PHARMACOKINETIC/PHARMACODYNAMIC CHARACTERIZATION OF JNJ-Q2, A NOVEL FLUOROQUINOLONE

By

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To Swami

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For antimicrobial agents, selection of optimized dosing scheme for specific pathogens not only increases chances of cure but also reduces probability of resistance development. A tool helpful in dose selection is pharmacokinetic/pharmacodynamic (PK/PD) model. JNJ-Q2, a novel fluorinated quinolone, with activity against key pathogens associated with complicated skin & skin structure infections including methicillin-resistant S.aureus (MRSA). Most MRSA infections that are community acquired present as skin and soft-tissue infections and the standard care used in treating these infections such as linezolid is effective, but its prolonged use can cause resistance. To characterize the time-course of JNJ-Q2 at 400mg single dose, concentrations not only in plasma, but also in skeletal muscle and subcutaneous adipose tissue, where the site of action for infections is, a microdialysis study was conducted in healthy volunteers. For best correlation to clinical outcome, PK/PD index ratio of 24h area under the curve and MIC (AUC_{24}/MIC) was evaluated. The time course effect of JNJ-Q2 was defined using a modified Emax model using *in vitro* time-kill data. Population PKPD model was developed to elucidate the dynamic relation between clinical PK and *in vitro* PD studies for drug development and dosage recommendation.

The project explored the usefulness of PK/PD relationships to support drug development and appropriateness of use of 400mg once a day oral dosing regimen for JNJ-Q2. Development of novel antibiotics such as JNJ-Q2 is necessary in order to have an effective armamentarium against adapting bacteria.

CHAPTER 1
FLUOROQUINOLONES AND THE APPLICATION OF PHARMACOKINETIC/PHARMACODYNAMIC APPRAOCHES IN THEIR DEVELOPMENT

Fluoroquinolones

Introduction

The emergence of a new class of antibacterial agents began with the synthesis of nalidixic acid which was introduced for clinical use in 1962 [1]. Modification of the nalidixic acid structure led to the synthesis of the first fluoroquinolone; norfloxacin, which exhibited enhanced activity for Gram-negative organisms, including *Pseudomonas aeruginosa* [1]. Further substitutions of the fluoroquinolone molecule resulted in the development of ciprofloxacin with high activity against Gram-negative bacilli but with questionable utility against important Gram-positive pathogens. Over the years we have seen numerous manipulations of the fluoroquinolone structure in order to synthesize a group of new fluoroquinolones with important advantages over their predecessors which exhibit enhanced potency against Gram-positive organisms and/or anaerobes, while maintaining Gram-negative activity. These agents demonstrate significantly improved pharmacokinetic profiles, with oral and intravenous formulations and the ability to concentrate in tissues and fluids at concentrations that exceed serum drug concentrations [2].

Mechanism of Action

Fluoroquinolones exert their bactericidal effect by inhibiting the type II topoisomerases DNA gyrase and topoisomerase IV. DNA gyrase introduces negative superhelical twists into bacterial DNA, and thus is essential for replication and transcription. Topoisomerase IV appears to separate interlinked daughter DNA circles

following a round of DNA replication, allowing segregation of the replicated chromosomes. Resistance to the fluoroquinolones is mediated by mutations in the quinolone resistance determining regions of the genes coding for the target enzymes. It has also been suggested that resistance occurs by active efflux of fluoroquinolones [1, 3].

Classes of Fluoroquinolones

The quinolones can be classified into four generations based on antimicrobial activity [Table 1-1] [4, 5]. First-generation agents have moderate gram negative activity and minimal systemic distribution. Second-generation quinolones have expanded Gram-negative activity and atypical pathogen coverage, but limited gram-positive activity. These agents are most active against aerobic Gram-negative bacilli. Ciprofloxacin remains the quinolone most active against *Pseudomonas aeruginosa* [2, 5, 6]. Third-generation quinolones retain expanded Gram-negative and atypical intracellular activity but have improved Gram-positive coverage. Finally, fourth-generation agents improve Gram-positive coverage, maintain gram-negative coverage, and gain anaerobic coverage [5].

Pharmacokinetics

Quinolones exhibit concentration-dependent bacterial killing. Bactericidal activity becomes more pronounced as the serum drug concentration increases to approximately 30 times the minimum inhibitory concentration (MIC) [5, 7]. Higher drug concentrations paradoxically inhibit RNA and protein synthesis, thereby reducing bactericidal activity[5]. Quinolones have a post antibiotic effect of about one to two hours [5].

Absorption

Fluoroquinolones are well absorbed after oral administration, with bioavailability ranging from approximately 70% for ciprofloxacin to 99% for levofloxacin [1, 7]. The majority of new fluoroquinolones are absorbed quickly, attaining their maximum concentration in plasma (Cmax) within approximately 1 to 2 hours after oral administration. A number of investigations into the effect of food on the pharmacokinetics (PK) of the new fluoroquinolones have demonstrated that although food intake can slow absorption, it does not cause clinically significant alterations in the extent of absorption [i.e. area under the plasma concentration time curve (AUC)] or overall bioavailability of gatifloxacin, gemifloxacin, grepafloxacin, levofloxacin, moxifloxacin, sitafloxacin, sparfloxacin, and trovafloxacin. Therefore, all of these new fluoroquinolones can be given orally without regard to food intake [3, 8-12]. However, quinolones chelate with cations such as aluminum, magnesium, calcium, iron, and zinc. This interaction significantly reduces absorption and bioavailability, resulting in lower serum drug concentrations and less target-tissue penetration [5, 7].

Serum drug levels achieved after oral administration are comparable to those with intravenous dosing, which allows an early transition from intravenous to oral therapy and a potential reduction of treatment costs [13].

Distribution and protein binding

One of the most attractive PK characteristics of fluoroquinolones is their large volume of distribution (Vd). Distribution of fluoroquinolones to tissues is very good, owing to their physicochemical properties. Tissue penetration is higher than the concentration achieved in plasma, stool, bile, prostatic tissue, and lung tissue. Quinolones also penetrate well in urine and kidneys when renal clearance is the route of

drug elimination. Penetration into prostatic fluid, saliva, bone, and cerebrospinal fluid is not known to exceed serum drug levels. The new fluoroquinolones have Vd ranging from 1.1 to 7.7 L/kg for gatifloxacin, levofloxacin, moxifloxacin, gemifloxacin, trovalfoxacin including ciprofloxacin [3]. In regard to inflammatory fluid penetration, the new fluoroquinolones all demonstrate fluid to serum ratios in the range of 0.9 to 1.3. This is not surprising as they have large Vd [14-23].

Plasma protein binding of the quinolones varies, with the newer quinolones less bound to plasma proteins than nalidixic acid, ranging from 5 to 73%. Clinafloxacin and gatifloxacin have 5 and 20% protein binding respectively while levofloxacin and moxifloxacin shown 31 and 47% and trevofloxacin of 73% [3, 12].

Metabolism

The degree of the metabolism of fluoroquinolones varies widely [24]. Biotransformation reactions involve predominantly the piperazinyl ring and its substituents. Most of the fluoroquinolone primary metabolites are active against bacteria however, these metabolites have a shorter elimination half-life than their parent compound [24].

Elimination

Newer fluoroquinolones exhibit longer half-lives than that of ciprofloxacin, ranging from 4.7 hours for sitafloxacin to 18.7 hours for sparfloxacin. Hence, most are administered every 12 to 24 hours.

The excretion of fluoroquinolones is primarily via the kidney and secondarily via the liver such as in case of sparfloxacin, moxifloxacin, and trovafloxacin. To avoid toxicity, dosages often need to be adjusted in patients with renal or hepatic impairment [5, 7]. Clinafloxacin, gatifloxacin, levofloxacin, and sitafloxacin are excreted in the urine

as the parent compound (>50%), indicating that these agents primarily undergo renal elimination. Conversely, gemifloxacin, grepafloxacin, moxifloxacin, sparfloxacin, and trovafloxacin are all eliminated predominantly by non-renal pathways [3, 18, 21]. A number of PK studies have been conducted in special populations. In the elderly, it has been demonstrated that the PK and bioavailability of levofloxacin, gatifloxacin, moxifloxacin, and sparfloxacin are not appreciably affected; however, grepafloxacin displays delayed absorption, a 31% increase in the peak plasma concentration, and a 48% increase in AUC in the elderly [8, 11, 25]. Excretion is decreased in individuals suffering from the renal failure and fluoroquinolones should be used in such patients with caution [24].

Pharmacodynamics

Understanding of clinical pharmacodynamics (PD) of anti-infective agents has dramatically increased during the past decade [26]. PD characteristics that best describe fluoroquinolones are concentration dependent bactericidal activity and a significant post-antibiotic effect for both Gram-positive and Gram-negative bacteria [7, 27].

The parameter most commonly used to quantify the antimicrobial activity of antibiotics against a certain bacterium is usually the minimum inhibitory concentration. It is defined as the lowest concentration of drug that prevents visible growth of the organism as detected by the unaided eye. Although the MIC is a well-established PD parameter, this parameter has several disadvantages. For instance, the MIC does not provide information on the rate of bacterial kill. MIC determination depends on the number of bacteria at a single time-point therefore doesn't represent the true course of bacterial activity and kill *in vivo* over time. A two-fold variability in the results is

acceptable and it is not uncommon to see the MIC reported as a range of values instead of a single number. Hence, any PK/PD approach based on this PD indicator will carry with it the same amount of variability and uncertainty.

An alternative PD approach, bacterial time–kill curves, has been proposed to offer detailed information about the antibacterial efficacy as a function of both time and antibiotic concentration [3]. Time–kill curves of many antibacterial agents have been studied in both *in vitro* kinetic models and animal infection models. The major limitation associated to the kill-curve approach is that it is not a very practical method. Obtaining a complete set of kill-curves that would allow a good evaluation of the concentration-effect and time-effect relationships between drug and bacteria is very labor-intensive and time-consuming.

The presently available fluoroquinolones with *in vitro* activity against *Streptococcus pneumoniae* (including current penicillin-resistant strains) are levofloxacin, sparfloxacin, gatifloxacin, moxifloxacin, and trovafloxacin. Levofloxacin and sparfloxacin exhibit inferior *in vitro* streptococcal activity compared with gatifloxacin, moxifloxacin, and trovafloxacin. Gatifloxacin is two to four times more active than levofloxacin against *S. pneumoniae in vitro*, and moxifloxacin is four to eight times more active [5]. Compared with ciprofloxacin and levofloxacin, the fluoroquinolones gatifloxacin, moxifloxacin, and trovafloxacin have greater *in vitro* activity against *S. aureus* and some Enterococcus strains [5]. Although gatifloxacin and moxifloxacin have *in vitro* anaerobic activity, only trovafloxacin is labeled for the treatment of anaerobic infections. Ciprofloxacin, ofloxacin, and the newer fluoroquinolones have exceptional intracellular concentrations.

Overall, these pharmacodynamic properties allow infrequent dosing of fluoroquinolones because prolonged activity, even when serum and tissue concentrations fall below the MIC, prevents bacterial re-growth. In addition, optimal pharmacodynamic values are associated with preventing the development of bacterial resistance.

JNJ-Q2

Physicochemical Properties

JNJ-Q2 [Figure 1-1] is one of the most potent anti-staphylococcal agents in a series of aminoethylidenylpiperidine fluoroquinolones [28]. The hydrochloride salt of JNJ-Q2 has a low molecular weight of 455.8 and acceptable solubility profile and lipophilicity [29]. The experimentally determined value of pKa (6.13), pKb (8.59), and the logarithm of the distribution coefficient (*D*) at pH 7.4 is 0.37 for JNJ-Q2 which is suggestive of absorption and permeability characteristics equivalent to those of currently approved fluoroquinolones. The aqueous solubility of 4.52 mg/mL for the hydrochloride salt of JNJ-Q2 is consistent with the development of oral and parenteral dosage forms [28].

Antimicrobial Activity

JNJ-Q2 is indicated for its use in complicated skin and skin structure infections (cSSSIs), including diabetic foot infection, community-acquired pneumonia (CAP) and other infections. JNJ-Q2 is a broad-spectrum, bactericidal fluoroquinolone antibiotic with potent activity against Gram-positive pathogens, including methicillin-resistant *S. aureus* (MRSA), Gram-negative and anaerobic pathogens [29].

JNJ-Q2 has demonstrated potent *in vitro* microbiologic activity against *S. pneumoniae*, including penicillin-resistant (Pen-R), erythromycin-resistant (Ery-R), and

levofloxacin-resistant (Lvx-R) isolates, with MIC values against these organisms of ≤ 0.12 µg/mL, a value that is 4-fold to 8-fold lower than gemifloxacin. JNJ-Q2 also demonstrated potent activity against clinical ciprofloxacin-non-susceptible *S. pneumoniae* with characterized mutations in at least 2 DNA gyrase or topoisomerase IV genes. The MIC value for JNJ-Q2 against these mutant *S. pneumoniae* isolates was 0.25 µg/mL, 32-fold lower than moxifloxacin, and 8-fold lower than gemifloxacin. In addition to the potent anti-pneumococcal activity, JNJ-Q2 displayed more potent activity than marketed fluoroquinolones against other Gram-positive pathogens, including methicillin-resistant *S. aureus* (MRSA). The newer fluoroquinolones (moxi- and gemi-) are currently not indicated for the treatment of MRSA related infections due to their insufficient activity against the target pathogens. The activity of JNJ-Q2 was 16-fold better than moxifloxacin against MRSA, with a MIC value of 0.5 µg/mL. Among the tested fluoroquinolones, JNJ-Q2 was the most potent compound against methicillin-resistant *S. epidermidis* (MRSE), *Enterococcus* spp, and *S. pyogenes* (MIC values of 0.25, 4, and ≤0.015 µg/mL, respectively). Against Gram-negative pathogens, JNJ-Q2 generally exhibited comparable activity to moxifloxacin and gemifloxacin, including potent activity against the respiratory pathogens *H. influenzae* and *M. catarrhalis* with MIC values of 0.015 and ≤0.015 µg/mL, respectively. JNJ-Q2 displayed potent bactericidal activity against *S. pneumoniae*, MRSA, and *E. coli* in time-kill studies exhibiting a 3 $\log_{10}$ kill at 2 times the MIC. The *in vivo* efficacy of JNJ-Q2 was superior to ciprofloxacin and comparable to moxifloxacin in lethal systemic infection models with methicillin-susceptible *S. aureus* (MSSA). Against a community-acquired MRSA isolate (CA-MRSA), JNJ-Q2 was superior to linezolid and vancomycin when dosed

subcutaneously. In a murine *S. pneumoniae* lower respiratory tract infection model, JNJ-Q2 was more potent than moxifloxacin [29].

CAP is a leading cause of death in the world, and the sixth most common cause of death in the United States. *S. pneumoniae* is the commonest pathogen responsible for 30% to 50% of CAP cases [30]. Although CAP caused by *S. aureus* is uncommon, it is important because of emergence of the community acquired MRSA (CA-MRSA). Current strategies for management of CAP are directed toward treating patients on an outpatient basis with accurate empirical therapy (e.g. monotherapy with either a β-lactam, a macrolide or a fluoroquinolone) whenever possible, by reducing the duration of therapy, or switching early on from intravenous to oral therapy. However, the incidence of infections caused by *S. pneumoniae* resistant to penicillin, macrolides and other anti-microbial agents has increased at an alarming rate during the past two decades. Fluoroquinolones such as levofloxacin, moxifloxacin, gatifloxacin, and gemifloxacin have become some of the most widely used and most effective antibacterial agents in the world in treatment of respiratory tract infections (RTIs) due to their improved activity against major respiratory pathogens including multi drug resistant *S. pneumoniae*. Novel agents have been recommended by several recent guidelines as first-line therapy for the management of most CAP patient categories [31]. The newer fluoroquinolones play an important role in achieving these goals as they are highly bioavailable in both plasma and at the sites of infection and potent against major respiratory pathogens and generally safer. However, increased use of fluoroquinolones in RTIs has also led to increasing resistance, especially to those most frequently prescribed fluoroquinolones, e.g., ciprofloxacin, moxifloxacin and levofloxacin.

Resistance of *S. pneumoniae* to fluoroquinolones has been reported in localized outbreaks and is associated with clinical failures [32, 33]. Therefore, concern is rising worldwide about the increased use of these agents that will result in increasing emergence of cross-resistance to the whole class. Therefore, development of newer agents like JNJ-Q2 with more potent activity is valuable.

JNJ-Q2 is anticipated to provide acceptable safety and tolerability with broad spectrum activity against Gram-positive and Gram-negative pathogens.

Applications of PK/PD in Fluoroquinolone Development

The selection of the correct dose and dosing regimen is a fundamental step for therapeutic success with any pharmacological agent. For antimicrobial agents the selection of the best drug and dosing scheme for a specific pathogen not only increases the chances of cure while preventing toxic side effects, but also decreases the probability of the infecting agent becoming resistant to the drug [26, 34-39]. However, deciding on the best drug and most effective dosing scheme is not an easy task and is often done based on trial and error rather than on rational design. Also for older drugs dose was used as the surrogate for antibiotic exposure, being adjusted solely on a milligram-per-kilogram basis leading to a high rate of clinical failure since it did not take into consideration the inter-subject variability in the PK parameters of the patient population [40]. Dose alone is an incomplete surrogate for drug exposure [41]. In addition, while the understanding of the time course of the drug in the body is a necessary step for dose optimization it alone cannot predict the time course or magnitude of the pharmacological effect [42]. Today, rational drug design is strongly based on principles of PK/PD modeling. PK/PD modeling has also become an extremely important tool in dose optimization [43]. By integrating the time-concentration

relationship of the antibiotic in the body and its concentration-effect relationship at the infection site, PK/PD modeling allows clinicians to establish an educated selection of best drug and optimum dosing range in an attempt to maximize efficacy, minimize toxicity, limit development of resistance and reduce costly trial-and-error approaches[39]. Much progress has been made in recent years towards the understanding the PK/PD relationship of anti-infective agents. Several different strategies have been developed such as MIC-based PK/PD indices, time-kill analysis, and advanced modeling and simulation techniques, such as population pharmacokinetics and Monte Carlo simulations. Following is an overview of the most commonly used PK/PD approaches for fluoroquinolones and their potential for the evaluation and optimization of drug therapy.

MIC-based PK/PD Indices

The two most important PK/PD indices associated with successful clinical and microbiologic outcomes for fluoroquinolones are the ratio of maximum serum concentration to the minimum inhibitory concentration (Cmax/MIC) and ratio of 24h area under the curve and MIC (AUC_{24}/MIC) [44-46]. They are highly correlated with each other, particularly when a fixed dosing interval is used in clinical trials. If the dosage is increased, both Cmax and AUC are increased. Preston et al [47] suggested that Cmax/MIC is most likely correlated to clinical outcome as long as it is greater than 10. However, when the ratio is less than 10, AUC_{24}/MIC is most important. The AUC_{24}/MIC has been used to evaluate the efficacy of fluoroquinolones.

The AUC_{24}/MIC target for fluoroquinolone therapy has been subject of debate over the years. Forrest el al [48] initially proposed a target of AUC_{24}/MIC > 125 for the treatment of Gram-negative infections with ciprofloxacin in elderly hospitalized patients,

has since then become a standard clinical breakpoint for the treatment of Gram-negative agents with fluoroquinolones although values between 100 – 125 are suggested to be sufficient [49]. The target for treating infections caused by Gram-positive agents with fluoroquinolones is believed to be lower. For *S. pneumoniae* an AUC_{24}/MIC > 30 has been associated with high probability of success in patients with community-acquired infections [50]. Schentag et al [51] suggested that there should be no differentiation between the targets for Gram-negative and Gram-positive bacteria and that area under the inhibitory curve (AUIC) values (calculated as the AUC that remains above the MIC during a 24 hr interval) should be above 250 to assure rapid killing of the infecting agent [52]. However, there has been a lot of discussion on this approach and in general not been very well accepted.

PK/PD Evaluation Based on Kill Curves

Kill curves provide a more dynamic description of the effect of antimicrobials hence allowing for more precise predictions [35]. An Emax model has been successfully applied to describe the relationship between concentration and effect for fluoroquinolones [35, 36, 53]. There have been some attempts to evaluate antimicrobial activity with an adapted Emax [54]. Parameters such as the maximum number of bacteria (Nmax) at the end of the growth phase, adaptation rate terms (*x*, *y*, *z*) and Hill factor (*h*) have been included in a PK/PD model to optimize the models [7]. Once these models have been established and validated, it is then possible to simulate the expected kill curves for different doses and dosing regimens of the antibiotic against the bacteria of interest by substituting the concentration term in the mathematical model by the fitted concentration versus time profile of the drug.

Summary

The new fluoroquinolones are a valuable addition to the antibiotic group. Their broader spectrum antimicrobial activity to include both Gram-positive and Gram-negative pathogens and favorable PK make these agents attractive alternatives to traditional therapy. Optimization of fluoroquinolone pharmacodynamics leads to maximizing pathogen eradication and clinical cure and minimizing the development of resistance. PK/PD indices are useful predictors of antibacterial therapy and the higher these parameters are the greater is the efficacy [45]. PK/PD based concepts can be used as decision making tools in different stages of dug development leading to reduction in development cost and time [39]. PK/PD characterization of JNJ-Q2 will allow streamlining drug development and selection of an appropriate dosage regimen.

Hypothesis

JNJ-Q2 has great potential for treating infections due to its broad spectrum activity against pathogens. However, in order to establish safe and effective dosing regimens for JNJ-Q2, it's PK and PD properties need to be explored and validated. Using an integrated PK/PD approach which combines both the free target site concentrations of the drug and the *in vitro* antibacterial activity will provide good insights towards understanding the potential of JNJ-Q2 for the treatment of cSSSIs, including diabetic foot infection, CAP and other infections.

The study hypothesizes, the recommended dosing regimen of 400mg administered as a single oral dose, is efficacious at the site of action based on the pharmacodynamic endpoints of AUC_{24}/MIC ratio and simulated kill curves.

Specific Aims

The first aim is to perform *in vitro* microdialysis experiments to validate the feasibility of using microdialysis as a sampling technique for JNJ-Q2. The second aim is validate an LC-MS/MS method for the analysis of JNJ-Q2 in saline. The third aim is to perform *in vivo* microdialysis study to evaluate the PK profiles of JNJ-Q2 in interstitial space fluid of soft tissues (skeletal muscle and subcutaneous adipose tissue) and plasma of healthy subjects after single oral dose of 400mg of JNJ-Q2. Next aim is to develop population pharmacokinetic (POPPK) model to characterize JNJ-Q2 PK in healthy subjects after 400 mg oral dose. The fifth aim is developing a PD model to describe time kill profiles of JNJ-Q2 against different bacterial strains. Finally the last aim is developing a PK/PD model to optimize an appropriate dosage regimen for JNJ-Q2.

Table 1-1. Classification of Fluoroquinolones [4]

Quinolone Generations	Antibiotic
First generation	Nalidixic acid
	Cinoxacin
Second generation	Lomefloxacin
	Norfloxacin
	Enoxacin
	Ofloxacin
	Ciprofloxacin
Third generation	Levofloxacin
	Sparfloxacin
	Gatifloxacin
	Moxifloxacin
	Grepafloxacin
Fourth generation	Trovafloxacin

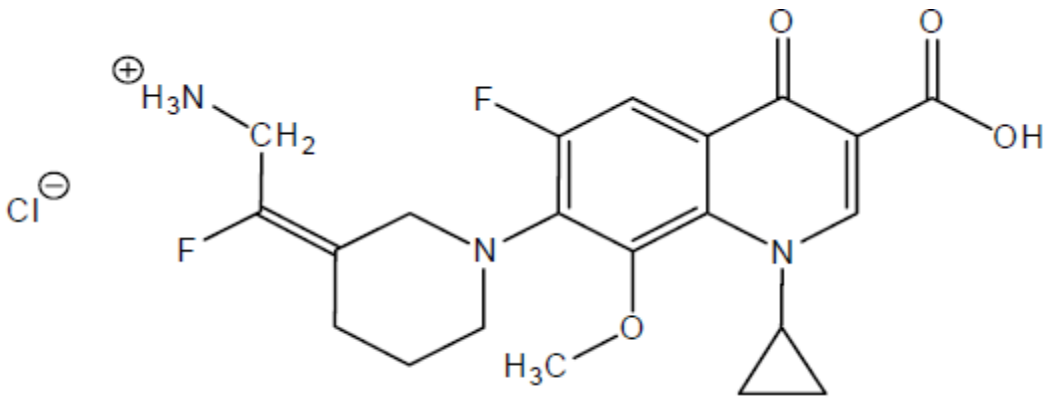

Figure 1-1. Chemical structure of JNJ-Q2

CHAPTER 2
APPLICATIONS OF MICRODIALYSIS IN PHARMACEUTICAL SCIENCES: CLINICAL MICRODIALYSIS IN SKIN AND SOFT TISSUES

Microdialysis (MD) allows the measurement of actual free drug concentrations in different tissues and organs and subsequently to relate these PK findings with PD observations to predict clinical efficacy. The ability to measure the free concentrations at the site of drug action over time makes microdialysis a very valuable tool for assessment of bioavailability and bioequivalence and has been recognized by industry and regulatory authorities such as the United States Food and Drug Administration (FDA) [55]. Earlier, PK research was restricted to measurement of drug concentrations from relatively easy to obtain biological matrixes such as tissue biopsies, urine, saliva or skin blister fluids, but the emerging knowledge of microdialysis and its benefits have slowly shifted the focus off of these methods and with good reason [56]. In the following chapter, we shall discuss various examples that help us understand why microdialysis is not just an important technique in the assessment of drug distribution in skin and soft tissues but also in general very crucial in the clinical drug development process.

Misconceptions about Tissue Drug Distribution

Until recent years, research on PK was limited for practical reasons to concentration measurements derived from matrices which were easy to obtain, such as blood and total tissue specimens. These approaches, however, caused considerable confusion, as their interpretation was flawed by a few misconceptions:

Drug-based or plasma-based models refer to the process of penetration into hypothetical compartments as “tissue penetration.” This concept is misleading, as it does not take into account the complexity of separate organ systems and disease processes. Therefore, although plasma-based modeling may provide useful information

in many cases, it must be kept in mind that it assumes rapid, unrestricted, and homogeneous diffusion processes in hypothetical spaces, assumptions that do not always hold true.

A second misconception was the reasoning that tissue is a uniform matrix. Measuring total antibiotic concentration measurements from biopsy specimens may be misleading for several reasons. Most importantly, it must be considered that the actual target space for anti-infective agents, with few exceptions, is the interstitial space fluid (ISF) [57]. If only overall tissue drug concentrations are measured, then the effective site concentrations of drugs that equilibrates exclusively with the extracellular space, such as β-lactams, may be underestimated [57]. This situation, in turn, will lead to an overestimation of the effective site concentrations of intracellularly accumulating drugs, such as quinolones or macrolides [57]. Thus, homogenization of various tissue fluids and cells will lead to a hybrid tissue drug concentration which is difficult to interpret [57].

Another misconception was the notion that the entire drug fraction present in various tissue spaces is responsible for pharmacologic activity. In fact, it has been shown that only the unbound drug concentrations at the infection site has the ability to exert anti-infective efficacy, both *in vitro* and *in vivo* [57]. Besides the fact that only the free fraction exerts activity, it is also only the free drug which has the ability to be distributed to the target site. This information was experimentally shown by various investigators, who found that differences in penetration were directly related to the free drug concentrations in serum. Although this concept is best described for antibacterial agents, one may safely assume that similar concepts hold true for many other classes of drugs.

It follows from these three considerations that an appropriate definition of tissue drug concentrations should imply in most cases the meaning of unbound interstitial drug concentrations and that a method which is to be considered the “gold standard” for the measurement of tissue drug concentrations should allow for the direct measurement of unbound antibiotic concentrations in the ISF of a given organ.

Microdialysis in the Skin: Tissue Bioavailability

MD is an excellent method to assess local drug exposure at any site where a probe can be placed. Obviously, this includes the skin, where many microdialysis studies have been performed.

The influence of changes in skin-barrier properties on drug penetration was tested using the MD method [58, 59]. Tissue levels of salicylic acid after application of 5% solution in ethanol to intact skin or skin that was treated with 1% or 2% sodium laureth sulfate (SLS), repeated tape-stripping and drying by acetone wipe were measured [58]. Skin irritation was highest in the 2% SLS and tape-stripped regions, moderate in the 1% SLS treated skin and mild after treatment with acetone. While AUC of salicylic acid in tissue was only mildly increased after acetone treatment compared to intact skin, it increased 46-fold in moderately and > 140-fold in highly irritated skin. The authors also assessed, whether application site had an influence on drug penetration and found slight differences which were not significant. The authors conclude that MD is a valuable tool for the assessment of concentrations of drugs in skin of various states of irritation and inflammation and for the evaluation of bioavailability of different study drug formulations. Acyclovir and penciclovir penetration through skin that was either intact, tape stripped and/or vasoconstricted with noradrenalin was tested [59]. The results showed that after application of drug creams to intact, not vasoconstricted skin, there

were no concentrations measurable above the lower limit of detection. When intact skin was vasoconstricted, concentrations became measurable and mean (± S.E.M.) AUC_{0-5} was 13.3 ± 2.9 and 27.6 ± 10.6 ng*mL^{-1}*h for penciclovir and acyclovir, respectively. After tape-stripping the skin to the extent of removal of the stratum corneum (45 tape strippings) the mean (±S.E.M.) AUC_{0-5} became 17.5 ± 3.8 and 12.2 ± 5 µg*mL^{-1}*h. The authors concluded that for the two drugs under investigation, which are small molecules, water soluble and having logP values of -1.8 (acyclovir) and -2.12 (penciclovir) the stratum corneum thus presents the biggest barrier to drug penetration. Transepidermal water loss (TEWL) was used as a measure of the disruption of the stratum corneum barrier function. When TEWL was measured after every 5 tape strippings, water loss increased with increasing numbers of strippings until the stratum corneum had been removed completely after 40-45 strippings. Comparing the logarithm of the concentration of penciclovir per hour with the logarithm of the TEWL showed a strong positive correlation ($r^2 > 0.9$). Since no *in vivo* recovery for both drugs was measured, values given here are relative concentrations only, which serve to compare and show relative changes.

To assess the feasibility of using microdialysis for drugs that are either highly protein bound or highly lipophilic, tissue levels of fusidic acid (protein binding 97%) and betamethasone-17-valerate were evaluated [60]. In a human clinical trial with twice daily application for two days, no measurable levels for either drug was found.

Microdialysis was used to assess skin penetration of acyclovir and salicylic acid into intact and tape-stripped skin (acyclovir only) [61]. For acyclovir, determinable dialysate levels were only found in tape-stripped skin, whereas salicylic acid levels were

found in intact skin. At the concentrations used, salicylic acid has keratolytic effect, so a certain amount of skin penetration is to be expected, the authors, conclude.

The concentration-time course of penciclovir in skin-blister fluid and dialysate after an oral dose of its prodrug famciclovir was followed in a study by Borg et al. [62]. Comparing plasma, blister and dialysate levels shows that plasma Cmax levels are similar to recovery adjusted dialysate Cmax levels. Cmax values found in blister fluid were slightly lower. The time to reach maximum concentrations in tissue (blister and dialysate) is about 60 min longer than in plasma, and tissue half-life is also about 60 min longer compared to plasma. The authors point out that microdialysis measures free tissue concentrations only. For highly protein bound drugs, the concentration in tissue could then be much smaller than total concentrations in plasma. In the case of penciclovir, it is pointed out, which has a protein binding of about 20%, plasma and tissue levels can be comparable. The study also tested for the influence of vasoconstriction on penciclovir concentrations in tissues. When adrenalin was added to the perfusate or the area above the probe site was cooled using a cooling pack, AUC values for penciclovir in skin tissue was reduced to about one third of the value achieved without interference.

The tissue concentrations of 8-methoxypsoralen after oral or topical application was also explored using microdialysis [63]. The compound is used for psoralen plus ultraviolet A (PUVA) therapy for a number of dermatoses. The study aimed to compare oral treatment and two topical treatments (bath and cream) to assess how to optimize treatment in regards to side effects such as the development of skin cancer after long-term PUVA therapy. The three treatments were compared in eight healthy volunteers in

an open, randomized, three-way crossover study. For three volunteers lower doses were given, the rest received higher doses to guarantee measurable concentrations, due to the assumption that 8-methoxypsoralen recovery may be low, because of relatively high protein binding and lipophilicity. Maximum skin tissue concentrations after application of lower doses of 8-methoxypsoralen (i.e. 0.6 mg/kg oral dose, 0.001% cream or 1 mg/L bath) were highest in volunteers treated by bath compared to cream and oral application, what is more, total plasma concentrations were up to a thousand-fold higher after oral application compared to cream or bath application. Compared to tmax after oral application, the maximum concentrations in tissues after topical applications were reached faster (one hour compared to one-4 hours) and with less variability. Maximum plasma concentrations were reached at 1-4 hours after oral application and after 1-3 hours after cream and 1.5 hours after bath application. Tissue concentrations of 8-methoxypsoralen were measurable after only 20 minutes and peaked within one hour. The authors conclude that topical treatment achieves high efficacy while having low systemic exposure, which could make topical treatment more desirable than oral application.

Microdialysis in Soft Tissue: Antimicrobial Agents

Infections of skin and soft tissue can be caused by a variety of Gram-positive and Gram-negative pathogens and are routinely treated with antibiotics. Whereas penicillins and cephalosporins are drugs of first choice, agents of different classes (e.g. oxazolidinones, glycopeptides, macrolides, tetracyclines, etc) have to be used in case of adverse events or emergence of β-lactam resistance. In order to increase the chances of clinical success and to decrease the likelihood of toxic side effects as well as resistance development, selection of an appropriate antibiotic dosing regimen becomes

extremely important [55]. The most rational approach is to link active drug concentrations to the respective pharmacodynamic outcome. However, efficacy predictions based on total plasma concentrations might be misleading, as most infections are not located in the bloodstream but rather in the ISF of tissues, which is the usual target site for bacterial infections [64]. In fact, it is the free, unbound drug in the ISF that is responsible for antimicrobial efficacy. The chapter shall discuss in detail different examples of how this can be achieved by use of microdialysis as a pharmacokinetic sampling technique and how it has the potential to streamline the decision process on proper drug dosing in drug development.

Moxifloxacin is clinically used in the treatment of uncomplicated skin and skin structure infections. A study by Burkhardt et al [65] used microdialysis to compare the free protein unbound moxifloxacin concentrations in normal as well as infected subcutaneous tissue. A single oral dose of 400mg moxifloxacin was administered to patients with spinal cord injury and decubitus ulcers and drug concentrations were determined from serum, saliva and subcutaneous tissue. The study found that moxifloxacin reaches adequate concentrations in normal s.c. tissue and decubitus ulcer tissue in patients with spinal injury. The concentrations measured in the ulcer tissue (Cmax: 2.4 ± 1.6 mg/l) and healthy tissue (Cmax: 2.7 ± 2.3 mg/l) were similar. The mean Cmax in serum was found to be 4.4 ± 2.7 mg/l and in saliva 1.4 ± 0.4 mg/l. AUC for normal (9.2 ± 8.6 mg*h/l) and infected subcutaneous tissue (9.6 ± 6.8 mg*h/l) were approximately same as the free AUC in serum.[65] Also, the tissue levels measured in the study correspond to free protein bound fraction of Moxifloxacin in serum which is

about 50-60%, by which they concluded that poor blood flow does not affect tissue levels of moxifloxacin in patients with the injury.

Traditionally, plasma samples were taken to determine pharmacokinetic properties of a compound and make predictions on the efficacy. However, these drug concentrations were sometimes presented as total drug concentrations where as in reality only the free drug is pharmacologically active and using total concentrations would overestimate the target site concentrations and hence clinical efficacy. Microdialysis has proven useful in the measurement of free drug concentrations in muscle and s.c. adipose tissue [66-69]. A study by Barbour et al [70] looked at the tissue penetration of ceftobiprole from plasma into skeletal muscle and adipose tissue after a single i.v. dose of 500mg using microdialysis in healthy volunteers. The study measurements allowed showing that ceftobiprole distributes into the ISF of skeletal muscle (fAUC$_{muscle}$/fAUC$_{plasma}$ of 0.69 ± 0.13**)** and s.c. adipose tissue (fAUC$_{s.c.}$/fAUC$_{plasma}$ of 0.49 ± 0.28). The *f*AUC of plasma (76.0 ± 8.81 h.mg/l) and *f*AUC of both tissues were significantly different and a difference between the muscle (50.6 ± 10.9 h.mg/L) and s.c. tissue (34.3 ± 19.0 h.mg/L) was also noted. These findings confirmed that it is important to measure free active drug in each tissue and not make the assumption that free drug levels in plasma equal free drug levels in tissue, even in well perfused tissues. The reason for difference in penetration ratios of drug can be attributed to several factors such as perfusion of particular tissue, local capillary density, the degree of tissue binding, the possibility of active transporters, loss of drug from peripheral compartments and lipophilicity of the compound [70]. The MIC for ceftobiprole against methicillin-resistant *S. aureus* [71] and penicillin-resistant *S.*

pneumoniae [72] has been reported as 2 mg/l. Measurements from the study showed that concentrations in both skeletal muscle and adipose tissue met the efficacy breakpoint i.e. remained above 2 mg/l for about 50% of the dosing interval. Hence, ceftobiprole qualifies as a potential agent with penetration capabilities to treat complicated skin and skin structure infections, as it is the key determinant in clinical efficacy.

A comparison of the kinetic profiles of two cephalosporins (cefpodoxime and cefixime) in soft tissue given the same oral dose was conducted in six healthy volunteers [69]. In this study, AUC in plasma of cefpodoxime was 22.4 mg/L*h ± 8.7 and for cefixime, 25.6 mg/L*h ± 8.5, which was similar. Interestingly, their target site exposure was markedly different, i.e. AUC_{muscle} was 15.4 mg/L*h ± 5.1 and 7.3 mg/L*h ± 2.2 for cefpodoxime and cefixime, respectively. The Cmax in plasma for cefpodoxime and cefixime was found to be 3.9 mg/l ± 1.2 & 3.4 mg/l ± 1.1 and the in muscle Cmax was 2.4 mg/l ± 0.9 & 0.9 mg/l ± 0.3, respectively. The reason for the difference in target site exposure of the two drugs in spite of similar profile in plasma is the difference in the degree of protein binding. The study provided insight into the free interstitial levels of cefpodoxime and cefixime which is more meaningful and showed that it is not sufficient to only consider plasma concentration profiles when evaluating pharmacokinetic properties of anti-infective agents. Using total plasma concentrations may overestimate the therapeutic outcome because only the unbound fraction in plasma is able to cross the capillary membrane and reach the interstitial space where the infection is. This may be the reason why some antibiotic treatments fail despite good activity *in vitro* as well as resistance development [73, 74]. The use of total plasma concentrations in a PK/PD

approach for predicting the clinical efficacy of antibiotics is common where MIC values are compared with plasma concentrations of the antibiotic, usually without considering protein binding. It improves the accuracy of predicting clinical efficacy if free antibiotic tissue levels by use of microdialysis. Cefpodoxime with low protein binding of 25% had more than twice higher peak concentration in the muscle than cefixime (2.1 vs. 0.9 mg/l). The average tissue penetration ($AUC_{tissue,free}/AUC_{plasma,total}$) of cefpodoxime (70%) was much higher than that of cefixime (29%) which is consistent with their protein binding values, suggesting that higher protein binding of the drug would lower the tissue penetration [73].

Burkhardt et al [75] used microdialysis to measure and compare the free protein-unbound ertapenem concentrations in the interstitial space fluid of two soft tissues, skeletal muscle and subcutaneous adipose tissue, following the administration of 1 g infusion, and compared them with the respective plasma concentrations. Results from the study indicate that free, unbound ertapenem profiles in the ISF of both skeletal muscle and subcutaneous adipose tissue are lower than corresponding total plasma concentrations. While free ISF concentrations of the skeletal muscle correlated well with free, unbound concentrations in plasma (4%-16% of total plasma concentration), they were comparably higher than free ISF concentrations in subcutaneous adipose tissue. This was observed in other studies as well and differences in blood flow in these two tissues may be a possible explanation [76]. Free ertapenem concentrations of 1.13 ± 0.68 mg/L in the muscle, observed 12 h after single-dose IV infusion of ertapenem, exceeded the MIC of methicillin susceptible *S aureus* (0.25 mg/L), *Streptococcus spp* (0.5 mg/L), extended-spectrum b-lactamase (ESBL)- producing *Enterobacteriaceae*

(0.03-0.06 mg/L), *Bacteroides fragilis*, and other anaerobic bacteria (≤ 1.0 mg/L) for at least 50% of the entire dosing interval. In comparison, free levels of 0.31 ± 0.16 mg/L in the subcutaneous adipose tissue (at 12 h) exceeded the MIC of the same SSSI pathogens for at least 30% of the dosing interval [55]. The authors concluded that free, active ertapenem concentrations reached sufficient levels in non infected ISF of muscle and subcutaneous adipose tissue.

When a drug is given orally, it gets absorbed from the intestine following which it reaches systemic circulation. Depending on the oral bioavailability of the drug, oral doses need to be adjusted to compare it with the respective IV dose in order to achieve similar therapeutic drug levels in the body. As a case in point, microdialysis was employed to compare free, active ciprofloxacin concentrations in the ISF of skeletal muscle and subcutaneous adipose tissue, after IV(400mg) or oral ciprofloxacin(500mg) administration, respectively [55, 77]. Ciprofloxacin is a broad-spectrum anti-infective agent of the fluoroquinolone class used in the treatment of upper respiratory infection, urinary tract infections and skin infections. Free ciprofloxacin concentrations were measured in the ISF of skeletal muscle and subcutaneous adipose tissue, saliva, and cantharis-induced skin blister, as well as capillary plasma, and compared to total venous plasma concentrations. Mean *f*AUCs of both muscle and subcutaneous adipose tissue were significantly lower than the corresponding AUC for plasma after oral and iv administration. $C_{skin\ blister}/C_{plasma}$ ratio >4 is an indicated that ciprofloxacin mostly accumulates in the inflamed lesions, while saliva and capillary blood concentrations were similar to total plasma [55, 77].

In another study, Hollenstein et al [78] addressed the issue of tissue penetration of ciprofloxacin in obese subjects with a mean weight of 122kg using microdialysis. They found significantly lower $AUC_{tissue}/AUC_{plasma}$ ratios in obese subjects (0.45 ± 0.27 vs. 0.82 ± 0.36)[14]. The results helped them conclude that the penetration of ISF is highly impaired in obese subjects, most probably due to a reduced capillary permeability surface area in fat tissue.

An interesting application of microdialysis was extended to evaluate the effects of simulated microgravity (sμG) on the pharmacokinetics of ciprofloxacin [79]. Astronauts have been taking drugs during flights since the early days but little information is available regarding the efficacy of drugs administered during space flights. Astronauts run into the risk of infections due to long term confinement of the spacecraft and impairment of the immune system [80, 81]. Also, physiological changes induced by microgravity may affect the pharmacokinetics of antibiotics, resulting in altered concentrations at the infection site, thus affecting the way antibiotics are given in space. The study determined and compared the unbound soft tissue concentrations of ciprofloxacin(250 mg orally) by microdialysis in normal gravity (1G) and sμG and related them to plasma concentrations. Also, the disposition of ciprofloxacin in humans after 3 days of sμG in comparison to 1G was examined. It was observed that the free interstitial ciprofloxacin concentrations measured by microdialysis in the medial vastus muscle ($AUC_{free,tissue)}$ were slightly lower during sμG(1540 ± 879 ng/mL*h) than during 1G (1861 ± 1091 ng/mL*h). The free plasma concentrations were not different in sμG(2547 ± 274 ng/mL*h) and 1G (2518 ± 971 ng/mL*h), a slightly lower value of *f* was obtained in sμG(0.61 ± 0.36 vs. 0.92 ± 0.63) suggesting that tissue penetration could be

altered in microgravity [79]. However, the differences were not statistically significant, probably due to the small number of subjects that participated in our study.

Linezolid, an oxazolidinone, is approved for the treatment of nosocomial pneumonia and complicated SSSIs [8]. It shows good antimicrobial activity against various resistant Gram-positive bacteria, including methicillin- and glycopeptide-resistant *S. aureus.* Although only free, unbound data is considered for antimicrobial efficacy and most relevant pathogens are located in the ISF, most of the available linezolid PK data is based on total plasma concentrations [55, 66]. Hence, a clinical microdialysis study was performed that evaluated the penetration of linezolid into soft tissues of healthy volunteers after single- and multiple-dose administration.[66] After calibration and baseline determination, 600 mg linezolid was infused intravenously for 30 min and dialysate as well as blood samples were taken for up to 8 h. After withdrawal of the MD probes, volunteers were started on oral linezolid (600 mg) BID for 5 consecutive days [55]. The second set of MD experiments was started simultaneously with the last oral dose. Results show that after single IV administration of linezolid, $fAUC_{0-8}$ was 65.3 ± 18.2 mg*h/L and 75.8 ± 24.2 mg*h/L for skeletal muscle tissue and subcutaneous adipose tissue respectively which was significantly higher than the $fAUC_{0-8}$ of plasma (53.0 ± 11.6 mg*h/L) [66]. However, at steady state, no significant differences between concentrations in the ISF of skeletal muscle and subcutaneous adipose tissue could be detected. These findings further indicated that steady-state concentrations in both muscle ($fAUC_{24}$ muscle/MIC 58.9 ± 33.0 mg*h/L) and adipose subcutaneous tissue ($fAUC_{24}$ s.c./MIC 46.6 ± 15.9 mg*h/L) were sufficient to treat infections that are caused by pathogens with MICs of up to 4 mg/L.

PK/PD Indices

The minimum inhibitory concentration is a well-established and routinely determined susceptibility breakpoint parameter for antibiotics. Combinations of this PD marker with free unbound PK parameters to MIC-based PK/PD indices such as free time above the minimum inhibitory concentration ($fT_{>MIC}$), fAUC/MIC and fCmax/MIC have led to a much better understanding of antibiotic dosing [55]. The first PK/PD index was developed for penicillins. It correlates *in vivo* efficacy with the amount of time free drug levels stay above the MIC of the target organism ($fT_{>MIC}$)[55]. A common threshold of $fT_{>MIC} \geq 40\%$ seems to be sufficient for the clinical efficacy of β-lactam antibiotics and fCmax/MIC index values of 10 to 12 seem to be a good predictor for aminoglycosides [55]. Target $fAUC_{24}$/MIC values of 100 to 125 (Gram-negatives), 25 to 35 (gram positives), and fCmax/MIC index values of 10 have been identified for fluoroquinolones and $fT_{>MIC} \geq 85\%$ is a good outcome predictor for oxazolidinones [55]. Once these MIC-based PK/PD indices are identified, they can support the identification of optimized dosing regimens and the prediction of treatment outcome. Knowledge of the free antibiotic concentration time course in the ISF is necessary in order to establish the respective $fT_{>MIC}$, fCmax/MIC and $fAUC_{24}$/ MIC index values. While several techniques are available for determination of free, unbound concentrations, they are not all capable of characterizing dynamic changes in free ISF concentrations. Only microdialysis allows the combination of these two properties which is essential in predicting clinical efficacy with accuracy. Therefore, it is a very valuable sampling tool and has become an inherent part of evaluation and establishment of PK/PD approach.

Conclusion

The examples presented in this review show that microdialysis is applicable for a variety of purposes. Being that the method is comparatively young, there are several methodological issues still to be addressed. Analytical methods for analyzing samples gathered via microdialysis frequently have to be very sensitive due to the limited amount of sample volume available [82]. Probe calibration is another important part of any microdialysis experiment, especially when absolute interstitial space concentrations need to be reported. Recovery should be measured for each probe and individual experiment and is dependent on a variety of factors, e.g. the tissue surrounding the probe, the flow rate, length of the diffusible membrane, composition of the perfusion fluid, the molecule to be perfused, and many more [82, 83].

In summary, microdialysis is an exciting new sampling technique to evaluate pharmacokinetics of drugs in skin and soft tissues serving as an important contributor to PK/PD approaches.

CHAPTER 3
DETERMINATION OF JNJ-Q2 IN SALINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC-MS/MS)

Objective

To validate a High Performance Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) method for the determination of JNJ-Q2 in saline. The assay is designed to be used for a microdialysis study of JNJ-Q2 in soft tissues.

The validation parameters included inter/intra-batch accuracy and precision measured over 4 days, robustness, stability studies including freeze-thaw stability, stability at room temperature, in autosampler and long term storage at -80°C and stability studies.

Validation Procedure

Two standard curves (one at start and second at end of batch) and five of each quality control (QC) concentrations were assayed on each analytical day for four days to find the lower limit of quantification (LLOQ) and linear range using inter/intra-batch accuracy and precision. The acceptance criteria are defined as accuracy between 80-120% at the (LLOQ) and 85-115% for all other standards and QCs. Precision is defined as relative standard deviation and must be <20% at the LLOQ and <15% at all other concentrations. The accuracy% is calculated as follows:

$$\text{Accuracy\%} = \frac{[\text{Measured}]}{[\text{Expected}]} * 100 \qquad (3\text{-}1)$$

The acceptance criterion for accuracy of QC samples is 85%-115% except at LLOQ where it is 80%-120%. At least 67% (4 of 6) of the QC samples should be within the above limits; 33% of the QC samples (not all replicates at the same concentration) can be outside the limits. If there are more than 2 QC samples at each level, then 50%

of QC samples at each level should pass the above limits of deviation. A minimum of 75% standards (at least 6 nonzero points) should be within the limits (85%-115%) for the analytical run to qualify.

Chemicals and Equipment

Test Article

JNJ-Q2 was provided by Johnson and Johnson, Pharmaceutical Research and Development, L.L.C. The compound was stored in the original shipping vial at 2-8°C in the GLP refrigerator in an amber colored pouch.

Internal Standard (IS)

The internal standard used for the LC-MS/MS assay was JNJ-28312141-AAC (Molecular weight: 460), provided by Johnson and Johnson, Pharmaceutical Research and Development, L.L.C. The compound was stored in the original shipping vial at 2-8°C in a GLP refrigerator in an amber colored pouch.

Reagent Preparation

JNJ-Q2 Stock Standard Solution

1 mg/mL stock standard solution of JNJ-Q2 is prepared by weighing 10 mg of JNJ-Q2 and dissolving it in 10 mL methanol. This is done by adding the JNJ-Q2 to a 10 mL volumetric flask and filling with the solvent. A secondary stock of 10 µg/mL is prepared by adding 10 µL of stock standard solution to 990 µL of dilution solution (50:50 v/v methanol:saline). The amount of secondary stock can vary as long as the ratio of stock standard to saline remains the same.

JNJ-Q2 Working Standard Solutions

After the 10 µg/mL secondary stock solution is prepared, the working standards are prepared with serial dilutions using methanol:saline (1:1 v/v) [Table 3-10]. The

starting volume and total volume can vary as long as the ratio remains the same so that the same final concentrations are obtained. The highest concentration in the standard calibration curve will be 792 ng/mL.

JNJ-Q2 QC Samples

Five sets of quality controls (QC) are prepared, each set containing a lower limit of quantification, low, two mids, and high QC. The high QC should be between 75-90% of the highest standard. The mid QC should be 40-50% of the highest standard and the low QC should be no more that 3x the lower limit of quantitation (LLOQ). Additionally, there should be one QC at the lower limit of quantification. From the stock solution of 1 mg/mL a solution of 10 µg/mL is prepared.

From here dilutions are made to get the desired concentrations for the QCs [Table 3-11]. The starting volume and total volume can vary as long as the ratio remains the same so that the same final concentrations are obtained. All quality control solutions should be prepared fresh daily.

In this dilution scheme [Table 3-11] the concentrations of 3.12, 6.93, 69.3, 139 and 693 ng/mL would be used as the LLOQ, low, Medium1, Medium2, and High QCs respectively, if the standard curve concentration range is 3.1-792 ng/mL.

Internal Standard Stock Solution

1 mg/mL stock solution was prepared by weighing 10 mg of JNJ-28312141 and dissolving in

10 mL of methanol. IS stock1 solution of concentration 10 µg/mL was prepared by adding 10 µL of IS stock to 990 µL of methanol. IS stock 2 of concentration 250 ng/mL was prepared by adding 25 µL of IS stock 1 to 975 µL of methanol. The dilution scheme for IS preparation is shown in Table 3-12.

HPLC Mobile Phase

Mobile phase A: methanol with 0.05 % v/v formic acid

Formic acid was added to HPLC grade methanol in a 1 liter glass bottle to get a final concentration of 0.05 % v/v. The mobile phase was degassed for 20 min in a bath sonicator for 20 min prior to use.

Mobile phase B: ammonium acetate buffer (10 mM, pH4.0)

Accurately weighed ammonium acetate (enough to make 10 mM in 1L) and dissolved in 1L triple distilled water and adjusted the pH to 4 using glacial acetic acid. Filtered through a 0.22 micron filter and degassed by sonication for 20 min for use.

Mobile phase A and B were used in an isocratic 70:30 ratio for HPLC analysis at 0.5 mL/min flow rate.

HPLC wash solution

Mix 250 mL of triple distilled water with 2 mL Formic Acid and 50 mL Acetonitrile. To this add 700 mL methanol and mix well. The wash solution is degassed.

Sample Preparation

All samples that have saline as the matrix can be directly injected onto the HPLC column. However, samples obtained from microdialysis, both *in vivo* and *in vitro,* need to be diluted with methanol to obtain a sufficient sample volume for LC-MS/MS analysis. This is done by dividing the test sample into two equal aliquots of one with 20 µL and adding 20 µL of 10 ng/mL internal standard in methanol. The second aliquot is available as backup sample. The sample results found from the analysis need to be multiplied by two to get the true concentration.

Assay Procedure

Mass Spectroscopy Set Up

An API 4000 LC-MS/MS system consisting of PE200 series Perkin Elmer pumps and autosampler connected to the API 4000 triple quad mass spectrometer was used for the analysis. See Figure 3-1 for representative chromatograms of JNJ-Q2 and internal standard.

HPLC Pump Conditions

- Mobile Phase: (1) 70% Methanol with 0.05% v/v Formic Acid; (2) 30% Ammonium acetate buffer (10 mM, pH 4)
- Flow rate: 0.5 mL/min
- JNJ-Q2 Retention Time: ~1.4 min
- Internal Standard Retention Time: ~1.9 min

Autosampler Conditions

- Injection Volume: 10 µL
- Run time: 4 min
- 1 injection per vial (blanks may be injected more than once)
- 1 vial per sample

Analysis procedure

- Each batch of samples will be run with two calibration curves, one at the start and one at the end and quality control standards at 3 levels (Low, Medium and High) in duplicate placed throughout the run.
- With each run a blank injection of 50:50 v/v methanol:saline will be made at the start to ensure system equilibration.
- Blank 50:50 v/v methanol:saline injections can be made periodically throughout run.

Results

Reproducibility of Calibration Curve Parameters

The calibration curves (4 days) generated during the determination of inter- and intra-day precision and accuracy were linear. Consistently good correlation coefficients (r^2>0.99) were obtained in these experiments [Table 3-1]. A $1/X^2$ weighting scheme was used. This option provided a similar correlation coefficient to the linear model and a y-intercept closer to 0.

Lower Limit of Quantification

The limit of quantification (LLOQ) was identified as 3.12 ng/mL. It was defined as the lowest concentration of quality controls used in this validation for which the accuracy% was between 80-120%.

Intra-batch Variability for Quality Control Samples

Intra-batch evaluation of the analyte was performed on four different days. Intra-batch variability on day 1 was 5.88%, 7.31%, 4.50%, 2.08% and 2.39% for the 3.12, 6.93, 69.3, 139 and 693 ng/mL quality control samples, respectively (n=5, Day 1). Accuracy for day 1 of all samples ranged from 94.3% to 116 (Day 1, Table 3-2 for averages). Intra-batch variability on day 2 was 13.2%, 4.62%, 6.94%, 5.21% and 2.35% for the 3.12, 6.93, 69.3, 139 and 693 ng/mL quality control samples, respectively (n=5, Day 2). Accuracy for day 2 of all samples ranged from 105% to 113% (Day 2). Two LLOQ QC out of five fell out of the specified limit on validation day 2; therefore accuracy and precision for fourth day validation was determined. Intra-batch variability on day 3 was 5.64%, 4.76%, 2.54%, 3.78% and 2.88% for the 3.12, 6.93, 69.3, 139 and 693 ng/mL quality control samples, respectively (n=5, Day 3). Accuracy for day 3 of all samples ranged from 94.8% to 113% (Day 3, Table 3-2 for averages). Intra-batch

variability on day 4 was 1.98%, 7.09%, 5.29%, 3.09% and 4.12% for the 3.12, 6.93, 69.3, 139 and 693 ng/mL quality control samples, respectively (n=5, Day 4). Accuracy for day 4 of all samples ranged from 99.1% to 114% (Day 4, Table 3-2 for averages).

Inter-batch Variability for Quality Control Samples

Inter-batch variability was 7.25%, 5.97%, 6.45%, 4.00% and 4.42% for the 3.12, 6.93, 69.3, 138.6 and 693 ng/mL quality control samples, respectively [Table 3-3]. Mean accuracy was between 98.2% and 114% [Table 3-3]. The accuracy range based on individual QCs is already stated above.

Freeze-thaw Stability

Three cycles of freeze/thawing were performed on the LOQ, mid, and high quality controls (6.93, 138.6 and 693 ng/mL, n=3) between 05 Jan 09 and 10 Jan 09. The QCs were also injected with the standard curve before freezing to ensure correct preparation. The samples were stable for three freeze/thaw cycles [Table 3-4]. One each of the 6.93 and 693 ng/mL QC samples fell out of acceptance criteria after the first freeze/thaw cycle with an accuracy of 118 and 120%. One each MQC and HQC was out of acceptance criteria after second freeze/thaw cycle with accuracy 117 and 117%. No QC fell out of acceptance criteria after third freeze/thaw cycle. The ranges for accuracy are 106%-115%, 109%-113%, and 93.3%-98.8% for the 1st, 2nd, and 3rd, freeze/thaw cycles respectively. Therefore, it is concluded that the QCs are stable for 3 freeze thaw cycles.

To test freeze/thaw stability, standard curves were prepared fresh and analyzed with quality control samples. The standard curves were prepared fresh from the powdered compound on 07 Jan 09 for 1st and 2nd freeze/thaw cycles and on 10 Jan 07 for 3rd freeze/thaw cycle.

Stability at Room Temperature

The stability of JNJ-Q2 QCs was tested at room temperature. To do so a set of QCs were prepared and left out on the bench-top. Then after a defined length of time a fresh standard and second set of QCs were prepared from the stock standard, and injected onto the column. The concentrations of QCs left on bench top were determined using the standard curve injected. The stock standard QCs was shown to be stable for 6 hours at room temperature [Table 3-5]. The accuracy of each individual QC sample ranged from 101%-106%.

Refrigeration Stability of QCs

To test the stability of QC samples at 4ºC a set of QCs were prepared and stored at 4°C. Then after a defined length of time a fresh standard and second set of QCs were prepared from the stock standard, and injected onto the column. The concentrations of QCs stored at 4°C were determined using the standard curve injected. The QCs were shown to be stable for 8 hours at 4°C. The mean accuracy of QC sample after 48 hours ranged from 98.9% to 101% [Table 3-6].

Freezer (Long-term) Stability

Long-term stability of the stock standard solution was demonstrated for 62 days by comparing QCs stored at -70°C for 62 days with freshly prepared standards. An aliquot of each the samples prepared on 14 June 09 was frozen at -70ºC and analyzed on 15 Aug 09. The Mean accuracy at each QC level ranged from 107-111% [Table 3-7].

Auto-injector Stability of QCs

To test the stability of QC samples in auto-injector a set of QCs were prepared and stored in auto-injector. Then after a defined length of time a fresh standard curve was prepared from the stock standard, and injected onto the column. The concentrations of

QCs stored in auto-injector were determined using the standard curve injected. The QCs were shown to be stable for 8 hours in the auto-injector. The mean accuracy of QC sample after 8 hours ranged from 100% to 102% [Table 3-8].

Robustness

To test the ability of this method to endure changes a different column was used to test accuracy and precision. Water's Symmetry column no. 019138233136 was used instead of column no. 016835292102, which was used for most other validation experiments. The intra-batch precision was 10.8%, 7.72%, 4.53%, 6.04% and 3.19% for the 3.12, 6.93, 69.3, 139 and 693 ng/mL quality control samples, respectively (n=5) [Table 3-9]. Accuracy ranged from 102%-113% for individual samples.

Summary

- Range of Standard Curve – Linear over the range of 3.09–792 ng/mL
- Lower Limit of Quantification – 3.12 ng/mL
- Selectivity – No interfering chromatographic peaks were observed in injections with blank saline at the retention time of JNJ-Q2 and internal standard (JNJ-28312141)
- Intra-batch Precision of Quality Controls (QCs) – 1.98% -13.2% for the range of 3.09–792 ng/mL
- Inter-batch Precision of QCs – 4.00% -7.25% for the range of 3.09–792 ng/mL
- Intra-batch Accuracy of QCs – 94.3% -116% for the range of 3.09–792 ng/mL
- Inter-batch Accuracy of QCs – 98.2% -114% the range of 3.09–792 ng/mL
- Freeze/thaw Stability of QCs – Stable over 3 cycles for the range of 3.09–792 ng/mL
- Stability in saline at Room Temperature of QCs – Stable over 6 hours for the range of 3.09–792 ng/mL
- Freezer Stability of QCs – Stable over 2 month for the range of 3.09–792 ng/mL

- Auto Injector Stability of QCs – Stable over 8 hours for the range of 3.09–792 ng/mL
- Refrigeration Stability of QCs – Stable over 8 hours for the range of 3.09–792 ng/mL
- Robustness method: (1) Intra-batch Precision of QCs – 3.19% -10.8% for the range of 3.09–792 ng/mL; (2) Intra-batch Accuracy of QCs – 102% -113% for the range of 3.09–792 ng/mL

An LC-MS/MS method for the analysis of JNJ-Q2 in saline was developed for the analysis of microdialysis samples. The calibration curve range, 3.09–792 ng/mL, is appropriately based on the expected *in vivo* concentrations. The compound is stable over 6 hrs at room temperature, can be frozen and thawed at least 3 times before stability becomes a concern. In conclusion, JNJ-Q2 was observed to be stable under the conditions which will be encountered during an *in vivo* clinical study and its analysis.

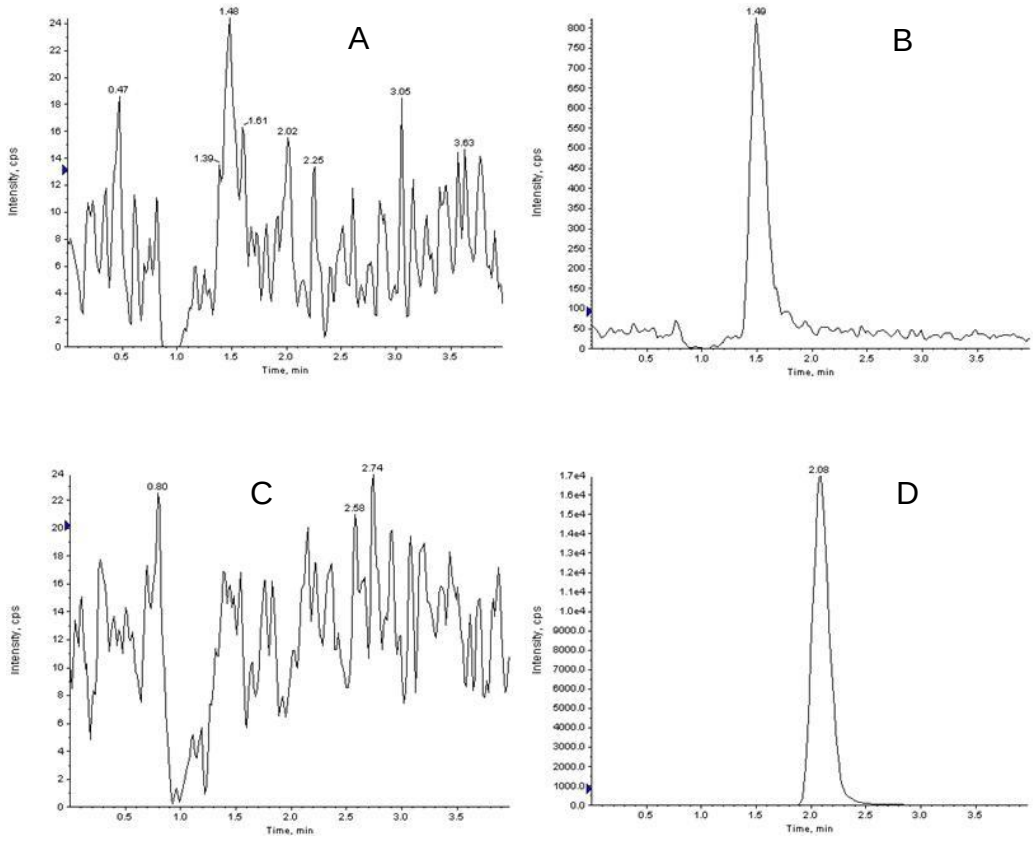

Figure 3-1. Representative chromatograms for (A) blank JNJ-Q2 (B) spiked JNJ-Q2 (3.09 ng/mL) (C) blank internal standard (D) spiked internal standard (5 ng/mL)

Table 3-1. Summaries of linearity data

	Slope	Intercept	r^2
Day 1	0.0105	-0.0090	0.9920
Day 2	0.0082	0.0002	0.9974
Day 3	0.0115	-0.0070	0.9952
Day 4	0.0105	-0.0038	0.9916
Mean	0.0102	-0.0049	0.9941
SD	0.0014	0.0040	0.0027

Table 3-2. Intra-batch variability of quality control samples (n=5 per day and per concentration)

QC		LLOQ	Low	Med 1	Med 2	High
Conc. (ng/mL)		3.12	6.93	69.3	139	693
Day 1	Mean	3.63	6.97	65.4	139	720
12/23/08	SD	0.21	0.51	2.94	2.88	17.2
	% CV	5.88	7.31	4.50	2.08	2.39
	% Accuracy	116	101	94.3	99.7	104
Day 2	Mean	3.52	7.29	72.5	146	783
01/02/09	SD	0.47	0.34	5.03	7.62	18.4
	% CV	13.2	4.62	6.94	5.21	2.35
	% Accuracy	113	105	105	106	113
Day 3	Mean	3.52	6.95	65.7	141	723
01/03/09	SD	0.2	0.33	1.67	5.33	20.8
	% CV	5.64	4.76	2.54	3.78	2.88
	% Accuracy	113	100	94.8	102	104
Day 4	Mean	3.58	7.23	68.7	143	744
01/05/09	SD	0.07	0.51	3.63	4.41	30.6
	% CV	1.98	7.09	5.29	3.09	4.12
	% Accuracy	115	104	99.2	103	107

Table 3-3. Inter-batch variability of quality control samples (n=20 per concentration)

QC	LLOQ	Low	Med 1	Med 2	High
Conc. (ng/mL)	3.12	6.93	69.3	139	693
Mean	3.56	7.11	68.1	142	742
SD	0.26	0.42	4.39	5.69	32.8
% CV	7.25	5.97	6.45	4.00	4.42
% Accuracy	114	103	98.2	103	107

Table 3-4. Freeze/thaw stability of JNJ-Q2 (n=3)

QC	Low	Med 2	High
Concentration (ng/mL)	6.93	139	693
Cycle 1			
Mean	7.35	153	795
SD	0.76	4.91	31.2
CV (%)	10.3	3.21	3.92
Accuracy (%)	106	110	115
Cycle 2			
Mean	7.76	151	786
SD	0.17	9.77	23.1
CV (%)	2.14	6.49	2.94
Accuracy (%)	112	109	113
Cycle 3			
Mean	6.71	129	684
SD	0.39	4.25	45.9
CV (%)	5.88	3.29	6.7
Accuracy (%)	96.8	93.3	98.8

Table 3-5. Room temperature Stability of JNJ-Q2 (n=3). Time= 6hr

QC	Low	Med 2	High
Concentration (ng/mL)	6.93	139	693
Mean	7.38	145	697
SD	0.66	7.54	25.3
CV (%)	8.88	5.22	3.63
Accuracy (%)	106	104	101

Table 3-6. Refrigeration stability (4°C) of JNJ-Q2 (n=3). Time= 8hr

QC	Low	Med 2	High
Concentration (ng/mL)	6.93	139	693
Mean	7.02	139	685
SD	0.11	6.63	33.8
CV (%)	1.51	4.77	4.93
Accuracy (%)	101	100	98.9

Table 3-7. Freezer stability (-70°C) of JNJ-Q2 (n=3).Time=62 days

QC	Low	Med 2	High
Concentration (ng/mL)	6.93	139	693
Mean	7.70	148	770
SD	0.17	1.59	13.3
CV (%)	2.20	1.07	1.73
Accuracy (%)	111	107	111

Table 3-8. Auto-injector stability of JNJ-Q2 (n=3). Time=62 days

QC	Low	Med 2	High
Concentration (ng/mL)	6.93	139	693
Mean	7.10	140	694
SD	0.61	9.85	44.6
CV (%)	8.63	7.04	6.43
Accuracy (%)	102	101	100

Table 3-9. Intra batch variability of JNJ-Q2 using a different column to check robustness (n=5)

QC	LLOQ	Low	Med 1	Med 2	High
Conc. (ng/mL)	3.12	6.93	69.3	139	693
Mean	3.50	7.51	70.9	146	781
SD	0.38	0.58	3.21	8.82	24.9
% CV	10.8	7.72	4.53	6.04	3.19
% Accuracy	112	108	102	105	113

Table 3-10. Calibration standard preparation of JNJ-Q2

CS Code	CS 9	CS 8	CS 7	CS 6	CS 5	CS 4	CS 3	CS 2	CS 1
Vol of DS (µL)	1840	1000	1000	1000	1000	1000	1000	1000	1000
Vol of CS added (µL)	160 of SS1	1000 of CS9	1000 of CS8	1000 of CS7	1000 of CS6	1000 of CS5	1000 of CS4	1000 of CS3	1000 of CS2
Final Conc (ng/mL)	792	396	198	99.0	49.5	24.8	12.4	6.19	3.09

Note: Dilution Solution (DS): Methanol:Saline (50:50 v/v)
JNJ-Q2 Primary Stock: 1 mg/mL in Methanol
JNJ-Q2 Secondary Stock1(SS1): 10 µg/mL

Table 3-11. Quality control sample preparation of JNJ-Q2

QC	High	Medium 2	Medium1	Low	LLOQ
Vol of DS (µL)	930	800	500	900	550
Vol of QC added (µL)	70 of SS1	200 of QC H	500 of QC M2	100 of QC M1	450 of QC L
Final Conc (ng/mL)	693	139	69.3	6.93	3.12

Table 3-12. Internal standard spiking solution

Solution ID	Prepared from Stock	Volume added (µL)	Total Volume (mL)	Solution Conc
IS Stock1 added (µL)	1mg/mL IS stock solution	10	1	10 µg/mL
IS Stock 2 added (mL)	IS Stock1	25	1	250 ng/mL

Note: 10 µL of 250 ng/mL IS Stock2 solution was added to all calibration standards and QC samples to have a final concentration of 5 ng/mL of IS. The starting volume and total volume can vary as long as the ratio remains the same so that the same final concentrations are obtained.

CHAPTER 4
IN VITRO MICRODIALYSIS OF JNJ-Q2

Objective

The aim of this study was to determine the ability of JNJ-Q2 to cross the MD membrane using two dose ranging, *in vitro* microdialysis experiments. The two experiments performed used the extraction efficiency (EE) and retrodialysis (RD) methods. In both of these methods, the percent recovery (R%) was determined. The R% had to be more than 10% if microdialysis was to be used for sampling JNJ-Q2 from soft tissues.

Microdialysis

MD is a unique tool for antibiotic sampling because the samples are taken from soft tissues, the most common site of infection, and because only the free, pharmacologically active drug is able to pass through the probe membrane. In this technique, the MD probe is placed into the tissue of interest and continuously perfused with a physiological solution (perfusate). Based on diffusion the drug passes from the tissue into the probe and is collected (dialysate). Ideally, an absolute equilibrium between the tissue and perfusate will be established. In reality, due to the fact that the MD probe is perfused at a constant flow rate of 1.5µL/min, an absolute equilibrium will not be reached. The ability of the drug to pass through the membrane and establish equilibrium at this flow rate will be established. It is termed the recovery (R) and this value has to be known to back-calculate the actual concentration at the sampling site, C_{tissue}, from the concentration in the dialysate, $C_{dialysate}$. This is done by using the following equation:

$$C_{tissue} = 100 \times C_{dialysate} \times R\%^{-1} \quad (4\text{-}1)$$

In MD, the sampling time is determined by the flow rate. A higher flow rate would lead to a shorter sampling time since there is a minimum volume requirement, but the recovery is decreased because there is not enough time for equilibration between the solution inside the probe and the surrounding media to occur. Therefore, a compromise has to be made between sample volume and flow rate. Depending on the sensitivity of the assay, the sample volume should not be smaller than 20µL. Therefore, at a flow rate of 1.5µL/min and a sampling time of 30min, 45µL are collected. The equilibration period is determined by the dead volume of the MD probe tubing which can be calculated from the manufacturer's specifications. Since this experiment will be performed using five different concentrations, all of the tubing has to be completely flushed before the sampling procedure can start. The equilibration time is the product of dead volume multiplied by flow rate.

Extraction Efficiency Method (EE)

In the EE method, blank saline is pumped through the MD probe at a flow rate of 1.5µL/min. The MD probe is then placed into the calibration tube containing analyte solution, starting with the lowest concentration. Drug will diffuse from the calibration tube into the MD probe, and the dialysate is collected for analysis. Each sample is collected for 30min after the end of a 25min equilibration period. To ensure that the prepared solution is the concentration expected within the calibration tube, two samples are taken from this tube, one before the sampling period and one after. It is important to sample from the calibration tube since it is critical to know the actual concentration in the tube to perform the calculations. The same procedure is done for the remaining five samples. After the highest concentration is completed the probe is flushed for two hours

with blank saline. All experiments are performed in triplicate. The percent recovery, R%, for the EE method is calculated as follows:

$$R\% = 100 \times \frac{C_{dialysate}}{C_{perfusate}} \quad (4\text{-}2)$$

Retrodialysis (RD)

In the RD method, the syringe contains the analyte solution that is pumped through the MD probe at a flow rate of 1.5µL/min. The MD probe is placed into a calibration tube that is filled with approximately 10mL of saline. The analyte diffuses out of the probe into the calibration tube. The loss of analyte through the membrane can then be determined from the $C_{dialysate}$. Samples are taken for 30min after the end of the 25min equilibration period. To ensure that the prepared solution is the concentration expected within the syringe, a sample is taken from the syringe before and after the sampling period. Again the lowest concentration is sampled first. In this method, the calibration tube, which contains a small amount of analyte after the sampling period, has to be exchanged with a new tube containing fresh blank saline. The same procedure is done for the remaining five samples. After the highest concentration is sampled the probe is flushed for two hours with blank saline. All experiments are performed in triplicate. The percent recovery, R%, for the RD method is calculated as follows:

$$R\% = 100 - \left(100 \times \frac{C_{dialysate}}{C_{perfusate}} \right) \quad (4\text{-}3)$$

Retrodialysis under Light-Dark Conditions

To check if JNJ-Q2 shows any instability due to its exposure to light during the *in vitro* MD study, experiments were performed in presence and in the absence of white laboratory light (set up was covered with amber films to avoid laboratory light) using RD technique. RD was chosen as it is represents the method to be used for *in vivo* experiments. This comparison was performed at a concentration level, which is the expected maximum tissue concentration after protein binding. All other conditions were kept identical.

Chemicals and Equipment

Test Article

JNJ-Q2 was provided by Johnson and Johnson, Pharmaceutical Research and Development, L.L.C. The compound was stored in the original shipping vial at 2-8°C in the GLP refrigerator in an amber colored pouch. The chemical structure of JNJ-Q2 (Molecular weight: 455.88) is shown in Figure 4-1.

Internal Standard

The internal standard used for the LC-MS/MS assay was JNJ-28312141-AAC (Molecular weight: 460.3), provided by Johnson and Johnson, Pharmaceutical Research and Development, L.L.C. The compound was stored at 2-8°C in a GLP refrigerator in an amber colored pouch.

Reagent Preparation

JNJ-Q2 Standards and Quality Control Solutions

JNJ-Q2 was provided as a pure powder. To obtain a 1 mg/mL primary stock solution, 10 mg of JNJ-Q2 was accurately weighed out and dissolved in 10 mL of pure methanol. The 1 mg/mL primary stock solution was diluted to a secondary stock solution

of 10 µg/mL with dilution solution (50:50 v/v methanol:saline). Calibration standards were then prepared by serial dilution with dilution solution. The standard curve ranged from 3.09 -792.08 ng/mL. Quality controls (QC) standards of 3.12, 6.93, 69.31, 138.61 and 693.07 ng/mL were used for LLOQ, LOQ, Medium1, Medium2, and High, respectively. The dilution scheme for the calibration standards (CS) and QCs are shown in Table 4-1 and Table 4-2. Preparation scheme and addition of IS is described in Table 4-3.

Mass Spectroscopy Mobile Phase

Mobile phase A: methanol with 0.05 % v/v Formic Acid

Formic acid was added to HPLC grade methanol in a 1 liter glass bottle to obtain a final concentration of 0.05 % v/v. The mobile phase was degassed for 20 min in a sonicator bath prior to use.

Mobile phase B: ammonium acetate buffer (10 mM, pH4.0)

Accurately weighed ammonium acetate (enough to make 10 mM in 1L) and dissolved in 1L triple distilled water and adjusted the pH to 4 using glacial acetic acid. Filtered through a 0.22 micron filter and degassed by sonication for 20 min for use.

Mobile phase A and B were used in an isocratic 70:30 ratio for HPLC analysis at 0.5 ml/min flow rate.

Sample Preparation

Calibration Solution for Microdialysis

Five calibration solutions of JNJ-Q2 over concentration range of 37.4– 513.7ng/mL were prepared. From the 1mg/mL stock solution of JNJ-Q2 in pure methanol a 10 µg/mL solution was prepared using the dilution solution (DS). This solution was used for

making further dilutions. These concentrations were selected for vitro studies as the expected maximum concentration of JNJ-Q2 in soft tissues is ~300 ng/mL.

Dialysate Samples

During the sampling period approximately 45 µL of sample were collected. Accurately 30 µL of the sample was diluted (1:1 by volume) with 10 ng/mL internal standard in methanol. The samples taken from the calibration tube and/or the syringe were also diluted 1:1 with 10 ng/mL internal standard in methanol, before and after the dialysis procedure (dilution factor of 2 for the samples).

Apparatus Setup

Prior to the start of experiments, each MD probe was checked for functionality. To do so, the inlet of the MD probe was connected to a syringe containing blank saline. The probe was then flushed manually. The probe was functional and ready to use when no liquid drops appeared on the MD probe membrane. The solution should only exit the probe from the outlet tubing. If droplets appeared on the membrane, the probe could not be used.

To control temperature, the sampling device was assembled on a heated stir plate and was maintained at 37^{o}C. During setup a 5mL syringe was filled either with blank saline solution (EE method) or analyte solution (RD method) and the enclosed air was cleared from the syringe. The syringe was put in place on the syringe pump and fastened. The pump was then connected to the inlet of the probe and run at a flow rate of 1.5 µL/min for 25 min to allow for equilibration. The probe itself was in a 15 mL centrifuge, or calibration, tube containing either blank saline (RD method) or analyte solution (EE method). Attention was paid to ensure that the membrane of the probe was completely covered with fluid and that it did not touch the wall of the tube. The

dialysate was collected in a microcentrifuge tube covered with parafilm, which helps to fix the outlet tubing from the microdialysis probe in place, and protected from laboratory light by aluminium wrap.

Sample Analysis

Mass Spectroscopy Set Up

An API 4000 LC-MS/MS system consisting of PE200 series Perkin Elmer pumps and autosampler connected to the API 4000 triple quad mass spectrometer was used for the analysis.

HPLC Pump Conditions

- Mobile Phase: 70% Methanol with 0.05% v/v Formic Acid, 30% Ammonium acetate buffer (10mM, pH 4)
- Flow rate: 0.5 mL/min
- JNJ-Q2 Retention Time: ~1.4 min
- Internal Standard Retention Time: ~1.9 min

Autosampler Conditions

- Injection Volume: 10 µL
- Run time: 4 min
- 1 injection per vial (blanks may be injected more than once)
- 1 vial per sample

Analysis Procedure

- The test samples were run with a calibration curve and quality control standards at 5 levels (LLOQ, Low, Two Medium's, and High) placed throughout the run.
- A blank injection of methanol:saline::1:1 was included.

Results

Method Validation Summary

- Range of Standard Curve – Linear over the average concentration range of 3.09 - 792.08 ng/mL

- Lower Limit of Quantification – 3.09 ng/ml (% error < 20%)
- Selectivity – No interfering chromatographic peaks were observed in blank saline
- Procedure – QCs (LLOQ, LOQ, Medium1, Medium2, and High) were used to test the accuracy and precision. The analysis was performed using a $1/X^2$ weighted linear regression.
- Accuracy and Precision – the mean accuracies for the LLOQ, LOQ, Medium1, Medium2, and High were 114.2%, 102.6%, 98.2%, 102.5% and 107.1% respectively. The average precision (CV %) was less than 7%.

Analytical runs were accepted if the correlation coefficient for the calibration curve (R^2) was at least 0.98, the calculated concentration of the low standard was within 20% of the nominal concentration, the calculated concentrations for all other standards were within 15% of their nominal concentrations, the low, mid-range and high quality controls were within 15% (20% at the LLOQ) of their nominal concentrations and at least three out of five of the QCs met the acceptance criteria at each concentration.

***In Vitro* Results**

In this experiment two *in vitro* microdialysis methods were used, the extraction efficiency (EE) and the retrodialysis (RD) methods. Both methods are acceptable to characterize how the compound interacts with the MD membrane and if the compound can freely pass through the membrane. These *in vitro* experiments were done as a preliminary study to the *in vivo* experiment. The retrodialysis method is the same method which will be used in the *in vivo* experiment for MD probe calibration. See Table 4-5 and Table 4-6 for results. The results from the comparison of exposure to light and under dark conditions for concentration ~300 ng/mL by retrodialysis are shown in Table 4-7.

Conclusions

The *in vitro* experiments confirmed that JNJ-Q2 has the ability to freely cross the microdialysis membrane. The R% was well over 10% (77.8% - 105.6%) and therefore microdialysis may be used as a sampling technique to obtain a PK profile in soft tissues. Also it was noted that carrying out the *in vitro* microdialysis under exposure to laboratory light had no significant impact on the drug stability. The recovery was found to be similar under light exposure and with no light.

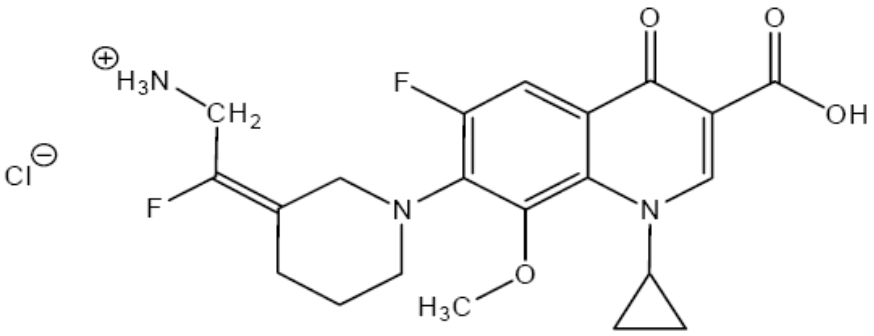

Figure 4-1. Clinical structure of JNJ-Q2

Table 4-1. Dilution schemes for calibration standard.

CS Code	CS 9	CS 8	CS 7	CS 6	CS 5	CS 4	CS 3	CS 2	CS 1
Vol of DS (µL)	920	500	500	500	500	500	500	500	500
Vol of CS added (µL)	80 of SS1	500 of CS9	500 of CS8	500 of CS7	500 of CS6	500 of CS5	500 of CS4	500 of CS3	500 of CS2
Final Conc (ng/mL)	792.08	396.04	198.02	99.01	49.50	24.75	12.38	6.19	3.09

*Dilution Solution (DS): Methanol:Saline (50:50 v/v).
JNJ-Q2 Primary Stock: 1 mg/mL in Methanol.
JNJ-Q2 Secondary Stock1 (SS1): 10 µg/mL (990µL DS + 10µL of Primary Stock).

Table 4-2. Dilution scheme for quality controls

QC	**High**	**Medium 2**	**Medium1**	**LOQ**	**LLOQ**
Vol of DS (µL)	930	800	500	900	550
Vol of QC added (µL)	70 of SS1	200 of QC H	500 of QC M2	100 of QC M1	450 of QC L
Final Conc (ng/mL)	693.07	138.61	69.31	6.93	3.12

Table 4-3. Internal standard spiking solution

Solution ID	Prepared from Stock	Volume added (µL)	Total Volume (mL)	Solution Conc
IS Stock1 added (µL)	1mg/mL IS stock solution	10	1	10 µg/mL
IS Stock 2 added (mL)	IS Stock1	25	1	250 ng/mL

*10 µL of 250 ng/mL IS Stock2 was added to all calibration standards and quality control samples to have a final concentration of 5 ng/mL of Internal standard.

Table 4-4. Inter-batch variability of quality control samples (n=20 per concentration)

QC	LLOQ	LOQ	MQC1	MQC2	HQC
Conc. (ng/mL)	3.12	6.93	69.31	138.61	693.07
Mean	3.45	7.04	68.21	141.17	729.78
SD	0.32	0.35	4.09	5.53	36.75
% CV	9.33	5.00	6.00	3.92	5.04
% Accuracy	114.26	102.58	98.21	102.53	107.13

Table 4-5. Recovery - retrodialysis method

Average Observed Concentration (ng/mL)	Mean R% (n=3)	SD
37.4	77.8	4.8
53.1	89.7	2.0
325.3	95.3	2.2
513.7	95.7	0.6
Average	89.6	*

Table 4-6. Recovery - extraction efficiency method

Average Observed Concentration (ng/mL)	Mean R% (n=3)	SD
66.9	105.6	7.79
111.6	100.7	9.12
168.3	93.1	10.97
318	96.2	9.36
485	100.6	5.37
Average	99.2	*

Table 4-7. Recovery - light & dark experiments by retrodialysis method

Average Observed Concentration (ng/mL)	Dark-Mean R% (n=3)	Average Observed Concentration (ng/mL)	Light-Mean R% (n=3)
307.7	89.7	325.3	96.6
	96.1		92.7
	98.7		96.4
Average	94.8	Average	95.3
SD	4.6	SD	2.2

CHAPTER 5
CLINICAL TISSUE DISTRIBUTION STUDY OF A NOVEL FLUOROQUINOLONE, JNJ-Q2 USING MICRODIALYSIS IN HEALTHY SUBJECTS

JNJ-Q2 is a novel fluorinated 4-quinolone oral antimicrobial agent. It is a broad spectrum fluoroquinolone with potent *in vitro* bactericidal activity against a diverse set of Gram-positive bacteria including the methicillin-resistant *S. aureus* strains (MRSA), Gram-negative, atypical respiratory and anaerobic pathogens. It is indicated for its use in treating community-acquired bacterial pneumonia and complicated skin and skin structure infections (cSSSIs), including diabetic foot infection. JNJ-Q2 displays a mechanism of action that is consistent with other members of the fluoroquinolone class, in that the compound inhibits the target enzymes DNA gyrase and topoisomerase IV *in vitro*, with IC50 values comparable to those of marketed fluoroquinolones [29].

The *in vitro* MIC value against MRSA is 0.25 µg/mL, 32-fold lower than moxifloxacin, and 8-fold lower than gemifloxacin. The *in vivo* efficacy of lethal infection efficacy models with methicillin-susceptible *S. aureus* strains (MSSA) was comparable to moxifloxacin and improved over ciprofloxacin. JNJ-Q2 was more potent against community acquired MRSA (CA-MRSA) isolate rather than linezolid or vancomycin when dosed subcutaneously. In a murine *S. pneumoniae* lower respiratory tract infection model JNJ-Q2 was more potent on an exposure basis than moxifloxacin in a murine MRSA skin infection model,JNJ-Q2 reduced the bacterial levels and skin lesion volumes to a greater extent than linezolid or vancomycin [29].

The planned clinical lead indication for JNJ-Q2 is cSSSIs including diabetic foot infection. Efficacy predictions based on total plasma concentrations might be misleading, as most infections are not located in the bloodstream but rather in the interstitial fluid (ISF) of tissues, which is the usual target site for bacterial infections [84].

In fact, it is the free, unbound drug in the ISF that is responsible for antimicrobial efficacy.

The tissue penetration and distribution of antibiotic is of great importance, since most of the infections occur in the tissue. At the infection site, the free, unbound fraction of the antibiotic is responsible for the anti-infective effect. [85] It is therefore important to access the *in vivo* penetration of JNJ-Q2 into the interstitial space fluid (ISF) of soft tissues such as s.c. adipose tissue and skeleton muscles. A technique that has been proven useful for the measurement of unbound concentrations in target tissues in human is MD.

Microdialysis is a minimally invasive sampling technique which allows the measurement of actual free drug concentrations in different tissues and organs and subsequently uses these PK findings to relate with PD observations to predict clinical efficacy. The ability to measure the free concentrations at the site of drug action over time makes microdialysis a very valuable tool for assessment of bioavailability and bioequivalence and has been recognized by industry and regulatory authorities such as the FDA [86, 87].

Furthermore, guidance from both European Medicines Evaluation Agency and the Unites States Food and Drug Administration (FDA) support the exploration of PK/PD relationships in plasma and target tissues that support dose and dose regimen selections. As such, tissue distribution studies are recommended to evaluate the PK/PD relationships for anti-infectives.

Objectives

The overall objective of this study is to evaluate penetration of the JNJ-Q2 into the ISF of s.c. and skeletal muscle tissues in healthy volunteers at a projected human

efficacious dose (400 mg) using the microdialysis technique. This study is divided into 2 parts Pilot and Main study. The hypothesis for testing is that JNJ-Q2 concentrations are measurable in the target tissues indicating the tissue penetration of the study drug.

Methods

Demographics

A total of 15 subjects participated in the study (3 in the pilot and 12 in the main). Of these subjects 10 were male and 5 were female. The ages of subjects ranged from 19-43 years old. This study enlisted 1 Asian, 2 Black, and 12 Caucasians [Table 5-1].

Study Drug

JNJ-Q2 was supplied as tablets by the sponsor, Johnson & Johnson Pharmaceutical Research and Development. Subjects received a 400mg single oral dose of JNJ-Q2 with 240mL of water. To ensure that an accurate dose is given, the study medication was administered directly into the subject's mouth under supervision of the investigator or designated study personnel.

Sampling Technique

Microdialysis is a sampling technique based on simple diffusion of free analyte through the semi permeable membrane at the tip of a microdialysis probe placed in the tissue of interest. The feasibility of this technique has been previously demonstrated in soft tissues [73,85, 88-90]. A physiological solution, i.e. saline, is continuously perfused through the probe at a low flow rate, i.e. 1.5 μl/min. Drug passes from the tissue (C_{tissue}) into the dialysate in the probe ($C_{dialysate}$) and is collected and analyzed. This principle is based on the fact that diffusion is equal in both directions across the semi permeable membrane of the microdialysis probe. However, the equilibrium between the tissue and dialysate is incomplete, $C_{tissue} > C_{dialysate}$, and the amount which is actually recovered has

to be calculated, typically by retrodialysis. [84]This is done by performing an *in vivo* calibration as described below.

$$in\_vivo\_\text{recovery}(\%) = 100 - \left(100 \times \frac{C_{\text{dialysate}}}{C_{\text{perfusate}}}\right) \quad (5\text{-}1)$$

The average of concentrations of calibration solution at the start of calibration and at the end of calibration was used for the $C_{perfusate}$ value. The concentration of the sample collected from 30 to 60 minutes after the start of the calibration time was taken as $C_{dialysate}$. Once the recovery value has been determined the concentration in the tissue is calculated as [84]:

$$C_{\text{tissue}} = 100 \times C_{\text{dialysate}} \times in\_vivo\_\text{recovery}(\%)^{-1} \quad (5\text{-}2)$$

The $C_{dialysate}$ represented here is the dialysate collected after dosing has started. Once the probes are implanted they remain in place until all study related procedures are completed. Therefore, each recovery value is specific for each probe implanted for use in the study.

Study Design

Study subjects were admitted to University of Florida Clinical Research Center (CRC). Refer to Appendix C for study criteria.The study was a single center, open-label, one-arm, non-randomized study in healthy subjects. It consisted of 2 parts: a pilot study and a main study.

Pilot

A pilot study with 3 subjects was conducted first to validate the microdialysis method with JNJ-Q2. At least 10% recovery is needed in the dialysate in order to quantify JNJ-Q2 in the target tissues. If the pilot study demonstrates a ≥10% recovery, it

were followed by the main study [55,87,88,91]. Pilot study subjects participated in a screening examination, a 1-day open-label treatment phase with end of study procedures, and a follow-up visit 1-2 weeks after the discharge [Table 5-2].

Three eligible subjects were admitted to the CRC for the microdialysis procedure on Day 1. Two microdialysis probes were inserted into the thigh, one into the medius vastus muscle and the other into the s.c. adipose tissue. JNJ-Q2 is locally administered via the MD probe at a concentration of approx. 2 µg/mL in saline at a flow rate of 1.5 µL/min for 60 minutes into the muscle and adipose tissue. Cumulative dialysate collected from 30 to 60 minutes after the start of the calibration and then individual dialysate samples every 30 minutes for 3 hours during the washout period. Pharmacokinetic plasma samples were collected before the start of the procedure, immediately following the equilibration period (1 hour) and upon completion of the washout period (4 hours).

Main

The main study involved 12 healthy male and female subjects. It consisted of a screening examination, 2-day open-label treatment phase, end-of-study procedures on Day 2, and a follow-up visit 1-2 weeks later to assess ongoing or new adverse events. The expected duration of the main study was approximately 5 weeks [Table 5-3].

Subjects were admitted on Day –1 to confirm eligibility to the study. On Day 1, microdialysis probes were inserted into the target tissues of subjects and the dialysis probe calibrated. Probe calibration was performed for 60 minutes followed by a washout period of a length determined in the pilot study. Following the washout period, microdialysis probes were continued to be infused with saline. Subjects received a single 400 mg oral dose of JNJ-Q2 on Day 1. Subjects were given a standardized lunch

by the CRC 30 min before dosing and allowed 20 min to consume it. Plasma samples were taken within 30 min predose and at 0.5, 1, 1.5, 2, 4, 6, 8, 10, 12, 16, 24 hours postdose. Blood samples for plasma protein binding analysis were also collected at 4 and 12 hours postdose. Dialysate samples were taken at 20-minute intervals for up to 12 hours. Concentrations of JNJ-Q2 in plasma (total drug concentration) and dialysate samples (free unbound concentration) were determined using the validated analytical methods.

Safety was assessed by review of adverse events, physical examination, vital signs, 12 lead ECGs, and clinical laboratory tests. Subjects were discharged from the unit on Day 2 following the 24-hour blood sampling and completion of safety assessments at the predefined time points.

A pharmacogenomic blood sample was collected from subjects who consented separately to the pharmacogenomic component of the study. Subject participation in pharmacogenomic research is optional.

While in the study unit the subjects were provided dinner on day -1; standardized breakfast, lunch, dinner and snack on day 1; and breakfast and lunch on day 2. Meals other than those standardized were provided from Shands Hospital Foodservice at University of Florida and were modified for the protocol restrictions.

Analysis

Sample analysis

The JNJ-Q2 concentration in dialysate was determined by a validated HPLC-MS/MS assay method. An API 4000 LC-MS/MS system consisting of PE200 series Perkin Elmer pumps and autosampler connected to the API 4000 triple quad mass spectrometer was used for the analysis. The mobile phase consisted of a mixture of

70% methanol with 0.05% v/v Formic Acid, and 30% ammonium acetate buffer (10mM, pH 4). The flow rate was set to 0.5 mL/min and injection volume to 10 µL. The lower limit of quantification (LLOQ) for this method was 3.12 ng/mL. The linearity for calibration curve was 3.09-792 ng/mL. To ensure system functionality throughout each run, quality controls standards (QCs) were injected at three concentration levels.

Sample preparation:

Dialysate samples obtained from MD study were diluted with methanol to obtain a sufficient sample volume for LC-MS/MS analysis. This is done by by a 1:1 dilution of dialysate with 10 ng/mL internal standard in methanol. Following the analyses, free adipose and muscle ISF tissue concentrations were calculated from measured dialysate concentrations by correcting for determined recovery% values using the formula C_{tissue}= 100 x $C_{dialysate}$ x recovery%$^{-1}$, where C_{tissue} is the drug concentration in the ISF of respective tissue.

Data analysis

Plasma and microdialysis fluid concentrations of JNJ-Q2 were subjected to pharmacokinetic analyses using non-compartmental methods. Pharmacokinetic parameters were determined by noncompartmental analysis using the commercially available software program WinNonlin 5.2 (Pharsight Corp., Mountain View, CA). The points selected for analysis were chosen using the best fit as supplied by WinNonlin. The area under the concentration–time curve from 0 to the last measured value ($AUC_{0–last}$) was calculated using the linear trapezoidal rule and $AUC_{0–\infty}$ was calculated as $AUC_{0–last}$ + C_{last}/ λ_z, where *C*last is the last concentration measured. Clearance (CL) was calculated as the dose divided by the $AUC_{0–\infty}$, and the volume of distribution (Vd) was calculated as the CL divided by λ_z. The plasma concentrations were corrected for the

free fraction based on individual protein binding. As a measure of tissue penetration, ratios of free muscle AUC to free plasma AUC (fAUC$_{muscle}$/AUC$_{plasma}$) and free adipose AUC to free plasma AUC (fAUC$_{adipoe}$/AUC$_{plasma}$) concentrations will be determined.

Results

Mean (%CV) *in vivo* recovery values of JNJ-Q2 from subjects in the pilot study for the adipose and muscle tissues were 70.8% (6.7) and 73.7% (5.5), respectively [Table 5-4]. In the pilot study, one probe malfunctioned after insertion and therefore the mean recovery value for the muscle was calculated from two subjects. These recoveries were considered high and sufficient (i.e. measurable and reproducible) to proceed to the main study. Additionally, the average concentrations at the end of the 3 hr wash out were higher than the effective LOQ of 6 ng/mL and therefore wash out was increased to 4 hrs.

Mean (%CV) *in vivo* recovery in the main study was 56.5% (29.3) and 68.1% (13.8) in adipose and muscle, respectively and the measured concentrations were adjusted accordingly. [Table 5-5]

Mean pharmacokinetic parameters are summarized in Table 5-6. MD data for adipose was calculated from only 11 subjects owing to probe failure during the experiment. Individual protein binding estimates were used to calculate unbound plasma concentrations. Mean Cmax (± SD) was 1.95 (±0.45), 0.67 (±0.14), 0.83 (±0.39) and 0.89 (±0.32) ng/mL for total plasma, free plasma, subcutaneous, adipose and skeletal muscle, respectively. t_{max} values were 4.08 (±1.72), 4.80 (±1.11), and 4.81 (±1.02) hrs for plasma, adipose and muscle, respectively. The drug's t½ in plasma was 13.6 (±5.48). Mean CL/F and Vd/F values were 12.5 (±3.87) L/hr and 223.9 (±42.8) L, respectively.

The mean protein binding was 65% and mean (±SD) free fraction in the plasma was 0.35 (±0.049). The mean concentration-time profiles for plasma, free plasma, free skeletal muscle ISF, and free s.c. adipose tissue ISF are presented in Figure 5-1. The mean $fAUC_{0\text{-}12}$ (%CV) ratios of tissue ISF compared to plasma were 1.15 (30.8) and 1.06 (44.7) for skeletal muscle and s.c. adipose tissue, respectively [Table 5-7]. Based on the AUC ratios, JNJ-Q2 penetrates the skeletal muscle and s.c. adipose tissues very well. JNJ-Q2 was well tolerated at 400mg single dose.

No serious adverse events or discontinuations from the study due to adverse events were observed in the study.

Discussion

The ability to penetrate the soft tissue at the infection site for JNJ-Q2 is important for the desired clinical outcome [92]. Thus, this MD study was designed to measure free, unbound JNJ-Q2 concentrations in the ISF of muscle and s.c. adipose tissue of 12 healthy volunteers following a single oral dose of 400 mg.

The results of these studies revealed that free unbound concentrations in the ISF of muscle and adipose tissues are comparable to the free concentrations in the plasma which is typically expected. [75, 93-95]

The study shows that JNJ-Q2 penetrates well into muscle and s.c. adipose tissue based on the $fAUC_{muscle}/fAUC_{plasma}$ and $fAUC_{adipose}/fAUC_{plasma}$ values calculated as 1.15 and 1.02, respectively. The degree of tissue penetration for JNJ-Q2 correlates well with other fluoroquinolones which are known to have good distribution in soft tissues. The respective $fAUC_{tissue}/fAUC_{plasma}$ ratios in muscle and adipose tissues for the following fluoroquinolones are: levofloxacin 0.85 and1.1 [96], moxifloxacin 0.86 and 0.81[21], gemifloxacin 1.7 and 2.4 [95], ciprofloxacin 0.57 and 0.57 [77].There is no significant

difference between the *f*AUC of plasma and *f*AUC of both tissues and between the *f*AUC of muscle and the *f*AUC of adipose tissue. There is evidence that tissue penetration by antibiotics depends on several key characteristics and chief among those are chemical and physical properties of the drug, including lipophilicity or hydrophilicity, the molecular weight, and plasma protein binding. Molecular weights are comparable between fluoroquinolones, but substantial differences with respect to hydro- and lipophilicity may be detected. The plasma protein binding of JNJ-Q2 has been demonstrated to be independent from the drug concentration similar to other fluoroquinolones in the class [97]. It was measured in each individual in this study. Peak concentrations were reached at 4.08 (±1.72) hrs which correlated to the expected gastrointestinal symptoms observed in the subjects. It has a moderate half- life time of 13.6 hrs. The high volume of distribution suggests a good tissue distribution of JNJ-Q2 into ISF which is highly advantageous because most infections are usually located here. One potential reason for high Vd is related to the ability of fluoroquinolones to concentrate intracellularly in human cells [98-100]

The concentrations of antimicrobial agents at the site of infection play an important role in the therapeutic efficacy and clinical outcome of the patient. However, caution must be taken when attempting to predict tissue penetration in patients due to the possibility of pathophysiological changes that may result in altered distribution and penetration.

In conclusion, the major advantage to the microdialysis technique is the ability to measure the free drug at the site of action, such as the ISF of soft tissues in regards to

skin and skin structure infections. JNJ-Q2 penetrates well into the ISF for these target soft tissues.

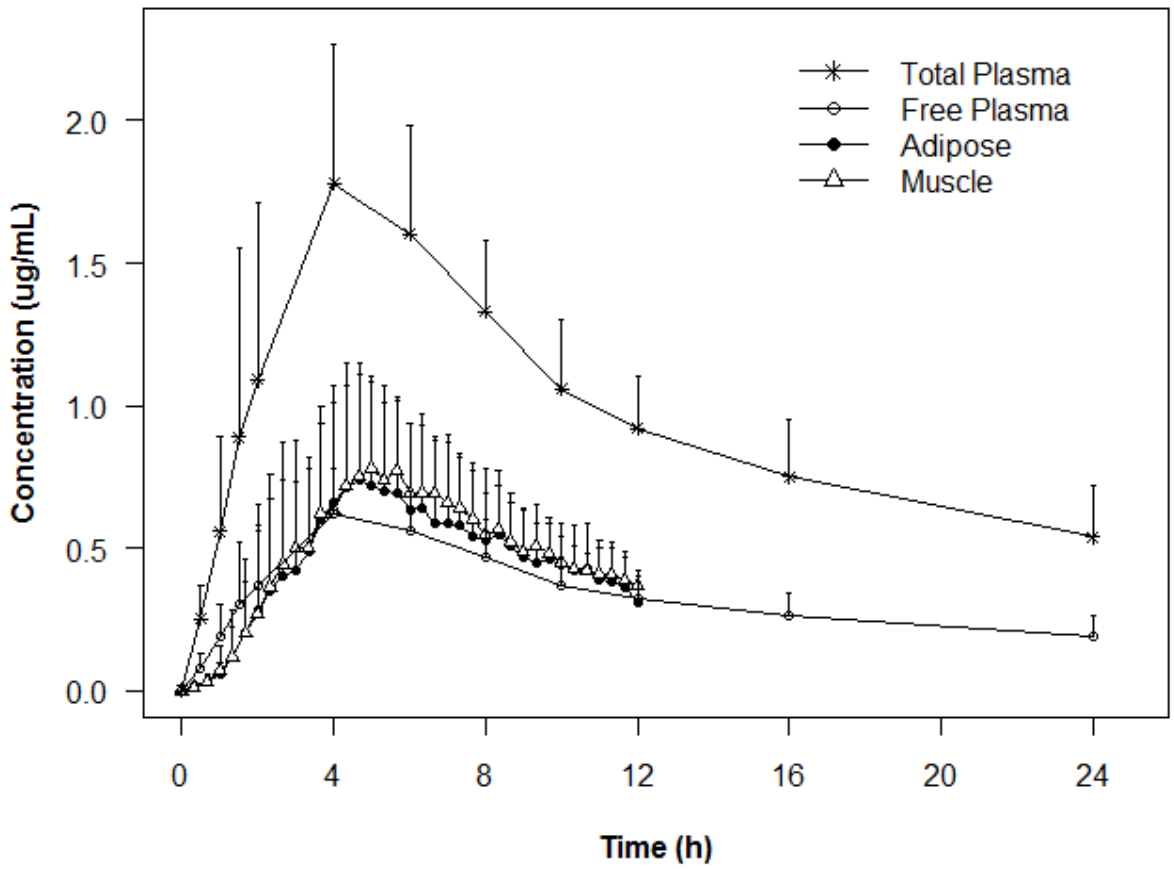

Figure 5-1. Mean JNJ-Q2 concentration-time profiles in plasma (unbound concentration) and the interstitial fluids of subcutaneous adipose and skeletal muscle following single oral dose of JNJ-Q2 (400 mg) in healthy subjects.

Table 5-1. Patient demographics: main study

Subject	Age (years)	Sex	Weight (kg)
1	26	M	70.3
2	21	M	56.5
3	26	M	90.6
4	24	F	60.1
5	25	M	100
6	44	M	79.5
7	25	F	71.7
8	23	M	87.3
9	36	M	90.6
10	23	F	48
11	43	F	61.5
12	20	F	56.1

Table 5-2. Study procedure chart for pilot study

Study Procedures	Screening (-21 to Day-1)	Pilot Study (Day 1)	Follow-Up (Day 7-14)
Signing informed consent	x		
Medical history	x		
Physical exam	x		x
Clinical lab evaluations	x	x	x
12-Lead ECG	x	x	x
Vital signs	x	x	x
Microdialysis probe insertion		x	
JNJ-Q2 administration		x	
Dialysate collection		x	
Microdialysis probe removal			x
AE evaluation		x	x

Table 5-3. Study procedure chart for main study

Study Procedures	Screening (-21 to Day-1)	Main Study (Day 1)	Follow-Up (Day 7-14)
Signing informed consent	x		
Medical history	x		
Physical exam	x		x
Clinical lab evaluations	x	x	x
12-Lead ECG	x	x	x
Vital signs	x	x	x
Microdialysis probe insertion		x	
JNJ-Q2 administration		x	
Dialysate collection		x	
Microdialysis probe removal		x	
Plasma sample collection		x	
Protein Binding sampling		x	
Pharmacogenomic sampling		x	
AE evaluation	x	x	x

Table 5-4. Mean (%CV) perfusate concentrations, cumulative dialysate concentrations, and the percent recovery of JNJ-Q2 in interstitial fluids of adipose and skeletal muscle during the pilot study

Matrix	Perfusate Conc. (µg/mL)	Dialysate Conc. (µg/mL).	Recovery (%)
Adipose n=3	1.77 (11.2)	0.515 (18.3)	70.8 (6.7)
Muscle n=2	2.06 (10.3)	0.547 (25.6)	73.7 (5.5)

Table 5-5. Mean (%CV) perfusate concentrations, cumulative dialysate concentrations, and the percent recovery of JNJ-Q2 in adipose and skeletal muscle in the main study

Tissue	Perfusate Conc. (µg/mL)	Dialysate Conc. (µg/mL)	Recovery (%)
Adipose n=11	1.54 (20.9)	0.634 (28.3)	56.5 (29.3)
Muscle n=12	1.48 (20.6)	0.456 (25.2)	68.1 (13.8)

Table 5-6. Mean (±SD) pharmacokinetic parameters of JNJ-Q2 in plasma, adipose, and muscle following a single oral dose of JNJ-Q2 (400 mg) in healthy subjects

Parameter	Total plasma[a]	Free plasma[a]	Muscle[a]	Adipose[b]
Cmax (µg/mL)	1.95 (±0.45)	0.67 (±0.14)	0.89 (±0.32)	0.83 (±0.39)
t_{max} (h)	4.08 (±1.72)	4.08 (±1.72)	4.81 (±1.02)	4.80 (±1.11)
AUC_{0-12} (h.µg/mL)	14.6 (±2.79)	5.09 (±1.05)	5.80 (±1.97)	5.43 (±2.29)
AUC_{0-24} (h.µg/mL)	23.1 (±4.29)	8.07 (±1.78)	NA	NA
$t_{½}$ (h)	13.6 (±5.48)	13.6 (±5.50)	NA	NA
V_d/F (L)	223.9 (±42.8)	648.6 (±137.4)	NA	NA
CL / F (L/h)	12.5 (±3.87)	36.0 (±10.4)	NA	NA

[a]n=12, [b]n=11, NA: Not Applicable

Table 5-7. Mean (%CV) ratio of tissue to free and total plasma JNJ-Q2 concentrations

Ratio	Cmax µg/mL	$AUC_{0-12\ h}$ h. µg/mL
Adipose/Total Plasma	0.425 (47.2)	0.372 (49.0)
Muscle/Total Plasma	0.472 (34.8)	0.406 (38.1)
Adipose/Free Plasma	1.20 (43.1)	1.06 (44.7)
Muscle/Free Plasma	1.33 (27.0)	1.15 (30.8)

CHAPTER 6
PHARMACOKINETIC/PHARMACODYNAMIC MODELING

Pharmacokinetic Modeling

Population pharmacokinetics (POP PK) is the study of the sources and correlates of variability in drug concentrations among individuals who are the target population receiving clinically relevant doses of a drug of interest [101]. Sheiner and Beal [102] introduced this approach approximately 30 years ago which then gained momentum not until the late 1980s and the early 1990s. Today many pharmaceutical companies use this approach routinely, to differing extents, during their drug development process. Advocacy by the FDA for PK screening is an important factor in the widespread adoption of this approach. FDA has a guidance document for the industry on population pharmacokinetics which serves as a standard in the drug development process [103].

POP PK seeks to identify the measurable pathophysiologic factors that cause changes in dose-concentration relationship and the extent of these changes so that, if such changes are associated with clinically significant shifts in the therapeutic index, dosage can be appropriately modified. POP PK analysis is an approach which can be used to obtain important PK and PD information not just from sparse data sets but also from relatively dense data or a combination of spare and dense data [104]. In contrast to the traditional PK evaluation, the POP PK approach collects relevant PK information in patients who are representative of the target population to be treated with the drug. Also identifies and estimates the magnitude of explained and unexplained variability in target population during evaluation. Residual variation includes intra-individual variability (random changes in a patient's parameter values over time), inter- occasion variability (change in a patient's parameter from one occasion [period] to another), drug

concentration measurement error, and model misspecification errors. These variations arise because all mathematical calculations for estimating or predicting parameter values are oversimplifications of reality [104]. This is another reason the POP PK approach has gained popularity due to the realization that the approach can be cost effective in revealing clinically important information about the determinants of inter-patient PK and PD variability in target populations. Sources of variation that contribute to differences between expectation and outcome are usually categorized as inter-individual and residual in nature. Good therapeutic practice should always be based on an understanding of PK variability. POP PK ensures that dosage adjustments can be made to accommodate differences in PK due to genetic, environmental, physiological or pathological factors. Success has been measured in terms of the increased efficiency in dosage adjustment, usually based on a subsequent Bayesian feedback procedure [105].

Recognition of the importance of developing optimum dosing strategies has led to a surge in use of POP PK approach in drug development and regulatory process. Strategies involving specifically the population approach, which are based on statistical methods such as nonlinear mixed effects modeling (NONMEM) have been advocated for investigating PK and PD variability as well as dose-concentration-effect relationships. Population approaches, if designed carefully and early, as part of the planning of the drug development program, are expected to play a significant role in contributing to drug development [106].

Population-based studies require fewer design criteria than other methods and are adaptable to the clinical setting. Individual-based PK studies can be divided into 2 types

with respect to their evaluation: compartmental and non-compartmental investigations. The latter type of study was originally thought to require fewer assumptions than the former but subsequently it has been shown that non-compartmental analyses are more restrictive and are basically compartmental in their approach. These studies estimate parameters which the compartmental investigation does not usually consider such as area under the moment curve and mean residence time [107].

Pharmacodynamic Modeling

With a good understanding of the exposure-response relationship, or PD, it may be possible to identify a quantitative link between the dose and drug effect. For antibiotics, this link has been established by correlating PK parameters that are based on free (*f*) plasma or serum concentrations to the MIC of the respective pathogen. For fluoroquinolones, the parameter that better correlates to therapeutic outcome is $fAUC_{24/}$MIC ratio [44-46, 108].

However, the MIC as single point in time estimate is not capable of characterizing the time course of neither growth nor antibiotic-induced kill or the antibiotic effect at concentrations besides the MIC [109]. In addition, the methodology to determine the actual MIC value has not yet been internationally standardized and is a source of variability between different MIC determination methods [110]. To overcome these limitations, other susceptibility breakpoints, such as, the EC50 have been suggested as the PD input for PK/PD indices. Additionally, to truly characterize the antimicrobial effect over time, time-kill experiments should be performed.

The FDA has recognized that model-based drug development provides an opportunity to streamline the drug development process. There have been numerous cases in which model-based development has saved money and time by aiding in dose

selection, clinical trial design, or support of a given dosing regimen. Therefore, this chapter will present the PK/PD techniques used to evaluate the currently recommended dosing regimen of JNJ-Q2, 400mg administered orally as a single dose. This will be done by developing a POP PK model to simultaneously fit concentration-time curves in three different tissues, i.e. plasma, skeletal muscle ISF, and s.c. adipose tissue ISF, based on plasma and microdialysis data. This will be then used to predict the target attainment rate using traditional endpoints, the *f*AUC_{24}/MIC and time-kill curves.

Materials and Methods

Population Pharmacokinetic Model Development

The PK data was obtained from a clinical microdialysis study, details of which are presented in chapter 5. Plasma data from the microdialysis experiment were adjusted for individual protein binding and compiled with the microdialysis data, which represents free concentrations, and used as full data set. A model was developed using NONMEM VII software with the ADVAN4 subroutine using First Order Conditional Estimation. To model the concentrations in plasma and tissues, a compartmental model was developed. Inter-individual variability was included in the model using exponential error model (Equation 6-7). An additive error model was used to describe the residual error (Equation 6-8). A covariate analysis was done by assessing the impact of covariates on inter-individual errors and parameters. Due to the fact that this population is comprised of twelve individuals, who were selected on the basis of a stringent protocol, elucidating any covariate impact was difficult. The specific compartments were labeled by using a FLAG indicator. In this case FLAG=1, FLAG=2, FLAG=3 indicates the central, adipose and muscle compartment, respectively. The fits of the model for the plasma concentrations are shown in Figures 6-2 and 6-3. The fits for the tissue compartments

are shown in Figures 6-4 to 6-7. The fits for plasma tissue are generally better than those for the free tissues. Model fit was assessed using visual inspection of the diagnostic (Figure 6-1, 6-8 and 6-9). Model fit was assessed by visual inspection. Lower concentrations, particularly in the absorption phase are not very well predicted by the model.

PK/PD Analysis

AUC/ MIC ratio

In vitro MIC values for various isolates of pathogens were taken from Morrow et al [28] study. The *f*AUC_{24} values were taken from the PK analysis of JNJ-Q2 after a single oral dose of 400mg (see Chapter 5). To calculate a clinically relevant target the *f*AUC_{24} was divided by MIC for the different isolates.

Time-kill curve approach

Data for time-kill analyses performed at 6 time points when exposed to JNJ-Q2 were taken from the study by Morrow et al [28] for the strains MRSA OC 8525, MRSA OC 11696 and MRSA OC 2838. An Emax model has been successfully applied to describe the relationship between concentration and effect [36, 53]. The study used a modified Emax model to evaluate antimicrobial activity using Scientist software.[54] The MIC for the MRSA strains is 0.25 ug/mL.

Results

Population Pharmacokinetic Modeling

A two-compartment body model with elimination from the central compartment was able to accurately fit the data [Figure 6-1]. This model was developed using

concentrations from plasma and both tissues. The deferential equations for this model are as follows:

$$\frac{dA(1)}{dt} = -Ka * A(1) \quad (6\text{-}1)$$

$$\frac{dA(2)}{dt} = -K23 * A(2) + K32 * A(3) - Ke * A(2) + Ka * A(1) \quad (6\text{-}2)$$

$$\frac{dA(3)}{dt} = K23 * A(2) - K32 * A(3) \quad (6\text{-}3)$$

In the above equations, Ka and Ke represent the absorption and elimination rate constants.K12 and K21 are transfer rate constants. Also, since volumes in ISF of tissues may not be equal an additional parameter was added to the model to account for this, termed the distribution factor for muscle or adipose (FM and FA). Therefore, the equations for the concentration in plasma, skeletal muscle, and s.c. adipose tissue respectively are:

$$Cplasma = \frac{A(2)}{Vc} \quad (6\text{-}4)$$

$$Cadipose = \frac{A(3) * K32 * FA}{K23 * Vc} \quad (6\text{-}5)$$

$$Cmuscle = \frac{A(3) * K32 * FM}{K23 * Vc} \quad (6\text{-}6)$$

The inter-subject variability was modeled assuming exponential error as follows:

$$P_i = P_{pop} \exp(\eta_i) \quad (6\text{-}7)$$

where P_{pop} is typical value for population, $\eta \sim N(0,\omega^2)$ is a normally distributed inter-individual random effect.

The residual variability was modeled using an additive error:

$$C_{obs} = C_{pred} + \varepsilon_i \quad (6\text{-}8)$$

Where C_{obs} the observed concentration, C_{pred} the predicted concentration by individual parameter, $\varepsilon_i \sim N(0,\sigma^2)$ is the residual random effect. Individual patient PK parameters

for which random effects were included in the model were calculated using the posterior conditional estimation technique using first-order conditional estimation. Diagnostic plots and objective function values were used to check for model fitting and selection. Model stability was confirmed by 800 successful non parametric bootstrap replicates which generated 95% confidence intervals which were then compared with the original estimates.(Table 6-1)

AUC/MIC Index

Table 6-2 lists the *f*AUC_{24}/MIC ratio for JNJ-Q2 against different strains of bacteria. *f*AUC_{24}/MIC ratios for Gram-negative pathogens, *Pseudomonas aeruginosa , Klebsiella pneumonia* and *Haemophilus influenzae* were 4.04, 32.3 and 538, respectively. Gram-positive isolates of ciprofloxacin resistant *S. pneumoniae*, MRSA, ciprofloxacin resistant MRSA, ciprofloxacin resistant MRSE and *Streptococcus pyogens* had *f*AUC_{24}/MIC ratio of 32.3 and for *S. pneumoniae* was 67.3. Literature suggests for fluoroquinolones, an AUC_{24}/MIC ratio of 100 to 125 correlates with optimal clinical and microbiological outcomes in seriously ill patients infected with Gram-negative pathogens and *Pseudomonas aeruginosa* and is said to be much lower for Gram-positive bacteria[111]. However, there has been considerable controversy as to whether or not this PD target applies to all patient populations and all organisms. Data from *in vitro* and animal models of infection that have recently emerged suggest that, for *S. pneumoniae*, the optimal AUC_{24}/MIC ratio is much lower than 100 to 125. In a study by Lister and Sanders for levofloxacin and ciprofloxacin, AUC_{24}/MIC ratios of 32 to 64 were associated with eradication of *S. pneumoniae* from an *in vitro* model of infection [112]. Also other studies in animal models for pneumococcal infection had AUC_{24}/MIC ratios

of 25 to 34 for ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, moxifloxacin, and sitafloxacin [111].

Like all fluoroquinolones JNJ-Q2 belongs to the class of concentration-dependent antibiotics, therefore the ratio AUC_{24}/MIC is the most relevant PK or pharmacodynamic (PD) parameter to determine antibacterial effects [113] and to predict clinical efficacy [22]. It needs to be pointed out that breakpoint values are generally based on total drug concentrations, whereas free concentrations of JNJ-Q2 in tissues and plasma were used for PK and PD calculations in the present study. Based on PK and PD calculations, it is tempting to speculate that JNJ-Q2 will effectively combat bacteria in the extracellular space fluid in soft tissues. However, tissue PK data should also be derived from patients with cSSSIs, because tissue penetration might be significantly altered by infection.

Therefore, though this index may not be solely used to base dosing decisions on but can be combined with stochastic modeling techniques to predict efficacious breakpoints for fluoroquinolones.

Modeling of Time Kill-Curves

A modified Emax model was developed to describe the bactericidal properties of JNJ-Q2 over time. Parameters such as the maximum number of bacteria (Nmax) at the end of the growth phase, adaptation rate term (x) and Hill factor (h) have been included in a PK/PD model to optimize the models.

$$\frac{dN0}{dt} = \left[k\left(1 - \frac{N0}{Nmax}\right)\right] * N0 \quad (6\text{-}7)$$

$$\frac{dN}{dt} = \left[k\left(1 - \frac{N}{Nmax}\right)(1 - exp^{-xt}) - \left(\frac{Emax * C^h}{EC50^h + C^h}\right)\right] * N \quad (6\text{-}8)$$

The results are shown in Table 6-3 and in Figures 6-10 to 6-12. The model predicted the kill and growth curves well. It was observed that the killing effect is more pronounced with increasing the concentration. No re-growth was seen up to 8 hours in all the strains. Model selection was based on the model selection criterion provided by the program, as well as visual inspection of the fitted curves.

The parameters generated from the PD model were successfully used to simulate the different MIC strengths ranging from 0.25 up to 16 times MIC. No re-growth is seen at 4 x MIC strength for MRSA OC 8525 and at 2 x MIC for MRSA OC 11696. A concentration of 16 x MIC is needed to avoid re-growth of bacteria for MRSA OC 2838 strain. The minimum MIC strength to prevent bacterial re-growth is between the range 2-16 for the 3 MRSA strains simulated. (Figures 6-13, 6-14, 6-15)

No re-growth was observed with the different simulated doses of 200mg, 300mg, 400mg and 500mg for MRSA OC 8525 and 11696 strains but re growth seen in MRSA 2838. At the 400 mg once a day oral dosing regimen a bacterial kill of 3 log units is observed at 24 hrs for MRSA OC 11696 and a 2 log kill for MRSA OC 8525 strain. For MRSA OC 2838 re-growth is seen after 10 hrs and hence a higher dose (greater than 500mg) may be needed to see any effect. (Figures 6-16, 6-17, 6-18)

400mg oral dose once a day seems to be an adequate dosing regimen for certain clinical strains of MRSA..It should be stressed, that the resulting simulations are valid only for expected outcome in the *in vitro* system but do not necessarily reflect therapeutic outcome *in vivo* for the same concentrations.

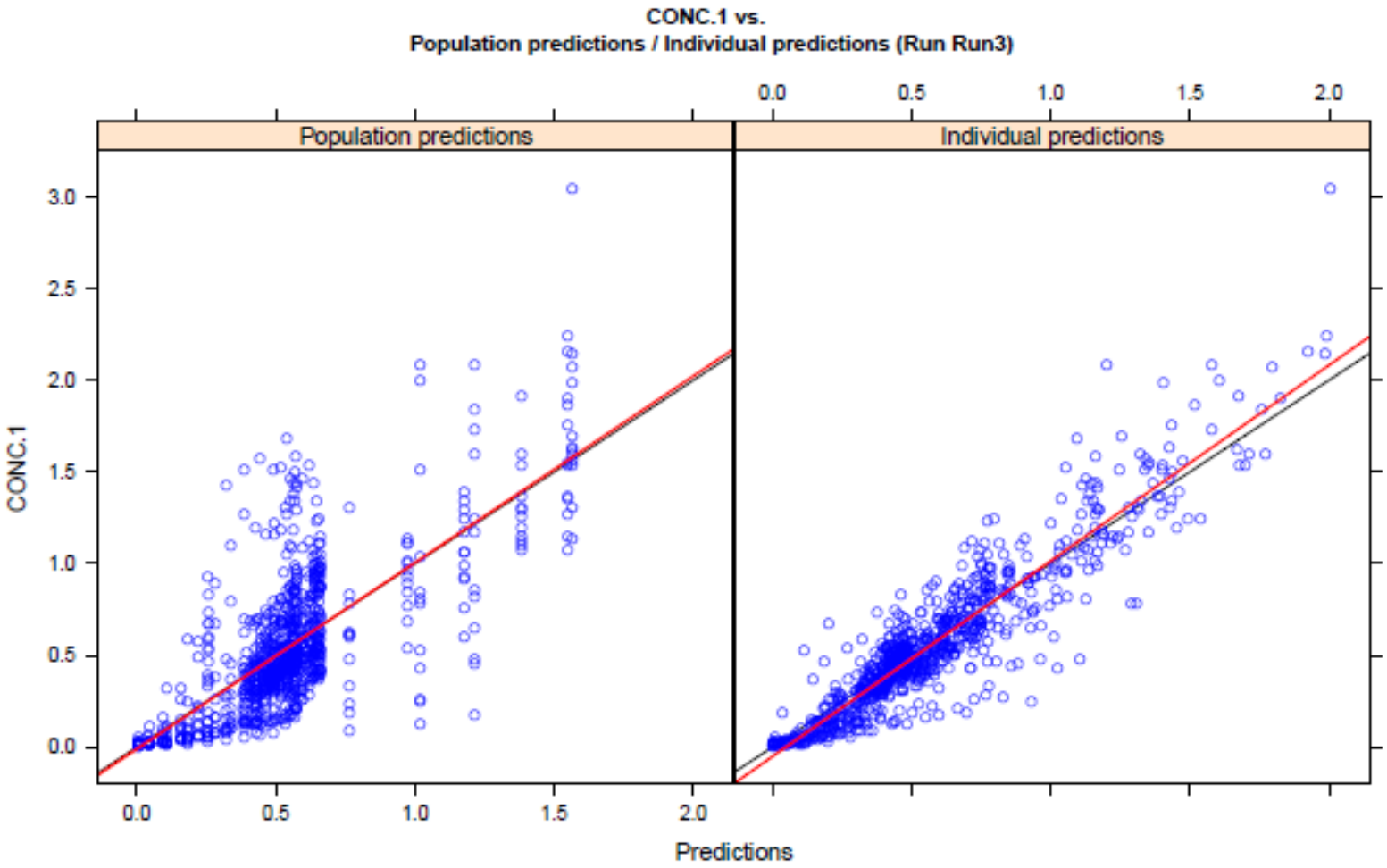

Figure 6-1. Observations versus population and individual predictions for all compartments. Predicted concentrations (μg/mL): x- axis and observed concentrations (μg/mL): y-axis

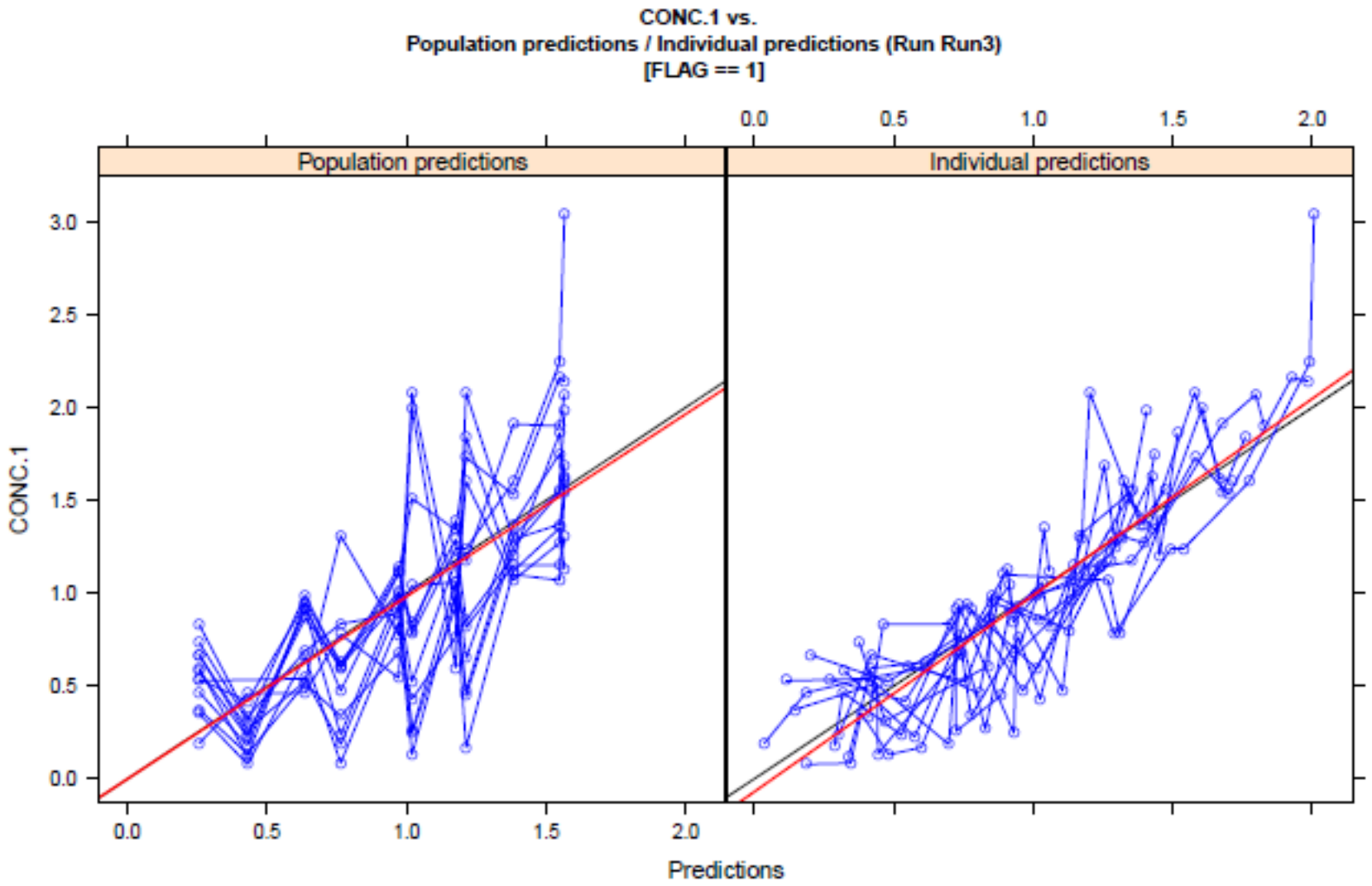

Figure 6-2. Observation versus population and individual predictions for plasma concentrations. Predicted concentrations (μg/mL): x- axis and observed concentrations (μg/mL): y-axis

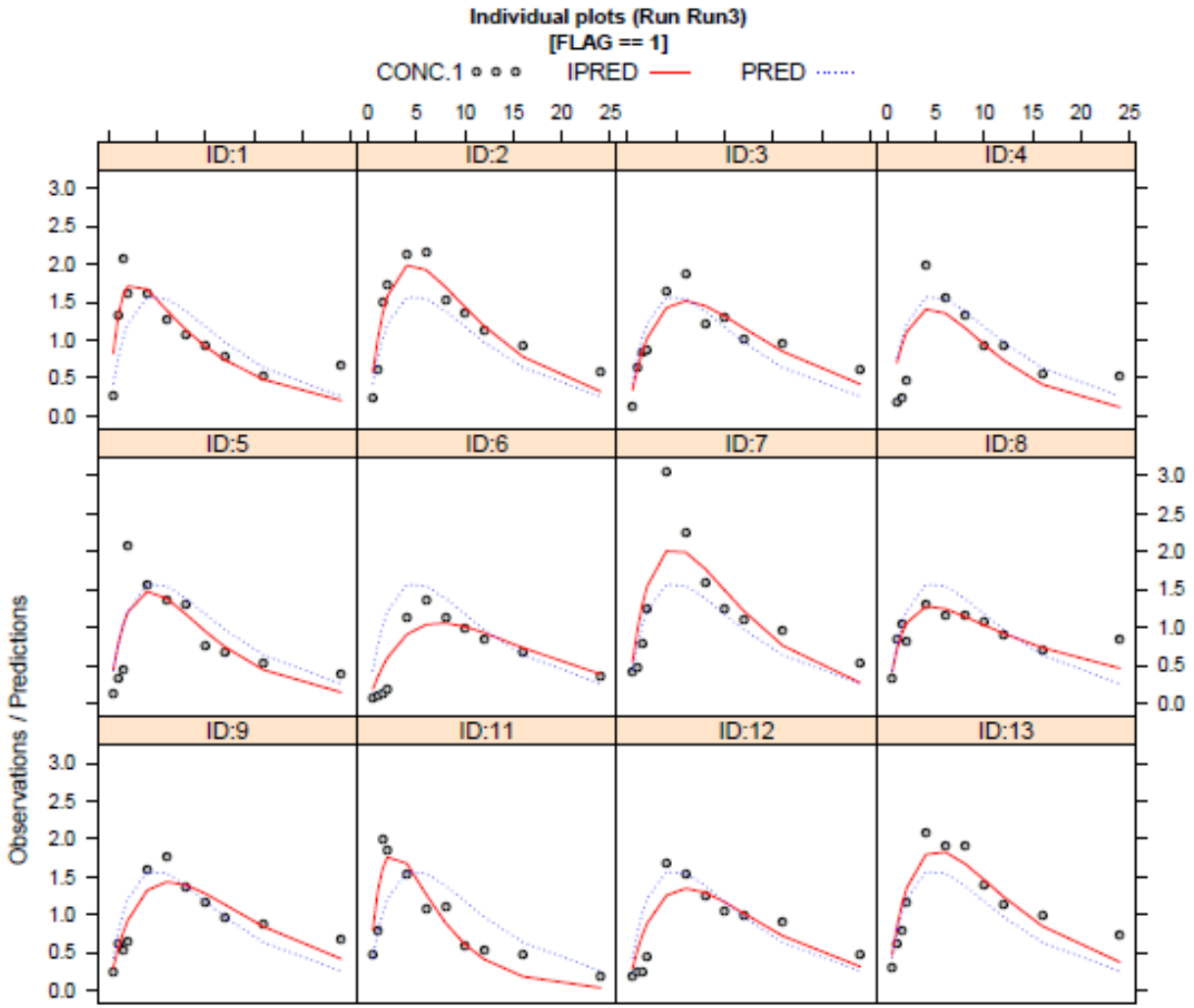

Figure 6-3. Individual fits for plasma. Time (hr): x- axis and observed/ predicted concentrations (μg/mL) : y-axis

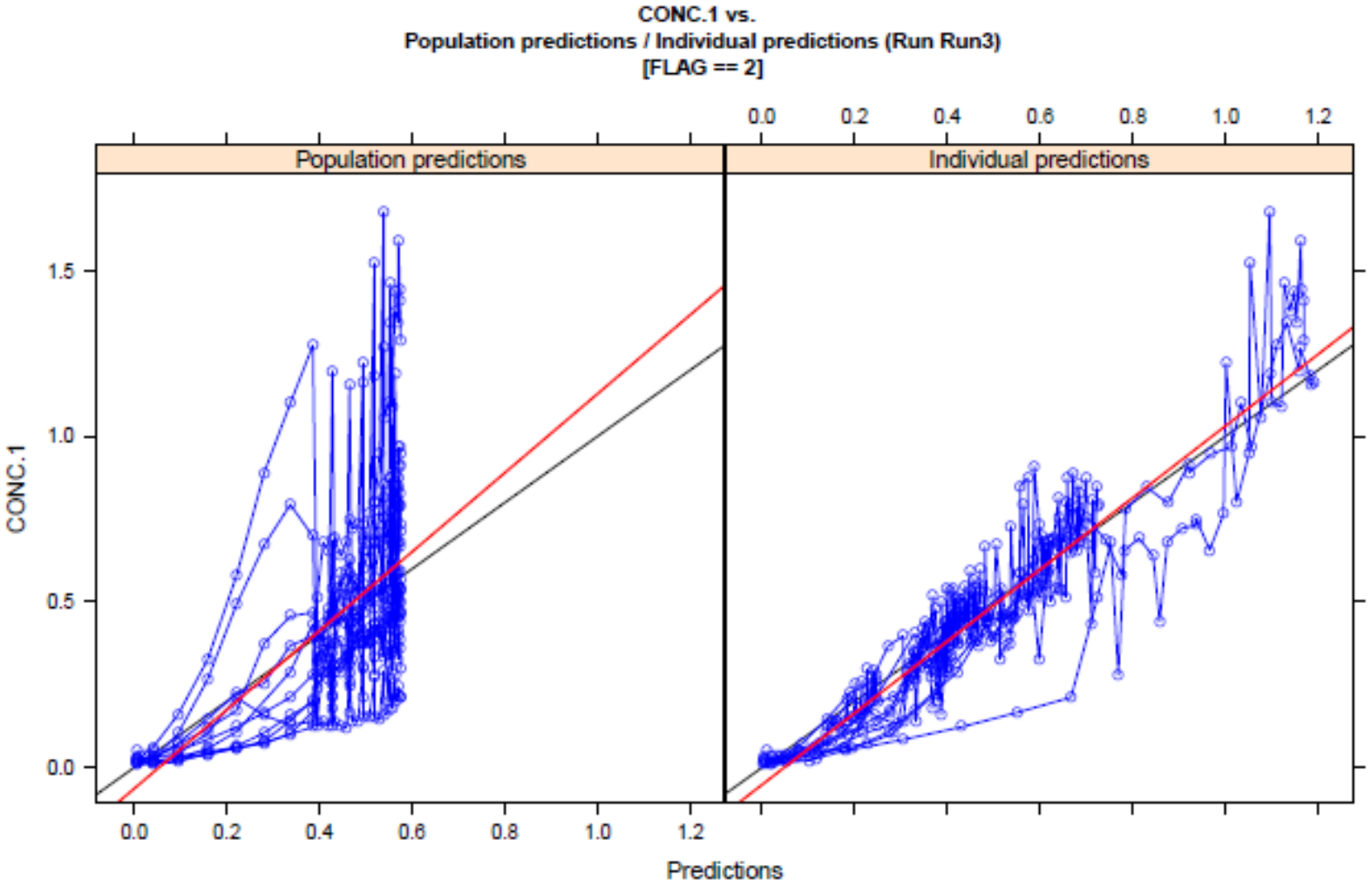

Figure 6-4. Observations versus population and individual predictions for adipose tissue. Predicted concentrations (μg/mL): x- axis and observed concentrations (μg/mL): y-axis

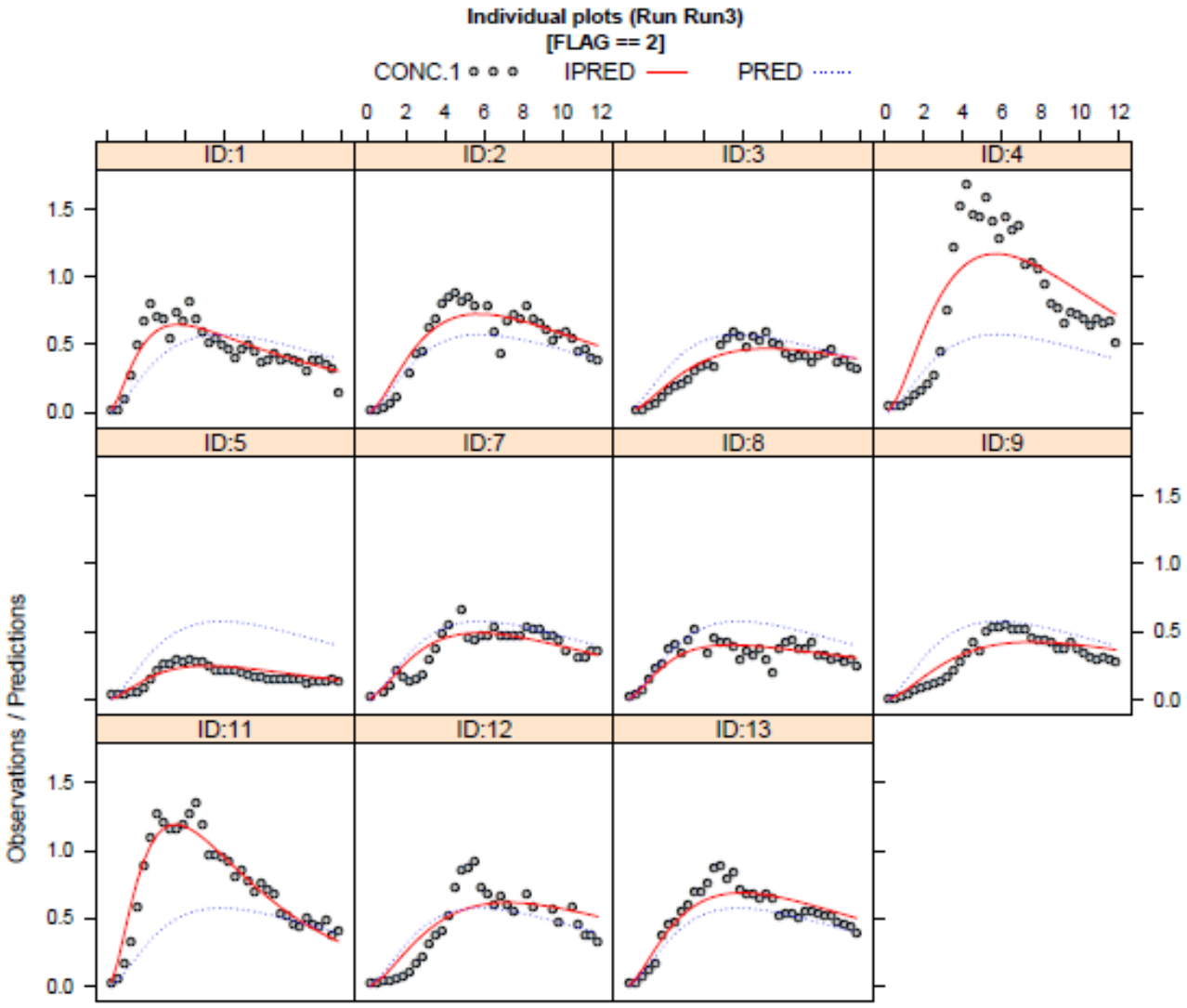

Figure 6-5. Individual fits for adipose tissue. Time (hr): x- axis and observed/ predicted concentrations (μg/mL) : y-axis

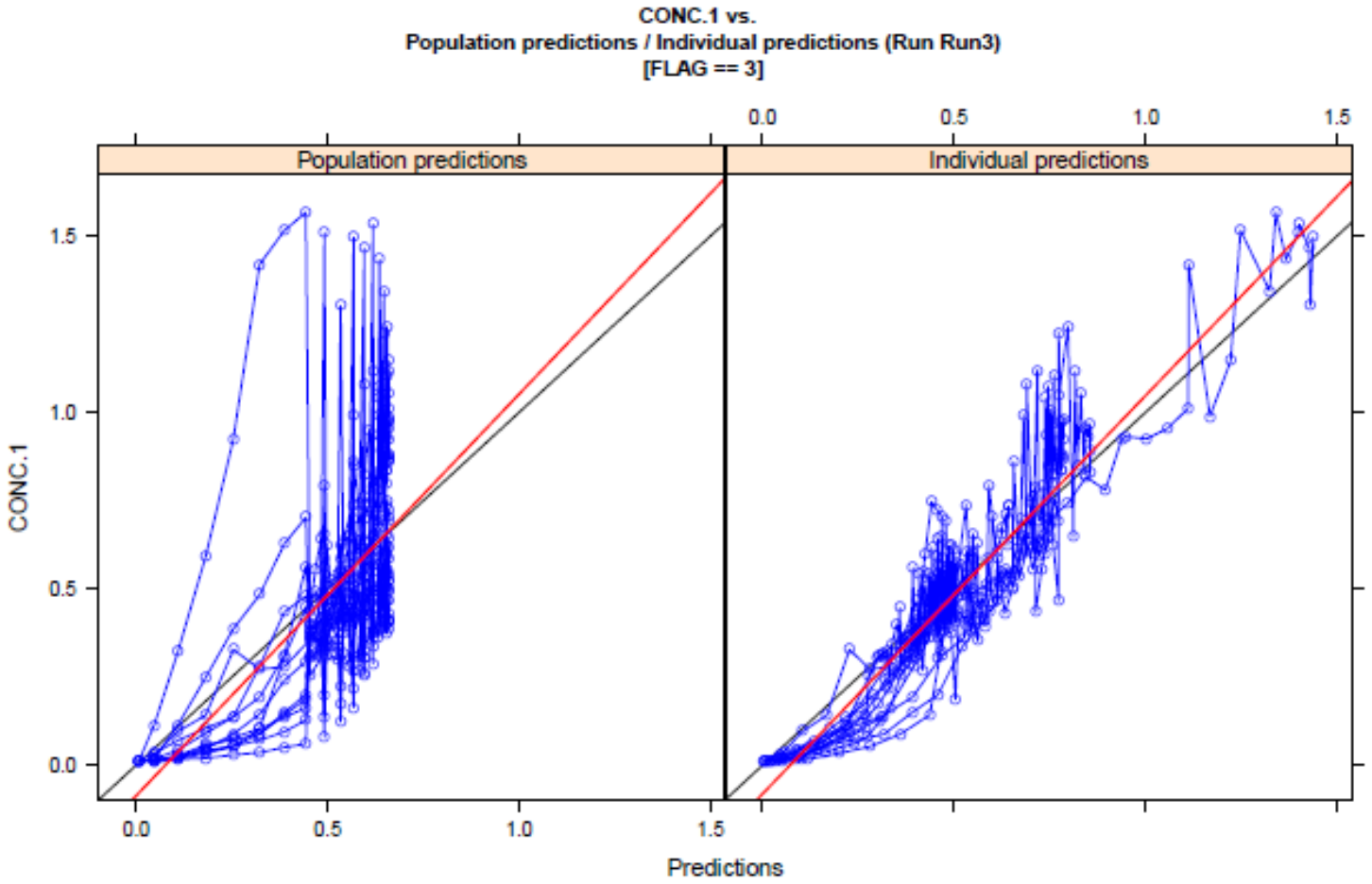

Figure 6-6. Observations versus population and individual predictions for muscle. Predicted concentrations (μg/mL): x-axis and observed concentrations (μg/mL): y-axis.

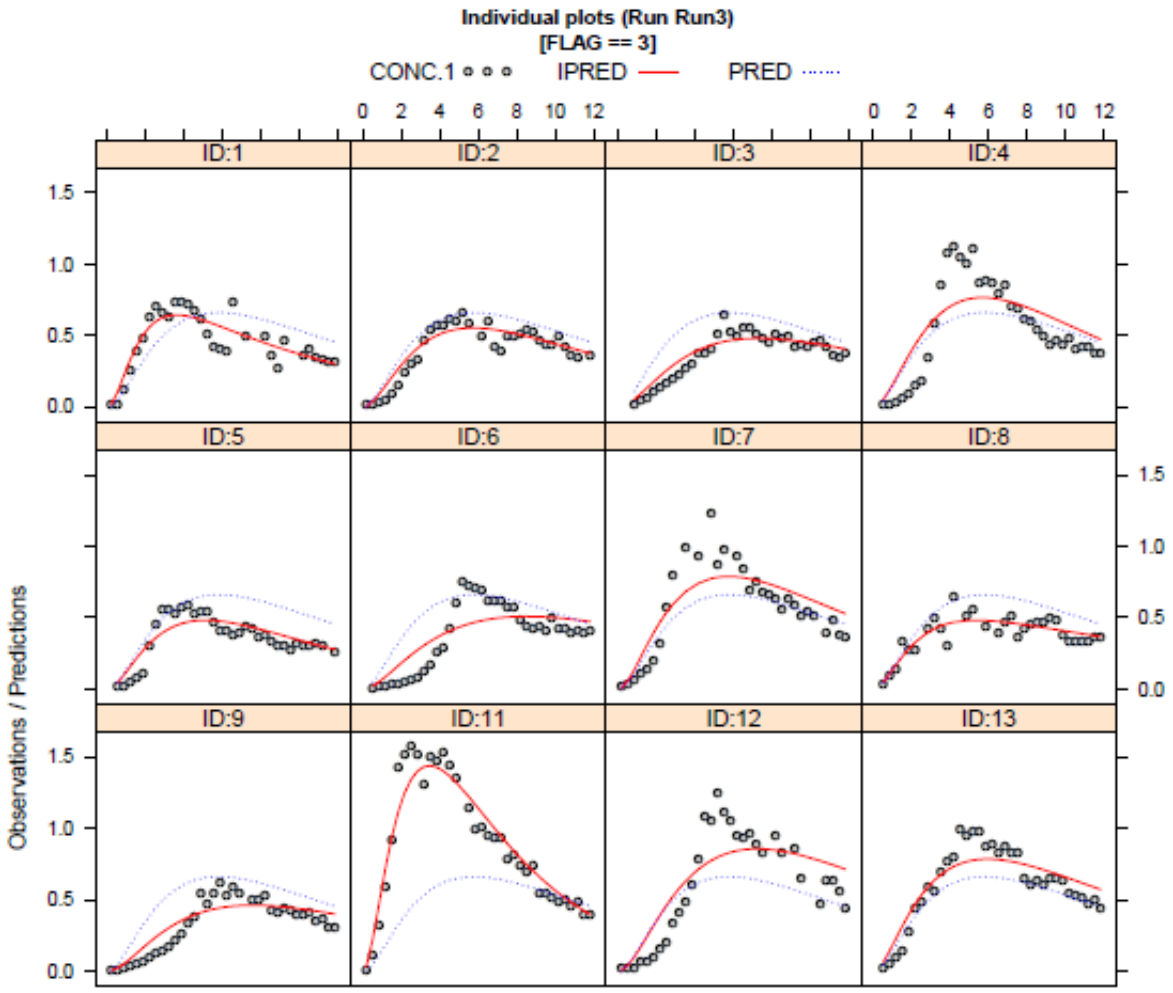

Figure 6-7. Individual fits for muscle. Time (hr): x- axis and observed/ predicted concentrations (μg/mL) : y-axis.

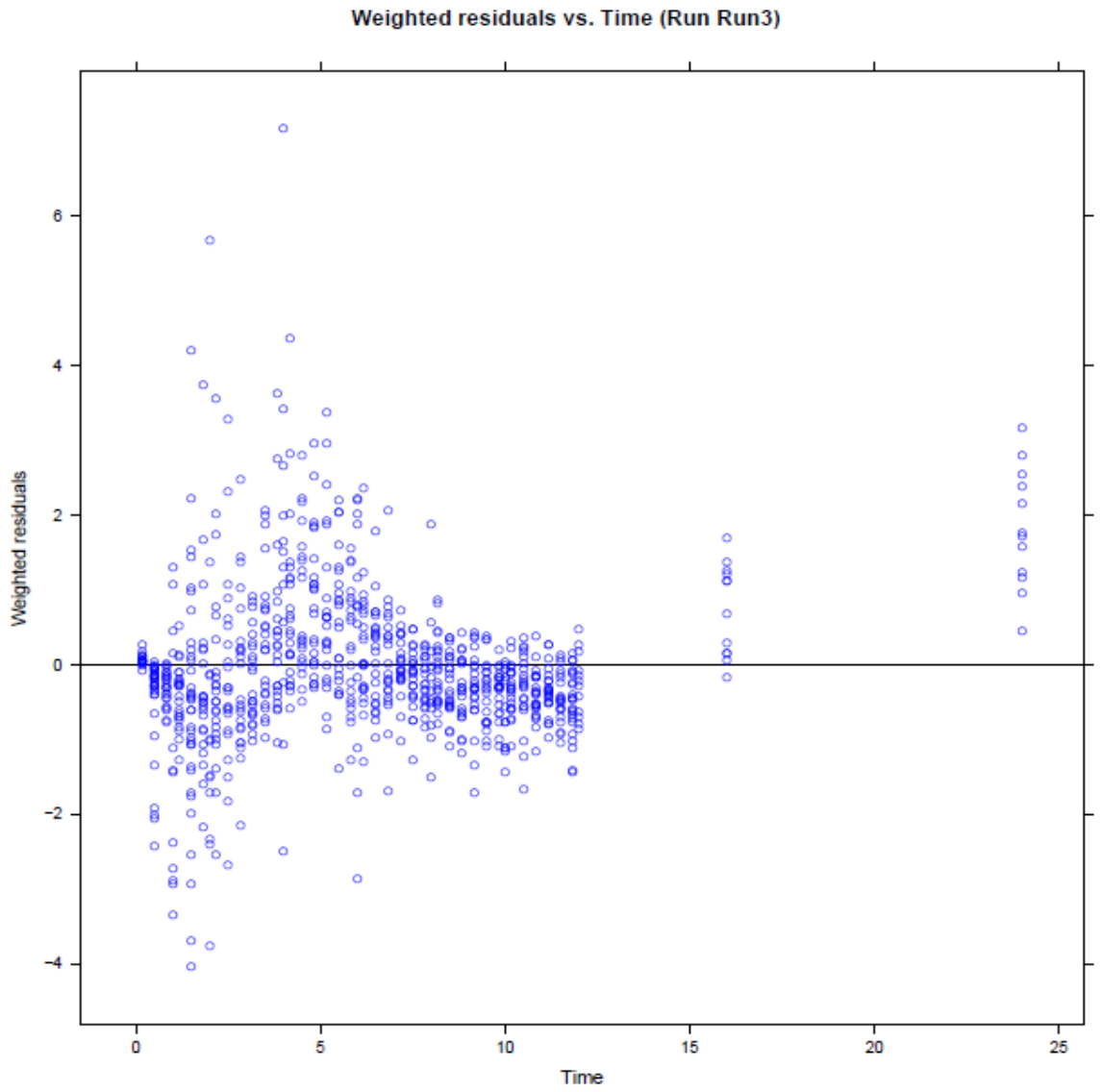

Figure 6-8. Weighted residuals plot versus time for all compartments

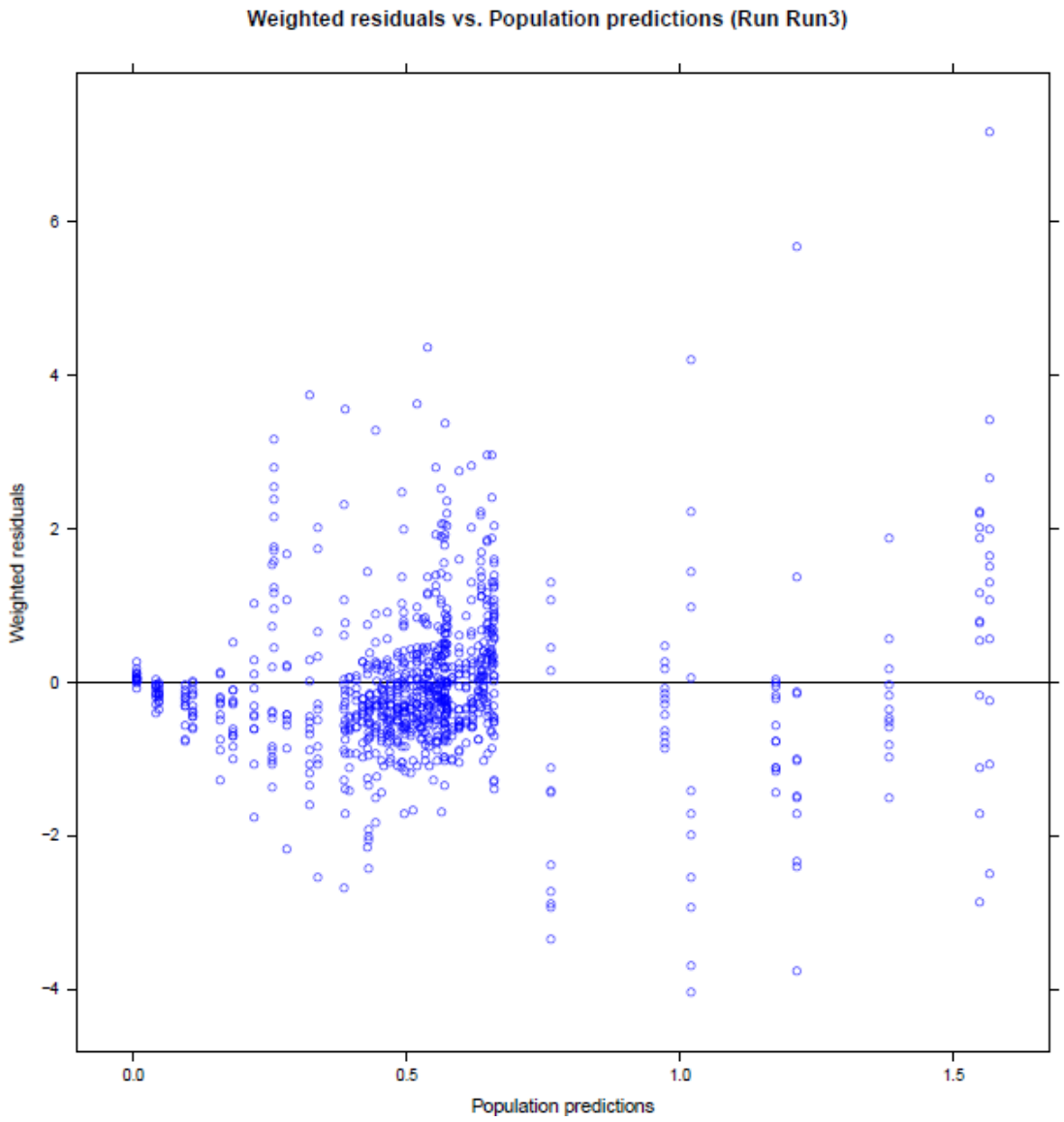

Figure 6-9. Weighted residuals versus population predictions for all compartments

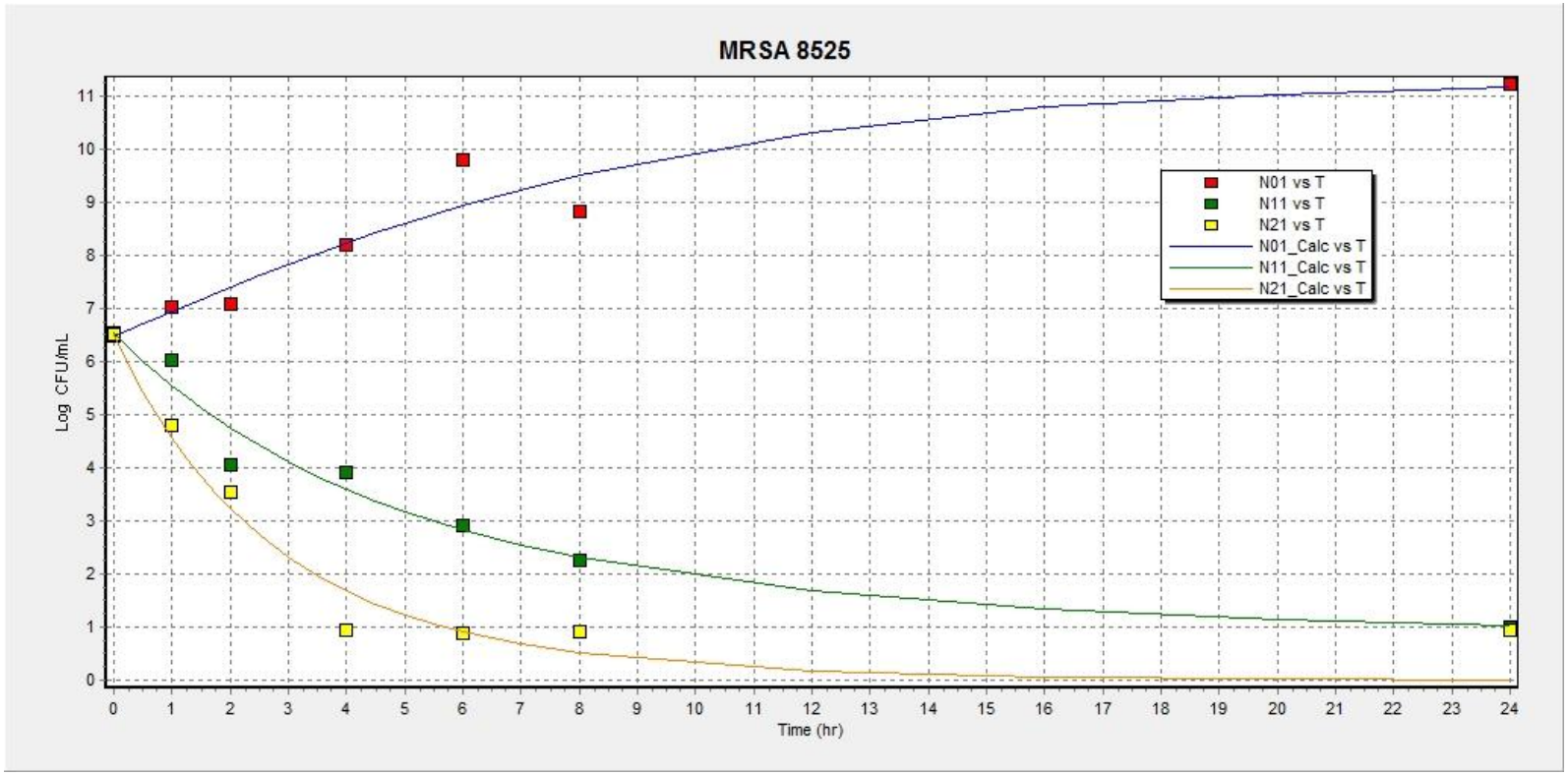

Figure 6-10. Curve fit for MRSA OC 8525

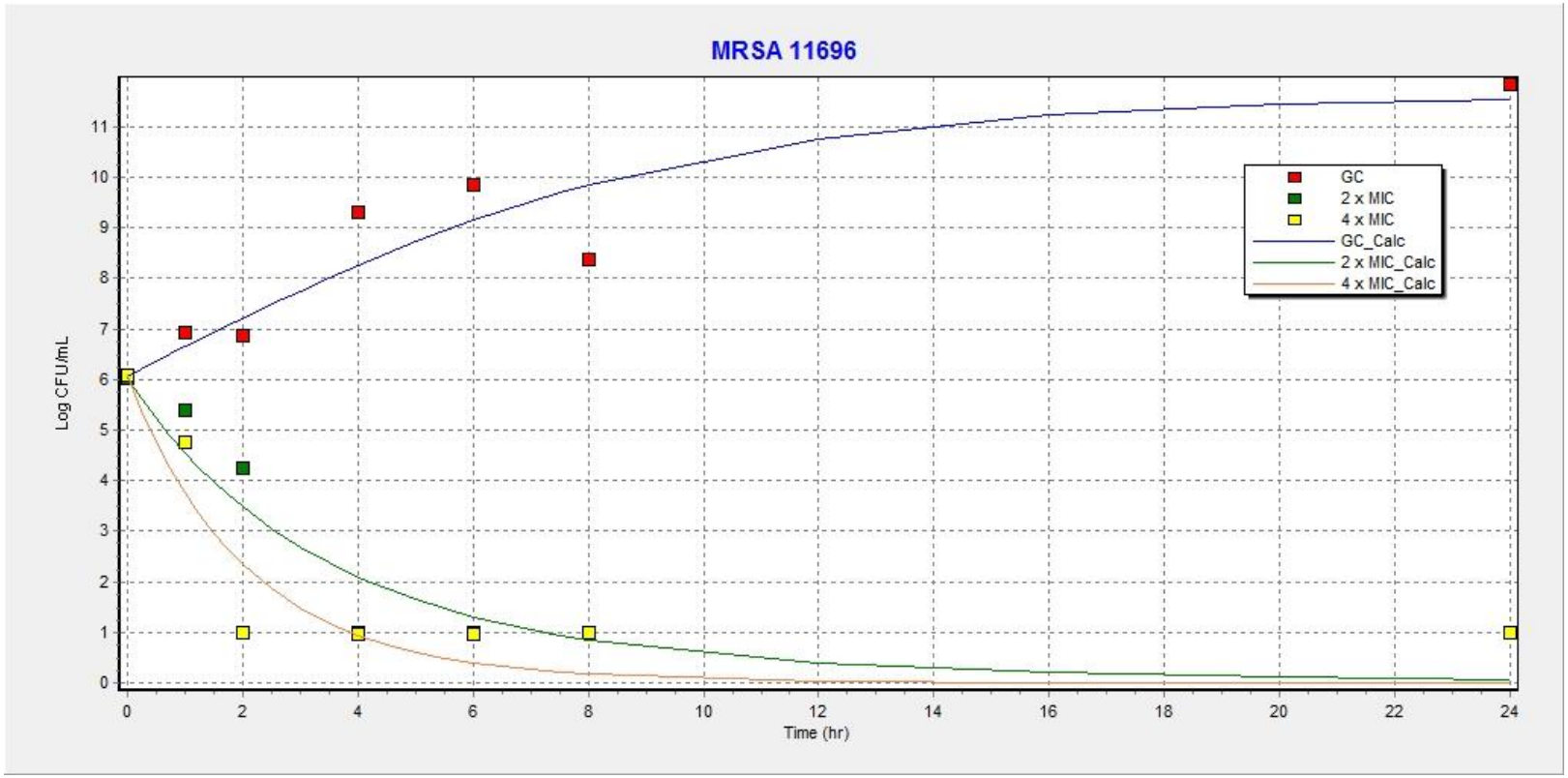

Figure 6-11. Curve fit for MRSA OC 11696

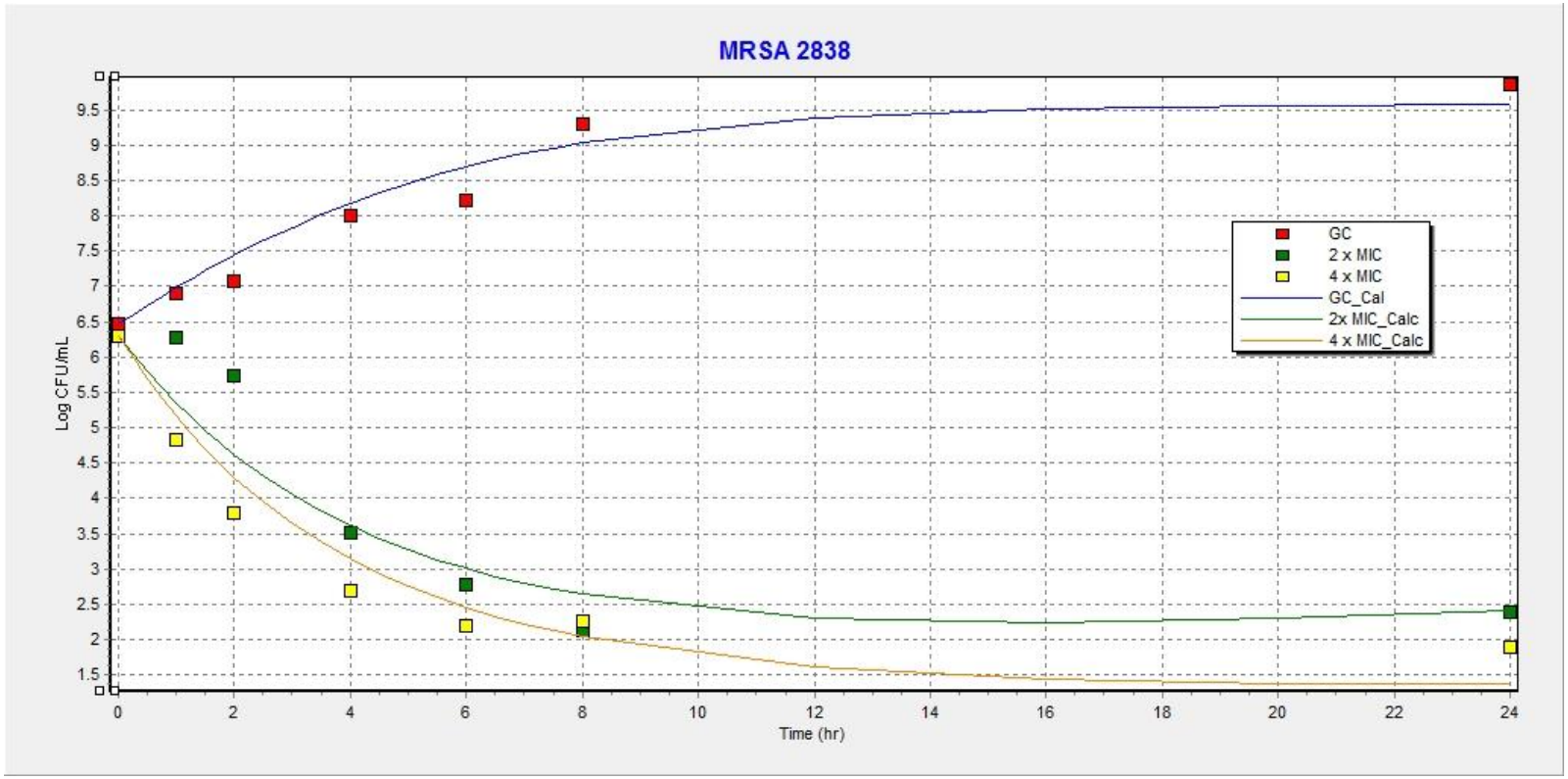

Figure 6-12. Curve fit for MRSA OC 2838

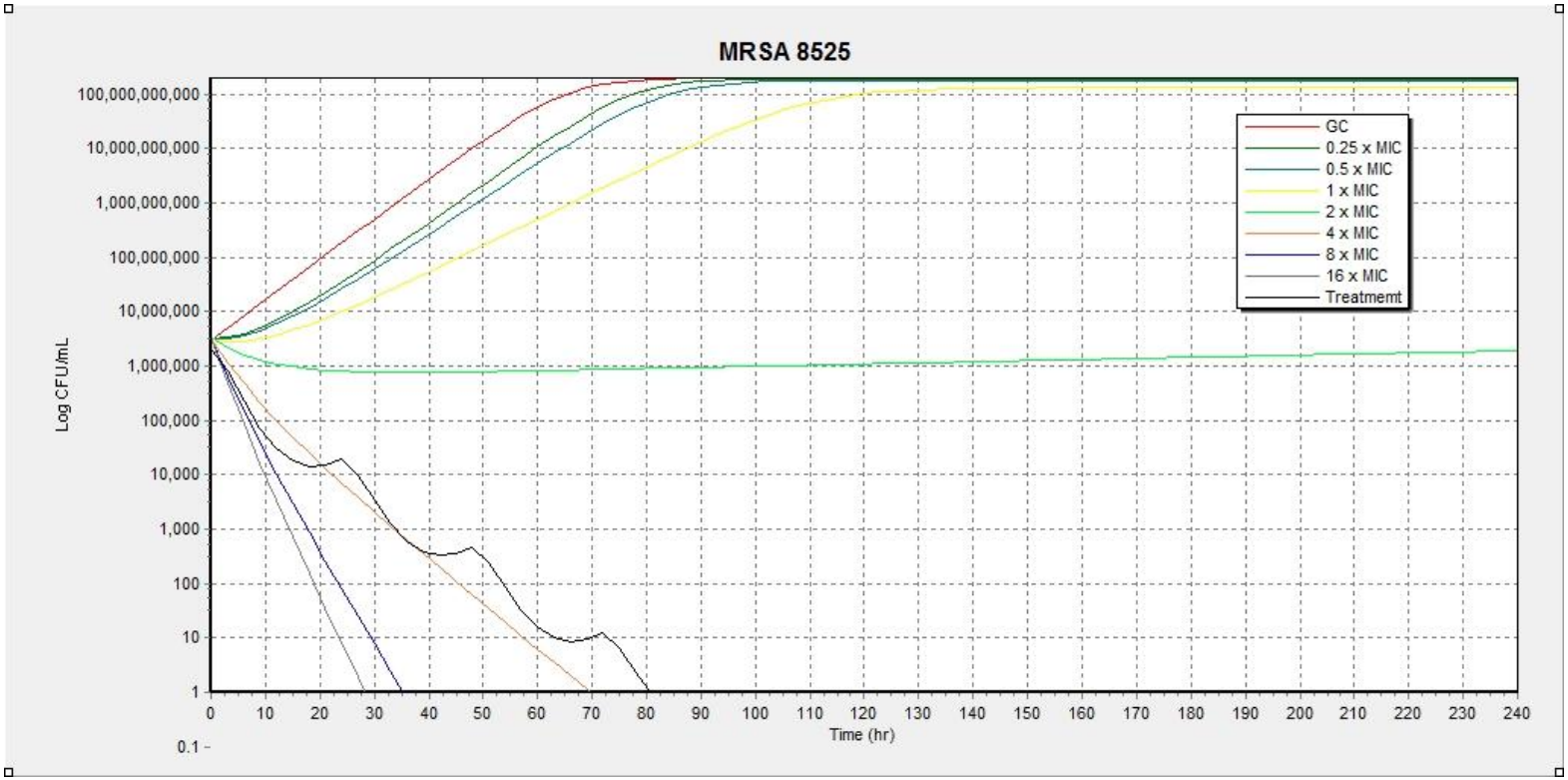

Figure 6-13. Simulated curves for bacterial kill over a range of MIC strengths for MRSA OC 8525 & once a day oral administration of 400mg JNJ-Q2

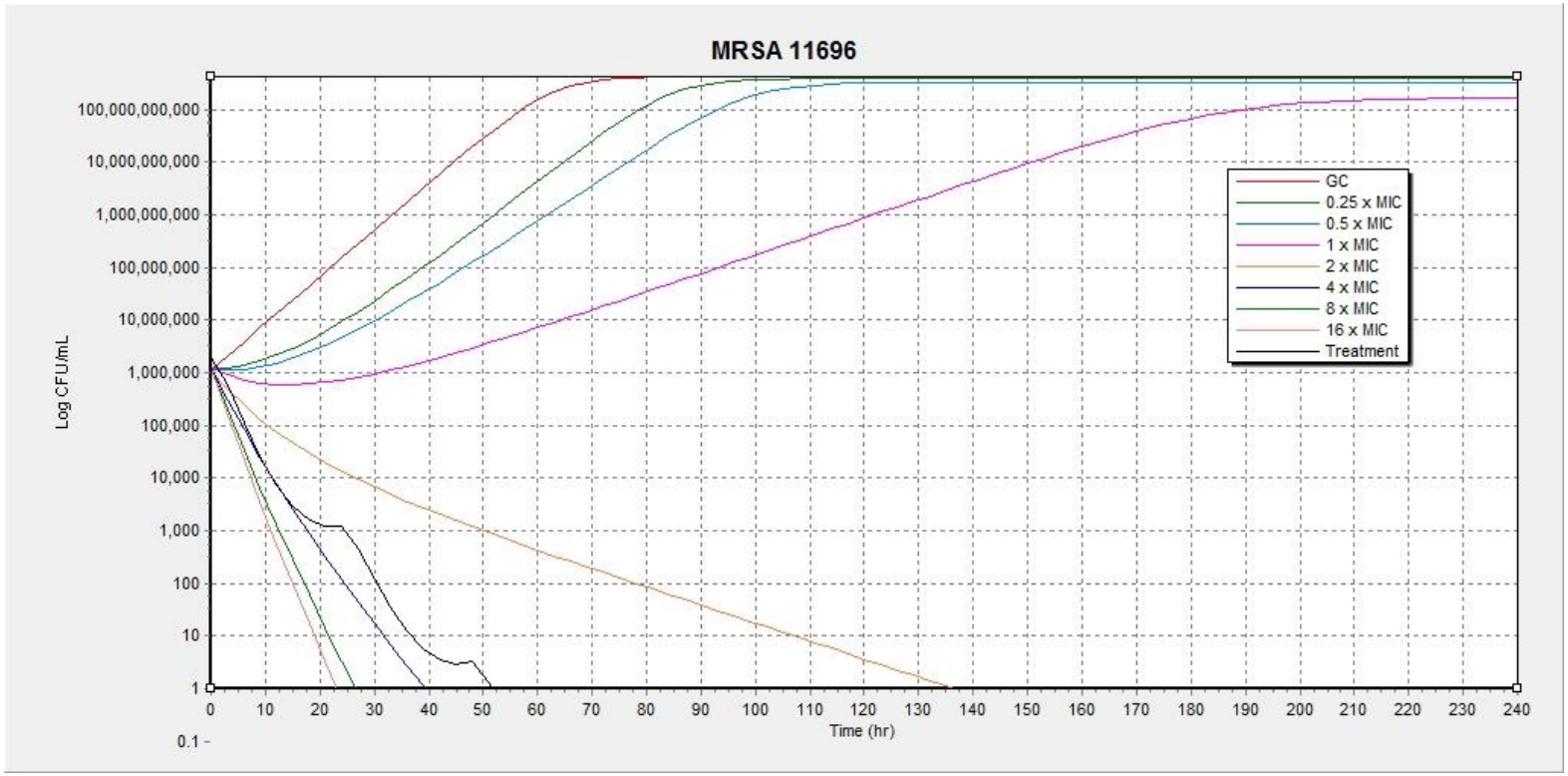

Figure 6-14. Simulated curves for bacterial kill over a range of MIC strengths for MRSA OC 11696 & once a day oral administration of 400mg JNJ-Q2

Figure 6-15. Simulated curves for bacterial kill over a range of MIC strengths for MRSA OC 2838 & once a day oral administration of 400mg JNJ-Q2

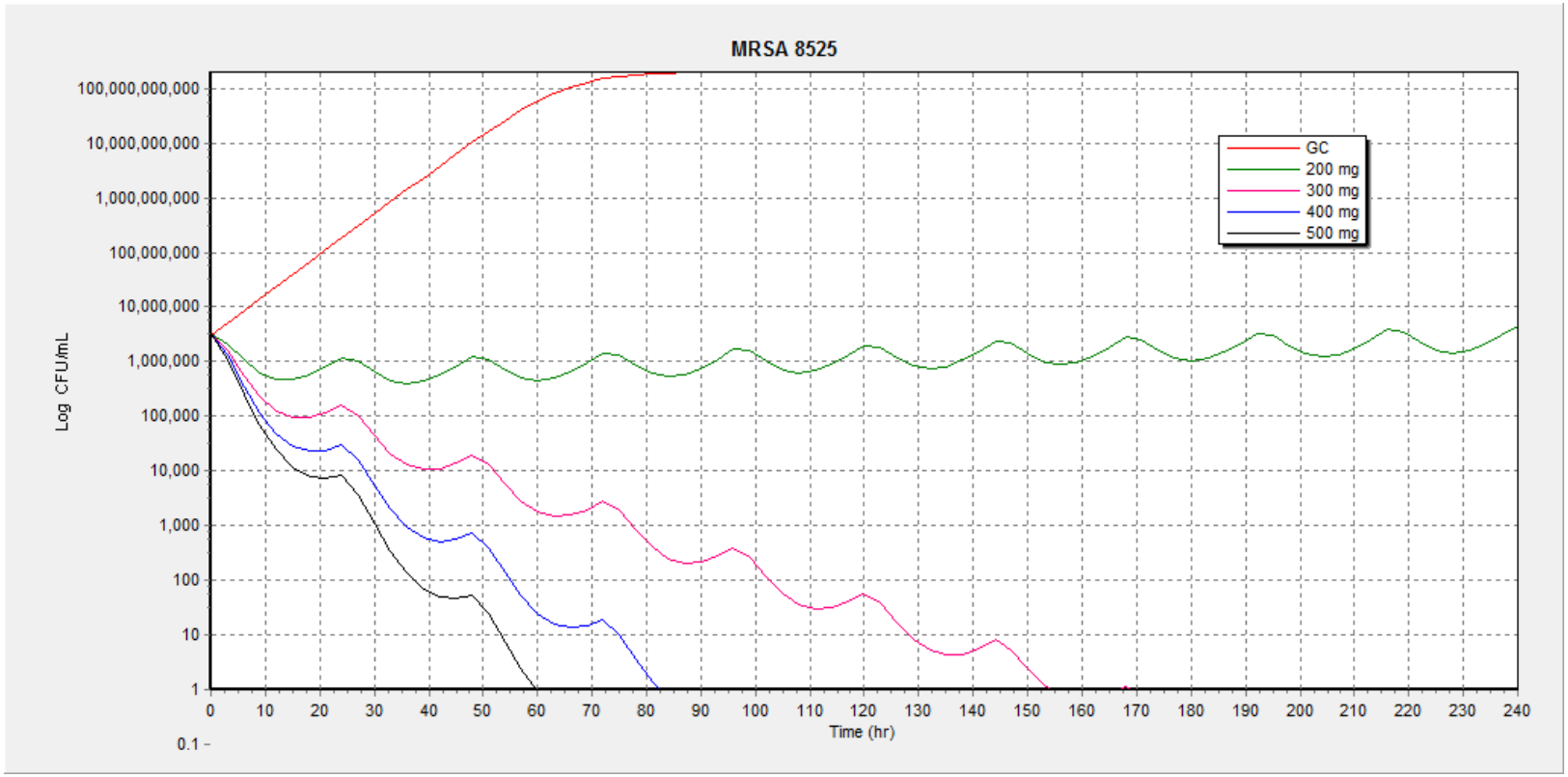

Figure 6-16. Simulated PK/PD of JNJ-Q2 against MRSA OC 8525 after once daily oral administration of 200mg, 300mg, 400mg and 500mg JNJ-Q2

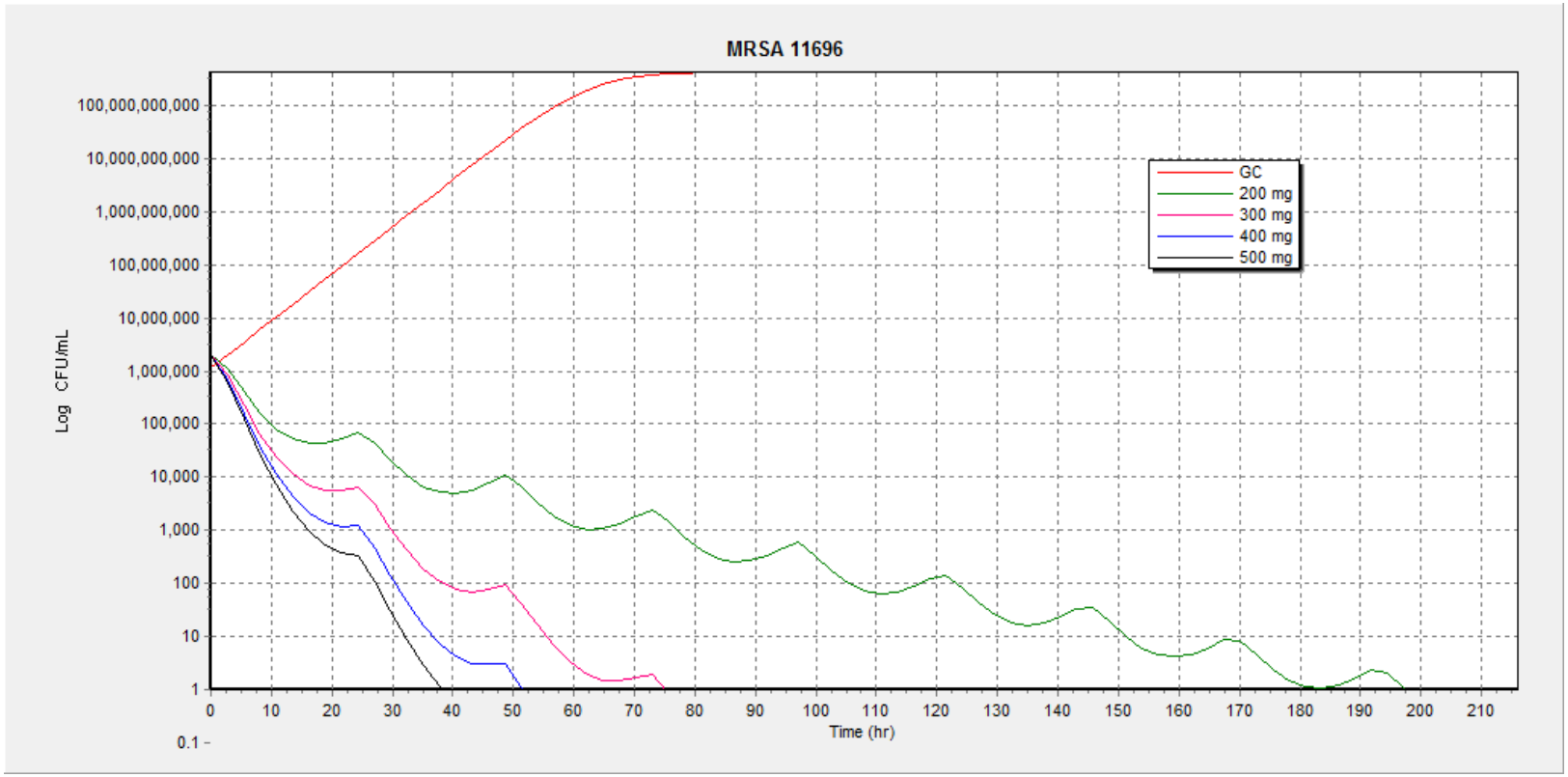

Figure 6-17. Simulated PK/PD of JNJ-Q2 against MRSA OC 11696 after once daily oral administration of 200mg, 300mg, 400mg and 500mg JNJ-Q2

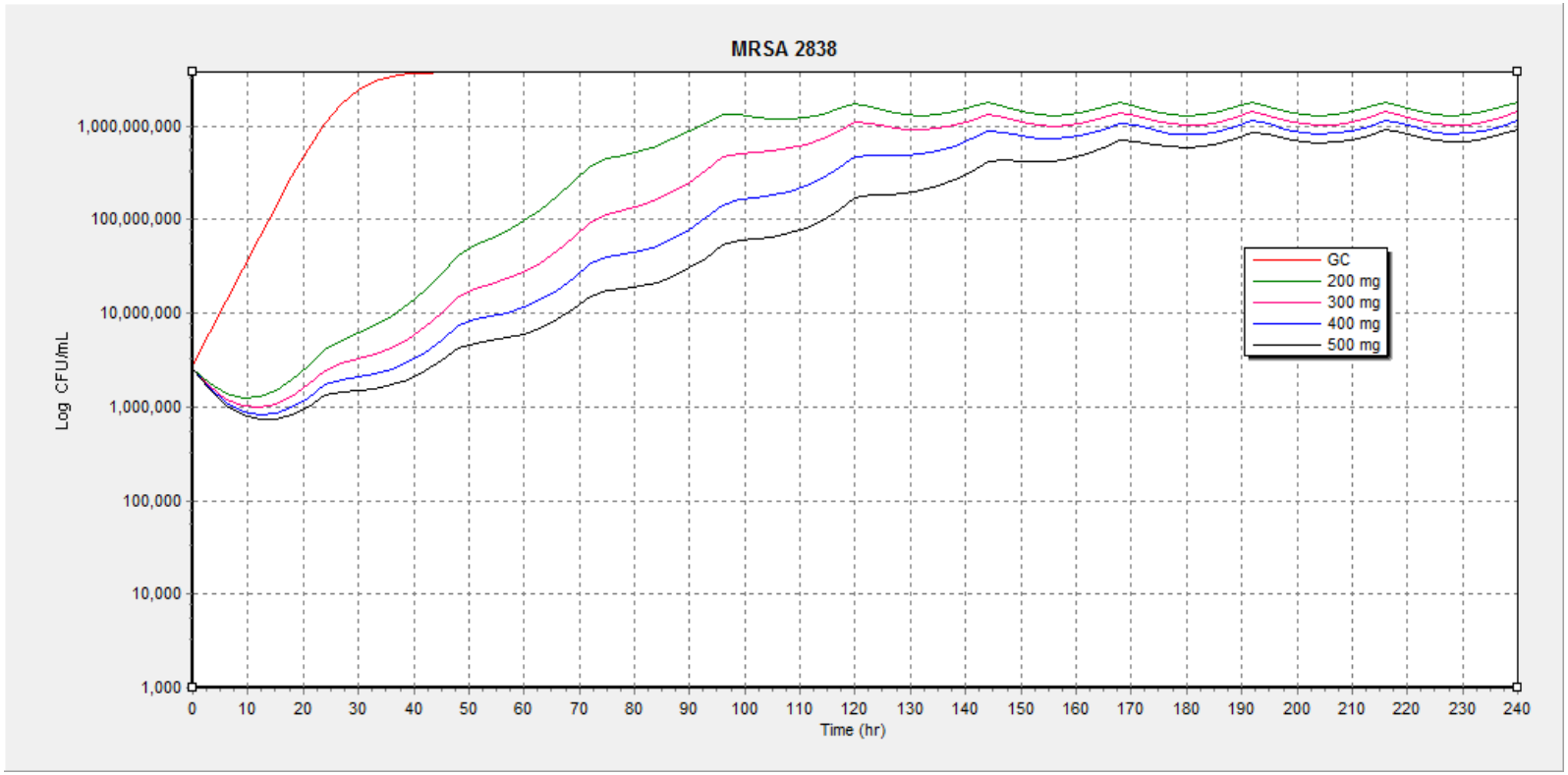

Figure 6-18. Simulated PK/PD of JNJ-Q2 against MRSA OC 2838 after once daily oral administration of 200mg, 300mg, 400mg and 500mg JNJ-Q2

Table 6-1. Estimated PK model parameters with bootstrap 95% confidence intervals

Parameter	Estimate	Bootstrap 95 % CI
Vc	138	95.2- 168
Vp	5.2	5.2-9.2
CL	16.8	14.55- 19.4
Q	6.27	5.16- 7.95
KA	0.34	0.21-0.48
FA	0.37	0.28-0.47
FM	0.42	0.35- 0.51
Btwn. Subj. Variability Vc	0.283	0.008-0.42
Btwn. Subj. Variability Vp	0.166	0.003-0.38
Btwn. Subj. Variability CL	0.224	0.13-0.31
Btwn. Subj. Variability Q	0.262	0.0003 – 0.36
Btwn. Subj. Variability KA	0.511	0.002 – 0.65
Btwn. Subj. Variability FA	0.436	0.21-0.59
Btwn. Subj. Variability FM	0.316	0.15-0.42
Residual Variability	0.158	0.12-0.19

Table 6-2. *f*AUC_{24}/MIC ratio for JNJ-Q2

Pathogen	*f*AUC_{24}/MIC ratio	Type
S. pneumoniae, all isolates	67.3	Gram-positive
Ciprofloxacin resistant *S. pneumoniae*	32.3	Gram-positive
MRSA*	32.3	Gram-positive
Ciprofloxacin resistant MRSA*	32.3	Gram-positive
Ciprofloxacin resistant MRSE	32.3	Gram-positive
Streptococcus pyogens	538	Gram-positive
Haemophilus influenzae	538	Gram-negative
Klebsiella pneumoniae	32.3	Gram-negative
Pseudomonas aeruginosa	4.04	Gram-negative

*isolates collected from 2004-2006

Table 6-3. Parameter estimates for 3 strains of MRSA

Strain / Parameter	OC 8525	OC 11696	OC 2838
k (hr^{-1})	0.17	0.20	0.26
Nmax (CFU/mL)	10^11.3	10^11.6	10^9.6
Emax (hr^{-1})	1.12	0.74	0.32
EC50 (μg/mL)	0.95	0.68	0.44
x (hr^{-1})	0.09	0.06	0.12
h	1.8	1.6	0.69

CHAPTER 7
CONCLUSION

JNJ-Q2 is a novel fluorinated 4-quinolone oral antimicrobial agent, with activity against key pathogens associated with CAP and cSSSIs, including diabetic foot infection. They target the DNA topoisomerases showing potent activity against Gram-negative and positive bacteria including MRSA strains. It is important, therefore, to assess the *in vivo* penetration of JNJ-Q2 into ISF of soft tissues, such as subcutaneous adipose tissue and skeletal muscles.

The selection of the correct dose and dosing regimen is a fundamental step for therapeutic success with any pharmacological agent. In the past, pharmacokinetic and pharmacodynamic assessment of antimicrobials were based on measuring plasma concentrations, however, most infections occur at tissue sites. Microdialysis is currently the most appropriate sampling technique that can provide continuous sampling of free drug in tissues to estimate active drug profiles at site of action. For antimicrobial agents, the selection of the best drug and dosing scheme for a specific pathogen not only increases the chances of cure while preventing toxic side effects, but it has been shown that suboptimal dosing can lead to bacterial resistance development and should be avoided. One approach to make an educated dose selection is PK/PD approach. PK/PD modeling has become an important tool to streamline drug development and is encouraged by regulatory agencies such as the FDA.

The traditional PK/PD model for dose selection in antimicrobial drug development was based on the relationship of drug kinetics to minimum inhibition concentration, from which the maximum efficacy can be classified into either time-dependent or exposure (AUC or Cmax) dependent. PK/PD characterization of antimicrobial agents has allowed

a better understanding of why a particular dosing regimen achieves clinical success or failure. Over the years, dose selection has become a much more sophisticated process over previous empiric methods. *In vitro* and *in vivo* experiments are used to define a relationship between drug concentration (PK) and effect (PD) and allow for a clear target to be identified so that efficacy is achieved in the clinical setting. The PK/PD indices typically used for fluoroquinolones include the ratio $fAUC_{24}$/MIC**,** which is based on a direct comparison of plasma concentrations of the antibiotic and MIC of the respective bacteria. The efficacy of the antibiotic can be predicted by this ratio where higher the value greater the efficacy. The *in vivo* PK parameters are usually determined from plasma drug profiles while *in vitro* PD parameters commonly use culture media drug concentrations. Fluoroquinolones which display concentration-dependent killing produce an increasing effect as the concentration increases as seen in case of JNJ-Q2.

This project explored the usefulness of PK/PD relationships to support drug development and selection of a dosage regimen for JNJ-Q2.

The *in vivo* PK study conducted was a single center, open-label, one-arm, nonrandomized study in healthy men and women. The study was designed to assess the *in vivo* penetration of JNJ-Q2 into ISF of soft tissues, such as s.c. adipose tissue and skeletal muscles, after administration of 400 mg single oral dose, since unbound concentration of an antimicrobial agent in the ISF at target site is responsible for its efficacy. Following single oral 400 mg JNJ-Q2 administration, unbound JNJ-Q2 was rapidly distributed and equilibrated to target sites, s.c. adipose and muscle tissues, in healthy subjects. Exposures in ISF of target tissues were slightly greater than that of unbound drug in plasma. Single oral dose of 400 mg JNJ-Q2 was well tolerated in

volunteers. The peak plasma levels were reached within 4-5 hours post dose and protein binding in plasma for JNJ-Q2 assessed was found to be consistent with other fluoroquinolones in the class. It may be reasonable to conclude that free, unbound plasma concentrations can be used as surrogate for unbound concentrations in ISF of s.c. adipose and skeletal muscle tissues.

The data from this study was used to develop a POP PK model so that the concentration in all three tissues could be predicted simultaneously. It was determined that a two-compartment body-model with elimination from the central compartment fit the data well. Additionally, due to the fact that free concentrations in the ISF were not equal to free plasma concentrations, a distribution factor for each soft tissue was included in this model.

For fluoroquinolones, the PK/PD parameter with the best correlation to clinical outcome is $fAUC_{24}$/MIC ratio. The high ratio value beyond the accepted break points for JNJ-Q2 indicates the high efficacy of bacterial kill *in vitro.* This information combined with the high penetration of JNJ-Q2 into ISF where infections are expected give us a good heads up on the promising of use of this antibiotic.

The major PD parameter used for antimicrobials is the MIC. This parameter, however, has some limitations i.e. it does not show the antimicrobial activity over time.

Time-kill curves, whereas, display the change in the number of bacteria over time offering a more dynamic effect profile than the static MIC parameter. Data was taken for the *in vitro* PD studies for JNJ-Q2 at different concentrations of the MIC against 3different strains of MRSA, from the work done by Morrow et al.[28] By developing a mathematical model corresponding to the change in bacteria over time given the

concentration a much more precise pharmacodynamic picture can be obtained than with the MIC PD parameter. A modified Emax model was developed to define the antibacterial activity over time and the pharmacodynamic parameters were calculated.

The PD parameters for JNJ-Q2 derived from time-kill model fit was combined with *in vivo* PK data in an integrated PK/PD model that best described the antibiotic's activity as a function of time and concentration and different dosing regimens simulated.

In conclusion, PK/PD characterization of JNJ-Q2 allowed a better understanding of dosing regimen of 400 mg currently recommended for use. This can be further used in optimizing the dosing regimen to have an efficacious drug in the treatment of CAP, cSSSIs and other infections.

APPENDIX A
REAGENTS AND EQUIPMENT FOR LC-MS/MS STUDY

Reagents

- Triple distilled water – Filtered in house by Corning AG-3
- Methanol – Fisher Scientific A452-4
- Formic Acid – Fisher Scientific A118^{P}-100
- Saline solution – Baxter 2B2323
- Ammonium Acetate – Fisher Scientific A639-500

Equipment and Disposables

- Weighing Balance – Mettler AE240
- Vortex – Kraft Apparatus Inc. Model PV-5
- Micropipettes – Eppendorf Research
- Pipette tips (1-200 µL) – Fisherbrand 22-707-500
- Pipette tips (100-1000 µL) – Fisherbrand 21-197-8F
- Autosampler vials – Sun Sri 200 046
- Aluminum seals – Sun Sri 200 100
- Bath Sonicator – Fisher Scientific FS110H
- 15mL Centrifuge tubes – Corning 430052
- 50 mL Centrifuge Tubes – Corning 430828
- Microcentrifuge tubes – Fisherbrand 05-408-129
- Work Station – Dell Precision 390
- LC-MS/MS system – API 4000 LC-MS/MS SYSTEM, J1940112
- Column – Symmetry C18 3.5 µm, 4.6mm x 50mm, SNo. 016835292102; 019138233136
- Analytical software – Analyst Software 1.4.2 for LC- MS/MS Systems
- Autosampler – Elmer Series 200, 293N5020903
- Pump – Perkin Elmer Series 200, 291N7100502A
- Manual Crimpers – Fisher 03-375-7
- Volumetric flask – Pyrex 5640
- Microsyringes – Tyco Healthcare 8881501400
- Plastic Cannula – BD 303345
- Graduated Cylinder – Pyrex 2982

APPENDIX B
REAGENTS AND EQUIPMENTS FOR IN VITRO MICRODIALYSIS STUDY

Reagents

- Triple distilled water – Filtered in house by Corning AG-3
- Methanol – Fisher Scientific A452-4
- Formic Acid – Fisher Scientific $A118^{P}$-100
- Saline solution – Baxter 2B2323
- Ammonium Acetate – Scientific A639-500

Equipment

- Weighing Balance – Mettler AE240
- Vortex – Kraft Apparatus Inc. Model PV-5
- Micropipettes – Eppendorf Research
- Pipette tips (1-200 µL) – Fisherbrand 22-707-500
- Pipette tips (100-1000 µL) – Fisherbrand 21-197-8F
- Autosampler vials – Sun Sri 200 046
- Aluminum seals – Sun Sri 200 100
- Bath Sonicator – Fisher Scientific FS110H
- 15mL Centrifuge tubes – Coning 430052
- 50 mL Centrifuge Tubes – Corning 430828
- Syringes – Becton Dickinson 309603
- Needle – Becton Dickinson 305167
- Microcentrifuge Tubes 1.5mL – Fisherbrand 05-408-129
- Microcentrifuge Tubes 0.5mL– Fisherbrand 05-408-120
- Syringe Pump – Harvard Apparatus Model 55-4150
- Heated Stir Plate – Fisherbrand Isotemp
- Thermometer – Fisherbrand 76mm Immersion 14-997
- Microdialysis Probes – CMA-60 P000002
- Amber IV Tubing Cover – Health Care Logistics 7617
- Mass Spectroscopy:
- LC-MS/MS – API 4000 LC-MS/MS SYSTEM, J1940112
- Perkin Elmer HPLC: (1)Autosampler – Perkin Elmer Series 200, 293N5020903; (2) Pump – Perkin Elmer Series 200, 291N7100502A; (3) Work Station – Dell Precision 390; (4) Column – Symmetry C18 3.5 µm, 4.6mm x 50mm, WAT200625; (5) Analytical software – Analyst Software 1.4.2 for LC- MS/MS Systems

APPENDIX C
STUDY CRITERIA

Inclusion criteria:

- Men or Women between 18 and 55 years of age, inclusive
- BMI between 18.5 and 32 kg/m2, inclusive [BMI = weight (kg)/height (m2)]
- Non-smoker (not smoked for ≥3 months prior to screening) and have a urine cotinine level indicative of a non-smoker
- Healthy on the basis of physical examination, medical history, vital signs, and 12-lead ECG performed at screening.
- Healthy on the basis of clinical laboratory tests performed at screening
- Women must be: (1)Postmenopausal (defined as having had the last menstrual period greater than 12 months prior to screening, or greater than 6 months prior to screening with a serum follicle stimulating hormone (FSH) concentration greater than 40 mIU/mL) or surgically sterilized (defined as being at least 6 weeks post-surgical bilateral oophorectomy, bilateral tubal ligation, or hysterectomy), and (2) Women of childbearing potential must be practicing an effective method of birth sterilization) at least 3 months before the Screening visit, and (3) Non-pregnant based on a negative serum beta-human chorionic gonadotropin (β-HCG) test analysis at screening and a negative serum or urine HCG test at admission
- Men must agree to use a double barrier method of birth control and to not donate sperm during the study and for 3 months after receiving the last dose of study drug.
- Agree not to consume any products containing quinine, grapefruit juice, sugar substitutes or Seville oranges, within 2 days prior to admission until discharge from the unit.
- Agree not to consume any methylxanthine products (such as tea, coffee, cola and chocolate, cocoa) within 2 days prior to admission until discharge from the unit.
- Agree not to consume any alcohol within 2 days prior to admission to the clinical research unit and until discharge from the unit.
- No clinically significant medical history according to the investigator.
- Subjects must agree to abstain from any strenuous exercise that is more excessive than normal routine, 72 hours prior to the first dose until the completion of the study.

- Willing/able to adhere to the prohibitions and restrictions specified in this protocol
- Subjects must have signed an informed consent and are willing to participate in the study.

Exclusion criteria:

- Clinically significant abnormal values for hematology, clinical bio-chemistry, coagulation, hematology, or urinalysis at screening.
- History of or current significant medical illness including (but not limited to) cardiac arrythmias or other cardiac disease, hematological disease, lipid abnormalities, bronchospastic respiratory disease, diabetes mellitus, renal or hepatic insufficiency, thyroid disease, Parkinson's disease, infection, or any other illness that the Investigator considers should exclude the subject.
- Known allergies, hypersensitivity, or intolerance to JNJ-32729463 or its excipients
- Clinically significant abnormal physical examination, vital signs (e.g. SBP >140 mmHg, DBP >90 mmHg, heart rate >100 bpm and <45 bpm) or 12-lead ECG (e.g. QTc >450 msec) at screening or prior to first dose of study drug.
- Family history of long QT or short QT syndrome
- Received an investigational drug (including vaccines) or used an investigational medical device within 30 days before the planned start of treatment or are currently enrolled in an investigational study
- Pregnant or breast-feeding
- Recent history of surgery; within the past 3 months prior to screening.
- Sexually active men, who have not been surgically sterilized, and who are unwilling to use a condom during intercourse or to refrain from sexual intercourse through the study and for up to 3 months after the last dose of study medication.
- Serology positive for hepatitis B surface antigen, hepatitis C antibodies or HIV antibodies.
- Positive urine screen for drugs of abuse.
- Positive alcohol breath test.
- Recent history (within previous 6 months) of alcohol or drug abuse.
- Drinks, on average, more than 8 cups of tea/coffee/cocoa/cola per day.
- Clinically significant acute illness within 7 days prior to study drug administration.

- History of drug and/or food allergies.
- Subjects who developed SAEs or experienced intolerance following administration of quinolone antibiotics.
- Donation of 1 or more units (approximately 450 mL) of blood or acute loss of an equivalent amount of blood within 90 days prior to study drug administration.
- Exposure to any new investigational agent within 90 days prior to study drug administration.
- Use of any prescription or over the counter (OTC) medication, herbal medication, herbal teas, vitamins, or mineral supplements within 14 days prior to study drug administration (not including paracetamol).
- Psychological and/or emotional problems, which would render the informed consent invalid, or limit the ability of the subject to comply with the study requirements.
- Any condition that, in the opinion of the investigator, would compromise the well-being of the subject or the study or prevent the subject from meeting or performing study requirements.
- Employees of the investigator or study center, with direct involvement in the proposed study or other studies under the direction of that investigator or study center, as well as family members of the employees or the investigator.

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BIOGRAPHICAL SKETCH

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