampicillin has poor oral bioavailability. Only 30-40% of drug is absorbed post oral administration of drug. Lafforgue et al. [27] performed ex-vivo experiments to determine whether the PepT1 transporter played a role in the absorption of ampicillin. The everted gut sac model failed to show the influence of PepT1 transporter in the absorption of ampicillin, and the study concluded that the reason behind low absorption of ampicillin could be due to the hydrophilic nature of the drug thereby limiting passive diffusion across intestinal membrane. As ampicillin has poor oral bioavailability it is administered via IV route.

Distribution and Clearance

The volume of distribution for ampicillin is approximately 15L [28]. This indicates ampicillin penetrates poorly into intracellular space. Ampicillin is primarily cleared by renal excretion (92%) with limited (8%) biliary excretion and metabolism [25].

Higher ampicillin plasma AUC (area under the curve) has been observed in elderly subjects (37.63 $\mu g.h.ml^{-1}$) compared to adults (16.39 $\mu g.h.ml^{-1}$) when tested at the same dose (500mg single oral dose) [26] This difference was attributed to decreased renal excretion of ampicillin in elderly as the kidney function decreases with increase in age. This result indicates that a large amount of ampicillin is excreted via renal route unchanged.

Chapter 2
Specific Aims

The common cause for infective endocarditis in patients is *Enterococcus faecalis* [29]. A recommended treatment for endocarditis is combination therapy of a cell wall inhibiting drug such as penicillin or ampicillin and a drug with bactericidal activity like aminoglycoside. However, this regimen is limited due to the development of aminoglycoside resistance [30]. This creates an issue for patients suffering from endocarditis caused by a highly resistant aminoglycoside enterococci strain. Previous results from animal studies utilizing an endocarditis model provided inconclusive data for the administration of ampicillin monotherapy as an effective treatment [31]. Therefore, there is a need to explore new combination therapies to overcome aminoglycoside resistance for this pathogen. Ampicillin and ceftriaxone are regarded as an alternative combination therapy against the highly resistant aminoglycoside *Enterococcus faecalis* strain. According to national guidelines, ampicillin and ceftriaxone were added as an option to treat both high-level aminoglycoside resistant and non-high level aminoglycoside resistant endocarditis.

An in vitro experimental model of antimicrobial efficacy provides an important basis for clinical investigations in humans. The pharmacokinetics of antibiotics may differ in humans as compared to other animals due to species differences in drug disposition mechanisms [32]. To overcome this drawback, alternative models such as in vitro pharmacodynamic models are being explored. An in vitro pharmacodynamic model simulates in vivo plasma profile of the medication while monitoring bacterial growth and death overtime [33]. The mathematical background and in vitro model suggested by Blaser [34] is appropriate for exploring the time-course profile for monotherapy or combination therapy for drugs with similar elimination half live, but the model fails for combination therapy for drugs with different half lives. One of the aims of this thesis is to

develop an in vitro model which would support the study of combination therapy of drugs having two different half lives, using ceftriaxone as a model drug. Further, the research aims to build a physiologically based pharmacokinetic-pharmacodynamic model for the combination therapy of ampicillin and ceftriaxone using the Simcyp simulator platform. This model will help elucidate the pharmacodynamic response, specifically the decrease in the systemic bacterial count after administration of combination drug therapy.

2.1 Aim 1

To create an in vitro pharmacodynamic model that allows for simultaneous evaluation of two antibiotics with different half live using ceftriaxone as a test drug

An alternative in vitro pharmacodynamic model for combination therapy was built to attain accurate concentrations of a test drug with a longer half-life. The modified IVPD model was set to mimic combination therapy of drugs with two different half-lives. The exit flow rate of the system was set according to drug with shorter half live. Computer simulations performed prior to performing the experiment confirmed that if the exit flow rate of the in vitro system is set based on the clearance of drug with shorter half-life it could achieve the concentration profile for the drug with shorter half-life as expected in vivo. Therefore, the IVPD model was set to simulate combination therapy with two different half-lives focused on attaining the accurate concentration of drug with longer half live. Ceftriaxone was used as a test drug for the in vitro studies. The modified in vitro study supported a combination of IV bolus administration of the drug in the central vessel with supplementation of drug from the supplemental vessel via a continuous infusion rate. The rate at which the drug was supplemented to the central vessel was varied at predetermined time point to attain the in vivo plasma concentration of the drug. The simulation

was verified by measuring drug concentrations over time in the central vessel using a validated high-performance liquid chromatography (HPLC) method.

2.2 Aim 2

Physiologically based pharmacokinetic-pharmacodynamic (PBPK-PD) model of ampicillin and ceftriaxone combination therapy against *Enterococcus faecalis* in a Sim-Healthy Volunteer population

Physiologically based pharmacokinetic models for ampicillin and ceftriaxone in healthy volunteer were developed using Simcyp simulator. The models were verified against published clinical data. Simcyp does not provide a built in PD model to simulate combination effect of drugs acting synergistically. Therefore, the other aspect of this research was to develop a pharmacodynamic model for the combination therapy of drugs acting synergistically. The verified PBPK model for ampicillin and ceftriaxone was interlinked to the custom PD model that was created using lua scripting based on a published PD model. The PBPK-PD model was verified by comparing the simulated pharmacodynamic response of ampicillin and ceftriaxone to data generated from an in vitro pharmacodynamic experiment published in the literature.

2.3 Aim3:

Application of PBPK-PD model to optimize the dosing regimen of ampicillin in a severe renal impaired population when given in combination with ceftriaxone against *E. faecalis*

The goal of this aspect of research was to apply the PBPK-PD model verified in healthy volunteers to optimize the dosing frequency for ampicillin when given in combination with ceftriaxone in a severe renally impaired population. PBPK models for ampicillin and ceftriaxone were verified in

severe renal impaired population against the published data. The verified PBPK model was then linked to PD model to simulate two dosing regimen of combination therapy, i.e., ampicillin 2g q 8 hours along with ceftriaxone 2g q 12 hours and ampicillin 2g q 6 hours along with ceftriaxone 2g q 12 hours. The optimal dosing strategy was evaluated based on the regimen that, based on model simulations, provided complete eradication of bacteria in severe renal impaired population. All simulations were performed using Simcyp.

Chapter 3

An In Vitro Pharmacodynamic Model Allowing Simultaneous Evaluation of Two Antibiotics with Different Half-lives Using Ceftriaxone as a Test Drug

3.1 Introduction

As mentioned in Chapter 1, the IVPD method described by Blaser in 1985 [32] for simultaneously studying the in vitro pharmacodynamics of two drugs only works for medications with similar half-lives. Figure 7 depicts the experimental set up for the Blaser method. The Blaser method consists of a central vessel and a supplement vessel. The central vessel contains both drug(s) and bacteria in a predefined volume (*Vc*) of biological fluid. The vessel has three ports. One port is the inlet attached to the supplementation model and another reservoir which supplements media, the other port is where the drug exits out of the central vessel, and the third port is where both the drugs are administered and from which samples are taken at different time points. The flow rate at which the drug would exit from the central vessel (i.e., clearance) is determined based on the in vivo half-life of the drug with faster elimination. The flow rate (labelled Cl in Figure 7) is calculated by multiplying volume of fluid in the central vessel by the in vivo plasma elimination rate constant for the drug (equation 5).

The supplementation model is used to supplement the drug with the longer half-life, to compensate for the relatively rapid loss of the drug from the central vessel (a consequence of the rapid flow rate needed to mimic the in vivo profile of the other drug). The flow rate of the supplementation model is based on the difference in the flow rate from the central vessel and the flow rate based on the clearance of the drug with the longer half-life (calculated as the volume of the supplemental vessel multiplied by the in vivo elimination rate constant of the drug). As the drug with longer half-life is being supplemented at a higher constant flow rate leading to faster elimination of the drug from the system, the model fails to capture the in vivo systemic profile of the of drug with longer half-life.

For example, consider an IVPD model mimicking the Blaser method and simulating combination therapy of ampicillin (Drug A) 2000mg q 4hours (C_{maxss} [maximum concentration reached at steady state] 150µg/mL, $t_{1/2}$ 1.4 hour or 84mins) and ceftriaxone (Drug B) 2000mg 12 hours (C_{maxss} 280µg/mL,$t_{1/2}$ 7 hour or 420mins). If the volume in the central vessel (Vc) is 250mL, clearance of Drug A (exit flow rate) for in vitro system is calculated as:

$$Cl_A = k \times Vc => 0.00825 \times 250 = 2.1mL/min \quad \text{equation 5}$$

Similarly, the clearance of Drug B (Cl_B, flow rate of ceftriaxone) for the in vitro system is calculated and the value is 0.4mL/min. Drug A and Drug B are administered into the central vessel as an IV bolus dose. Based on Blaser's IVPD model set up (Figure 7), the exit flow rate of the entire IVPD system is based on the clearance of Drug A (Cl_A = 2.1 ml/min). Drug B is to be supplemented from the supplementation model to the central vessel at a flow rate which equals 1.7ml/min (2.1 mL/min- 0.4 mL/min).

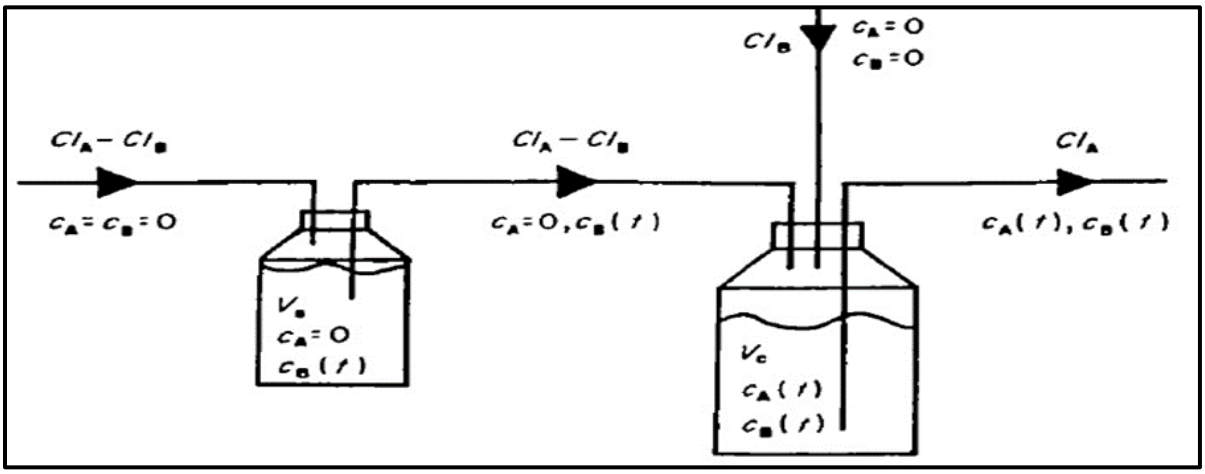


Figure 7. Schematic diagram of IVPD suggested by BLASER for simultaneous study of two drugs. [32]

The concentration of Drug A in the central vessel as a function of time over the duration of the experiment is described by the following monoexponential equation:

$$c_p = c_o . e^{-k_e t}$$ equation 6

The concentration of Drug B from the central vessel over time can be calculated as:

$$c_p = c_o . e^{-k_e t} + \frac{F k_a D_o}{V_c(k_a - k_e)}(e^{-k_e t} - e^{-k_a t})$$ equation 7

Where c_p is the concentration of drug at time t. c_0 is concentration at time zero, ka is 1st order rate constant for supplementation of Drug B into the central vessel, k_e is the elimination rate of constant of Drug A, V_c is volume of the central vessel, and D_0 is supplemental dose. It was observed that the supplementation model mimics the first order oral absorption model. Figure 8 describes how Blaser's supplementation model mimic first order oral absorption model.

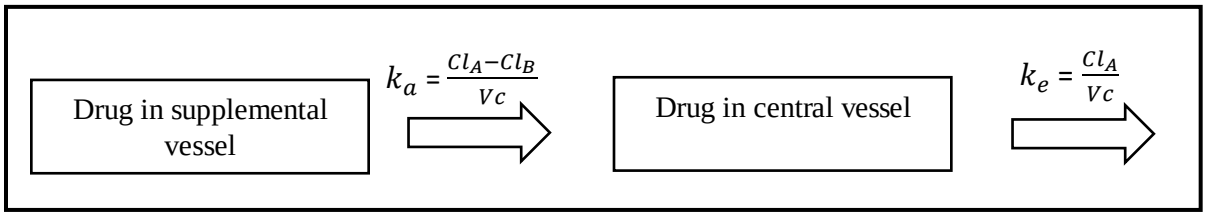


Figure 8. Flow chart depicting supplementation model of Blaser method

The amount of drug in the supplemental vessel at time t can be described by the following equation:

$$\frac{dD_{supp}}{dt} = D_0 e^{-k_a t}$$ equation 8

ka is the first order absorption rate constant, D_0 is the dose of the drug, D_{supp} is the amount of drug in the supplemental vessel at time t. The rate of drug eliminating from central vessel is described by $-k_e$D. Then, the amount of drug in central vessel at time t will be:

$$\frac{dD}{dt} = rate\ in - rate\ out$$ equation 9

$$\frac{dD}{dt} = D_0 e^{-k_a t} - k_e \mathrm{D}$$ equation 10

Integrating equation 10 and dividing amount by the volume of the vessel ($V_{c)}$ gives the overall concentration of drug in central vessel at time t:

$$c_p = \frac{k_a D_o}{V_c(k_a - k_e)}(e^{-k_e t} - e^{-k_a t})$$ equation 11

Thus, the overall concentration of Drug B in the central vessel (equation 7) involves the contribution of the concentration achieved post IV bolus dose in the central vessel (following the mono-exponential equation) and the amount of drug supplemented from the supplemental vessel (following first order oral absorption equation).

Computer simulations were performed to model the central vessel concentration vs. time profile based on Blaser method for Drug A (ampicillin) and Drug B (ceftriaxone) based on the standard dosing regimen for each drug. The first step was to calculate the input parameter values such as dose to be administered (based on Cmax), in vivo elimination rate constant, and flow rate (clearance) and the second step is to simulate concentration of ampicillin and ceftriaxone in the central vessel based on equations 6, 7, and 11.

Step 1:

$k_e = \frac{0.693}{84}$ = 0.00825 1/min equation 12

(in vivo plasma elimination rate constant for ampicillin, 84 minutes)

$k_e = \frac{0.693}{420}$ = 0.0016 1/min equation 13

(in vivo plasma elimination rate constant for ceftriaxone, 420 minutes)

$Cl_A = k \times Vc => 0.0082 \times 250 = 2.1 mL/min$ equation 14

(exit flow rate of the in vitro system based on 250 ml volume of central vessel)

$Cl_B = k \times Vc => 0.0016 \times 250 = 0.4\, mL/min$ equation 15

(flow rate for ceftriaxone)

$Cl_A - Cl_B => 2.1 - 0.4 = 1.7 mL/min$ equation 16

(flow rate at which ceftriaxone is supplemented into central compartment)

$k_a = \frac{Cl_A - Cl_B}{V_C} = \frac{2.1-0.4}{250} = 0.0068$ 1/min equation 17

(1st order rate constant for supplementation of ceftriaxone is supplemented to the central vessel)

$D_0 = fC_0 \times V_c => 78.81 \times 250 = 19702.5 \mu g$ equation 18

(IV bolus Dose for ampicillin administered in the central vessel)

$D_0 = fC_0 \times V_c => 20 \times 250 = 5000\ \mu g$ equation 19

(IV bolus Dose for Ceftriaxone administered in central and supplemental vessel)

Step 2:

Microsoft excel software was used to simulate concentration vs time profile for ampicillin and ceftriaxone based on equation 6 (concentration for ampicillin and ceftriaxone in central vessel), equation 7 (total concentration for ceftriaxone in central vessel including supplementation) and

equation 11 (concentration of ceftriaxone in central vessel supplemented from the supplemental vessel).

The concentrations attained for both the drugs via the Blaser method were compared to their expected in vivo concentrations. The expected concentration was calculated using the one compartment IV model equation.

Result from the Computer Simulation: When the concentration vs time profile was simulated in excel using equation 6 (concentration of ampicillin in central vessel using Blaser's equation) and expected concentration of ampicillin based on the one compartment IV model equation it was observed that the concentration of ampicillin achieved by Blaser method could mimic the expected in vivo plasma concentration (Figure 9).

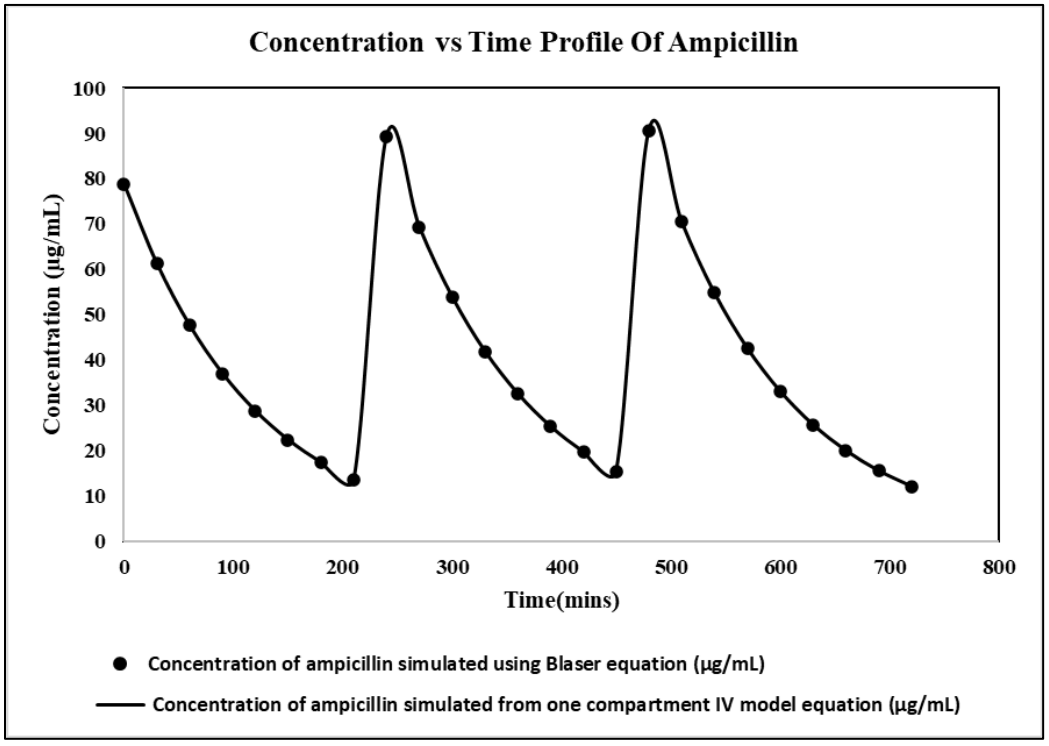


Figure 9. Comparing concentration vs time profile of ampicillin simulated using excel based on Blaser's equation with expected in vivo plasma concentration calculated based on one compartment IV model equation

The concentration of ampicillin is achieved based on the IVPD model suggested by Blaser because the exit flow rate of the IVPD model is set according to the clearance of ampicillin.

For ceftriaxone, the concentration achieved by Blaser method could not mimic the expected in vivo plasma concentration (Figure 10 c). This is because of the following reasons: 1) the amount of drug in the central vessel is being eliminated at a faster rate (Figure 10 b) as the IVPD model's exit flow rate is set according to ampicillin's clearance (i.e., 2.1mL/min). This exit flow rate is higher than the actual clearance of ceftriaxone (0.4 mL/min). 2) The supplementation model fails to supplement the concentration of drug required to compensate the excessive loss of drug from the central vessel as it is analogous to first order oral absorption model (figure 10 a).

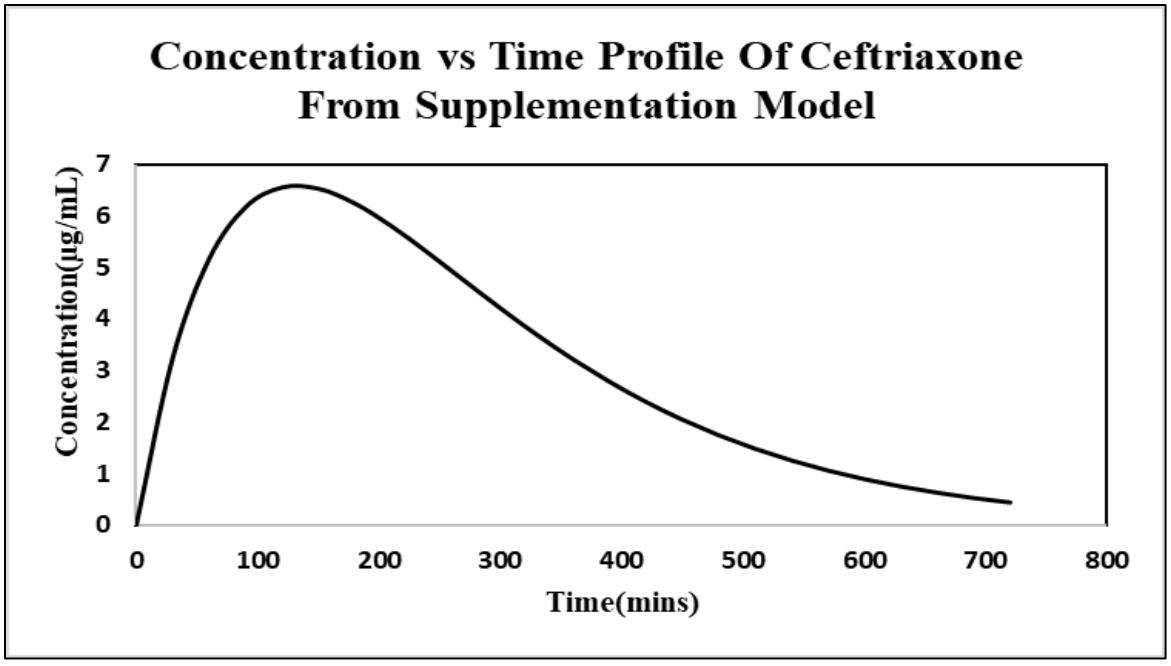


Figure 10 a Concentration vs time profile of ceftriaxone simulated in the central vessel after supplemented from the supplement vessel

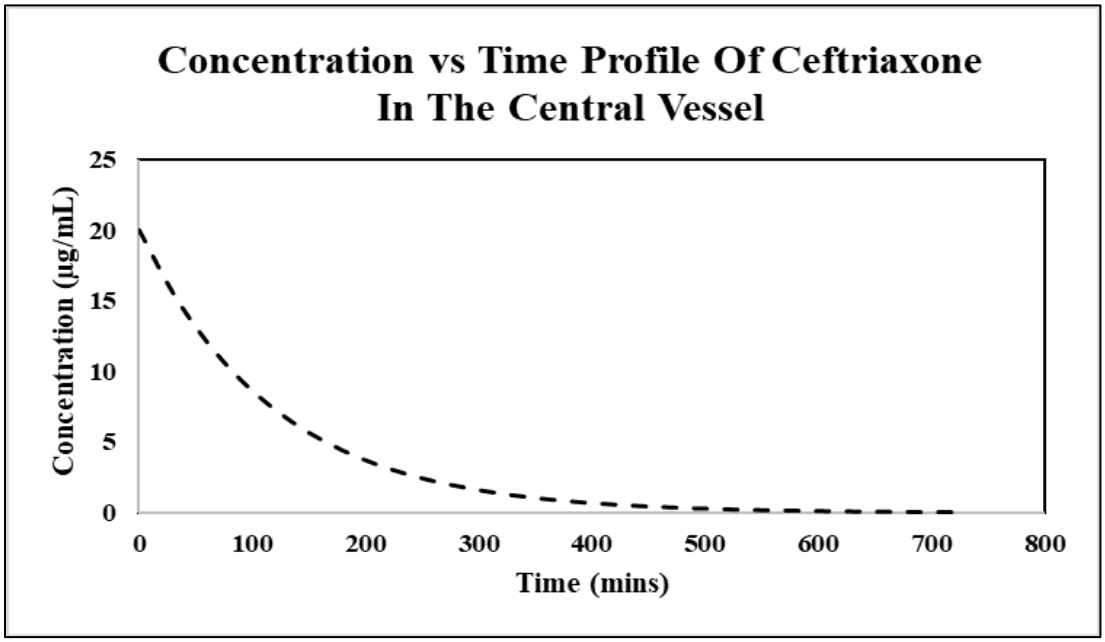


Figure 10 b Concentration vs time profile of ceftriaxone simulated in the central vessel when the exit flow rate is at 2.1mL/min

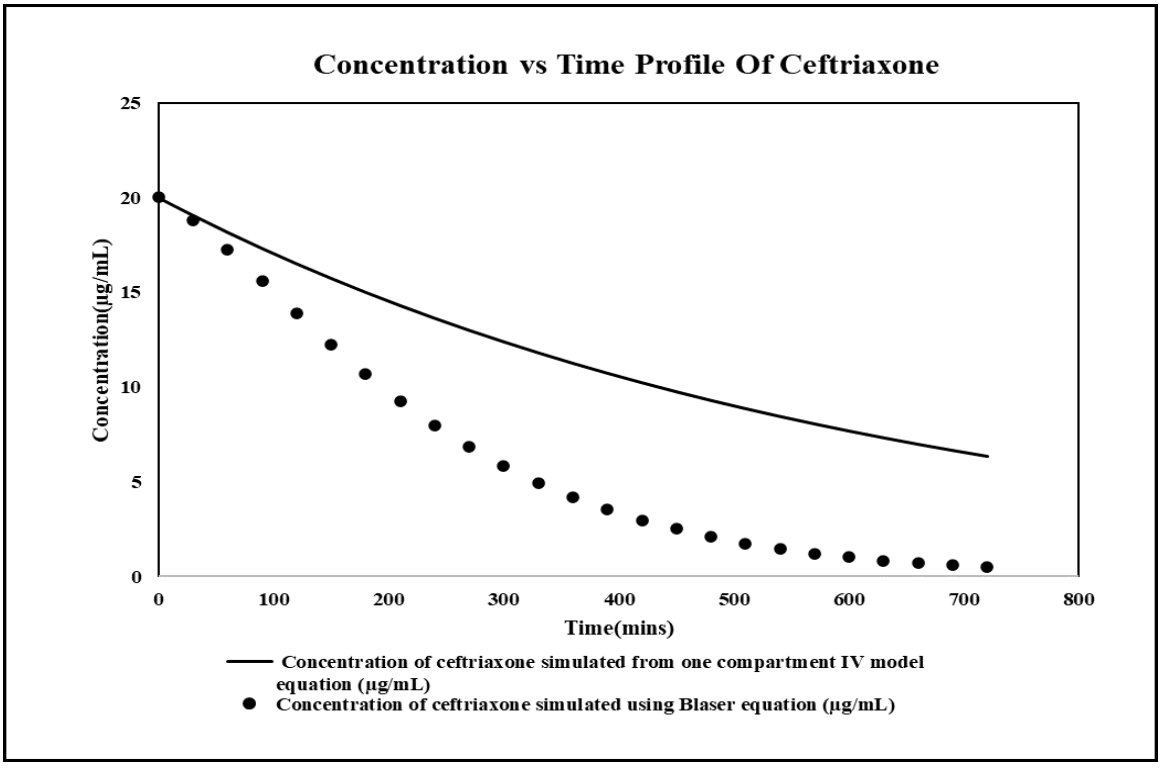


Figure 10 c Comparing concentration vs time profile of ceftriaxone simulated using excel based on Blaser's equation with expected in vivo plasma concentration calculated based on one compartment IV model equation

For example, the concentration of drug ideally to be supplemented to the central vessel at time 90 minutes should be 7.93 µg/mL to overcompensate the loss of excessive of drug from the central vessel. Instead, the drug concentration supplemented from the supplementation vessel was calculated to be 6.18 µg/mL. Table 1 summarizes ceftriaxone in vivo plasma concentration, the concentration of drug in the central vessel when the exit flow rate of the IVPD system is 2.1mL/min, the concentration of drug in the supplementation compartment using Blaser's equation (equation 11) and the ideal concentration that is to be supplemented to the central vessel for the IVPD model to mimic the in vivo concentration of ceftriaxone (named in the table as expected concentration from supplementation model). The expected concentration from supplementation model was calculated as the difference between the expected in vivo plasma concentration and the concentration of ceftriaxone achieved at higher flow rate, i.e. 2.1mL/min . All the concentrations were simulated using excel software.

Table 1 Ceftriaxone concentration achieved at different flow rates and in vivo plasma concentration

Time(mins)	Concentration from supplementation model (µg/mL)	Expected concentration from supplementation model (µg/mL)	Concentration @ 2.1mL/min	In vivo plasma concentration
0.00	0.00	0.00	20.00	20.00
30.00	3.25	3.52	15.54	19.06
60.00	5.17	6.09	12.08	18.17
90.00	6.18	7.93	9.39	17.32
120.00	6.57	9.21	7.30	16.51
150.00	6.54	10.06	5.67	15.73
180.00	6.25	10.59	4.41	15.00
210.00	5.82	10.87	3.43	14.29
240.00	5.30	10.96	2.66	13.62
270.00	4.75	10.91	2.07	12.98
300.00	4.21	10.77	1.61	12.38
330.00	3.70	10.54	1.25	11.80
360.00	3.22	10.27	0.97	11.24
390.00	2.78	9.96	0.76	10.72
420.00	2.39	9.63	0.59	10.21
450.00	2.05	9.28	0.46	9.74
480.00	1.74	8.92	0.35	9.28
510.00	1.48	8.57	0.28	8.84
540.00	1.25	8.22	0.21	8.43
570.00	1.05	7.87	0.17	8.03
600.00	0.89	7.53	0.13	7.66
630.00	0.74	7.20	0.10	7.30
660.00	0.62	6.88	0.08	6.96
690.00	0.52	6.57	0.06	6.63
720.00	0.43	6.27	0.05	6.32

Thus, the concentration of ceftriaxone attained in the central vessel following Blaser method is much lower than the target profile based on in vivo plasma concentrations. This indicates that the Blaser method is unable to attain the concentration vs time profile for the drug with a longer half-life.

To overcome this experimental design limitation, an alternative approach was tested and implemented in the proposed research, a method that will allow accurate simulation of drug with longer half-life (ceftriaxone). The modified IVPD model (LIU method) was designed to simulate the combination therapy of drugs with different half live. The exit flow rate was set according to the clearance of the short half-life drug (ampicillin). The IVPD model modification was to set the supplementation model to follow continuous infusion and change the infusion rate at a predefined time at which ceftriaxone will be supplemented to the central vessel. Ceftriaxone must be supplemented to compensate the loss from the central vessel as the exit rate is faster than the input flow rate of the drug (clearance of ceftriaxone). Although the suggested IVPD model was set to simulate combination therapy, in this research only ceftriaxone concentrations in the central vessel were experimentally measured. Because the exit flow rate of the modified IVPD model is based on the clearance of ampicillin, one would expect to attain the concentration of ampicillin as expected in vivo, as illustrated in Figure 9.

3.2 Methodology

Mathematical Background

Our hypothesis was that the infusion rate of Drug B (longer half-life) from the supplementation model to the central vessel must be adjusted during experimentation to achieve the desired concentration vs. time profile. The test medication for this research was ceftriaxone, an antibiotic with a relatively long elimination half-life in humans when compared to ampicillin; the IVPD model parameters were established based on this drug. The table below lists the parameters used for the experimental infusion rate schedule for ceftriaxone. The fCo (fraction unbound initial concentration) value is based on the typical plasma unbound Cmax reported in humans. (20)

Table 2 Ceftriaxone input parameter value

Parameter	Values
Volume of central vessel (V)	250 ml
Flow rate out of central vessel (Cl)	0.4 mL/min
k_{out} (ratio of Cl and V)	0.0016 1/min
$t_{½}$ (in vivo plasma half-life)	426.87 1/ min
Dose	5000 μg
fCo(fraction unbound initial concentration)	20 μg/ml

Ceftriaxone is administered as a bolus dose at time zero in the central compartment. The IVPD model's exit rate was to set to drug with shorter half-life (ampicillin. flow rate 2.1 ml/min). This would lead to faster elimination of ceftriaxone compared to in vivo. To compensate the loss of ceftriaxone a supplementation model was set to follow continuous infusion model. The rate at

which ceftriaxone would be supplemented would vary at a predefined time point to match the in vivo plasma concentration. The following equation describes the total concentration of ceftriaxone in the central vessel over time.

$$C = C_{max}\, e^{-kt} + \frac{k_o}{Cl}\,(1 - e^{-kt}) \qquad \text{equation 20}$$

C_{max} = Maximum concentration of drug in the central vessel – this is the concentration at the start of the experiment (t =0).

k_0 = infusion rate of drug from supplemental to central compartment

Cl= clearance (the flow rate from the central vessel)

In the case where the infusion rate (k_0) needs to be adjusted at different time points during the experiment, the equation above needs to be expanded accordingly. The change in dosing rate was optimized to match the in vivo plasma concentration of ceftriaxone. Computer simulations were used to optimize the change in rate of infusion using equation 20. The infusion rate was changed every 3 hours, 4 hours and 6 hours. The results suggested that more frequent rate adjustment (i.e., every 3 hours) could better capture the in vivo plasma concentration profile(Figure 11), but this was not practically feasible when executing the experiment. Therefore, the supplementation model involved adjusting the infusion rate from the supplemental vessel every 4 hours.

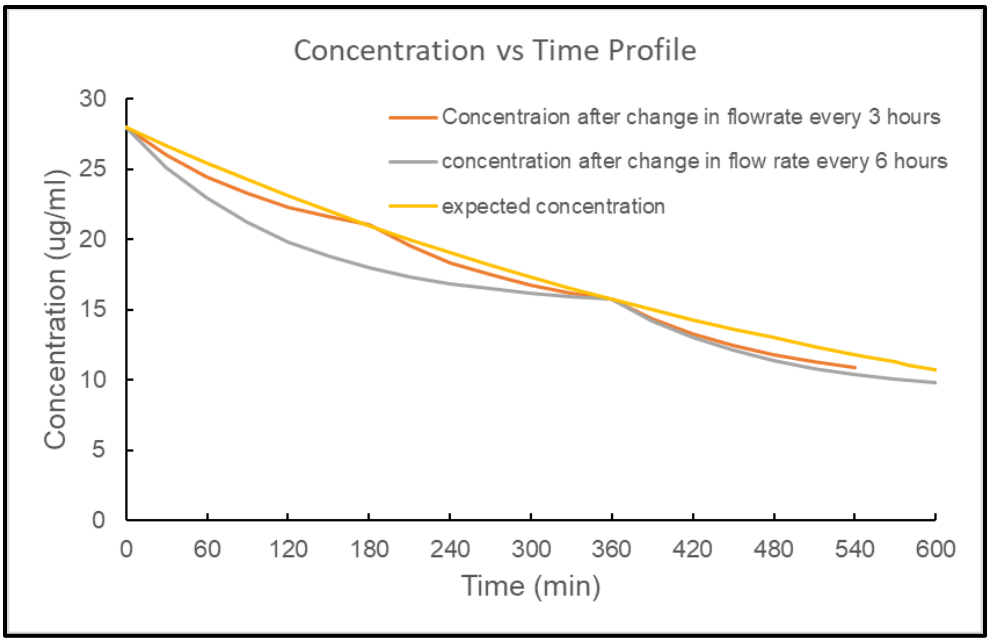


Figure 11. Depicting concentration vs time profile of ceftriaxone with change in infusion rate from the supplementation model after every 3, 4 and 6 hours.

Based on equation 20 change in infusion rate after every 4 hours for a 12 hour study was calculated as follows:

<u>From 0- 240 mins (Rate 1:- 26.54 µg/min)</u>

$$13.62 = 20 \times e^{-0.0084*240} + \frac{Rate_1}{2.1} \times (1 - e^{-0.0084*240}) \quad \text{equation 21}$$

<u>From 240- 480 mins (Rate 2:- 18.08 µg/min)</u>

$$9.27 = 20 \times e^{-0.0084*480} + \frac{Rate_2}{2.1} \times \left(1e^{-0.0084*(480)}\right) \quad \text{equation 22}$$

<u>From 480- 720 mins (Rate 3:- 12.31 µg/min)</u>

$$6.32 = 20 \times e^{-0.0084*720} + \frac{Rate_3}{2.1} \times \left(1 - e^{-0.0084*(720)}\right) \quad \text{equation 23}$$

Figure 12 depicts the computer simulation of the predicted 12 hour concentration profile for ceftriaxone in the central vessel using this modified approach (referred to as ceftriaxone concentration after change in flow rate) compared to the profile based on the in vivo PK of the drug (blue line).

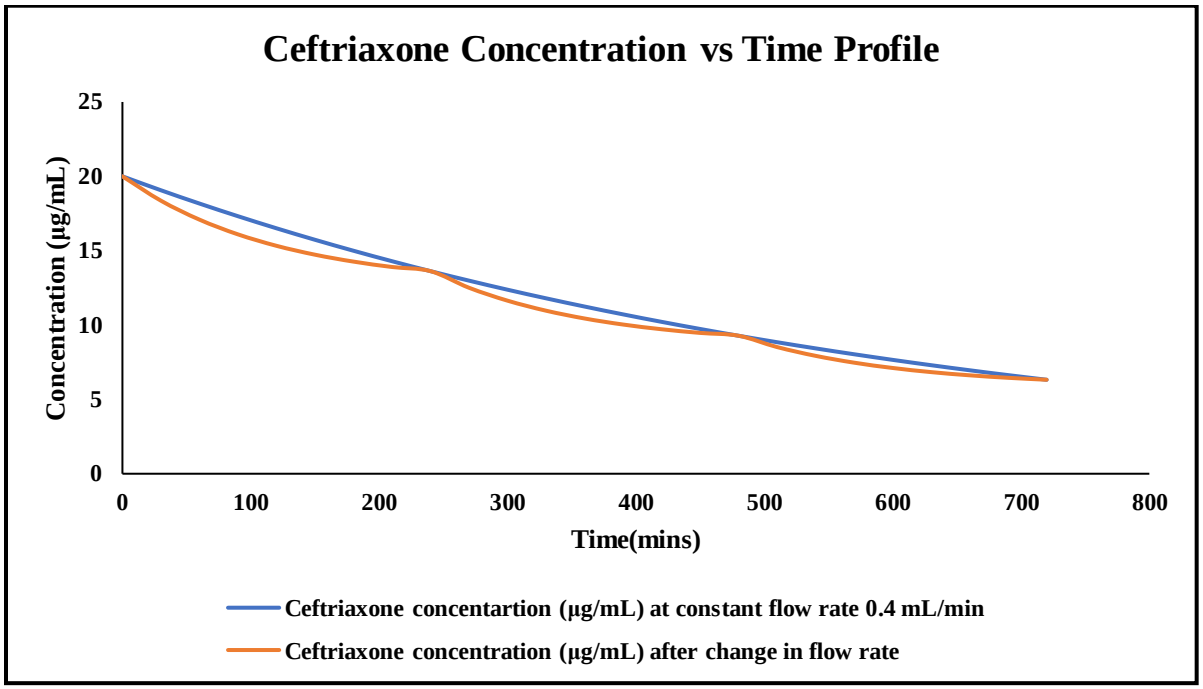


Figure 12. Graph generated from excel showing concentration of ceftriaxone having constant rate 0.4ml/min (blue line) and the concentration of the drug after change in flow rate (orange line)

To confirm and validate these predictions, a 48-hour study of IVPD model (LIU method) simulating combination therapy of ampicillin and ceftriaxone was performed in duplicate. The concentration of ceftriaxone was quantified using a validated HPLC method.

Materials

All the chemicals purchased were of analytical grade. Ceftriaxone sodium, disodium hydrogen phosphate heptahydrate, sodium dihydrogen phosphate monohydrate was purchased from Sigma-Aldrich (Milwaukee, Wisconsin). Trifluoro acetic acid (TFA) and methanol were purchased from Fisher Scientific. Acetonitrile was obtained from VWR (Bridgeport, New Jersey) Deionized water (18 M Ω resistivity) was used in all experiments.

In vitro pharmacodynamic model (LIU method)

A 48-hour IVPD experiment was run in duplicate.. The IVPD model is intended to test drugs with two different half-lives, and as observed in Figure 9, the model is able to accurately capture the systemic profile of short half-life drug (Drug A) since the exit flow rate from the central vessel is based on the clearance of that drug. Therefore, these experiments focused on optimizing the model to accurately mimic in vivo concentration profile of the drug with a longer half-life, ceftriaxone.

Figure 13 depicts the experimental set up of a one compartment IVPD model running combination therapy of ampicillin and ceftriaxone. The IVPD model consists of the following components: two reservoirs (reservoir A and B), two pumps (pump 1 and 2,Cole Parmer Masterflex L/S pumps), central vessel, sampling port, and a waste container. Reservoir A contained a ceftriaxone stock solution (38.5μg/mL) and was in series with pump 1. Reservoir B contained phosphate buffer saline (PBS, pH 7.4) and was in series with pump 2. Both pumps were connected via a Y shaped plastic tube to the central vessel. The central vessel had 3 ports: an inlet port connected to the pumps, a sampling port, and an exit port connected to the waste container. The central vessel was vacuum sealed and prefilled with 250mL of phosphate buffer saline (V_c = 250 ml).

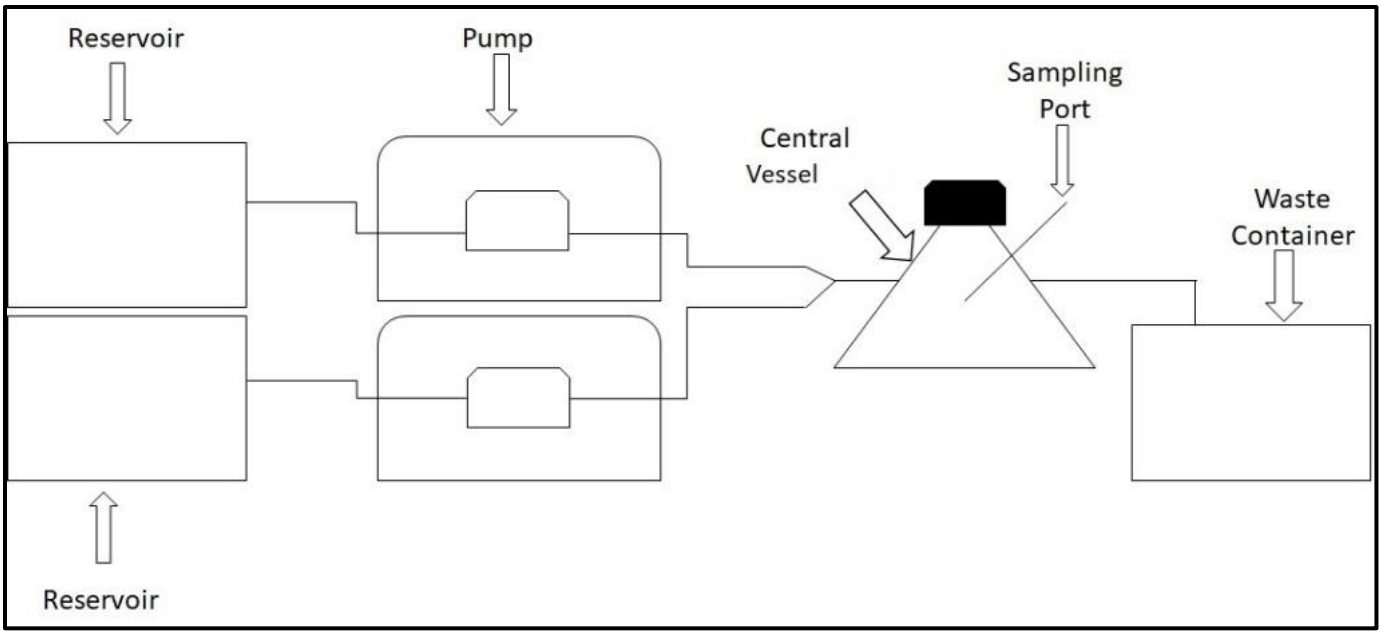


Figure 13. Experimental set up of IVPD model for combination therapy of drugs having two different half-live

The exit rate of the IVPD model was set according to the clearance rate of the short half-life drug, ampicillin (2.1mL/min). An IV bolus dose of 5 mg ceftriaxone was administered into the central vessel via the sampling port (targeting a Cmax of 20 µg/mL). A magnetic stirrer ensured uniform mixing of the drug in the central vessel. Pump1 supplied ceftriaxone from reservoir A (supplemental compartment) to the central vessel. A stock solution of 38.35 µg/mL stock solution of ceftriaxone was prepared as the highest infusion rate calculated was 38.35 µg/min. The rest of the infusion rate were then calculated as mentioned in equation 24. Pump 2 supplied PBS maintained at pH 7.4 from reservoir B. The flow rate at which ceftriaxone was supplemented to the central vessel was changed after every 4 hours.

For the 48 hour experiment, four IV bolus doses of ceftriaxone were administered at an interval of every 12 hours. The drug was infused into the central vessel from Reservoir A at a constant rate

after IV dose administration, and the rate was changed every 4 hours within the 12 hour dosing frequency, In total, there were four dosing periods (0, 12, 24, and 36 hours) consisting of a bolus dose with simultaneous infusion (where the rate was adjusted every 4 hours) based on the equations 21 through 23.

The adjusted rates of infusion over the 48-hour experiment are presented in Table 3. The flow rate of the pump supplementing ceftriaxone (pump 1) was adjusted based on the calculated rate of infusion. For example, reservoir A contained 38.35 µg/mL stock solution of ceftriaxone in phosphate buffer saline. If the flow rate of the pump is 1mL/min the rate of infusion achieved will be 38.35 µg/min. To achieve the rate of infusion for the first 4 hours i.e., 26.54 µg/min, the flow rate was calculated as follows:

38.35 µg/min = 1ml/min equation 24

26.54 µg/min = x mL/min

x = 0.69mLmin

The flow rate at which PBS was supplemented was calculated as the difference of 2.1mL/min-0.69mL/min = 1.41mL/min. As the flow rate for ceftriaxone changed every 4 hours so did the flow rate of PBS.

The right side of the central vessel is connected to a plastic tube through which the solution passes into the waste container. A ceftriaxone bolus dose was administered every 12 hours, consistent with a multiple dosing regimen. Samples were collected from the central vessel at the following time points: 0.5,0.6, 2, 3.5, 4.5, 6, 7.5, 8.5, 10. 11.5, 24.5, 26, 27.5, 28.5, 30, 31.5, 32.5, 34, and 35.5 hrs. The time points were selected to capture three data points for each rate adjusted interval: one sample was taken 30 minutes after the change of rate, a second sample was collected at the

midpoint of the 4 hours interval, and third sample was collected 30 minutes before the next change in flow rate. Ceftriaxone concentrations were measured by HPLC. All samples were stored at -80℃ prior to analysis.

Table 3 Summary of calculated change infusion rate, flow rate of pump 1 and 2 after every 4 hours

Time(mins)	Infusion rate	Flow rate for pump 1	Flow rate for pump 2
0-240	26.54	0.69	1.41
240-480	18.08	0.48	1.62
480-720	12.31	0.32	1.78
720-960	34.93	0.93	1.17
960-1200	23.79	0.63	1.47
1200-1440	16.20	0.43	1.67
1440-1680	37.35	1	1.1
1680-1920	25.44	0.68	1.42
1920-2160	17.32	0.46	1.64
2160-2400	38.35	1.00	1.10

2400-2640	26.12	0.68	1.42
2640-2880	17.79	0.46	1.64

Note:- The infusion rate calculated was for pump 1. The flow rate for pump 2 was calculated so the sum of the flow rate for pump 1 and pump 2 equals 2.1mL/min (2.1mL/min is the clearance of drug with shorter half-life)

HPLC Method for Ceftriaxone

The assay was conducted using an Agilent 1100 HPLC based on a published method [90,92]. Separation was performed using a YMC ODS AQ reversed phase column (250mm x 4.6mm) packed with 5µm diameter particles[90]. The volume of injection was 20µL. The mobile phase consisting of acetonitrile and phosphate buffer 0.05M, pH 3.8 with 0.1%TFA in ratio 20:80 was delivered at a flow rate of 1.2mL/min. Total run time was set to 6mins. Ceftriaxone was monitored at a wavelength of 260nm. Triplicate injections were evaluated for each sample. The column was equilibrated for one hour with mobile phase before the first injection.

Validation of Method

Method validation was established based on the following criterial linearity of calibration standard curve, precision, and system suitability. A stock solution for ceftriaxone was prepared in buffer at a final concentration 200 µg/mL. Calibration standards were prepared by diluting the stock solution to final concentrations of 5, 10, 20, 25, 50, and 100 µg/mL. A calibration curve was constructed by plotting mean peak area (based on triplicate injections) vs. concentration from which R^2 value and regression equation were computed.

Precision was measured by injecting lowest concentration (5µg/mL) and highest concentration (100 µg/mL) in triplicate on the same day. System suitability for the method was performed by repeated injection of a target concentration (25 µg/mL) (number of injections=6). Acceptance criteria for linearity of standard curve were based on goodness of fit value and for system suitability, and precision %relative standard deviation (%RSD) value was set to $\leq$ 2% [90].

3.3 Results & Discussion

Method Validation

For ceftriaxone, a linear relationship between peak area and concentration was observed. The R^2 value for the calibration curve was calculated to be 0.995.

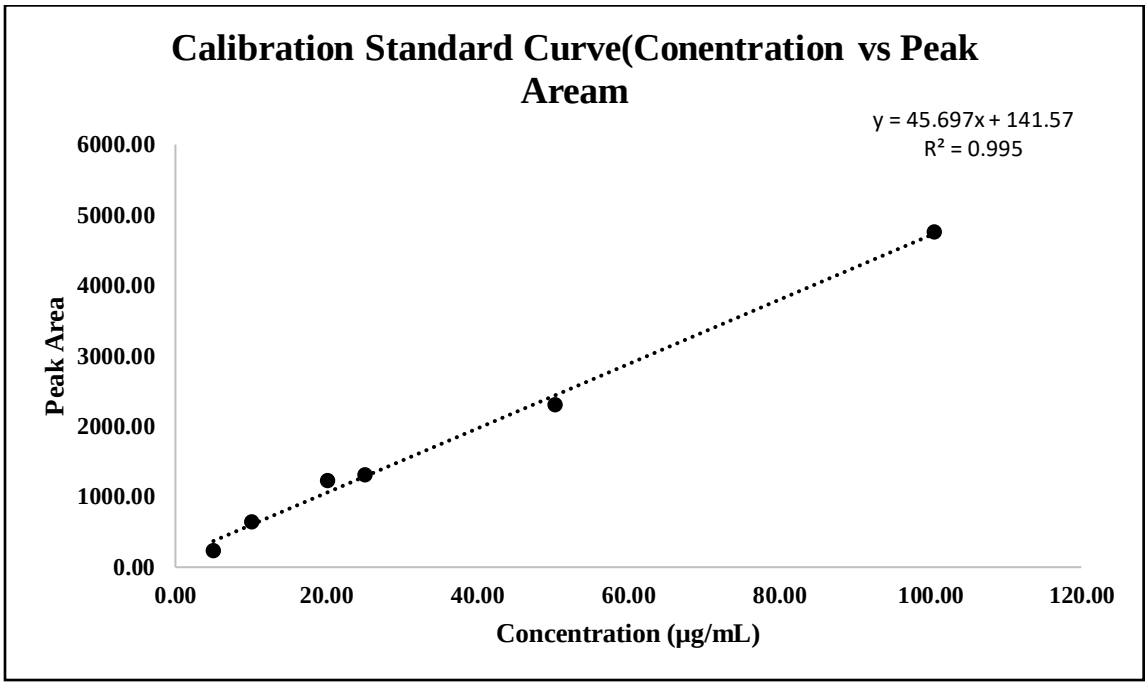


Figure 14. Linearity Curve for Ceftriaxone

Precision

Precision is the measure of degree of agreement between individual values when the method is run repeatedly [91] . Intraday precision was measured for 5µg/mL and 100µg/mL (Table 5). The %RSD value for 5µg/mL and 100µg/mL was 1.84 and 0.26 respectively.

System Suitability

System suitability was performed to check if the system can reproduce consistent result under the same condition [91]. The (%RSD) value for system suitability was 0.081% (Table 6).

Table 4 Precision data for ceftriaxone

Parameter	Concentration = 5µg/mL	Concentration = 100µg/mL
Area	241.30	4773.80
	237.30	4763.00
	232.60	4749.10
Mean	237.07	4761.97
Std deviation	4.35	12.38
%RSD	1.84	0.26

Table 5 System suitability for ceftriaxone

Parameter	Injection	Area
	1	1314.60
	2	1312.10
	3	1312.00
	4	1312.00
	5	1312.00
	6	1311.80
Mean		1312.40
SD		1.07
%RSD		0.08

In vitro pharmacodynamic model (LIU method)

The concentration of ceftriaxone in central vessel over time using the proposed model captured the expected in vivo plasma concentration (Figure 15). In the figure, the black dots are the ceftriaxone concentration measured from the LIU model, the dashed line represents the computer simulated value for the LIU IVPD model, and the solid black line reflects the expected in vivo profile. Ceftriaxone measured concentrations were lower than expected for several time points. This was due to an observed increase in volume in the central vessel (initial volume 250mL). The volume in the central vessel was readjusted back to 250mL by pushing air into the sample port via an empty syringe.

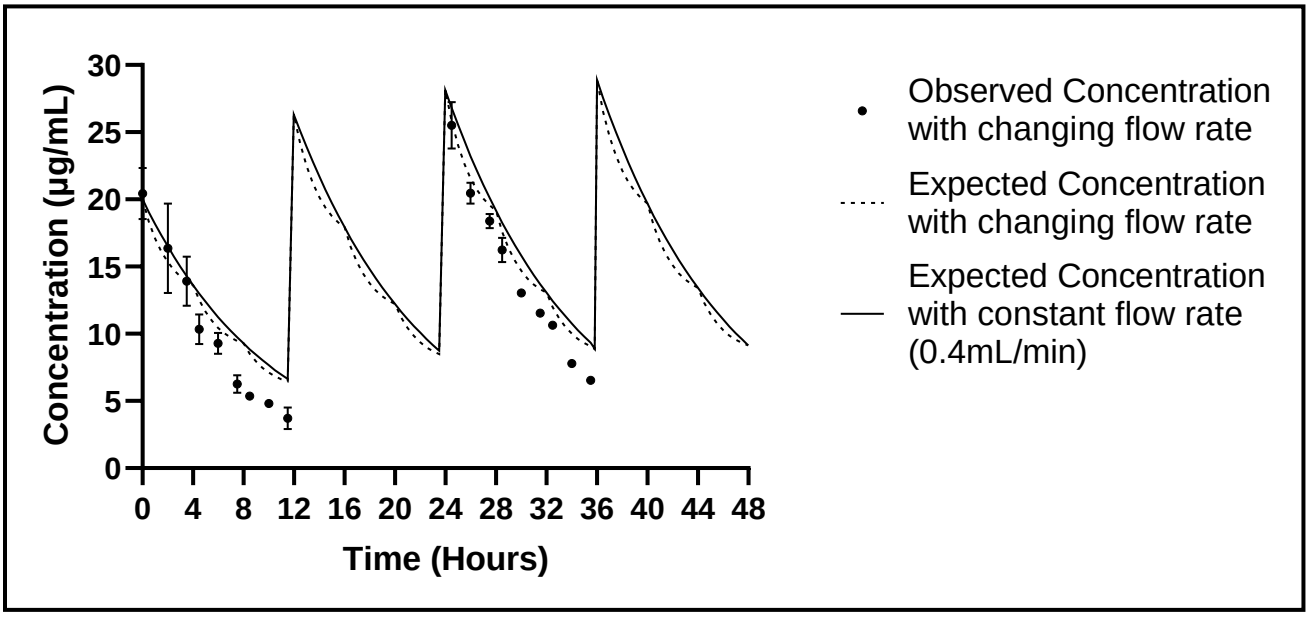


Figure 15. Concentration time profile of ceftriaxone after change in infusion rate every 4 hours (48 hours study, observed time points depict standard error bars)

The presented LIU model can mimic the target in vivo plasma concentration of a drug with longer half-life when given in combination therapy with a drug having shorter half-life in an IVPD model, which overcomes a limitation of the Blaser method. However, lower than expected concentrations in the central vessel were observed during experimentation and was attribute due to the increase in volume in the central vessel over the period of experiment. This experimental error results from the loss of vacuum. Loss of vacuum can be caused if the rubber stopper covering the sampling port is not sealed properly. It is difficult to spot this issue visually during the course of the experiment.

To overcome the increase in volume, one can inject empty air into the central vessel via an empty syringe while clamping the inlet tube and bring the volume in the central vessel to the initial volume, i.e. 250mL. As the difference between the observed and expected values were marginal (Appendix 3), the results demonstrate that the LIU IVPD model can capture the target profile for a drug with a longer half-life when testing combination therapy of two drugs with different half live.

A literature search found a 2007 publication that suggested an alternative to Blaser method for combination of drugs having two different half live[48]. This model employed a one compartment IVPD model, and the flow rate of the pump was adjusted to the clearance of drug having shorter half-life. The drug with longer half-life was supplemented to the central compartment via a syringe. The syringe was connected to a computer controlled dosing pump which delivered the drug at an exponentially decreasing rate [48]. Although this method was able to attain the desired concentration for the longer half-life drug, it requires a special software to control the dosing pump, which may not be accessible to all research laboratories. The LIU method developed and verified in this dissertation does not require this software and can be easily reproduced. Although the IVPD model can be time consuming and labor intensive, and the bacterial growth in vitro can be different

than in vivo, the IVPD model is an important pre-clinical tool. This model can evaluate antimicrobial PK/PD indices, monitor bacterial resistance, and optimize drug dosing regimens. However, in-silico models can bridge the gap between clinical research and *in vitro* studies. PBPK-PD models can be utilized to study the pharmacodynamic response of antibiotics which can help to design clinical trials. The next part of this dissertation explores development of a PBPK-PD model for the combination therapy of ampicillin and ceftriaxone against *Enterococcus faecalis*.

CHATPER 4

Physiologically Based Pharmacokinetic-Pharmacodynamic (PBPK-PD) Model of Ampicillin and Ceftriaxone Combination Therapy Against Enterococcus Faecalis In Sim – Healthy Volunteer

4.1 Introduction

The use of combination therapy of antibiotics has been rising over the past decade. Combination therapy provides broad-spectrum activity against the bacteria, helps reduce the dose of a drug, reduces the recurrence of the resistant bacterial population, and reduces the bacterial load [77,60]. Several factors are to be considered for optimizing dose for combination therapy. Factors such as the exact amount of dose and the dosing intervals at which the drugs are administered and altered pharmacokinetics of drugs in disease state that can affect the drug's efficacy over time [77]. Physiologically based pharmacokinetic and pharmacodynamic (PBPK-PD) modeling based on in vitro monotherapy and combination therapy data can be an essential asset for optimizing dose for combination therapy [77]. PBPK modeling is a mechanistic tool that defines the distribution of drugs based on blood flow rate, organ size, and tissue volume[78,79]. Applications of the PBPK model include but are not limited to, are to understand the pharmacokinetics of the drug in the special population (pediatrics, renal population, etc.) and the extrinsic (drug-drug interaction), intrinsic factors (age, genetics, organ dysfunction) that affect the pharmacokinetics of drug [80,81]. Therefore, when integrating the PBPK model with the PD model, various dosing regimens in different populations can be simulated without the need for running labor-intensive and time-consuming in vitro experiments such as time-kill experiments repetitively [79].

The first step towards building a PBPK-PD is to develop and validate the substrate profile of the drug in a healthy volunteer and special population with the published literature [79]. The second step is to develop a PD model by extracting data from in vitro time-kill curve experiments. Time-kill curve experiments assess the change in a colony-forming unit (CFU) of bacteria over time against the different concentrations of the drug. Other information required for simulation is the dosing regimen of the drug, plasma concentration of drug at the site of action, route of

administration, and sampling time points [56]. The next step is to understand which mathematical equations fit the data best to simulate the growth model of bacteria in the absence of a drug, the effect of monotherapy and combination therapy against the bacteria.

For this study, the PBPK-PD model was developed for combination therapy of drugs acting synergistically (ampicillin and ceftriaxone) using Simcyp® simulator (version 18, Certera Sheffield, UK). However, Simcyp version 18 did not have the innate capability to simulate pharmacodynamic response for combination therapy of drugs acting synergistically. Therefore, a custom PD model was developed using a lua script to overcome this drawback. The developed PBPK-PD model was verified by observing how well the simulated results can capture the in vitro data published in the literature[62,84]. Once the PBPK-PD model was verified in healthy volunteers, the next step was to use the model to optimize the dose frequency of ampicillin in the special population (severe renal impaired patients).

When administered together, ampicillin and ceftriaxone act synergistically against *E. faecalis*. *E. faecalis* can cause diseases such as urinary tract infection, meningitis, bacteremia, and infective endocarditis. Bactericidal therapy is required to achieve a clinical cure in infective endocarditis caused by enterococci. Enterococcal bacterial growth depends upon penicillin-binding protein (PBP) enzymes which link precursor pentapeptide molecules to the peptidoglycan cell wall. Ampicillin is a structural analog of precursor pentapeptide molecules and binds to PBP-4, which leads to the inhibition of cell wall growth due to the production of reactive oxygen species. However, β-lactam tolerance was observed due to an increase in low-affinity PBPs (PBP-4 in *E. faecalis*), which weakly binds to β-lactam, and due to abolition of reactive oxygen species enzyme superoxide dismutase [2]. The low affinity of PBP-4 stems from mutation in pbp-4 gene A617T that facilitates the binding of β-lactam binding[70]. Thus, ampicillin monotherapy has been seen

to achieve a poor bactericidal effect, whereas combination therapy of ampicillin and ceftriaxone can achieve bactericidal activity. Ampicillin binds to PBP-4, whereas ceftriaxone binds to PBP-2 and PBP-3, leading to the inability of pentapeptide to link with the peptidoglycan cell wall, which causes inhibition of cell wall growth, leading to bacterial death [4].

A dosing regimen of 2000 mg IV q 4 hours of ampicillin and 2000 mg IV q12 hours of ceftriaxone in healthy volunteers was simulated using the PBPK-PD model and verified against the published in vitro pharmacodynamic study [62,84].

4.2 Methodology

Developing PBPK models for ceftriaxone and ampicillin in healthy volunteers

A minimal PBPK model was developed for ceftriaxone and ampicillin using Simcyp version 18. The parameter values are summarized in Table 6 (ceftriaxone) and Table 7 (ampicillin). The plasma concentration time profile for each drug was extracted from published studies [20,24] by digitization from a published figure using Web Plot Digitizer (version 4.2, https://apps.automeris.io/wpd/).For ceftriaxone, blood: plasma ratio and steady state volume of distribution was calculated using the parameter estimation tool in Simcyp. Published data suggests that concentration of ceftriaxone is dependent on protein binding [20]. However, it was found that the area under the free drug concentration time curves increased proportionally with increase in dose ranging from 0.5g-2g [20]. This was also substantiated by another study where proportional increase in free plasma concentration time curve was observed, i.e., 10.1 to 106 μg.h/ml over a 0.15- to 1.5-g dose range [21]. Therefore, the study concluded that the impact of non-linear pharmacokinetics of ceftriaxone was anticipated to be insignificant [20]. The other parameter values were taken from the website Drug Bank and published literature. [20, 21, 24].

Table 6 Input parameter values of ceftriaxone for Simcyp Substrate profile [20,21,24]

Physiochemical Properties	**Values**	
Molecular weight	661.66	
Log P	2.12	
Pka	3.36	
B/P	0.82	
fu	Concentration(mg/L)	fu
	25	0.047
	100	0.073
	200	0.115
	300	0.154
	400	0.213
	600	0.279
	800	0.358
Distribution		
Model	Minimal PBPK	
Vss (L/Kg)	0.116	
Kp scalar	1	
Clearance		
Renal clearance (L/h)	0.533	
Biliary Clearance (L/h)	0.657	

Table 7 Input parameter values of ampicillin for Simcyp Substrate profile [24]

Physiochemical Properties	**Values**
Molecular weight	349.05
Log P	0.88
Pka	3.24
B/P	0.63
Fu	0.104
Distribution	
Model	Minimal PBPK
Vss(L/Kg)	0.30
Kp scalar	1
Clearance	
Renal clearance (L/h)	16.92

The virtual clinical trial design for ceftriaxone and ampicillin is summarized in Table 8. For ampicillin, a dose of 1000mg (30-minute infusion) was evaluated. For ceftriaxone, a dose of 2000mg(30-minute infusion) was evaluated. The virtual clinical trial was set to mimic the published clinical data [20,24].

The PBPK model was verified by visual predictive check, which compared the simulated plasma concentration vs time profile with published literature[20,24]. The clinically observed Cmax and AUC values were then compared with the model-predicted values as a further verification measure. Fold error was calculated as the ratio of model predicted value and observed value of

these parameters. Fold errors between 0.5 and 2.0 represent the generally accepted range for model verification.

Table 8 Input values for Trial design of ceftriaxone and ampicillin [20][24]

Parameters	Ceftriaxone	Ampicillin
Single Dose	2000 mg	1000mg
Route of administration	IV infusion	IV infusion
Infusion time	0.5 hours	0.5 hours
Age	20-50	20-50
Male: Female	0.5	0.5
No. of subjects in each trial	12	10
Duration of study	24 hours	24 hours
No. of trials	10	10

Developing PBPK-PD models for ceftriaxone and ampicillin in healthy volunteers

The PD model was developed based on a published in vitro pharmacodynamic study (IVPD) performed for combination therapy of ampicillin and ceftriaxone against *E.faecalis* [62,84]. The study performed a 72 -hour IVPD experiment against three strains of *E.faecalis* namely W04, W07 and, W151. The results reported the minimum inhibitory concentration (MIC) value for all three strains of bacteria and the bacterial count vs. time profile graphs. The graphs represented bacterial count in absence of drug, the effect of monotherapy, and the effect of combination therapy [84].

The first step towards developing the PD model was to extract the bacterial count vs time profile values from the graphs in the published research article[84]. This was done by using Web plot Digitizer version 4.2 (https://apps.automeris.io/wpd/). The next step was to develop the PD model for the bacterial count in absence of a drug, i.e., the growth model. For the growth model the following equation was used [58]:

$$\frac{dS}{dt} = k_{growth} * \left(1 - \frac{S}{S_{max}}\right) * S \qquad \text{equation 25}$$

Smax is the maximum bacterial count in the absence of a drug, and kgrowth is the bacterial growth rate [58]. Smax is the parameter that explains the controlled growth of bacteria in an in vitro experiment. Maximum bacterial count is achieved due to the limitation of nutrients and stress [58,85,86]. As the information present in the published data was limited to the bacterial count vs time graph, the value for the growth model parameter (kgrowth) was acquired using the software Berkeley Madonna. The bacterial count vs time data in the absence of drug obtained from the published data was imported to the software. A PD script was written defining equation 25 in the equation window of the software:

```
STARTTIME = 0
STOPTIME= 72
DT=0.02
; Parameter definitions
kgrowth = 0.1
Bmax = 9.98
d/dt(S)= kgrowth*(1-S/Bmax)*S
Init S = 8.18
```

Start and stop time in the PD script defines the duration of study of the in vitro experiment. DT is the differential time points; this means the software would simulate results after every 0.02 mins. The values for Bmax (maximum bacterial count) and Init S (initial bacterial count) were obtained

from the published bacterial count vs time graph. To obtain the value for kgrowth, first, an arbitrary number was assigned to the parameter. The software was then run to simulate the graph. The graph window in the software showed the imported graph of the bacterial count vs time profile from the literature and the graph simulated by the software. Then the parameter slider was used to optimize the value for kgrowth. Figure 16 shows a screenshot of the software explaining the above process.

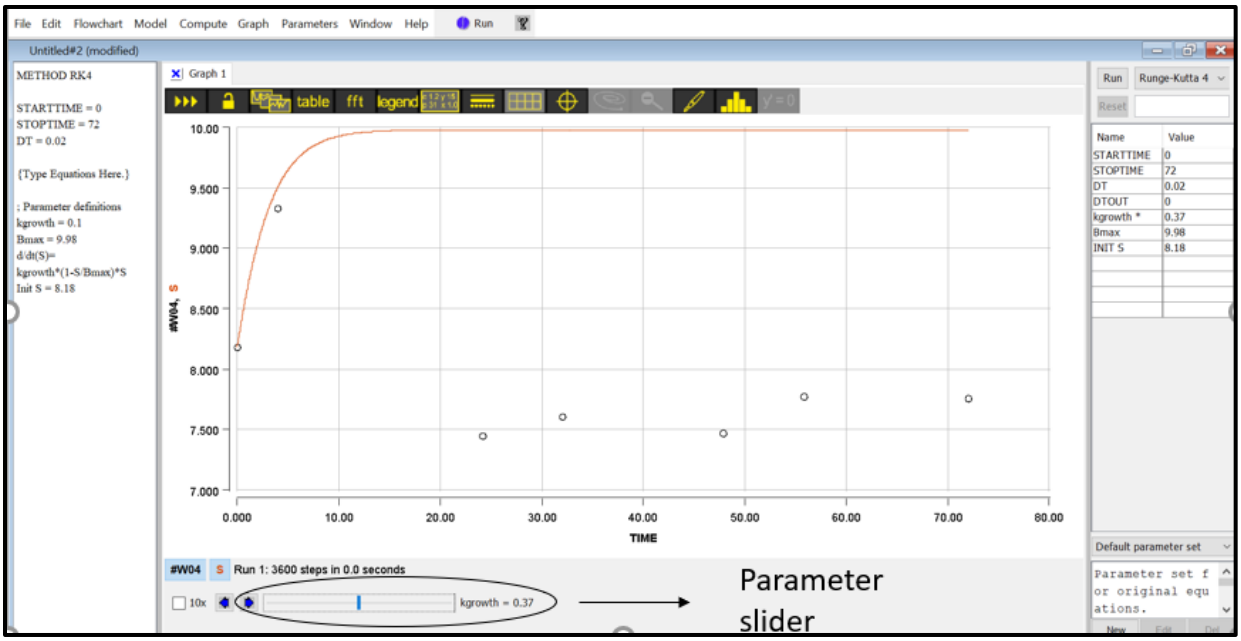


Figure 16. Screenshot of Berkeley Maddona software showing optimizing parameter for kgrowth for growth model(Black dots represent the published data and the orange line is the data points simulated by the software)

It was observed that bacterial count in the published data in absence of drug increased and then decreased [84]. To investigate this further, a growth model reported in another publication was simulated [62]. This in vitro study was performed by Nele Wellinghausen et al [62]. For the in vitro study an initial inoculum of 10^7 CFU/mL was inoculated in the flask containing Todd-Hewitt broth with 1% yeast extract. For generation of aerobic growth conditions, culture flasks were incubated with shaking (200 rpm) at 36°C, the flask was then incubated for 48 hours. The aliquots

were taken at different time points throughout the incubation period. To determine the CFU bacteria were plated on CASO sheep blood agar in duplicate after serial dilution of the samples [62].

Once the growth model was developed, a PD model to simulate the effect of monotherapy was built. A modified Emax equation published in the literature[58], was used to calculate the change in bacterial count over time in presence of monotherapy of ampicillin and ceftriaxone:

$$\frac{dS}{dt} = kgrowth * \left(1 - \frac{S}{Bmax}\right) * S - \left(\frac{Emax0 * cps^h}{EC50^h + cps^h}\right) * S \qquad \text{equation 26}$$

where kgrowth is the growth rate of bacteria in absence of drug, S is the bacterial count, Bmax is the maximum bacterial count in absence of drug, Emax0 maximum killing rate, cps is the concentration of antibiotic, EC50 is the concentration of the antibiotic where 50% of the Emax0 is obtained and h is the hills coefficient. Berkeley Madonna was used to obtain the value of EC50, Emax, kgrowth, and h for each strain of bacteria in a similar fashion as the parameter value was obtained for the growth model. The Berkeley Madonna script for estimation of the above-mentioned parameter is mentioned in Appendix 1. All the values for the parameters are summarized in Table 9.

Table 9 Parameter values optimized for W04, W07, and W151 of Enterococcus faecalis

Strain	Parameter	Ampicillin	Ceftriaxone	Without drug
W04	**Init S (log cfu/mL)**	8.18	8.18	8.18
	Bmax (log cfu/mL)	9.98	9.98	9.98
	Kgrowth (1/hour)	0.37	0.37	0.37
	Emax0 (1/hour)	0.48	0.16	-
	EC50 (µg/mL)	48	48.49	-
	h	0.5	1.2	-
Strain	**Parameter**	**Ampicillin**	**Ceftriaxone**	**Without drug**
W07	**Init S (log cfu/mL)**	8.19	8.19	8.19
	Bmax(log cfu/mL)	9.98	9.98	9.98
	Kgrowth (1/hour)	0.37	0.37	0.37
	Emax0 (1/hour)	0.42	9.00E-02	-
	EC50 (µg/mL)	39.4	45	-
	h	4.06	1.64	-
W151	**Init S (log cfu/mL)**	8.17	8.17	8.17
	Bmax (log cfu/mL)	9.98	9.98	9.98
	Kgrowth (1/hour)	0.37	0.37	0.37
	Emax0 (1/hour)	0.34	0.1	-
	EC50 (µg/mL)	37	54	-
	h	1.4	1.2	-
Data from Nele	**Init S (log cfu/mL)**	-	-	7.15

Wellinghausen et al study[62]				
	Bmax (log cfu/mL)	-	-	8.10
	Kgrowth (1/hour)	-	-	1.1

After the estimation of kgrowth, EC50, Emax, and h parameter a custom PBPK-PD model was built in Simcyp® version 18 with the help of lua script.

Developing PBPK-PD model in Simcyp using lua script

The parameter values obtained from Berkeley Madonna were then used as input values to develop the PBPK-PD model in Simcyp. PBPK-PD model was built to define the growth model, the effect of monotherapy of ampicillin and ceftriaxone against *E.faecalis*, and the combined synergistic effect of the drugs against the bacteria. The dosing regimen for monotherapy and combination therapy were kept the same as in the in vitro study published in the literature[84].

For the growth model following lua script was used:

```
function popSimSetup(...)
   sc:setNUserOdes(1)
end
function odeRateStep(t, xin, su, gu, P, ...)
Su[1] = 8.18
kgrowth = 0.37
Bmax = 9.99
 gu[1] = ((kgrowth*(1-S/Bmax)*S)
 return gu[1]
end
```

The command function popSimSetup(...) defines that the simulation will be considered for the population. sc:setNUserOdes(1) indicates that the script has one differential equation. A similar PD script was written for monotherapy equation(Appendix 2). For combination therapy a mixed

framework equation for drugs acting synergistically was utilized [60]. In the equation below Drug indicates ampicillin and DrugB indicates ceftriaxone:

$$\text{Drug} = \left(\frac{\text{Emax0} * \text{cps}^{\text{h}}}{\text{EC50}^{\text{h}} + \text{cps}^{\text{h}}}\right) \quad \text{equation 27}$$

$$\text{DrugB} = \left(\frac{\text{Emax01} * \text{cps}^{\text{h}}}{\text{EC502}^{\text{h}} + \text{cps}^{\text{h}}}\right) \quad \text{equation 28}$$

$$\frac{\text{dS}}{\text{dt}} = \left(\text{kgrowth} * \left(1 - \frac{\text{S}}{\text{Bmax}}\right) * \text{S} - (\text{Drug}) * \text{S}\right) - \left(\text{kgrowth} * \left(1 - \frac{\text{S}}{\text{Bmax}}\right) * \text{S} - (\text{DrugB}) * \text{S}\right)$$

equation 29

Simcyp cannot simulate the PD effect for drugs acting synergistically. This is because the current in-built model cannot consider the concentration of two drugs unless there is a drug- drug interaction taking place. To overcome this challenge the following custom PD model was developed. Ampicillin was run as a substrate and ceftriaxone were designated as the inhibitor. lua script for substrate was coded to attain the blood: plasma ratio for ampicillin.

```
Substrate (ampicillin)
function individualCompoundSetup(...)
        BP = sc:getCompoundIndivBP()
        sc:setIndivXtra(1, BP)
end
function directAlgebraicStep(xin, P, ...)
        xout = 1 * BP
        return 0
end
```

Next was to code for inhibitor(ceftriaxone) :

```
Inhibitor(ceftriaxone)
function odeInitStep(xin, su, P, ...)
   su[1] = 8.18
   return 0
end

function odeRateStep(t, xin, su, gu, P, ...)
   S = su[1]-- sensitive/susceptible bacteria
   BP = sc:getIndivXtra(1)
   sc:setParameter(7, BP)
   Cps = sc:getState(2)/BP
   Cpi = xin

   kgrowth = 0.37
   Bmax = 9.98
   Emax0 = 0.48
   EC50 = 48
   h = 0.5
   Emax01 = 0.16
   EC502 = 48.49
   ha = 1.2
   kg = 0.37
   DRUG = (Emax0*(Cps)^h)/((EC50)^h+(Cps)^h)
  DRUGB =(Emax01*(Cpi)^ha)/((EC502)^ha+(Cpi)^ha)
 gu[1] = ((kgrowth*(1-S/Bmax)*S)-(DRUG)*S)-((kgrowth*(1-S/Bmax)*S)-(DRUGB)*S)
 return gu[1]
end
```

The function getState(2) retrieves the total blood concentration of the ampicillin which then is divided by the individualized blood to plasma ratio to convert to plasma concentration. This function is the key to run the combination therapy as it helps to attain the concentration of the substrate.

The PBPK-PD model developed in Simcyp was verified in a healthy volunteer population for bacterial growth in absence of drugs, monotherapy, and combination therapy. Verification of the

model was performed by visual predictive check, the simulated bacterial count over time was overlaid with the change in bacterial count over time published in the literature [84].

4.3 Results & Discussion

Developing substrate profile of ceftriaxone and ampicillin in healthy volunteer

To evaluate the predictive performance of the PBPK models developed for ceftriaxone and ampicillin, model simulated plasma-concentration time profiles for each medication were compared to published data [20,24]. This comparison is provided in Figure 17 and Figure 18 for ceftriaxone and ampicillin. The black dots in the graph represent the clinically observed concentrations (mean values from the study) and the model-simulated mean concentration is depicted by the solid black line. The double black and dashed lines represent the 95^{th} percentile and the 5^{th} percentile values predicted by the PBPK models.

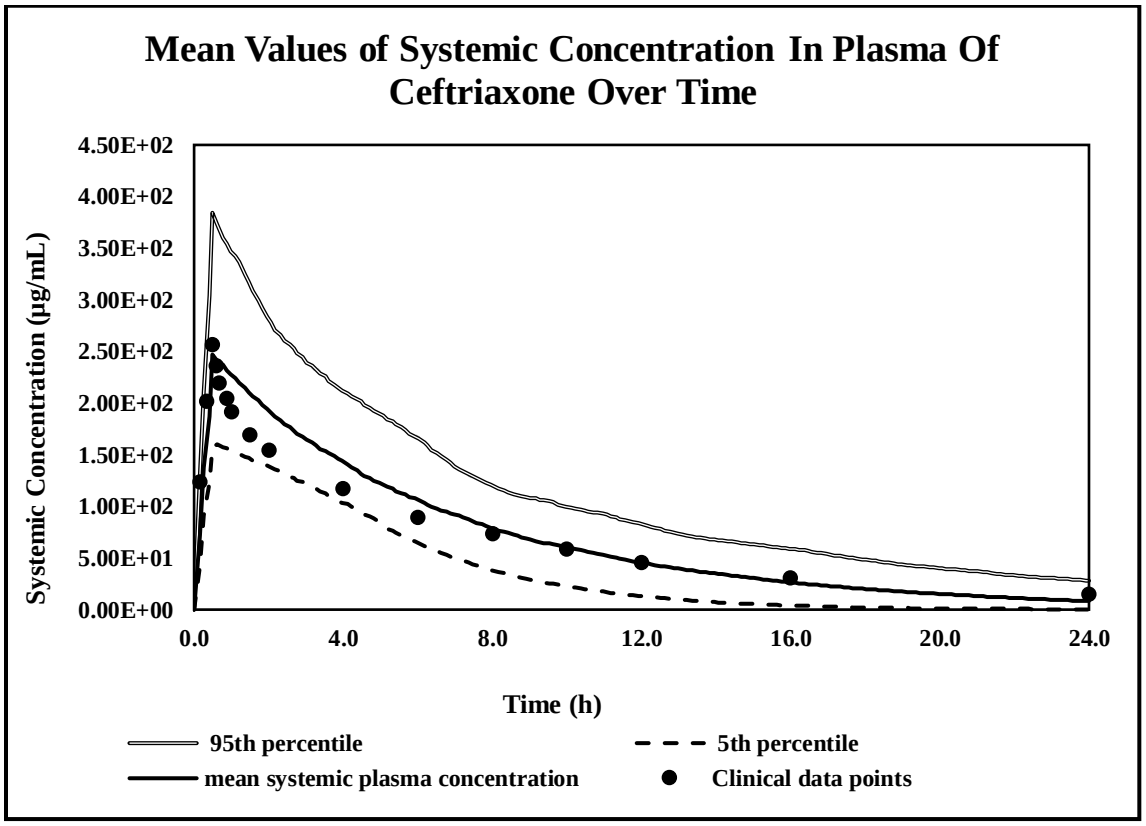


Figure 17. Verification of Ceftriaxone PBPK model in healthy volunteers' population

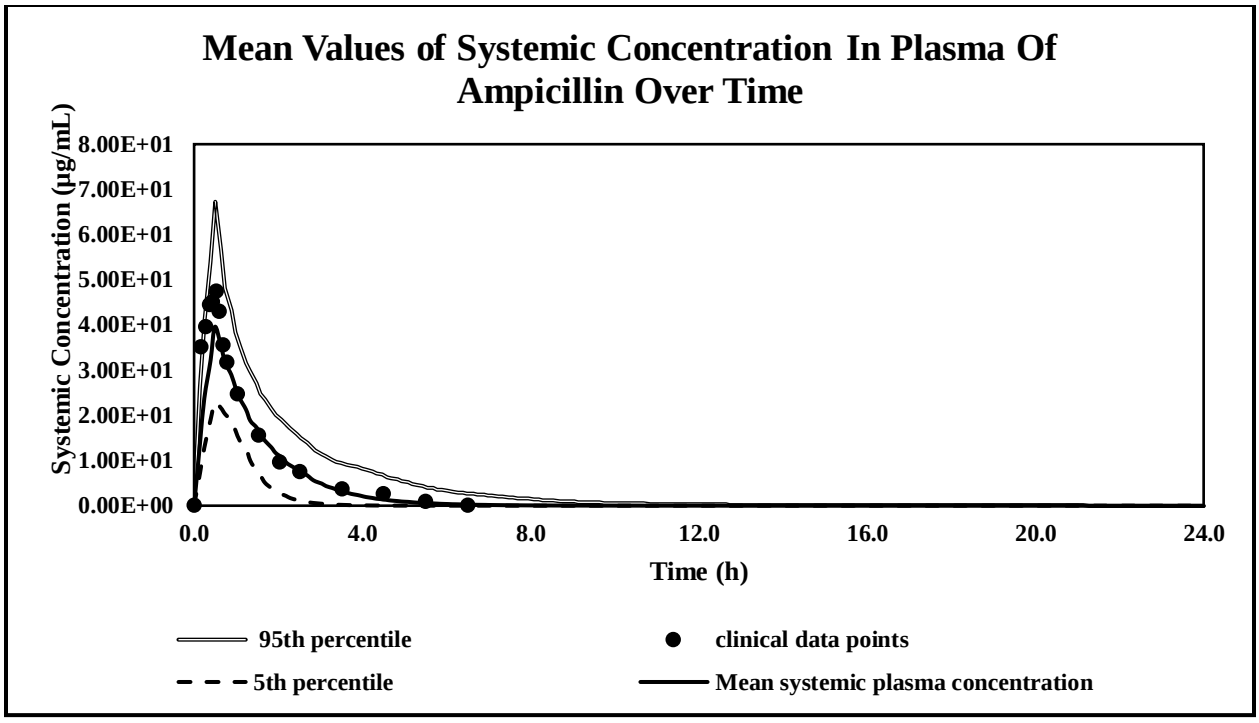


Figure 18. Verification of Ampicillin PBPK model in healthy volunteers' population

The pharmacokinetic parameters (Cmax, AUC) for ceftriaxone and ampicillin are presented in Table 10 and Table 11. The tables compare the model-predicted and observed mean parameter values the fold errors ranged from 0.85 to 1, indicating that the model predicts pharmacokinetics parameters successfully. This indicates that the model can be applied to different clinical settings.

Table 10 Comparison of predicted vs observed mean Cmax ad AUC estimated for ceftriaxone

Population	**Parameter**	**Predicted**	**Observed**	**Fold Error**
Healthy	Cmax(µg/ml)	249.2	256.9	0.96
	AUC(µg/ml*h)	1620	1703	0.95

Table 11 Comparison of predicted vs observed mean Cmax ad AUC estimates value for ampicillin

Population	Parameter	Predicted	Observed	Fold Error
Healthy	Cmax(μg/ml)	41.9	49	0.85
	AUC(μg/ml*h)	63.7	63.5	1

Growth Model

The PBPK-PD model adequately captured the bacterial count as observed in the published in vitro pharmacodynamic experiment [62, 84]. However, as seen in Figure 19 the growth model could not mimic the observed decline in the bacterial count and subsequent regrowth phase as observed for three different strains of bacteria W04,W07 and W151 [84].Therefore, another published study where an in vitro study was performed for the growth of bacteria in absence of drug by Nele Wellinghausen et al [62] was referred to investigate this further. The growth model simulated using Simcyp could capture the bacterial count in absence of drug as observed in the study performed by Nele Wellinghausen et al [62] (Figure 19 In vitro study performed by Nele Wellinghausen et al)

The growth model couldn't capture the bacterial count in absence of drug for three strains of bacteria namely W04, W07, and W151 could be because of the higher inoculum used at the beginning of the in vitro pharmacodynamic experiment [84] . This assumption can be made on the fact as the initial inoculum used for the in vitro pharmacodynamic experiment was ~10^9 CFU/mL [82] and the initial inoculum for the study performed by Nele Wellinghausen et al [62] was ~10^7 CFU/mL. Further investigation is required to ascertain this claim.

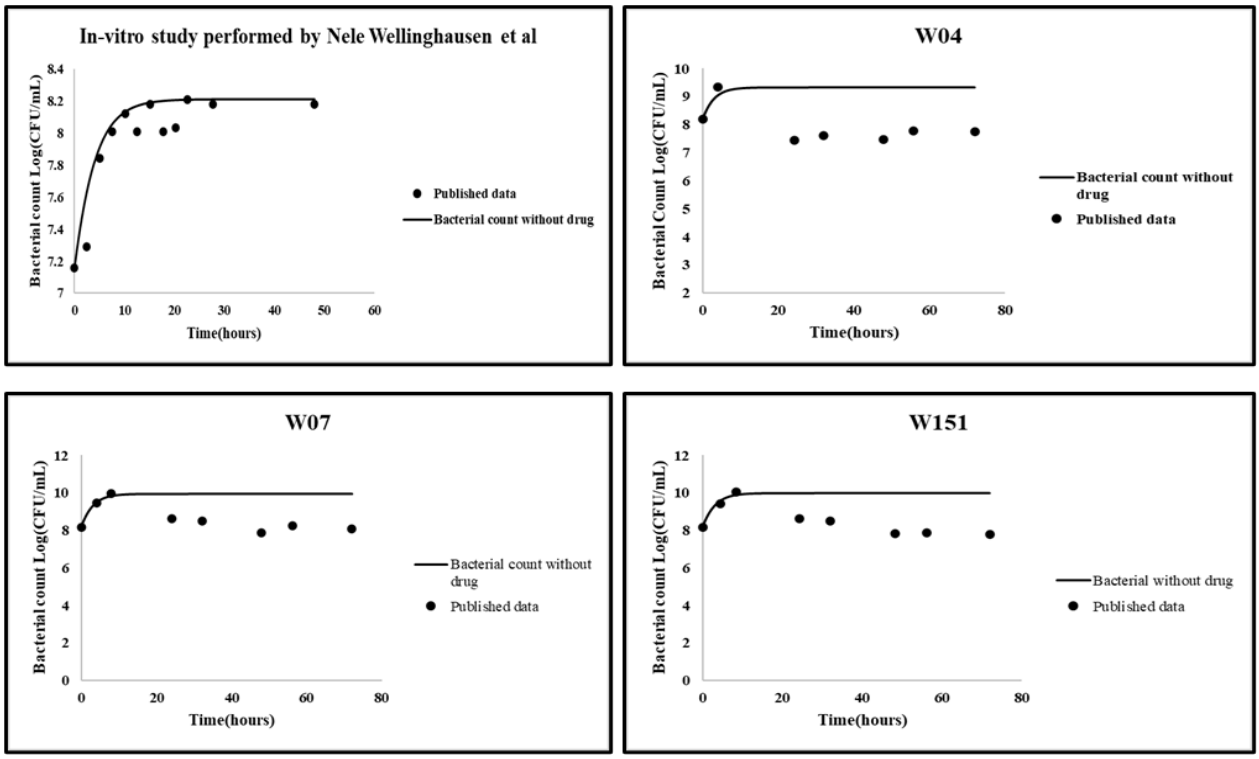


Figure 19. Describing the growth model for all the three variants of Enterococcus Faecalis and in vitro study performed by Wellinghausen et al (black dots are bacterial count in published literature and black solid line is the simulated bacterial count)

Bacterial Count after Monotherapy of Ampicillin and Ceftriaxone

The objective of this simulation was to verify if the PBPK-PD model can mimic the bacterial count over time in silico as observed in vitro after monotherapy of ampicillin or ceftriaxone against *Enterococcus faecalis*. Based on visual prediction the simulated data could capture in vitro data for both the drugs as seen in Figure 20 [53]. Monotherapy of ampicillin does show bactericidal effect against all the three variants of *Enterococcus faecalis.* This result contradicts the finding of the published data. In the published data ampicillin is bactericidal against W04 and W07 strain but have bacteriostatic effect against W151 strain. This can be attributed to the differences which can take place between bacterial growth in-vivo vs in vitro as discussed above. Regrowth of the

bacteria is observed when monotherapy of ampicillin is administered. This is because *Enterococcus faecalis* becomes resistant to ampicillin monotherapy over time by developing PBP 2 and PBP 3. Ceftriaxone monotherapy does not have any effect on the bacteria (Figure 20). Ceftriaxone acts on PBP 2 and PBP 3 which is only developed by the bacteria once it gets resistant to ampicillin. For this reason, combination therapy of ampicillin and ceftriaxone is recommended for complete eradication of *Enterococcus faecalis.*

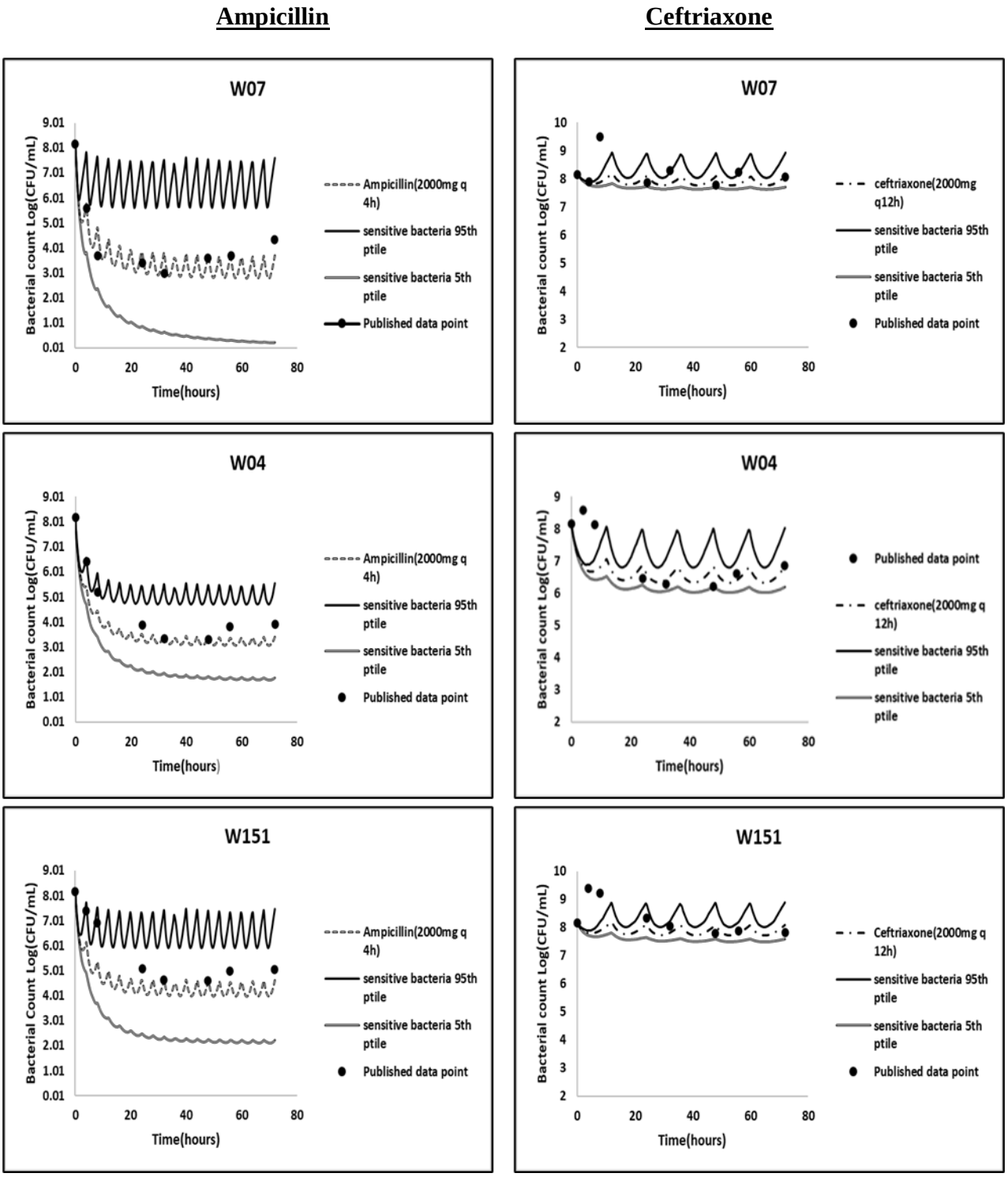


Figure 20. Graph of bacterial count over time for ampicillin and ceftriaxone monotherapy in healthy volunteer

Bacterial Count after combination therapy in healthy volunteers

When a combination of ampicillin 2000mg IV q4 hours and ceftriaxone 2000mg IV q12 hours was administered in healthy volunteers, the bacterial count over the period of 72 hours in W04, W07 and W151 was reduced to 0.029 log CFU/mL, 0.029 log CFU/mL and 0.009 log CFU/mL, respectively. This indicates synergistic effect of ampicillin and ceftriaxone was achieved against *E. faecalis.* Figure 21 depicts graphs showing bacterial count vs time for combination therapy against the different resistant strains. The PBPK-PD model assumed that the simulated bacterial count would be lower in comparison to the observed bacterial count achieved in vitro. This assumption was made as the bacterial count in vitro is counted by visual inspection and is difficult to record the bacterial count below 2 log (CFU/mL). The PBPK-PD model developed in healthy volunteers was verified with the published data [62,84]. The PBPK-PD model will further be extended in severe renal impaired population to justify the change in frequency of ampicillin when given in combination with ceftriaxone.

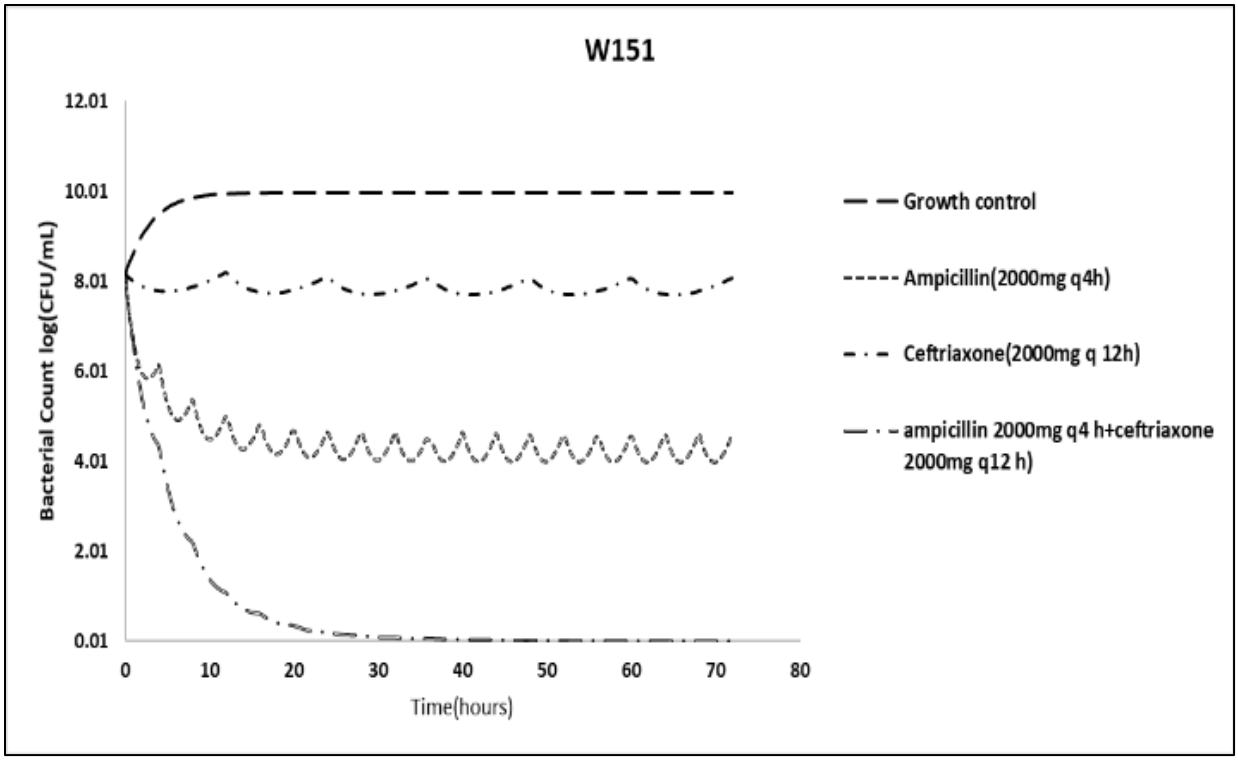


Figure 21 a Change in bacterial count over a period of 72 hours in healthy volunteers for growth model, monotherapy, and combination therapy for W151 variant

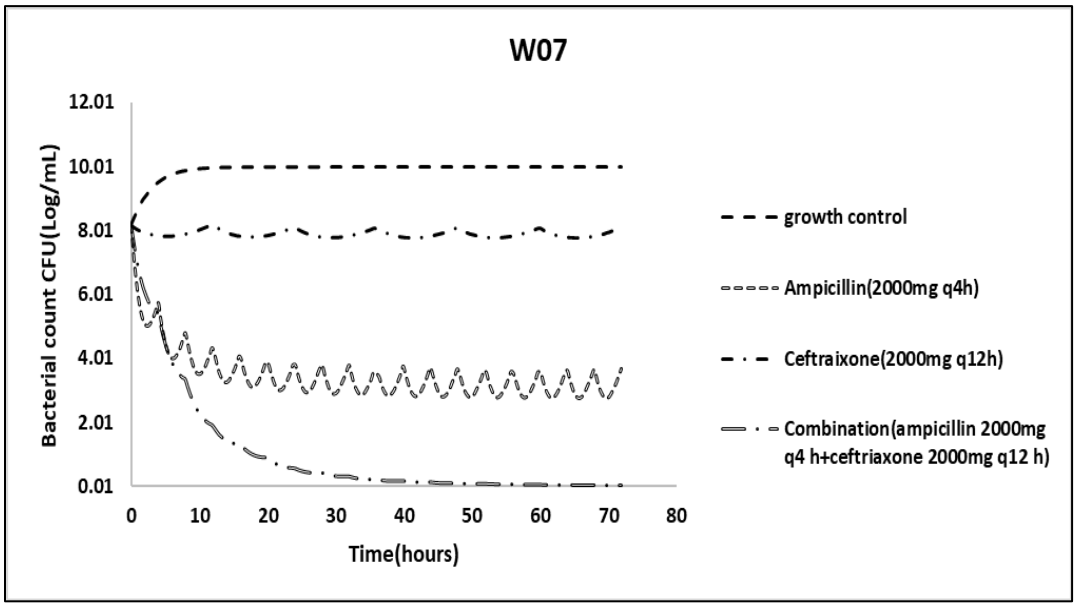


Figure 21 b Change in bacterial count of 72 hours in healthy volunteers for growth model, monotherapy, and combination therapy for W07 variant

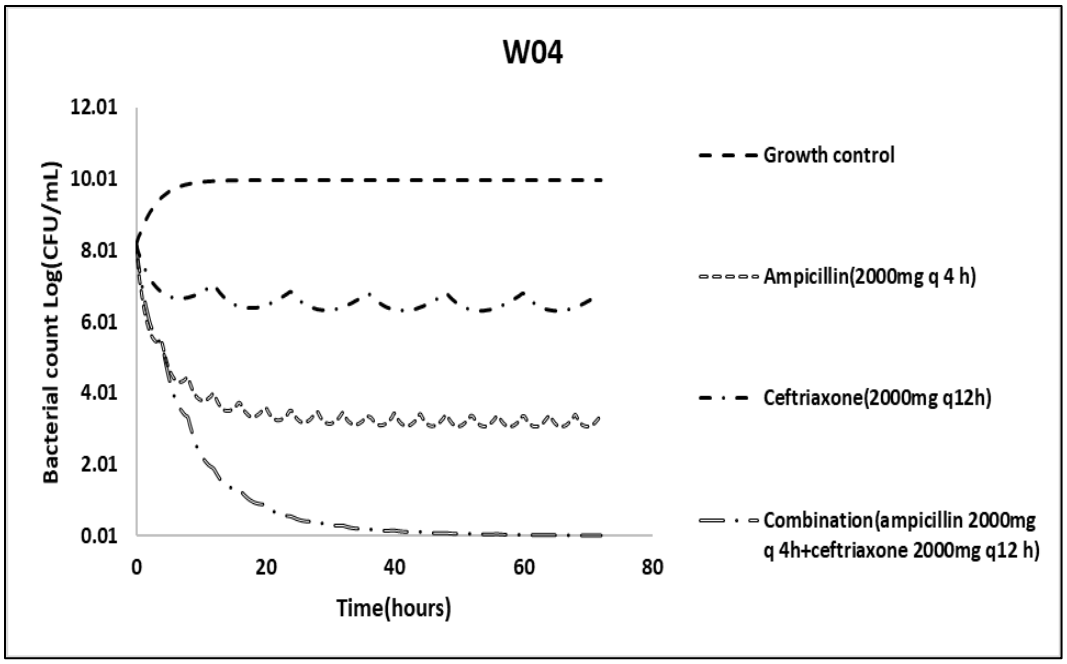


Figure 21 c Change in bacterial count over a period of 72 hours in healthy volunteers for growth model, monotherapy, and combination therapy

Chapter 5

Application of PBPK-PD model to optimize the dosing regimen of ampicillin in a severe renal impaired population when given in combination with ceftriaxone against E. faecalis

5.1 Introduction

Many antibiotics are primarily cleared through renal excretion, and their disposition can be affected in patients with chronic kidney disease (CKD). It is important to understand the change in pharmacokinetics of renally excreted drugs in patients suffering through various stages of CKD to prevent adverse drug reactions resulting from increased systemic exposure by adjusting the dosing regimen, if necessary [43]. PBPK modeling is an established tool for investigating the change in PK characteristics of such drugs in renal impaired patient and optimizing dosing regimens.

The application of PBPK modeling to evaluate a drug's systemic exposure in renal impaired patients has been rising over the years [35]. For example, Mitsuo et al developed a minimal PBPK model for glycopyrronium bromide using Simcyp simulator in patients suffering chronic obstructive pulmonary disease (COPD) with normal kidney function and for mild, moderate, and severe kidney renal impaired populations. Since only a slight increase in AUC was observed (range 1.2 – 1.6 fold) in mild, moderate, and severe renal impaired patients in comparison to patients with normal kidney function, this suggested that no change in dosing regimen required for glycopyrronium bromide drug in renal impaired patients [44]. Additionally, Li Zhou et al created a PBPK model for ceftazidime to assess the ability of the in silico tool to predict systemic exposure of the drug in renal and healthy volunteers [35].

In silico software platforms such as Simcyp have built-in patient populations that allows for investigation of alterations in pharmacokinetics of drugs due to changes in physiology. The chronic renal disease population in Simcyp is subdivided in two populations stratified by glomerular filtration rate (GFR): GFR 30-60 mL/min (moderate renal impaired population) and GFR<30

mL/min (severe renal impaired population). In Simcyp, GFR can be calculated based on two methods: Cockcroft-Gault and modification of diet in renal disease (MDRD). Cockcroft-Gault predicts creatinine clearance based on the follow equations:

$$Cl_{creatinine} = \frac{((140-age)*body\ weight)}{(plasms\ creatinine*72)} \quad \text{equation 30}$$

$$Cl_{creatinine} = \frac{((140-age)*body\ weight)}{(plasms\ creatinine*72)} \times 0.85 \text{ (if female)} \quad \text{equation 31}$$

MDRD calculates creatinine clearance as follows:

$$175 * plasma\ cereatinine^{-1.154} * age^{-0.203} * 0.72 \quad \text{equation 32}$$

Additionally, a user defined GFR calculation option is also available where the user can use lua scripting to model the GFR [17]. This gives user the flexibility to incorporate alternative approaches for estimating GFR.

Based on the FDA guidance for PBPK analysis, PBPK modeling can be used at any stage of drug development to answer when, where, and how to conduct certain clinical studies and recommend dosing regimen in product labeling. The application of PBPK modeling to support dose recommendation in special populations such as renal impaired patients is expected to increase in coming years [45].

For the purpose of this thesis, PBPK models were developed for ampicillin and ceftriaxone in severe renal impaired patient populations. The developed PBPK model was also linked to a pharmacodynamic(PD) model. In this investigation, the model was used as a predictive tool to help justify change in dose frequency of ampicillin when given in combination with ceftriaxone against *E.faecalis* in severe renal impaired population.

A published clinical study evaluated systemic exposure of ampicillin dosed 2000mg via the intravenous infusion over 15mins to healthy volunteer, patients suffering moderate renal impairment(creatinine clearance Cl_{CR} 30-60mL/min), and patients having severe renal impairment (Cl_{CR} 7-30mL/min). It was observed that AUC in moderate and in severe renal impaired patients increased by 36.7% and 140% respectively in compared to healthy subjects[37]. This reflects ampicillin's prolonged retention in the systemic circulation resulting from reduced clearance in cases of renal insufficiency. The study conclusion suggested a dose adjustment of ampicillin in severe renal impaired patients[37].

The aim of this chapter was to extend the verified PBPK-PD model in healthy population to a severe renal impairment population and to justify the change in dose frequency of ampicillin when given in combination with ceftriaxone. To attain this aim, PBPK models for ampicillin and ceftriaxone were verified in severe renal impaired patients based on published data. The verified PBPK model was then linked to the PD model, and two dosing regimens were simulated(both the dosing regimen are published in literature [82]): ampicillin 2g q8 hours with ceftriaxone 2g q12 hours and ampicillin 2g q6 hours with ceftriaxone 2g q12 hours. The dosing regimens were evaluated based on the target PD endpoint; that is, complete eradication of bacteria from the system.

5.2Methodology

Developing PBPK models for ceftriaxone and ampicillin in a renal impaired patient population

Once the PBPK model was verified in Sim-Healthy Volunteer population (chapter 4), it was then applied to the renal impaired population in Simcyp. The built in "Sim-RenalGFR_less_30" population was used to simulate the observe change in PK parameters for ampicillin and ceftriaxone. All input parameters were kept the same as those used in PBPK models in healthy volunteers. Details of the model trial designs are summarized in Table 12. The simulated dosing regimen for ceftriaxone (1gram administered via intravenous infusion for 30 mins) and ampicillin (2 grams via intravenous infusion for 15 mins) were based on the reference published clinical studies [35,36]. Verification of the PBPK model was performed as described previously in Chapter 4. AUC was compared between healthy volunteer and renal impaired patients for both the drugs (based on the same dosing regimens). For comparison the same dosing regimen was followed in healthy volunteer as in renal impaired patients. Statistical analysis (unpaired t-test) was performed for concentration of drug in healthy volunteer and in renal impaired patients for both the drug. The p value was then calculated using excel software. If the p value is ≤ 0.05 difference was considered to be significant.

Table 12 Input values for Trial design of ceftriaxone and ampicillin in renal patients [37][38]

Parameters	Ceftriaxone	Ampicillin
Single Dose	1000 mg	2000 mg
Route of administration	IV infusion	IV infusion
Infusion time	0.25 hours	0.25 hours
Age	21-47	21-47

Male: Female	0.5	0.5
No. of subjects in each trial	6	4
Duration of study	24 hours	24 hours
No. of trials	10	10

Applying PBPK-PD model to justify change in frequency of ampicillin when given in combination with ceftriaxone against *E.faecalis* in severe renal impaired patient population

The verified PBPK-PD model for combination therapy of ampicillin and ceftriaxone in healthy volunteer was extended to severe renal impaired patient population. The model was extended to severe renal impaired patients to justify the change in frequency of ampicillin recommended in this population. Recommended change in ampicillin's frequency for dose 2000mg in published literature [82] in severe renal impaired patients are q 6 hours and q 8 hours. Two dosing regimens for the combination therapy was simulated utilizing the PBPK-PD model, ampicillin 2g q 8 hours and ceftriaxone 2g q 12 hours, and ampicillin 2g q 6 hours and ceftriaxone 2g q 12 hours [82]. A plot for bacterial count vs time was plotted separately for each dosing regimen. The PD endpoint was to observe which dosing regimen achieved complete eradication of bacteria in severe renal impaired patients, To evaluate the difference in bacterial count vs time profile for the two dosing regimen, a PBPK-PD model was simulated for ampicillin monotherapy given at dose 2 g at a frequency of 8 hours and 6 hours. %Time>4*MIC(minimum inhibitory concentration) was also calculated for each change in frequency.

5.3 Results & Discussion

Developing PBPK profile of ceftriaxone and ampicillin in renal impaired patients

The purpose of this aspect of the research was to simulate changes in the PK parameters for ampicillin and ceftriaxone in patients with severe renal insufficiency (GFR <30 mL/min). The in built population Sim-RenalGFR_less_30 was utilized in Simcyp. The PBPK models that were developed and verified in a healthy volunteer population were applied to renally impaired population [39].

To evaluate the accuracy of the models developed for ceftriaxone and ampicillin, the simulated plasma-concentration time profile curves were compared to published data [37,38]. The results, presented in Figure 22 and Figure 23 (observed values (black circles), the simulated mean profile (black line)) illustrate that the PBPK models for both medications captured the clinical observed systemic exposure in renally impaired patients. These observations are supported by Tables 13 and 14, where the fold error for model predicted Cmax and AUC values are within acceptable limits (0.5 – 2.0).

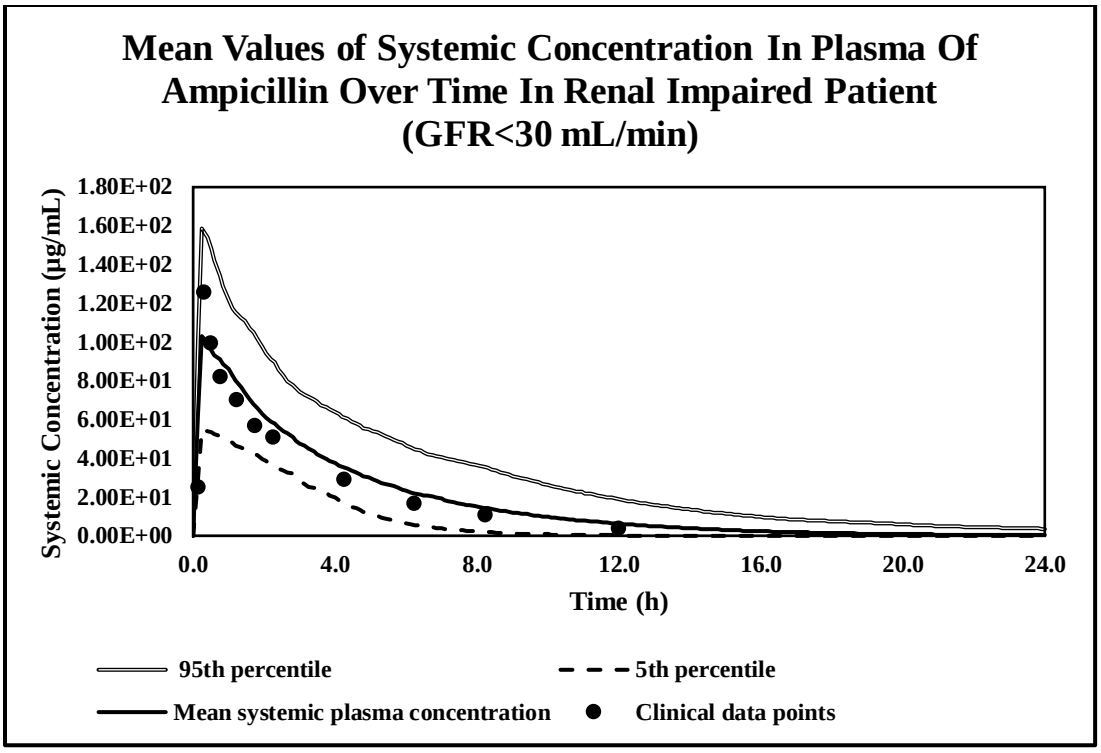


Figure 22. Verification of Ampicillin PBPK model in severe renal impaired population.

Table 13 Comparison of predicted vs observed mean Cmax ad AUC estimated for ceftriaxone

Population	Parameter	Predicted	Observed	Fold Error
Renal	Cmax(µg/ml)	106	125	0.85
	AUC(µg/ml*h)	239	379.7	0.63

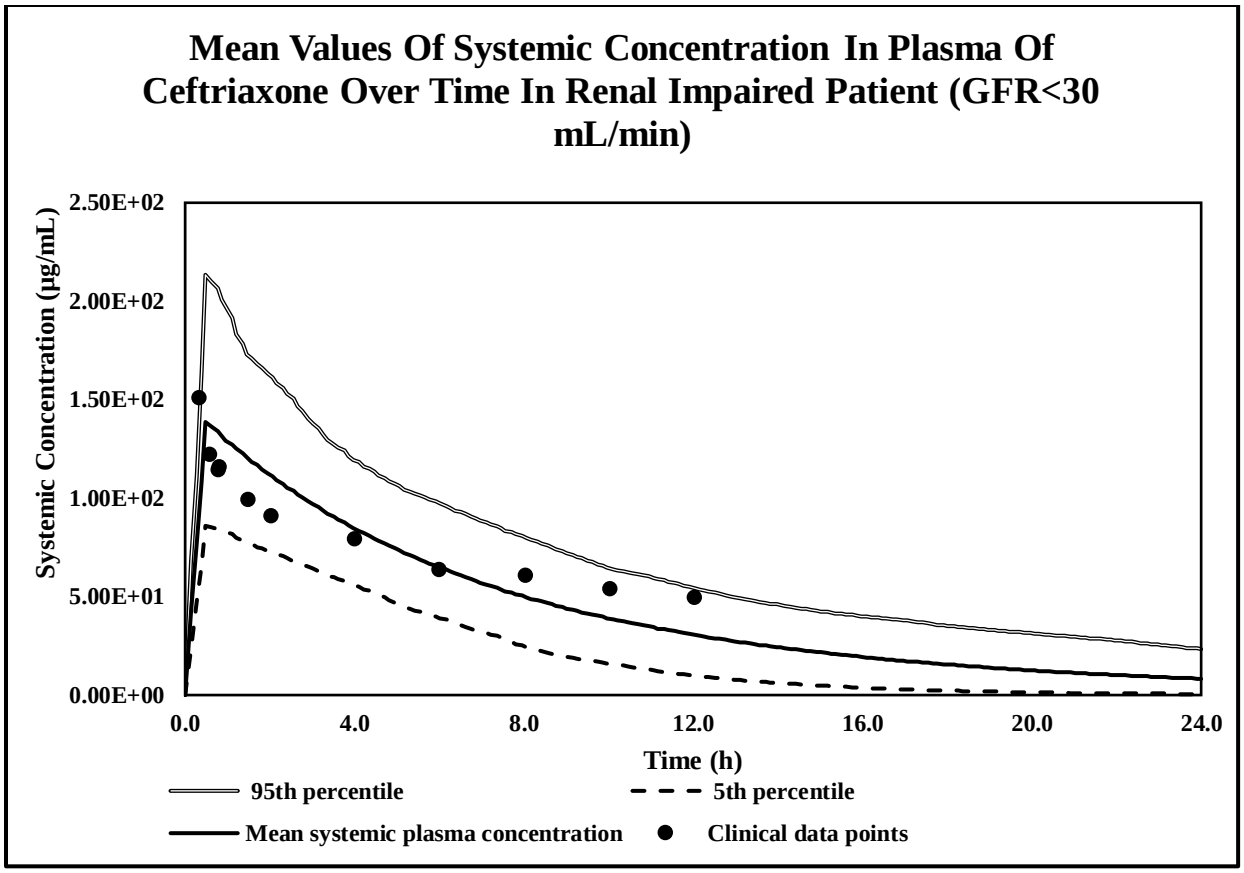


Figure 23. Verification of Ceftriaxone PBPK model in severe renal impaired population

Table 14 Comparison of predicted vs observed mean Cmax ad AUC estimated for ceftriaxone

Population	Parameter	Predicted	Observed	Fold Error
Renal	Cmax(µg/ml)	139	151	0.92
	AUC(µg/ml*h)	1040	839	1.2

Comparison of AUC parameter in healthy volunteer and renal impaired patients for ampicillin and ceftriaxone

The area under the curve predicted by PBPK model in healthy volunteers and severe renal impaired patient post intravenous administration of 2000mg ampicillin was 127 µg/mL*h and 379.7 µg/mL*h (Figure 24)[35]. This represents nearly a 200% increase AUC, and the difference was statistically significant ($p < 0.00001$) based on unpaired t-test calculation. This indicates that there is a significant difference systemic exposure of ampicillin between healthy volunteers and severe renal impaired population when administered the same dose. These findings suggest that a possible change in dosing frequency (extended) for ampicillin is recommended in renal impaired patient.

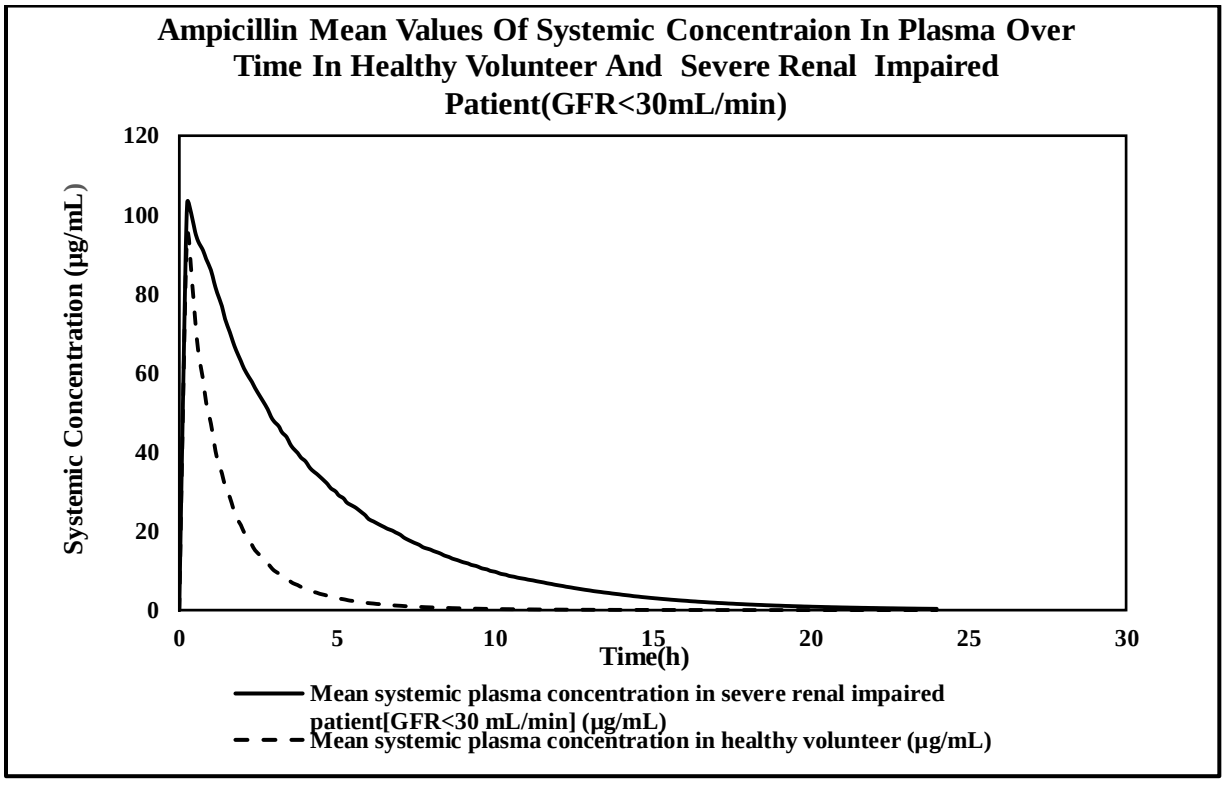


Figure 24. Comparison of plasma concentration in healthy volunteer vs renal impaired patient for ampicillin post administration of 2g dose via IV infusion

For ceftriaxone, there was no statistical difference (p value = 0.29) in exposure between populations when tested at the same dosing regimen.. Following administration of 1000mg of ceftriaxone, the mean AUC in healthy volunteer was 998 μg/mL*h and in renal impaired patients the value was1040 μg/mL*h (Figure 25) [35]. This indicates that no dosage adjustment is necessary for ceftriaxone in severe renally impaired patients.

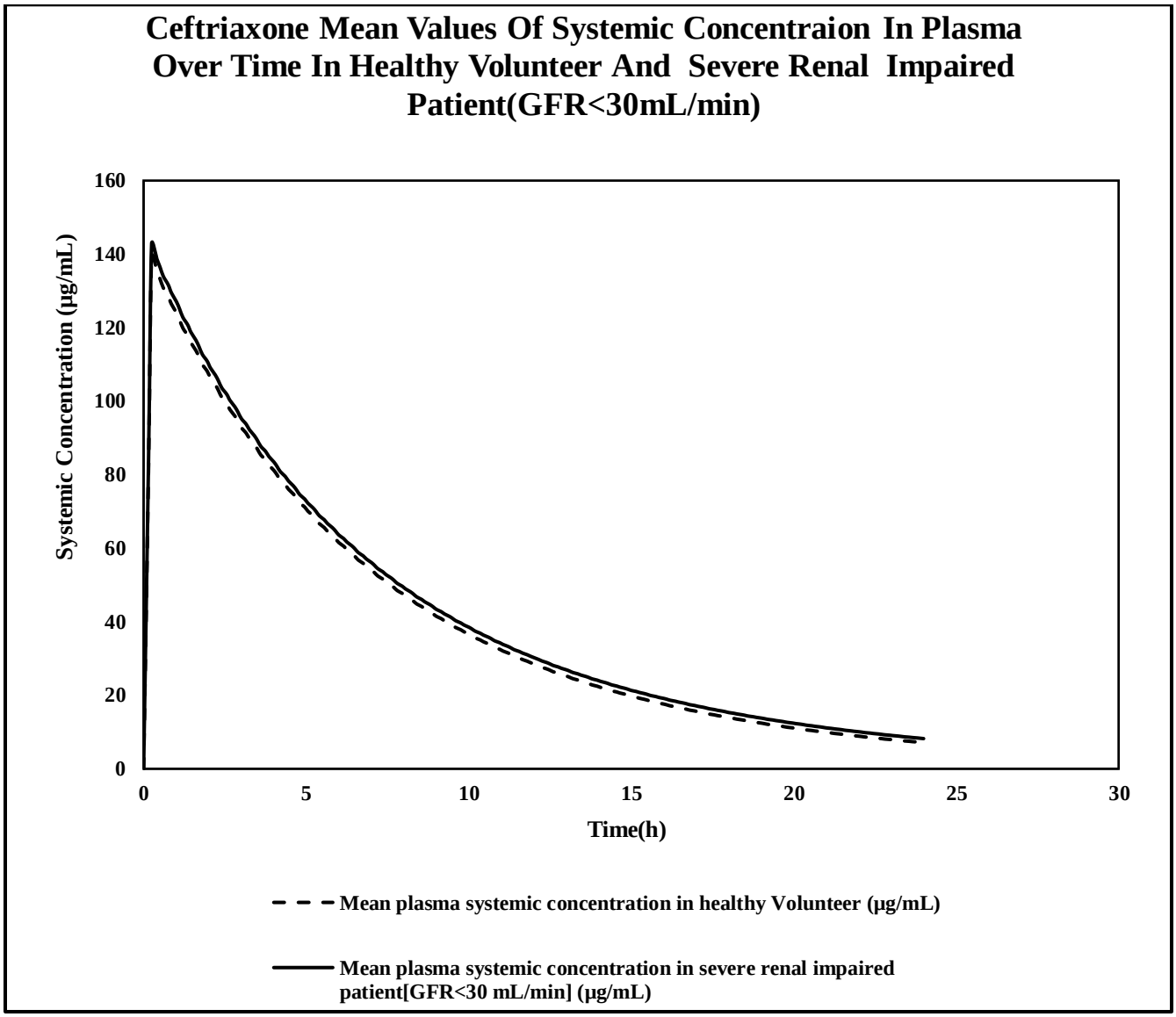


Figure 25. Comparison of plasma concentration in healthy volunteer vs renal impaired patient for ceftriaxone

Overall, the PBPK model adequately predicted exposure of ampicillin and ceftriaxone in a severe renal impaired population. However, the PBPK model does slightly under predict the AUC for ampicillin when compared to the published data. Several factors can be the cause for this. For example, the published study was carried out on only 4 patients [38] , potentially leading to an unreliable estimate mean AUC. In the published clinical study the lower limit of GFR was recorded to be 7mL/min [38], whereas the lower limit of Simcyp <30mL/min GFR population is 15mL/min. The dimensions of kidney can be different from patients enrolled in the clinical study for ampicillin in comparison to the virtual population in the simulator. Other factors such as patient's kidney volume can also affect the clearance of drug, as a published study by Jovanovic et al reported a decrease in kidney volume with reduced kidney function in chronic kidney disease function (CKD) patients [40].

Ampicillin systemic exposure AUC was nearly two fold higher (198% increase)in severe renal impaired patient compared to healthy volunteers after single dose IV administration of 2000mg. This difference suggests that ampicillin should be dosed less frequently in patients with GFR <30mL/min.

For ceftriaxone the differences in exposure between populations was minimal (4%) administration of 1000mg IV dose via intravenous route. This finding is not surprising as 30%-60% of ceftriaxone clearance involves biliary excretion.

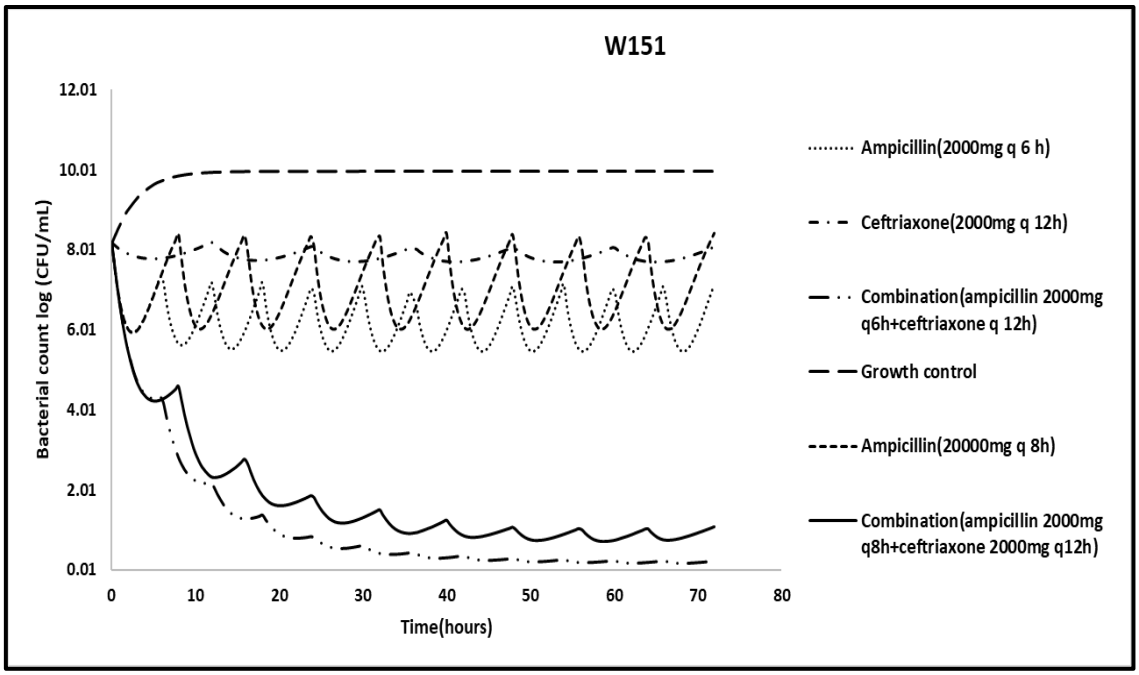
Applying PBPK-PD model for optimizing dose of ampicillin when given in combination with ceftriaxone against E.faecalis in severe renal impaired patients

The PBPK-PD model simulations in healthy volunteers showed that ampicillin 2000mg q 4 hours when given in combination of ceftriaxone 2000mg q 12 hours resulted in complete eradication of bacteria.

The verified PBPK-PD model in healthy volunteers was extended to severe renal impaired patients' population to justify a change in dosing frequency of ampicillin. The dosing regimen of ampicillin 2000mg IV q8 hours [82] and ceftriaxone 2000mg IV q12 hours was able to attain the synergistic effect, although. complete eradication of bacteria (the target PD endpoint) was not observed with this dosing regimen.

Another dosing regimen of ampicillin 2000mg IV q6 hours [82] and ceftriaxone IV 2000mg q12 hours was simulated. This dosing regimen eradicated the bacteria in a similar trend as observed in healthy volunteers (ampicillin dose 2000mg q 4 hours and ceftriaxone dose 200mg q 12 hours). To understand why complete eradication of bacteria was not attained when ampicillin was given less frequently, bacterial count was compared over time after administration of ampicillin 2000mg IV q8 hours and IV q6 hours. Figure 26 illustrates the change in bacterial count from the initial inoculum post monotherapy of ampicillin given every 8 hours(black dashed line) is less in comparison to the bacterial count attained at the end of ampicillin given every 6 hours(black dots).

a)



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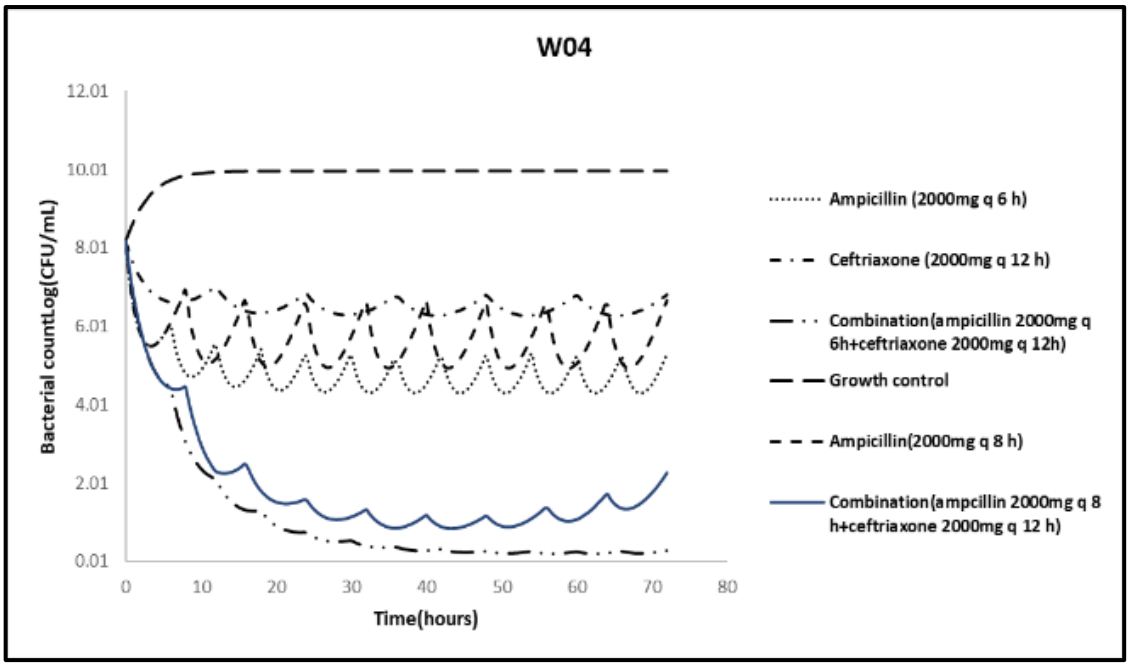

Figure 26 a,b Change in bacterial count over a period of 72 hours in renal patients for growth control, monotherapy of ampicillin 2000mg q-4 h, q-6 h, monotherapy of ceftriaxone 2000mg q-12 h, for combination therapy ampicillin 2000mg q-4 h + ceftriaxone 2000mg q-12 h, ampicillin 2000mg q-8 h + ceftriaxone 2000mg q-12 h and ampicillin 2000mg q-6 h + ceftriaxone 2000mg q-12 h for variant W151 and W04

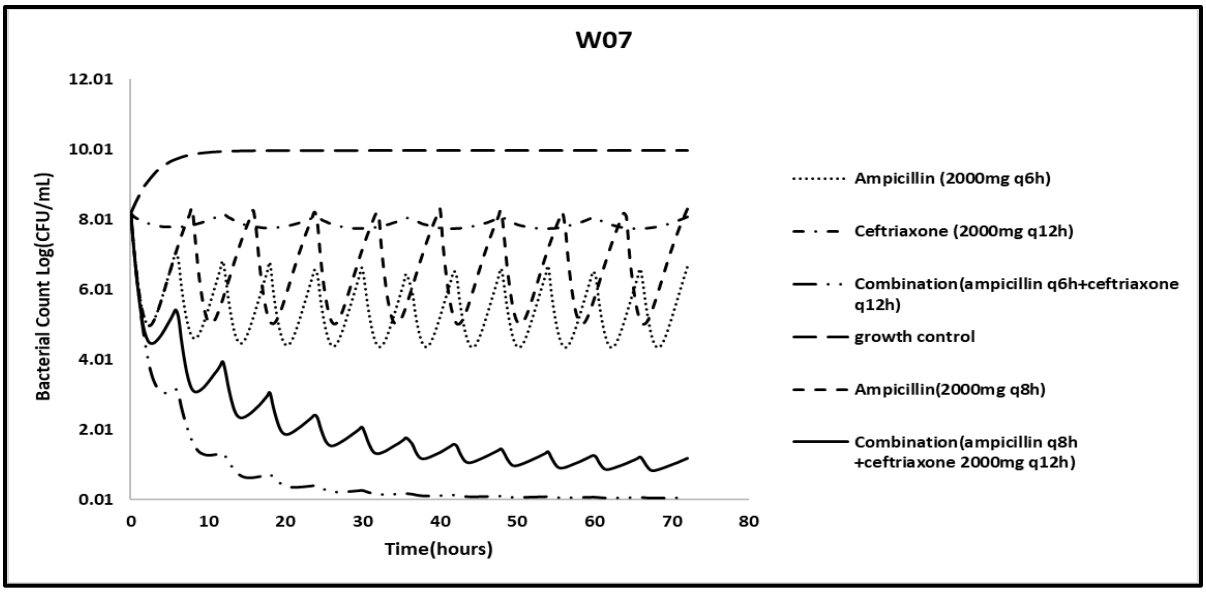

Figure 26 c Change in bacterial count over a period of 72 hours in renal patients for growth control, monotherapy of ampicillin 2000mg q-4 h, q-6 h, monotherapy of ceftriaxone 2000mg q-12 h, for combination therapy ampicillin 2000mg q-4 h + ceftriaxone 2000mg q-12 h, ampicillin 2000mg q-8 h + ceftriaxone 2000mg q-12 h and ampicillin 2000mg q-6 h + ceftriaxone 2000mg q-12 h for variant W07

Table 15 Change in percentage time above 4×minimum inhibitory concentration (MIC) value for all the three variant of Enterococcus faecalis

Bacteria	**MIC (Ampicillin)**	**%T>*f*4×MIC (q 4hours)**	**%T>*f*4×MIC (q 6hours)**	**%T>*f*4×MIC (q 8hours)**
W04	0.5	160	106	80
W07	2	60	40	30
W151	1	110	73.33	55

Table 16 Bacterial count at the end of 72 hours study for W04, W07 and W151 in renal impaired patients

Variant	Monotherapy of ampicillin (2000mg q6 hours)	Monotherapy of ampicillin (2000mg q8 hours)	Combination therapy (ampicillin 2000mg q 8 hours+ ceftriaxone 2000mg q 12 hours)	Combination therapy (ampicillin 2000mg q 6 hours+ ceftriaxone 2000mg q 12 hours)
W04	5.3	6.67	2.27	0.27
W07	6.65	8.3	1.19	0.053
W151	7.13	8.43	1.08	0.23

This could be because the bacteria are becoming resistant when ampicillin is given less frequently. One reason that the bacteria are getting resistant could be due to the suboptimal concentration of ampicillin achieved when ampicillin is given 2000mg IV every 8 hours. Suboptimal concentration is when the amount of active drug at the target is not sufficient to kill the bacteria with borderline susceptibility, allowing for the modification of the target and increase in resistant bacterial population [54].

In our study it might be possible that the concentration after administration of monotherapy of ampicillin given every 8 hours is not optimum at the target site, leading to rapid increase in PBP 2 and PBP 3 resulting in an increased resistant *E. faecalis* population. This can also be explained based on %T above *f*4*MIC calculated using published literature data [82].

As seen in Table 15 the %*f*T above 4*MIC for ampicillin given every 6 hours is closer to the value when ampicillin is given every 4 hours in healthy volunteers, whereas %*f*T above 4*MIC of ampicillin given after every 8 hours is half of that value. This also indicated that when ampicillin is given less frequently the concentration of drug remains below the MIC for prolong period in comparison to when ampicillin is given more frequently. This would provide the bacteria time to regrow. In other words if the %*f*T above 4*MIC is below 30% it won't lead to eradication of bacteria.

Therefore, ampicillin given every 6 hours would lead to better eradication of bacteria. Another observation from the overall simulation is that every variant of *E. faecalis* acts differently as the overall bacterial count for each of the three variant is different at the end of 72 hour. Further investigation must be done to understand the reason behind this.

Overall, the PBPK-PD model can simulate bacterial count as observed in the in vitro experiment and could justify the difference when ampicillin is administered in severe renal impaired patients along with ceftriaxone. The custom PD model can be utilized for optimizing dosing regimen not performed in clinical studies for any combination therapy acting synergistically.

Chapter 6

Summary

Bacterial resistance is a major global issue. Increased bacterial drug resistance is threatening the efficacy of life-saving antibiotics. The Centers for Disease Control and Prevention (CDC) has identified bacteria that pose a concerning threat to life and are contributing to clinical and financial burden on the healthcare system [65]. Therefore, this thesis is focused on evaluating novel in vitro and in silico methods that can help combat bacterial resistance by achieving optimum systemic concentration of drugs, bridging the gap between in vitro and in vivo studies, evaluating new dosing regimens, and monitoring of pharmacodynamic response.

A novel in-vitro pharmacodynamic model (IVPD) and a physiologically based pharmacokinetic-pharmacodynamic model (PBPK/PD) were developed for the combination therapy of ampicillin and ceftriaxone. Ampicillin plus ceftriaxone combination therapy is first line therapy against severe *Enterococcus faecalis* infections, particularly *Enterococcus faecalis* infective endocarditis [68]. Since ampicillin and ceftriaxone have very different elimination half live, a novel in-vitro pharmacodynamic model (IVPD) model was developed for testing two drugs with different systemic elimination half live. The flow rate novel of IVPD model is designed to mimic the in vivo plasma versus time profile of the drug with a shorter half-life. However, in order to capture the profile of the drug with the longer half-life, a novel approach was used whereby drug was supplemented into the central compartment by a rate adjusted continuous infusion. This supplementation approach compensates for rapid loss of drug from the central compartment (due to the experimental flow rate used to mimic the short half-life drug) and simulates physiologic plasma concentrations of the long half-life drug. The novel IVPD model was run for 48 hours,

with ceftriaxone as the test drug. Expected concentrations of ceftriaxone were achieved with the novel IVPD model.

The other aspect of the thesis focuses on developing PBPK-PD model in Simcyp for combination of drugs acting synergistically. Simcyp did not have a in built PD model which could simulate combination therapy of drugs acting synergistically, therefore a custom PD model was developed using lua script which could overcome this caveat. The model was verified in healthy volunteers against published in vitro data. The PBPK-PD was further extended to optimize dose frequency of ampicillin when given in combination with ceftriaxone in severe renal impaired patients (referred to as the Sim_Renal_less_30 denoting patients with GFR below 30 ml/min). As ampicillin is primarily cleared by the kidney, there is a need to optimize the frequency of ampicillin in renal impaired patients. The Simcyp® simulator was used to develop and test the PBPK-PD model. The first step was to build the substrate profile for ampicillin and ceftriaxone in Simcyp and verify the model using the built-in population of Sim-Healthy Volunteer and Sim_Renal_less_30. The model for Sim-healthy Volunteer for ampicillin and ceftriaxone was verified against published data [20,24] Once the model was verified by visual predictive check and fold error (ratio of predicted and observed values for Cmax and AUC) in Sim-Healthy volunteer, the model was extended to the Sim_Renal_less_30 population. The simulation results for ampicillin and ceftriaxone in Sim_Renal_less_30 population was also verified against clinical data [37,38].

The verified PBPK model in Sim-Healthy volunteer was then integrated with the PD model in Simcyp to monitor the bacterial growth over a period of 72 hours in the absence of drug and with the recommended dosing regimen of ampicillin 2000 mg every (q)4 hours and ceftriaxone 2000 mg q12 hours administered via intravenous bolus [84]. The pharmacodynamic endpoint was

bacterial count over time. The ampicillin dose was optimized to achieve a bactericidal effect (eg. >3 log10 difference from the initial log10 CFU/mL) and show no signs of bacterial regrowth.

A custom PD model was built in Simcyp using Lua script language. To depict the pharmacodynamic response for monotherapy, a modified Emax equation was used [59]. For combination therapy a mixed framework equation was utilized [60]. The PBPK-PD model was verified by visual predictive check by comparing the change in bacterial count simulated in silico and was compared with the bacterial count obtained from an in vitro study published. [84] *E. faecalis* showed regrowth at the end of 72-hour study with monotherapy of ampicillin, and no change in bacterial count was observed with monotherapy of ceftriaxone. These results were expected as the bacteria becomes resistant to monotherapy of ampicillin by developing penicillin binding protein (PBP) 2 and PBP 3. Ceftriaxone by itself does not show any bactericidal effect as it acts on PBP2 and PBP3 which are only expressed in *E. faecalis* after exposure to ampicillin. The combination therapy showed complete eradication of bacteria.

The next step was to monitor the bacterial count after change in dose frequency of ampicillin, in severe renal impaired patients [66,67]. The dosing regimen of ampicillin 2g q 8 hours with ceftriaxone 2g q 12 hours [82] did attain bactericidal effect, though regrowth of bacteria was observed at the end of 72-hour treatment period. Thus, alternative dosing strategies were tested in this population. Model simulations showed that ampicillin 2000 mg q6 hours and ceftriaxone 2000 mg q12 hours [82] resulted in complete eradication of *E. faecalis.*

Overall, this thesis was able to develop a new IVPD model which could simulate the expected in vivo plasma concentration vs time profiles of two drugs with different half live. It also focused on the importance of in silico approaches and showcased the capability of the Simcyp platform as a powerful tool to monitor pharmacodynamic response when integrated with the PBPK model. And

lastly, the PBPK-PD model could justify the change in dose frequency of ampicillin when given in combination with ceftriaxone in severe renal impaired patients with *E. faecalis* infection

Chapter 7
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Appendix 1: Berkeley Madonna Script

Ceftriaxone

```
{Type Equations Here.}

METHOD RK4

STARTTIME = 0

STOPTIME= 72

DT=0.02

; Parameter definitions

 dose= 2000 ; dose amount 2000mg from 0 h onwards

 dose_int= 12; dosing interval

 ndoses= 6; number of doses

 ; PK parameters

 ka=0.086; elimination rate constant

V = 7.78; L volume of distribution

Emax0= 0.1 ; 1/hour maximum bacteril kill rate

kgrowth = 0.37 ; 1/hour bacterial growth rate in abscence of drug

Bmax = 9.98; log cfu/ml maximum bacterial growth in absence of drug

EC50 = 54; mg/l concentration 50%
```

```
h = 1.2;hill coefficient

 ; dosing input. Set to zero after ndoses

 ; administered

 dosingperiod = if time < ndoses*dose_int-DT then 1 else 0

 input = pulse(dose,0,dose_int)*dosingperiod

 d/dt(comp1)=-ka*comp1 +input;dosing compartment

init comp1 = 0

CD = comp1/V

  DRUG = (Emax0*(CD)^h)/((EC50)^h+(CD)^h)

  d/dt(S)= kgrowth*(1-S/Bmax)*S -(DRUG)*S

init S = 8.18
```

Ampicillin

```
{Type Equations Here.}

METHOD RK4

STARTTIME = 0

STOPTIME= 72

DT=0.02
```

```
; Parameter definitions
 dose= 2000 ; dose amount 2000mg from 0 h onwards
 dose_int= 4; dosing interval
 ndoses= 18; number of doses
 ; PK parameters
 ka=0.36; elimination rate constant
V = 27.6; L volume of distribution
Emax0= 0.34 ; 1/hour maximum bacteril kill rate
kgrowth = 0.37 ; 1/hour bacterial growth rate in abscence of drug
Bmax = 9.98; log cfu/ml maximum bacterial growth in absence of drug
EC50 = 37; mg/l concentration 50%
h = 1.4;hill coefficient
 ; dosing input. Set to zero after ndoses
 ; administered
 dosingperiod = if time < ndoses*dose_int-DT then 1 else 0
 input = pulse(dose,0,dose_int)*dosingperiod
 d/dt(comp1)=-ka*comp1 +input;dosing compartment
init comp1 = 0
```

```
CD = comp1/V

   DRUG = (Emax0*(CD)^h)/((EC50)^h+(CD)^h)

     d/dt(S)= kgrowth*(1-S/Bmax)*S -(DRUG)*S

init S = 8.17
```

Appendix 2 : Lua scrip

Ceftriaxone monotherapy

```
function popSimSetup(...)
  sc:setNUserOdes(1)
  sc:setUserStateName(1, "sensitive bacteria")
end
function odeInitStep(xin, su, P, ...)
  su[1] = 8.18
  return 0
end
function odeRateStep(t, xin, su, gu, P, ...)
  S = su[1]  -- sensitive/susceptible bacteria

  kgrowth = 0.37
  Bmax = 9.98
  Emax0 = 0.16
  EC50 = 48.49
  h = 1.2


  DRUG = (Emax0*(xin)^h)/((EC50)^h+(xin)^h)

 gu[1] = kgrowth*((1-S/Bmax)*S -(DRUG)*S)


  return (S)
end
```

Ampicillin monotherapy

```
function popSimSetup(...)
  sc:setNUserOdes(1)
```

```
  sc:setUserStateName(1, "sensitive bacteria")
end
function odeInitStep(xin, su, P, ...)
  su[1] = 8.18
  return 0
end
function odeRateStep(t, xin, su, gu, P, ...)
  S = su[1]  -- sensitive/susceptible bacteria

  kgrowth = 0.37
  Bmax = 9.98
  Emax0 = 0.48
  EC50 = 48
  h = 0.5


  DRUG = (Emax0*(xin)^h)/((EC50)^h+(xin)^h)

 gu[1] = kgrowth*((1-S/Bmax)*S -(DRUG)*S)


  return (S)
end
```

Appendix 3 HPLC analysis

Time(hours)	Observed ceftriaxone concentration(µg/mL) Run 1	Observed ceftriaxone concentration(µg/mL) Run 2	CV%
0.50	21.78	19.09	9.31
2.00	18.71	14.01	20.55
3.50	15.20	12.63	13.07
4.50	11.13	9.55	10.79
6.00	9.84	8.73	8.49
7.50	6.72	5.78	10.64
8.50	5.54	5.18	4.84
10.00	4.92	4.71	3.01
11.50	4.28	3.13	22.10
24.50	24.29	26.75	6.82
26.00	19.91	21.02	3.84
27.50	18.01	18.77	2.94
28.50	15.61	16.87	5.49
30.00	13.10	12.98	0.66
31.50	11.67	11.38	1.75
32.50	10.64	10.64	0.00
34.00	7.90	7.66	2.17
35.50	6.67	6.43	2.61

CV = coefficient of variance (Standard Deviation / Mean) * 100)

Appendix 4 Curriculum Vitae

CAREER GOALS

- To develop into a highly valued PBPK/PD modeler that will apply strategic and technical expertise in quantitative analysis (dose-response, exposure-response) in dose selection using model-informed drug development approach.
- To be a valuable partner with clinical pharmacologist to provide scientific and operational insights into model-informed drug development program from first in human dosing through life cycle management.
- To integrate pharmacometrics expertise within program team with subject matter experts in clinical research, DMPK, regulatory, digital health, global outcomes/epidemiology, biostatistics, and other key data science disciplines.

STRENGTHS AND ACCOMPLISHMENTS

- Good expertise and experience in quantitative modeling such as PK/PD and physiological-based PK modeling.
- Excellent oral and written communication and inter-personal skills enabling seamless integration into new modeling teams, and other teams such as clinical development, clinical operation, and study teams.
- Effective influence skills with ability to drive collaborations and decisions in a cross-functional, multi-cultural organization.
- Fast and highly motivated learner on different aspects of new software and drug development.
- Had integrated in vitro DMPK and in vivo animal PK data to predict drug-drug interactions using PBPK on an oncology asset, enabling justification of study design of clinical DDI studies (Arvinas).
- Developed and applied a PBPK/PD model to optimize dosing regimen of ampicillin and ceftriaxone combination therapy in renal impairment patients (Long Island University).

SKILLS

- Simcyp
- Gastroplus
- Phoenix Winnonlin
- Ussing Chamber
- Scintillator
- Berkeley Madonna
- SigmaPlot, GraphPad Prism
- Sandwich type Elisa
- In-vitro Pharmacodynamic Model
- Franz diffusion cell

RESEARCH EXPERIENCE

September 2018 – est Fall 2021

PhD Project, Long Island University, NY, USA

In vitro and in silico approaches to evaluate the pharmacokinetics and pharmacodynamics of combination antibiotic therapy against drug-resistant bacteria

- Developed PBPK/PD model for ampicillin and ceftriaxone combination therapy in healthy volunteer.
- Utilized PBPK/PD model to optimize dosing regimen of ampicillin and ceftriaxone combination therapy in renal impairment patients.
- Optimized in-vitro pharmacodynamic model to attain the plasma concentration time profile of ceftriaxone when given in combination with ampicillin.
- Developed HPLC method for quantifying unknown concentration of antibiotics.

Apo-B in Prevention of cardiovascular disease (CVD) in persons living with HIV (PLWH) on Statin Therapy

- Performed sandwich type Elisa to quantify concentration of Apo-B a cardiovascular disease biomarker in HIV patient plasma sample.

Dec 2017 - May 2018

MASTERS PROJECTS, LONG ISLAND UNIVERSITY, NY

Bioequivalent Diclofenac Sodium Gel Formulation

- Gel formulation using Carbopol 980NF and 934NF, performing Franz diffusion studies, troubleshooting solubility issues.

Academic Capstone Project Submission for PKPD Modelling Software SIMCYP

- Etanercept Physiologic-Based Pharmacokinetics (PBPK) Analysis in Renal Impairment and Patients with Rheumatoid Arthritis: Modeling and Simulation Approach.

May 2021-August2021

Arivnas, Connecticut, USA

Clinical Pharmacology Intern

- Develop PBPK model for atorvastatin and apixaban using Gastroplus.
- Drug-drug interaction of atorvastatin and apixaban with in house PROTAC drug.

May 2020-August2020

PPDM Bioanalytical Intern, Merck & Co, Boston, USA

Evaluation of homogeneous proximity immunoassays for preclinical bioanalytical applications

- Performed thorough literature review to explore homogeneous proximity immunoassays such as AlphaLISA, SPARCL and TR-FRET.
- Compared the above-mentioned immunoassays with MSD and Gyrolab, based on assay run time and sensitivity.
- Applications of AlphaLISA, SPARCL and TR-FRET in the pharmaceutical field from PK/PD perspective.

June 2019- August 2019

Biopharmaceutics and Special Dosage Form Intern, Merck & Co, Pennsylvania, USA

- Performed Ex-vivo studies to understand various in-vitro and formulation effects of oral peptide drug delivery system.
- Developed a workflow to understand the apparent permeability of radio-labelled oral peptide across rat intestine using ussing chamber.

- Exploring advantages of Ussing chamber over other ex-vivo techniques.

Jan 2015 – Dec 2015

Undergraduate Project, Shobhaben Pratapbhai Patel School of Pharmacy and Technology management, Maharashtra, India

Solubility enhancement of Glipizide using cyclodextrins and spray drying technique

- Determining molar ratio required for the preparation of complex using jobs plot method.
- Developed nanocomposite complex of beta cyclodextrins encapsulating with glipizide using spray drying technique.
- Validation of the nanocomposite complex structure using following techniques: structure elucidation using NMR, FTIR, XRD tests.
- Solubility enhancement studies tests: Dissolution tests using multiple pH, and saturation solubility tests.

EDUCATION

Sept 2018- est Fall 2021

PhD in Pharmaceutics, Long Island University, NY, USA

Major in Pharmacokinetics 3.73 CGPA

Jan 2017-May 2018

Master's in pharmacy, Long Island University, NY, USA

Major in Industrial Pharmacy 3.78 CGPA

Jul 2012-May 2016

Bachelor of Pharmacy, Shobhaben Pratapbhai Patel School of Pharmacy and Technology Management, Mumbai, India

3.43 CGPA

PUBLICATION

- Eangoor, P., **Das S**., & Pucci, V. (2020). Evaluation of homogeneous proximity immunoassays for preclinical bioanalysis. Bioanalysis, 12(24), 1757–1766. https://doi.org/10.4155/bio-2020-0258

- **Das S**, D R Taft, J Cusumano, K Shah. Application of Physiologically Based Pharmacokinetics and Pharmacodynamics (PBPK/PD) model for optimizing dosing regimen of ceftriaxone and ampicillin against E. faecalis in severe renal impaired patient. [Manuscript in progress]

- Cusumano JA, **Das S**, Shah Z, Andrade J, Taft D. An update to in vitro pharmacodynamic modeling methodology of two drugs with different half live. J Antimicrob Chemother. [Manuscript in progress]

- B Fischetti, R J Cope, L Berkowitz, D R Taft, **Das S**, R Molla, D Rampersad, S Ye, Candidate Evaluation of Apolipoprotein B in Patients with HIV as a Potential Marker for Cardiovascular Disease. [Manuscript in progress].