DEVELOPMENT AND VALIDATION OF A BIOANALYTICAL METHOD FOR OCCIDIOFUNGIN

A Dissertation

by

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ABSTRACT

Fungal infections caused by opportunistic pathogens can be severe and have been growing in prevalence. Many clinically relevant pathogens have resistance to or are developing a resistance to the commonly used treatments. Occidiofungin is a novel cyclic peptide that is active against a wide range of fungi and has a novel mechanism of action. As such, occidiofungin is being developed for use in treating vulvovaginal candidiasis and its recurrent form. This study describes development and validation of bioanalytical methods for the quantification of occidiofungin in rat and rabbit plasma. These methods are used to accurately and precisely quantify small amounts of occidiofungin in rat and rabbit plasma. Validation of this method was performed within the linear range of 150 to 15000 ng/mL for accuracy and precision in rat and rabbit plasma. Calibration curve linearity, stability of drug in plasma was established in quality controls. Extract stability, matrix effects and recovery of drug in the extract was also determined. This validated method allows for further studies into the absorption, distribution, metabolism, and excretion in addition to other pharmacokinetic studies.

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NOMENCLATURE

OCF	Occidiofungin
VVC	Vulvo Vaginal Candidiasis
RVVC	Recurrent Vulvovaginal Candidiasis
IND	Investigational New Drug
FDA	Food and Drug Administration
CRO	Contact Research Organization
HPLC	High Performance Liquid Chromatography
UVVIS	Ultraviolet Visible Spectroscopy
LCMS	Liquid Chromatography – Mass Spectrometry
LC-MS-MS	Liquid Chromatography Tandem Mass Spectrometry
LC-HRMS	Liquid Chromatography- High Resolution Mass Spectrometry
ADME	Absorption Distribution Metabolism Excretion
SRM	Single Reaction Monitoring
MRM	Multiple Reaction Monitoring
ESI	Electron Spay Ionization
HESI	Heated Electron Spray Ionization
APCI	Atmospheric Pressure Chemical Ionization
HILIC	Hydrophilic Interaction Chromatography
LLOQ	Lower Limit of Quantification
SIL	Stable Isotopically Labeled
SPE	Solid Phase Extraction

QUCHER'S Quick Easy Cheap Effective Rugged and Safe

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CHAPTER I

GENERAL INTRODUCTION AND LITERATURE REVIEW

General Overview

Occidiofungin (OCF) is a novel antifungal compound that is being developed to treat recurrent vulvovaginal candidiasis (RVVC)(Smith, 2020). OCF is a non-ribosomally synthesized cyclic octa peptide with novel amino acids incorporated in its structure. OCF exhibits its antifungal activity by preventing polymerization of actin, leading to apoptosis. This is a unique mechanism of action that is different from existing antifungal drugs. The unique structure and mechanism of action makes OCF an attractive candidate for drug development as an antifungal agent. Several *in vitro* and *in vivo* toxicological studies are required to submit an Investigational New Drug (IND) application to the Food Drug Administration. Additional pharmacokinetic studies in animal models are essential in evaluating the safety and efficacy of any new drug candidate (Pandey et al., 2010).

One aspect of these animal studies is understanding the absorption, distribution, metabolism and excretion (ADME studies) of a drug candidate in suitable animal models. These ADME studies are usually supported by various analytical methods that are used to quantify the drug in plasma over time and this data is used to calculate various ADME parameters. The measured plasma concentration is then used to calculate various pharmacokinetic parameters such as C_{max}, T_{max}, AUC etc. Additional methods

may be required to measure the concentrations of drug and its metabolites in saliva, cerebrospinal fluid, tears, milk and other bodily fluids. The drug concentrations measured in other body fluids help understand how well a drug is distributed in the body and the associated effect. With a high cost of drug development and the need for pharmacokinetic studies, it is necessary to develop and validate an analytical method that will allow for accurate measurement of the drug. Thereby, a well-established bioanalytical methods are needed to support pharmacokinetic studies at stages of drug development. A successful IND is required for conducting Phase 1 clinical trials to establish safety of OCF as an antifungal drug. Phase 2 clinical trials are subsequently done to establish the efficacy of OCF for treating a fungal disease. The information learned in this study facilitated the development of a method that will enable consistent and reliable quantification of OCF throughout the different stages of drug product development.

Bioanalytical Methods

Bioanalytical methods are used to determine the concentration of drugs in various animal matrices such as blood, plasma, serum, saliva, tears, cerebrospinal fluid, etc. The measurement of drug concentrations, especially in blood, plasma or serum, in a given animal model is a necessary and an important step in evaluating a compound for its potential pharmaceutical use. Drug concentration versus time data is necessary to determine the effectiveness and relative safety of a potential therapeutic compound. It also provides insights into the toxicokinetics, pharmacokinetics and pharmacodynamics

of the drug. The effects of both systemic and organ specific drug exposure can be monitored using these methods. Data, thus acquired, provides insight into the toxicity of the drug (Walker, 2004). These bioanalytical methods require substantial method development and validation. They are designed and developed to be both reproducible with regards to method precision and accuracy across a wide range of concentrations and mediums before they can be validated for use (Whitmire et al., 2011).

The FDA guideline provides limits for precision, accuracy, stability, recovery, and selectivity/specificity for validating a bioanalytical method [FDA Guidance ("Bioanalytical Method Validation Guidance for Industry ", May 2018)]. Precision is the degree of scatter among a series of measurements from a single data set. Accuracy is the degree of closeness of the value determined to the nominal value. Stability is measured as the intactness of the analyte in a given matrix under varying conditions. Recovery refers to the efficiency of the analytical process in extracting the analyte from the biological matrix and is reported as a percentage. Selectivity references to the ability of the method to determine one compound from another. Specificity is the ability to assess the analyte in presence of other components that are expected to be there. The FDA guidance on developing a method that has these abilities has acceptance criteria of 15% for accuracy. There is a notable exception however when it comes to selectivity and matrix effects which the guidelines suggest accepting anything within 20% of the accepted value.

Instrumental Techniques and Methods used in Bioanalytical Methods

Liquid Chromatography Mass Spectrometry

Liquid chromatography tandem mass spectroscopy (LCMS) is a hyphenated technique that is widely used in bioanalysis and other fields. Hyphenated techniques combine chromatographic separation with mass spectrometry detection leading to enhanced quantitative/qualitative analyses (Patel et al., 2010). Additional hyphenated techniques include LC-HRMS, which is liquid chromatography coupled to high resolution mass spectroscopy. LC-HRMS has high versatility and performance that records a full scan, detecting all ions resolved (Rochat, 2018). However, there is some loss of sensitivity associated with full scans and HRMS instruments are very expensive. These hyphenated techniques have several variables that need to be considered when applying them to bioanalysis. Understanding the chemical properties of any given molecule is required for devising steps for extraction, enrichment and analysis. These steps have profound influence on the performance of the method. The chemical structure of a molecule is an important factor that dictates the choice of potential chromatography techniques. The structure of the analyte must be studied to understand its polarity, mass, hydrophobicity, and ionic grouping's all of which can influence the technique to be used.

The next part of this hyphenated techniques involves mass spectrometry, allowing for specific detection, quantitation and confirmation of molecular products (Patel et al., 2010). However, there are some considerations as to what type of

monitoring to use, and how to ionize the sample. Selected Reaction Monitoring (SRM) is used to monitor ions based on their mass, and then monitor their downstream fragmentation products. Multiple reaction Monitoring (MRM) scanning provides monitoring of multiple product ions from a single precursor ion in a similar manner to SRM, however it tracks multiple downstream product ions (1978, R. W. Kondrat, G. A. McClusky, R. G. Cooks). Once the monitoring type has been determined the ionization method needs to be considered as well. There are several modes of ionization that could be used to break the compound into its product ions. Electrospray ionization (ESI) is widely used to analyze samples that are already ionized in liquid form. The technique involves evaporating solvent from tiny droplets before solvent ions of interest entering the mass spectrometers source chamber. Additionally, the optimized use of ESI will not destroy the molecule of interest and will ionize a wide variety of chemical compounds. Heated Electron Spray Ionization (HESI) is a modified form of ESI that involves a heating device on the ESI source. This heats the gas to temperatures between 200 and 600 °C, this causes the droplets to evaporate rapidly causing increased efficiency. This can lead to higher sensitivity due to the reduction of the signal to noise ratio. However, using HESI can cause decomposition of certain compounds due to the heat that is involved with it. Since ESI is based on ions in solution it cannot be used to analyze non-polar molecules that are neutral (carry no charge for example steroids) in solution. Such molecules are better suited for atmospheric pressure chemical ionization (APCI). APCI involves the process of ionizing the neutral molecules in gas phase by colliding them with ionized gas molecules (such as nitrogen). The ionizing gas is ionized via a highly

charged electric field at atmospheric pressure. This ionization technique results in relatively intact molecular ions. However, APCI does involve vaporizing your sample using heat, and is not very tolerant to high salt concentrations (Siuzdak, 2004).

Chromatography:

An important component of LCMS is the use of liquid chromatography to resolve analytes of interest from other related compounds or matrix impurities in the sample. Part of this hyphenated technique involves separating components via a chromatographic method. Choosing a given type of chromatographic technique will depend on the molecule, its structure and physiochemical characteristics. Hydrophilic interaction chromatography (HILIC) excels at separating highly polar molecules using specialized silicas as the stationary phase. Ion exchange chromatography operates by containing a stationary phase that will exchange ions therefore weakly binding with the ionic mobile phase and analyte. By modulating the ionic conditions, you can cause the molecule to bind and release from the resin enabling sample purification. This excels at binding molecules with a known counter ion that is available to bind to the selected stationary phase. Reversed-phase chromatography functions by having a stationary phase that is less polar than the mobile phase, causing compounds to elute based on polarity. This type of elution makes it widely applicable to many sample types and thus is widely used.

Mass Spectrometry:

LCMS using a triple quadrupole instrument in MRM mode is by far the de facto method of choice for bioanalysis. A triple quad instrument operates by adding an

additional quadrupole mass analyzer in tandem to the first one. These two quadrupoles are separated by a collision cell, wherein precursor ions are subjected to collision induced dissociation using a neutral gas such as Nitrogen, Argon or Helium. This allows for the use of selective reaction monitoring or multiple reaction monitoring due to setting the two quadrupoles to scan for the precursor ion and one or more product ions. If the scan is targeted to a single molecule it is considered SRM or if targeting multiple masses MRM. One of the main advantages of LCMS using a triple quadrupole instrument is that it can be used to detect and quantify the low levels of analyte in a sample in MRM mode. This technique regularly provides lower limits of quantitation (LLOQ) down to nanograms per milliliter, and in certain cases it is possible to measure down to picograms per milliliter. Other advantages are selectivity and specificity as a result of which with minimum sample clean up one can measure an analyte in a complex matrix. However, one of the major limitation is variability in ionization of the analyte in any given sample or between two samples. This variability in ionization can be due to suppression or enhancement of ionization caused by interferences present in a matrix. This is the basis of matrix effects. Matrix effects can also be concentration dependent, thus producing nonlinear curves with the calibrator curves providing a lower response at higher concentrations. Matrix effect can adversely impact the measured concentrations of analytes. The impact of matrix effects is ameliorated by the use of internal standards. We shall briefly describe the various types of internals standards in the next section along with their advantages and disadvantages.

Internal Standards in LCMS

Most modern methods incorporate an internal standard that is added to samples and calibrators at a known concentration to help reduce variability due to changes in ionization of samples and also normalize for possible matrix effects. The ratio of response of analyte to response of the internal standards is used to calculate the concentration of analyte in the calibrators and samples. Chromatographic Assays in bioanalytical studies therefore routinely use an internal standard, that is added at a known and fixed concentration to all calibrators, quality control samples, and unknown samples that will be analyzed. The internal standard provides a reference response to which the analyte is compared. (Zou, 2020).

An internal standard ideally acts exactly like the analyte of interest while still being distinguishable by the mass spectrometer. There are generally two types of internal standards that are considered when using LCMS for bioanalytical method development, analog internal standards, and stable isotopically labeled (SIL) internal standards (Sargent, 2013).

Analog internal standards are compounds that have small chemical modifications as compared to the analyte of interest. These modifications could include a structural analog, an isomer, or even a homologue, so that they will act as close to the compound of interest as possible. Analogs are cheaper to produce as compared to the more expensive SIL counterparts and are also an alternative when a SIL is not available for a given analyte. Even though the analog internal standard may have similar physicochemical properties as the analyte of interest it may still fail as an effective

internal standard (i.e., it may not normalize the differences observed in the ionization of an analyte across; 1) several samples in a batch and 2) across data acquired over several days). Researchers have documented several cases where a structural analogs could only partially compensate for the variable ionization caused by components in matrices like plasma(I. Fu, 1998). The difference in response due to the analyte being in varying matrices when compared to a standard solution is known as matrix effects (Paul Kebarle, 1993). This can cause varying effects such as ion suppression or enhancement based on the matrix, instrument used for analysis, and sample preparation (Hong Mei, 2003)

SIL internal standards are isotopically labeled compounds, that are identical in chemical structure and elemental composition to that of the analyte. SIL internal standards are also called as Isotopologues. The main distinguishing feature of Isotopologues from the analyte is the difference in molecular mass that is detectable by the mass spectrometer. However, Isotopologues have the same physicochemical properties and act identically to the original compound. SIL internal standards are ideal for LCMS method as it can be used in pharmacokinetics and toxicology studies as it mirrors how the drug is used in the body while having a distinguishable mass (Schellekens et al., 2011). SIL internal standards are normally synthesized using starting materials enriched in ^{13}C, ^{15}N, or deuterium (Zou, 2020). Several factors are taken into account when determining which SIL internal standards are to be used. Deuterium is most commonly used in synthesizing SIL internal standards due to its relatively low cost compared to other labels. Hydrogen is practically present in every compound. One disadvantage of deuterated compounds is that these SIL internal standards can have a

different retention time as compared to the native analyte. This small change in retention time, in most cases is not a significant change, is attributed to differences in bonding forces exhibited by deuterium as compared to hydrogen, and while this effect is minimal, it is still noticeable (Wieling, 2002).

Deuterium incorporation should be undertaken at a position in a molecule that is a non-exchangeable site of the molecule. Labeling with deuterium on certain sites in the molecule where an incorporated deuterium exchanges with hydrogen in aqueous solvent is not optimal. The H-D exchange leads to loss of the internal standard in the sample thus leading to poor precision and accuracy of the measured concentrations of the analyte. An important step in generating labeled compounds are ensuring that there is enough of a mass difference or else there is a possibility of mass spectrum overlap. Spectrum overlap between an analyte and its SIL leads to cross talk across the MRM channels of analyte and the internal standard thereby impacting measurements(Sargent, 2013). This mass difference is approximately 3 Daltons for small molecules due to the low concentration of naturally occurring isotopes at these low weights. However, when working with larger molecules, chlorinated or brominated compounds a larger weight difference is required so that the SIL can be resolved from naturally occurring isotopologues of the analyte. There are additional limitations that come with making a SIL analog, the cost of production and getting consistent incorporation of the label into the SIL molecule. When making the material it is ideal that consistent incorporation of the label is ascertained. The presence of unlabeled compound must be minimized to prevent contribution of the unlabeled compound in the SIL into the analyte MRM

transitions (Sargent, 2013). Additionally, isotopically labeled internal standards are inherently expensive and can be complex to synthesize. The synthesis of SIL compounds can occur either via total synthesis or via fermentation using bacterial or fungal cultures. Total synthesis is done chemically from available precursors, in the case of SIL compounds allows for the addition of isotopes at specific sites (Goss et al., 2014). The other primary method is incorporating it in the *in vivo* production of the compound, such as fermentation or other natural product pathways. This has an increased cost compared to total synthesis as the isotopically enriched starting material has to be provided to the organism in the fermentation medium. The fermentation leads to the production of the SIL compound but a substantial amount of the labeled starting material is diverted toward other biochemical pathways of the organism. Hence fermentation and other natural product pathway methods to make SIL, are usually manufacturing methods of last resort due to the inherent inefficiency of isotope incorporation. Additionally, choosing the right labeled isotope is important as some labeled isotopes will interfere with organism's homeostasis, and even inhibit cell division (Zachleder et al., 2018). When using organisms to incorporate the isotopes into a compound one must also monitor and ensure consistency in labeling. Thus, several criteria need to be taken into consideration when choosing the process and methods to make SIL internal standards to ensure successful production. The primary drawback for SIL material made by culturing is that it can have limited availability due to difficulty in production and purification. In general, a SIL internal standard for bioanalytical applications is preferred, due to its

ability to act nearly as identical to the compound of interest while still being distinguishable by mass.

Other Techniques

Liquid chromatography, such as HPLC, can be coupled to various detection techniques to detect the analyte after chromatography. Commonly used techniques include hyphenated techniques (Liquid Chromatography-mass spectrometry LCMS), ultraviolet visible detection (UV-VIS), fluorescence detection, and radiolabeling detection. Each technique its advantages and disadvantages shall be discussed here.

HPLC-UVVIS

In this method HPLC is coupled to a UV-VIS detector. Ultraviolet-visible spectrophotometer measures the amount of UV radiation that is absorbed by specific compounds at various wavelengths, and while this technique is quick and simple it has moderate specificity (Dr.K. Bhavyasri, 2019). This technique cannot be used to detect very low concentrations of an analyte or distinguish between compounds that co-elute on the column.

HPLC Fluorescence Detection

This technique couples a fluorescence detector to an HPLC. The detector uses a monochromic light to excite fluorophores, in an analyte, to emit light at a different wavelength that can be measured. This measurement can then be compared to standards to determine concentration and purity. This technique requires that the compounds exhibit fluorescence at known wavelengths. Potential downside is the possible decomposition of the fluorophore upon excitation thus modifying the reading. (Bright,

1988). This technique is limited to compounds that exhibit fluorescence naturally or the analyte must be labeled with a fluorophore.

HPLC Radiolabel Detection

Radiolabeled compounds are synthesized by substituting a radioactive isotope of commonly occurring elements (for example ^{3}H, ^{14}C), the radioisotopes undergo radioactive decay at a constant rate. This rate is called as the half-life and is specific for a particular radioisotope. This decay allows leads to production of alpha or beta particles or gamma rays. The production of these particles is based on mechanism by which the radioisotope undergoes decay... Detection of these compounds is based on the radioisotope that is incorporated and the manner in which it decays.

Using radiolabeled compounds has an advantage compared to MS as it does not require ionization and can be used for assessing metabolites in circulation. However, the major downside of using radiolabeling is cost involved with using these radioactive compounds and potential safety concerns (Isin et al., 2012).

Extraction Techniques used in Sample Preparation for Bioanalysis

To analyze samples that are obtained from various matrices, an extraction method must be developed to selectively extract the analyte out of the matrix. Even though LCMS methods are very useful, the specificity and selectivity of the methods are usually greatly enhanced by adding a purification step before the actual LCMS analysis. The purification step is used to selectively purify the analyte and its internal standards away from all other matrix components. The choice of an extraction method, much like the HPLC method used, is governed by the structure of the molecule and its

physicochemical properties. This extraction can be done in a variety of ways: Solid Phase Extraction (SPE), Liquid-Liquid extraction, and protein precipitation. Solid phase extraction methods of sample purification are routinely employed, such methods are expected to be QUick, Easy, CHeap, Effective, Rugged, and Safe (QUECHERS). These QUECHERS methods can be based on ion exchange, reverse phase or Hydrophilic Lipophilic Balance (HLB)(Griffin, 1949; Kole et al., 2011).

SPE uses a solid particle material to separate the different component in a sample. This can be based on hydrophobicity or ionic exchange in addition to other chemical properties. SPE can be very selective and normally has high recovery due to the nature of resin structure. However, it can take a long time to develop suitable methods and to run the extraction itself. This is in addition to the high costs involved with the particle material (Anjana Vaghela; Ashok Patel, 2016). Most small organic molecules are extracted using a water immiscible organic solvent (ethyl acetate, hexane, chloroform etc.) and this technique is called liquid-liquid extraction (LLE). This method is easy and removes most of the matrix interferences away from the analyte and also allows for increasing the sensitivity of the methods by sample enrichment.

Liquid-Liquid Extraction relies on combining the aqueous base sample with another immiscible liquid solvent that will extract the analytes in the sample into the organic solvent ((Komal Patel, 2019). Liquid-Liquid Extraction has a relatively low cost due to its use of liquid solvents, however this use of large volumes of solvents can be a downside due to disposal concerns. Additionally using liquid-liquid extraction has a relatively low method development time due to the ease of use (S. Pandey et al., 2010).

Protein precipitation is commonly employed in biological matrixes when there are not good methods to selectively purify the analyte from the matrix. This method involves the addition of a water miscible organic solvent (for example methanol, acetonitrile or acetone) to the plasma or serum sample. The solvent operates by changing the conformation of serum proteins, mainly albumin, which causes them to aggregate and precipitate out of the solution. This causes the analyte of interest to unbind from the plasma or serum proteins and stay in the remaining aqueous organic solution. Protein precipitation gives the least clean sample as all the phospholipids and other small molecule components of the matrix that can cause interference in LCMS analysis are present in the supernatant extract. This method usually has an extremely high recovery for the analyte due to the theoretical removal of all proteins via precipitation and centrifugation, provided the analyte does not stick to the pellet. This sample preparation technique that has the lowest cost compared to other methods (Anjana Vaghela; Ashok Patel, 2016). Because of these advantages protein precipitation is widely used, and was used in our study based on the reasons above (Wujian et al., 2015).

Developing Bioanalytical Methods for Occidiofungin

We propose to develop two bioanalytical methods for OCF and validate using LCMS in rat plasma and rabbit plasma. Using plasma involves the addition of anticoagulant to stop clotting which prevents the release of these factors. However, anticoagulants when added can influence chromatography and cause changes in ionization when being analyzed by LCMS. The complexity of serum and plasma necessitates the use of HPLC to resolve the analyte from various potential interferences.

Reversed-phase chromatography was chosen to be used in this method as it allows for samples to be run without special preliminary treatment. Additionally Reversed-phase chromatography provides great resolution as it will resolve molecules of varying polarity. The instrument that will be used for developing the technique will be a triple quad instrument running MRM type scanning. This will provide unit resolution allowing for the separation of each integer mass. The MRM transition for OCF is a mass of 1216 and a charge of 1.

Preparation of Calibrators and Quality control samples

Calibrator curves, quality controls are prepared in the matrix by spiking the matrix with a known amount of analyte. The dynamic range of the calibration curve is dependent on the nature of the drug, the dosage given to the animal and the concentrations to be expected in the plasma or serum. Calibrators contain a known amount of analyte which can then be used construct a curve against which all other samples will be compared. (FDA ("Bioanalytical Method Validation Guidance for Industry ", May 2018). Quality Control samples (QCs) contain a known and consistent quantity of analyte that are used to ensure integrity of the data recovered for each test done. QC's are made in large quantities at several different levels and then stored and removed to either run experiments or to provide quality control for the duration of validation or actual study. To make both calibrators and quality controls the analyte, which is the compound of focus in the bioanalytical study is required. Study samples (experimental samples) are those samples that are acquired from an animal that was dosed with the drug.

Occidiofungin as a compound of interest

The primary compound of interest is OCF, which is a novel antimicrobial compound that has significant activity against fungal infections, mainly yeast (Emrick et al., 2013). OCF is derived from the soil bacterium *Burkholderia contaminans* MS14 and is a cyclic glycolipopeptide (Gu et al., 2009; Lu et al., 2009). This cyclic peptide is made of 8 amino acids, which contains a beta hydroxy tyrosine and a beta hydroxy asparagine. Additionally, a xylose sugar is attached via a glycosidic linkage to an 18 carbon novel amino acid. (Lu et el., 2009). OCF is produced via a non-ribosomal peptide synthetase type mechanism which involves multiple modular enzymes to produce it. With the increasing prevalence of fungal infections that do not respond to normal treatment strategies, an increase need for new antifungals is growing. Ideally, identifying and developing new antifungals have differing modes of action to the currently prescribed version and can increase treatment options (Emrick et al., 2013). Currently, there are four main groups of antifungals that are used therapeutically, polyenes, allylamines, azoles, and echinocandins. The first three groups mainly target ergosterol which is a critical component for maintaining cellular fluidity and permeability at homeostatic levels in fungal cells (Rice, 1999). Echinocandins however inhibit an enzyme complex that makes (1,3)-β-glucan polymers for fungal cell walls, which will degrade the structural integrity and shape of the fungal cell(Rice, 1999). OCF has a unique mechanism of action, allowing it to be effective against fungal species that have become resistant to current treatment methods. While the specific mechanism by which OCF exhibits is antifungal activity is unknown, initial research shows that it triggers a

mechanism similar to apoptosis in yeast, making it a prime candidate to be developed into an antifungal treatment (Emrick et al., 2013). Due to these factors OCF has prospects to treat a variety of conditions which includes both primary and recurring vulvovaginal candidiasis. Vulvovaginal candidiasis (VVC) is a condition that is caused by a *Candida albicans* in approximately 85-95 % of cases. Recurrent vulvovaginal candidiasis (RVVC) is a recurring infection of VVC which occurs three or more times within 12 months. Approximately 5-10% of women with VVC develop this condition (Alexia Matheson (2017); Sobel (2007). Developing RVVC can cause lost work hours and increase health care cost in addition to the medical consequences (Samuel Aballea, 2013). The medical consequences of VVC and RVVC can vary from inflammation and itching to severe discomfort and pain with abnormal vaginal discharge being possible (Samuel Aballea, 2013). Recurrent vulvovaginal candidiasis can occur for a variety of reasons but is commonly treated using over-the-counter (OTC) medications such as miconazole (Sobel, 1992). The current OTC medications used to be prescription only, however subsequent reclassification by the FDA has led to an increase in use of these antifungal drugs. This increase in use can be attributed to the increase in azole resistance in Candida species due to incorrect usage and diagnosis (Daron G. Ferris, 2002) . To further exacerbate this issue there have been little if any therapeutic developments for recurrent VVC that have been approved by the FDA (Smith, 2020). With the lack of further developments, it incentivizes further research and innovation to solve a growing issue. Additional experiments have demonstrated that it is not only active against yeast but other animal and plant fungal pathogens. Initial toxicology studies in mice also show

that OCF does not have any significant alterations on the functions of various organs. However, there was a decrease in body weight and organ weight over time at a 20mg/kg subcutaneous dose. While there were some effects on body and organ weights these effects were not serious and did not result in organ toxicity. This provides compelling evidence OCF should be pursued as a potential antifungal treatment. Further research into the antifungal effects of OCF *in vivo* should be further explored.

Potential Value of Research

This research will provide a new framework for OCF for future studies involving the bioanalytical measurement of OCF in rabbit and rat serum. This framework will allow for studying absorption, distribution, metabolism, and excretion in further toxicological and pharmaceutical studies. Methods developed from this study will support further animal studies investigating toxicology and pharmacokinetics. Further, these methods will allow for an IND application to be submitted to the FDA. This will allow for further research and knowledge into its effects through all phases of clinical research. Information provided by this study will influence a variety of factors in this application and influence its potential as an effective and safe drug.

CHAPTER II

DEVELOPMENT AND VALIDATION OF AN BIOANALYTICAL METHOD

Introduction

Fungal pathogens are a leading cause of disease in a variety of organisms from plants to humans. In humans, fungal infections are attributed to large medical costs in the way of treatments and lost productivity. The discovery and validation of new antifungal treatments is needed to provide treatment for fungal infections (Brown et al., 2012). Vulvovaginal candidiasis (VVC) and its recurrent form (RVVC) is one such fungal infection that effects a majority of women. It is commonly treated with over the counter drugs (OTC) but VVC is evolving to be more drug resistant and has thus become more difficult to treat. This necessitates significant demand for the development of new drugs that can treat these resistant infections. OCF is a novel antimicrobial compound that has activity against a wide range of fungi, such as the yeast responsible for VVC. OCF operates through a novel mechanism of action which mitigates many of the current resistance mechanisms arising in the clinic (Emrick et al., 2013).

Several regulatory requirements must be satisfied in order to get a drug into the clinic. One such regulatory requirement is filing an Investigational New Drug (IND) application with the Food Drug Administration for approval to initiate Phase 1 and Phase 2 clinical trials. The development of a typical drug can exceed one billion dollars, thus

developing better ways to evaluate a new drug for efficacy and safety is important (DiMasi et al., 2016; Pandey et al., 2010).

From the inception of the IND to the completion of clinical trials many technical aspects of drug development need to be addressed such as toxicology, mutagenicity, ADME (Absorption, Distribution, Metabolism and Excretion) etc. Many of these studies require that the drug and its metabolites are accurately quantified in various biological matrices such as blood, serum, plasma, bile, saliva and cerebrospinal fluid etc. In each matrix, a bioanalytical method must be developed and validated so that the bioanalytical method can be used to quantify the drug and/or its metabolites of interest (when deemed necessary). Several guidelines provided by the FDA are taken into consideration to ensure that the method developed will provide precise and accurate data as the method will be adopted for use in ongoing clinical development studies.

An important consideration for drug development is that FDA requires a rodent and a non-rodent animal models for toxicological testing. For this study, rats and rabbits were chosen as they represented both rodent and non-rodent animal model systems used in the development of a VVC antifungal product. Further, rabbit was chosen for the non-rodent animal model due its small size and its responsiveness to topical drugs (Auletta, 1994). . The main aim of this study is to develop a bioanalytical LC-MS/MS method that will be used to further the preclinical and clinical development of OCF. We specifically developed two methods to quantify OCF in rat and rabbit plasma. The development of these bioanalytical methods will support the toxicological studies on OCF in both rat and

rabbit animal models. The data from these studies will be used to support the IND application.

Materials and Methods:

Chemicals and Materials

All reagents were obtained from VWR unless otherwise indicated. Both the K_2EDTA Rat Plasma and the Sodium Citrate Rabbit plasma were obtained from Innovative Research (Innovative research, Novi, MI, USA). The internal standard and OCF were prepared by culturing *Burkholderia contaminans* MS14 using a process that was previously reported (Lu et al., 2009).

Occidiofungin Stable Isotope Internal Standard Production

When developing these LC-MS/MS methods, an internal standard is required, and there are several options available. For these methods a stable isotopically labeled (SIL) OCF was determined to be the ideal internal standard. This is due to its ability to act almost identically to the native compound and be distinguishable on the MS by virtue of its difference in mass. A Stable Isotopically Labeled compound of OCF was manufactured to be used as the internal standard in the bioanalytical methods. The SIL OCF was produced and purified as previously described(Lu et al., 2009). A notable modification to the process was the use of ^{15}N enriched ammonium chloride (Cambridge Isotope Laboratories) as the nitrogen source. ^{15}N label was chosen over other stable isotopes due to the elemental composition of OCF ($C_{52}H_{85}N_{11}O_{22}$), and the minimal growth media that is used to grow *Burkholderia contaminans* MS14. We anticipated that using an ammonium based ^{15}N has higher chances of getting incorporated into OCF, additionally

complete incorporation of ^{15}N would give enough resolution of the resulting SIL from the native OCF in the mass spectrometer. It must be noted carbon labeled compounds such as glucose are expensive in comparison to ^{15}N material, and we do not have appropriate knowledge as to where the labeled carbon will be incorporated in the molecule, also the efficiency and reproducibility of such incorporation is not known. Finally incorporating ^{15}N as an ammonium salt ensures that it will be used in all metabolic steps as it will be the sole nitrogen source (Chokkathukalam et al., 2014). After purification the SIL OCF was dried and submitted for mass spectrometry analysis. The mass of the fully substituted ^{15}N SIL OCF was predicted to be 1227 Daltons which is 11 higher than the standard 1216 OCF and this was the observed mass for the product made using ^{15}N enriched ammonium chloride. This indicated that the ^{15}N was fully substituted into the compound and the material those produced could serve as an ideal internal standard.

Preparation of Calibration Curves and Internal Standard Solutions

Calibrations Curve Solutions:

Calibrators are made using a known amount of analyte and are used to construct a curve that will be used to compare all other samples to allow for their quantification. Calibration curves were constructed from a native OCF stock solution composed of 6.5 mg of OCF dissolved in 25 mL of a 35% acetonitrile/65% water solution. This yielded a solution with OCF at a concentration of 200 ug/mL. This stock solution was stored in a 25 mL amber glass volumetric flask. The stock solutions was used to prepare spiking

solutions at eight different levels of concentration: 15 ng/mL, 30 ng/mL, 150ng/mL, 300ng/mL, 600ng/mL, 1500ng/mL, 2500ng/mL, and 15,000ng/mL (Table 1). All calibrator solutions were mixed and stored in 10 mL amber glass volumetric flasks at 4 °C. Subsequent dilutions from the calibrator solutions were performed using the same solution (35%:65% acetonitrile: water).

Internal Standard Solutions:

Two internal standard solutions were made for the experiments, a stock solution, and a working solution. The Internal Standard Stock solution (100 ug/mL) was made weighing 1 +/- 0.20 mg of ^{15}N labeled OCF and dissolving it in 10 mL of freshly prepared 35:65 acetonitrile: water solution. This solution was stored in a 10 volumetric flask and refrigerated at 4 °C.

The working internal standard solution (1000 ng/mL) was prepared by pipetting 0.1 mL of the internal standard stock solution into a 10 mL volumetric flask before the final volume was adjusted to 10 mL using the 35:65 acentonitrile:water solution. The final working solution was stored at 4 °C when not in use.

The working solution was spiked at a fixed volume into all calibrators and quality control samples for all experimental runs.

Quality Control Sample Preparation for Plasma Samples

Quality controls are the sample in matrix with a known concentration of the drug. It is independently prepared sample in the matrix as compared to the calibration curve. The QC sample is compared to calibration curve and is used to assess precision and accuracy

of the method. The quality control samples were prepared following the same procedure as outlined for both rat and rabbit plasma. Low QC is 3 times the lower limit of quantitation (LLOQ), medium QC is in the middle of the calibration curve, high QC is 80% of highest standard. Quality controls were prepared at 4 different concentrations to cover a large range of test samples and calibrators. The four QC samples are as follows: quality control low (QCL) 15 ng/mL, quality control middle (QCM) 200 ng/mL, Quality control high (QCH) 800 ng/mL, and quality control very high (QCVH) 12,000 ng/mL or 10,000 ng/mL in rat and rabbit plasma, respectively.

Preparation of QCVH:

This QC was made by adding 0.6 mL of the OCF stock solution to a 10 mL volumetric flask, and then adding plasma to a final volume of 10 mL. QCVH was then aliquoted out as 0.1 mL volume into 0.2 mL centrifuge tubes and stored at -80 °C.

Preparation of QCH:

This QC was made by adding 0.100 mL of the OCF stock solution into a 25 mL volumetric flask and then adding plasma to a final volume of 25 mL. QCH was aliquoted out as 0.2 mL into a 0.2 mL centrifuge tubes and stored at -80 °C

Preparation of QCM:

A retained sample (2.5 mL) of the OCH solution was used for making the QCM sample. This sample was added into a 10 mL volumetric flask and then plasma was added to a final volume of 10 mL. QCM was aliquoted out as 0.1 mL into a 0.2 mL centrifuge tubes and stored at -80 °C

Preparation of QCL:

A retained sample (0.075 mL of QCM was retained and placed into a 10 mL volumetric flask and then plasma was added to reach a final volume of 10 mL. QCL was then aliquoted out as 0.1 mL into a 0.2 mL centrifuge tubes and stored at -80 °C

Sample Preparation for Bioanalysis

Preparation of Calibrators for Analysis:

Blanks, calibration curve standards, and system suitability samples were prepared by adding 0.050 mL of the study plasma to 1.8 mL centrifuge tubes via a repeater pipette. The calibrators each received 5 µL of their respective calibrator spiking solution. Blanks received 5 µL of 35:65 acetonitrile:water solution to keep consistent volume with the calibrators Then all samples except for the blank received 10 µL of the internal standard working solution while the blank samples received 10 µL additional solvent to maintain consistent volume. Samples were capped and vortexed briefly for approximately 5-10 seconds to ensure homogeneity.

Preparation of QC for Analysis:

Six replicates of samples of quality controls at each level were thawed for 30 minutes at room temperature. While these samples thawed, the calibrators, internal standards, and plasma was removed from the fridge and given 30 minutes to come up to room temperature. After 30 minutes the calibrators, internal standards, and plasma were thoroughly mixed by inverting them multiple times. After 30 mins of thawing 0.050 mL of each respective QC sample was transferred to a 1.8 mL centrifuge tube using an air displacement pipette. 5 µL of 35:65 acetonitrile:water solution was then added to the QC samples to keep consistent volume with the calibrators. Then all samples except for the

received 10 µL of the internal standard working solution while the blank samples received 10 µL additional solvent to maintain consistent volume. Samples were capped and vortexed briefly for approximately 5-10 seconds to ensure homogeneity.

Protein Precipitation of all Samples:

Subsequently, 0.100 mL of methanol was added to each sample and vortexed for 5-10 seconds to precipitate the proteins out of solution. Samples were then centrifuged at 6,000 rpm using an Eppendorf 5415R rotor housed in an Eppendorf 5415D Centrifuge for 16-18 minutes to pellet the precipitate. After centrifugation, the clear upper liquid layer was transferred to a new 1.8 mL centrifuge tube taking care to minimize protein transference by disrupting the pellet. Samples were then stored at 4 °C until submitted for LCMS analysis.

LCMS-MS Instrumentation Setting and operating conditions

The liquid chromatography tandem mass spectrophotometry unit consisted of a ThermoFisher Quantum Access mass spectrometer with a HESI ion source in positive ionization mode. The ion source's spray voltage was set at 3800 +/- 100 volts, with a vaporizer temp of 300 °C. The LC-MS-MS is set to monitor in MRM and is monitoring OCF with a mass of 1216 at Q1 and its product ion 1084 at Q3. Additionally, the SIL internal standard was monitored with a mass of 1227 at Q1 and its product ion at 1095 Daltons. Dwell time for both samples was 1.000 seconds. The HPLC pumps and auto sampler were an Agilent 1200 system running a SinoChrom C18 Column, 3 micron, 4.0 x 50 mm column. Column temperature was set at 40 °C. HPLC flow rate was 0.85 mL a minute with a starting gradient of 95:5 (water:acetonitrile) with 0.1% formic acid.

Retention time for both the SIL compound and analyte was 10.2 minutes. Two sets of system suitability samples were run before every experimental run with N=7. The first set was using a separately prepared calibrator A and the second set used a separately prepared calibrator B. The first injection was dropped and the last six were used to determine residual standard deviation (RSD).

Results and Discussion

Production of an ^{15}N Labeled Internal Standard for Occidiofungin

As described in the method the ^{15}N stable isotope labeled (SIL) internal standard for OCF was produced using a slight modification to previous described methods for culturing non-labeled OCF. To check for consistent ^{15}N labeling of the internal standard, the purified internal standard preps and native OCF were analyzed using a ThermoFisher Exactive Orbitrap mass spectrometer was analyzed on the LCMS and the mass and chromatogram compared to that of OCF.

Occidiofungin has a mass of 1,216 Daltons with a chemical formula of $C_{52}N_{11}O_{22}H_{85}$, thus using the ^{15}N isotopologue and obtaining full incorporation would yield a mass that is increased by 11 Daltons thus weighing 1,227 Daltons. When initial ^{15}N samples were dried down and ran on the ThermoFisher Exactive Orbitrap mass spectrometer the significant mass returned was 1,227.5540 with a charge of +1 (Table 2). This confirmed that the incorporation of ^{15}N into OCF was very efficient under the culturing conditions used to make the SIL standard.

An aspect of SIL that makes it preferred over other internal standards is that it behaves identically to the analyte in many physicochemical aspects except for its molecular mass. HPLC was used to further evaluate our SIL internal standard and the base analyte for physiochemical similarities. Upon comparisons of chromatograms of ^{15}N labeled OCF and purified OCF, both chromatograms have similar retention times, peak shape, and area under the curves (data not shown).

Method Validation

Linearity and Sensitivity

The calibration curve for OCF was constructed to give a wide range of concentrations. Rat plasma linearity was established using the correlation coefficient for the calibrators, which on average was 0.98(Table 3). This value justifies and indicates acceptable levels of linearity within the range of 15 to 15,000 ng/mL. Rabbit plasma linearity was established in a similar manner with an average correlation coefficient of .97633 indicating that there are acceptable levels of linearity between 15 and 15,000ng/mL (Table 4).

Accuracy, Intraday and Inter-Day Precision (Rat Plasma)

Accuracy was expressed as a percentage residual standard deviation between 6 sets of each quality control sample. Precision relied on these same sets of quality control samples but used the average percent difference between the prepared concentration and the measured concentration. The Intraday RSD for QCL ranged from -19.23 to 84.45 % and the precision ranged from -245.05 to 43.65 % difference (Table 5). At 15 ng/mL QCL was neither reproducible nor precise in its measurements. Taking into account all six days of studies the inter-day precision and accuracy was 23.39 and 38.20 % respectively. QCM's intraday accuracy and precision ranged between 6.05 to 22.06 and -1.05 to -18.94 % respectively. Inter-day precision and accuracy were -5.64 and 12.77 % respectively. These values are within the guidelines that are set by the bioanalytical method without modification. QC high had an intraday precision that ranged from 1.26 to -25.37 % and an accuracy with a range 3.61 to 16.5 %. Inter-day precision for QCH was -6.84 % while accuracy was 16.32 %. QCVH precision ranged between -31.97 to 69.59 %, while its accuracy ranged from 9.48 to 109.35 %. The inter-day precision and accuracy was 12.86 and 84.09 % respectively. These results for the QC high and QC very high indicate that while the method is precise it is not maintaining reproducibility.

It was hypothesized that this was due to intraday inconsistency in calibrator's or the variability in the mass spectrometry system. Calibrators that had greater than +/- 15% difference from the nominal concentration were dropped and the data was reexamined. Further, in the event that multiple calibrators fell above the 15% difference, the individual sample with the highest % difference was dropped (Table 6). When these adjustments were made the intraday precision and accuracy improved for each quality control, however there were still runs that were not within 85% to 115% precision, and greater than 15% residual standard deviation. This indicated that there were outliers within each data set that were influencing negatively influencing the calibrators. Removing these outliers brought the inter-day precision within reasonable levels for all 4 quality controls. The inter-day accuracy for the quality control low, medium, high, very high was 77.82, 15.29, 10.25, and 22.65 % respectively. Thus, the inter-day accuracy of the quality controls even when taking into account calibration variation cannot be well established for QCL, QCM, and QCVH, while QCH inter-day accuracy can be confirmed. The effects seen on the inter-day and intra-day accuracy was determined to be due to the variability of the mass spectrometry instrument. This was confirmed and controlled for by running system suitability samples before each experimental sample to determine the deviation of the LCMS system.

Accuracy, Intraday and Inter-day Precision (Rabbit Plasma)

Intra-day and inter-day accuracy and precision was evaluated using the parameters set in the guidelines from the FDA. These guidelines indicate that precision and accuracy should be within 15%. The between run accuracy and precision were all within the 15% limit, indicating that the method might be usable for further validation experiments (Table 7). At 15 ng/mL, the between run precision and accuracy is outside of the range for validation, indicated that the 15 ng/mL is our lower limit of quantification, because at this point we are losing the ability to quantify samples with both accuracy and precision. QCL at 15 ng/mL continued to be the lower limit even when 7 calibrator samples were used instead of 8 samples. As seen in Table 8, even after dropping outliers, this approach does not bring QCL into precision and accuracy compliance.

Recovery and Matrix Effects

Matrix Effects (Rat Plasma)

Matrix effects were analyzed to determine the interference that could be caused by the matrix on the samples. This effect can increase or decrease the area counts for the analyte giving variable readings. To determine the matrix effect calibrators are made and ran using no plasma and compared to the calibrator C extracted from matrix. Matrix effects were tested on two calibrators, calibrator C and H at 150 and 15,000 ng/mL respectively (Table 9). The matrix effect observed for calibrator C and H was 0.86668

and 0.86364 %, respectively. This falls within the +/- .15 range for matrix effects indicating that there is an insignificant effect on the samples due to the matrix tested.

Matrix Effects (Rabbit Plasma)

These results have not been obtained yet

Recovery

Recovery from the plasma solution was tested using two calibrator solutions, Calibrator C and H at 150 and 15,000 ng/mL, respectively. At calibrator C the average percent recovery was 54 % which indicates that not all the analyte or spiked internal standard is being recovered from the extraction. There was a notable hook effect within the internal standard for calibrator H (Figure 1). When calibrator H was tested the average percent recovery was 118 % (Table 10). This indicates that the method was returning more of a response for the analyte and is than what was added to the sample. This is most likely due to the increase in analyte causing suppression within the response of the internal standard. This can cause distortion in the data which can lead to variation in results.

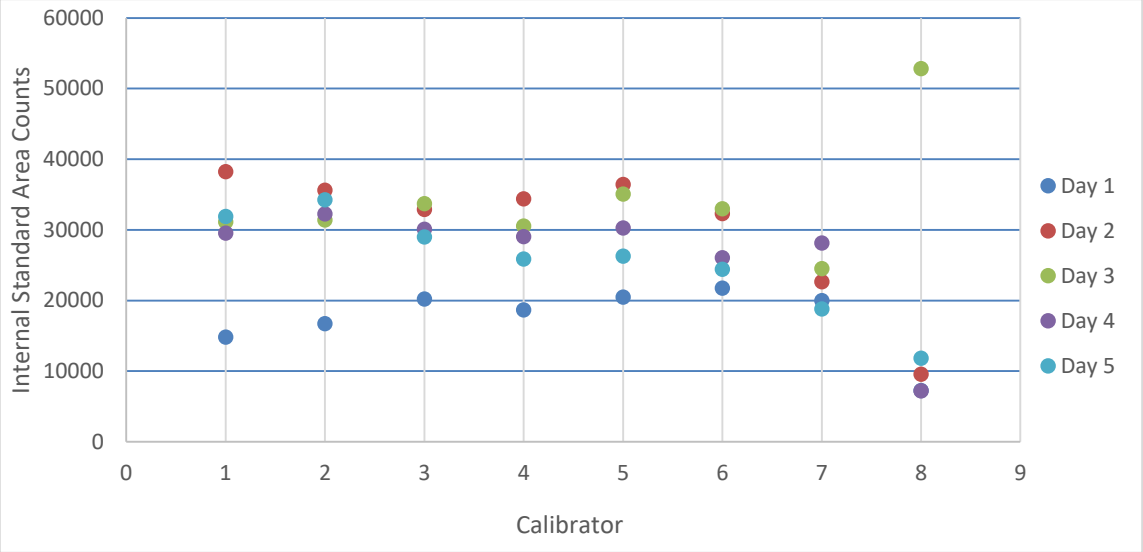

Figure 1: Graph showing drop in internal standard area counts as the amount of analyte increases. Max hook effect seen at Calibrator 8 (H) with 15,000 ng/mL of analyte

Stability Studies

Freeze Thaw Studies (Rat Plasma)

Freeze thaw stability was assessed over a course of two thaw schedules, with each study containing all QC's done in triplicate. After 3 cycles of freezing and thawing QC low, medium, and very high all saw significant reduction in the amount of material detected as indicated by the high percent difference from the predicted value for the QCs .(Table 11). In QCH, the percent difference was within the acceptable range of +/-15% with an RSD of 3%. The data after 6 freeze thaw cycles looked similar to that of the 3 cycles, with QCL, QCM, and QCVH showing a significant reduction in the amount of analyte as indicated by the high percent differences. QCH had a 3% difference with an RSD of

12% which indicated that it did not see the same level of degradation. However overall degradation in the freeze thaw study indicated that samples should be prepared fresh, and not frozen and thawed repeatedly.

Freeze thaw Studies (Rabbit plasma)

Freeze thaw stability for rabbit plasma was assessed in the same manner as rat plasma (Table 12). The overall accuracy and precision were within tolerable limits which had a range of 11% to 14% and 2.6% to 12.4% for 3 day and 6 day studies respectively. This indicates that within rabbit plasma the analyte will maintain stability for at least 6 thaw cycles. This allows for the freezing and unfreezing of samples multiple times without worry of major error. However, the effect of QC low is still returning values indicative of it being the LLOQ, due to the high variability and lack of precision.

Bench Top Stability (Rat)

Bench top stability was assessed after 24 hours sitting at ambient temperature to determine the change in accuracy and precision (Table 13). After 24 hours, the % difference for QCL, QCM, and QCVH were -77.55, -25.53, -16.35, -31.44 %, respectively. This is indicative that sitting overnight on a bench top had reduced the amount of analyte and thus the precision of the QCs to a level that would invalidate the analysis. Accuracy wise the bench top samples maintained less than 15% RSD indicating that the samples degraded at a similar rate. QCL however had a residual standard deviation of 93% indicating high variability among the analyzed samples.

Bench Top Stability (Rabbit)

Bench top stability for rabbit samples had similar results to that of the rat plasma (Table 14). This is indicative that when preparing samples, the samples should be appropriately stored at – 20 °C or sample degradation over the course of 24 hours will put them out of an acceptable QC range.

Conclusion

Based on what we have learned through the study OCF stability in matrix is not consistent for 24 hours at room temp indicating that samples should be extracted and quickly stored. Additionally, based on freeze thaw studies, samples should not be thawed and refrozen for rat plasma, and up to 3 times for rabbit plasma. We have learned that the current method of sample preparation and submission to LCMS will yield valid results. However, an adjustment of range should be considered, due to the high variability and lack of accuracy at 15ng/mL. This indicates that 15 ng/mL is our lower limit of detection for this method. Based on FDA guidance this is a valid method ranging from 30ng/mL to 15,000 ng/mL and should be used to support further studies.

Future studies should include developing and validating a QC for 30 ng/mL in addition to determining the OCF stability in matrix long term storage (3-6 months). A method will need to be validated for the use in lab mice and for studying gel product stability in

addition to other stability studies. Finally, a development of a method in human serum and plasma should be pursued as it will support further clinical work.

Overall, this work supports the development of a method for OCF in both rat and rabbit plasma. The bioanalytical methods developed have suitable levels of precision and accuracy and will be subsequently validated in future studies. The validated methods can be then used in analysis of samples from both rats and rabbits (toxicology, pharmacokinetic and metabolism studies). These methods will also serve as a starting point for future human clinical work to support regulatory filings of OCF to the FDA.

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Table 1: Calibration Spiking Solutions' Dilutions Scheme.

Calibration Standard	Occidiofungin Target Conc. In Rabbit Plasma (ng/mL)*	Amount of Primary Solution (A) to Pipet (mL)	Volume of Acetonitrile/Water (CS-1) (mL)
A	15	5.00 mL of Cal Std B	5
B	30	2.00 mL of Cal Std C	8
C	150	5.00 mL of Cal Std D	5
D	300	5.00 mL of Cal Std E	5
E	600	0.3	9.7
F	1500	0.75	9.25
G	2500	1.25	8.75
H	15000	7.5	2.5

Table 2 Elemental composition and mass determination of the ^{15}N labeled internal standard in comparison to isotopologues of occidiofungin

Isotopologue	Elemental composition	Exact mass (Observed Mass)
Native OCF	$C_{52}N_{11}O_{22}H_{85}$	1216.5533
$^{15}N_{11}$ OCF	$C_{52}N_{11}O_{22}H_{85}$	1227.5540
$^{15}N_{10}$ OCF	$C_{52}N_{11}O_{22}H_{85}$	1226.5635
^{15}N9 OCF	$C_{52}N_{11}O_{22}H_{85}$	1225.5794

Table 3: Rat Plasma Linearity**.**
Rat Plasma Linearity is shown by the regression lines R^2 value being greater than 0.095. This shows that the Calibrators are can provide reliable data across the whole range of analysis. Slope equation is reported as the provided by the instrument and analysis.

Day #	Number of Calibrators	Slope Equation	Regression R^2
Day 1	8	Y = 0.00333233*X	0.99
Day 2	8	Y = -0.0229739+0.0031187*X	0.99
Day 3	8	Y = 0.0984493+0.00277851*X	0.98
Day 4	8	Y = -0.0336339+0.00375003*X	0.96
Day 5	8	Y = -0.0205244+0.00312852*X	0.96
Day 6	8	Y = -0.0232323+0.00282903*X	0.99

Table 4: Rabbit Plasma Linearity
Rabbit Plasma Linearity is shown by the regression lines R^2 value being greater than 0.095. This shows that the Calibrators are can provide reliable data across the whole range of analysis. Slope equation is reported as the provided by the instrument and analysis.

Day #	Number of Calibrators	Slope Equation	Regression R2
Day 1	8	Y = -0.026694+0.0026762*X	0.99
Day 2	8	Y = -0.0175922+0.0027253*X	0.95
Day 3	8	Y = 0.00623984+0.00262997*X	0.99
Day 4	8	Y = -0.0128328+0.00269482*X	0.97

Table 5: Mean intra-day and inter-day precision and accuracy for rat plasma using all calibrators regardless of accuracy.
Accuracy and precision using all calibrators shows high variability in the data and importantly a failure to validate the precision shown in between run data using all calibrators. Day 1-6 do not represent actual days and samples were run approximately a week apart from each other.

Rat Plasma Accuracy and Precision 8 Calibrators							
Within-run 15 ng/ml Unmodified							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Between Run
Accuracy	16.58	35.57	-245.05	43.65	19.62	7.62	23.39
Precision	84.45	6.61	19.23	13.00	12.99	15.65	38.20
Within-run 200 ng/ml Unmodified							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Between Run
Accuracy	-9.56	-11.93	-6.16	-18.94	-2.14	-1.05	-5.64
Precision	17.35	6.05	6.68	22.06	15.19	17.18	12.77
Within-run 800 ng/ml Unmodified							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Between Run
Accuracy	-12.71	-10.22	-5.85	-25.37	1.26	10.16	-6.84
Precision	9.33	7.28	7.94	3.61	10.59	16.50	16.32
Within-run 12000 ng/ml Unmodified							
	Day 1	Day 2	Day 3	Day 4	Day5	Day 6	Between Run
Accuracy	N/A	N/A	24.61	-31.97	69.59	12.20	13.86
Precision	N/A	N/A	9.48	12.79	109.35	18.22	84.09

Table 6: Mean Intra-day and Inter-day precision and accuracy for rat plasma dropping a single calibrator with the least accuracy.
Rat plasma accuracy and precision are shown to be within acceptable limits when dropping a calibrator that was more than 15% different from its prepared value. The calibrator dropped varied between each experimental day. Day 1-6 do not represent actual days and samples were run approximately a week apart from each other.

Rat Plasma Accuracy and Precision 7 Calibrators							
Within-run 15 ng/ml 7 calibrator's							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Between Run
Accuracy	16.58	35.57	-78.77	-51.79	17.22	7.62	-17.55
Precision	84.45	6.61	117.87	41.54	12.42	15.65	77.82
Within-run 200 ng/ml 7 calibrator's							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Between Run
Accuracy	-9.56	-11.93	-4.46	-28.98	-7.92	1.94	-12.64
Precision	17.35	6.05	5.88	5.99	15.12	17.24	15.39
Within-run 800 ng/ml 7 calibrator's							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Between Run
Accuracy	-12.71	-10.22	-12.69	-21.99	-5.03	13.78	-12.78
Precision	9.33	7.28	7.68	3.70	10.58	16.51	10.25
Within-run 12000 ng/ml 7 calibrator's							
	Day 1	Day 2	Day 3	Day 4	Day5	Day 6	Between Run
Accuracy	N/A	N/A	8.75	-25.02	-11.83	5.10	0.67
Precision	N/A	N/A	7.22	11.39	16.24	11.44	14.18

Table 7: Inter and Intra Day Precision and Accuracy of Na Citrate Rabbit plasma Rabbit plasma accuracy and precision unmodified over 4 experimental days. The between run data fails to validate between 200 and 10000 ng/mL using 8 calibrators. . Day 1-4 do not represent actual days and samples were run approximately a week apart from each other.

Rabbit Plasma Accuracy and Precision 8 Calibrators					
Within-run 15 ng/ml Unmodified					
	Day 1	Day 2	Day 3	Day 4	Between Run
Accuracy	158.02	0.64	-33.74	270.33	143.00
Precision	18.99	16.07	32.90	64.99	72.15
Within-run 200 ng/ml Unmodified					
	Day 1	Day 2	Day 3	Day 4	Between Run
Accuracy	-6.98	-21.17	-4.88	25.28	-0.09
Precision	12.75	9.66	18.67	27.03	25.22
Within-run 800 ng/ml Unmodified					
	Day 1	Day 2	Day 3	Day 4	Between Run
Accuracy	-0.26	-8.36	-8.40	-2.97	-7.81
Precision	4.92	4.96	3.62	4.75	15.61
Within-run 10000 ng/ml Unmodified					
	Day 1	Day 2	Day 3	Day 4	Between Run
Accuracy	-3.51	3.17	-3.25	9.48	1.47
Precision	6.37	6.63	10.00	16.50	11.64

Table 8: Inter- and Intra-day precision and accuracy of Na Citrate Rabbit plasma with 1 Calibrator dropped

Rabbit plasma accuracy and precision when 1 calibrator that was more than 15% different from the prepared value was dropped from the analysis. Between run data shows that accuracy can be validated for 200-10000 ng/mL and precision between 800-10000ng/mL. Day 1-4 do not represent actual days and samples were run approximately a week apart from each other.

Rabbit Plasma Accuracy and Precision 7 Calibrators					
Within-run 15 ng/ml 7 calibrator's					
	Day 1	Day 2	Day 3	Day 4	Between Run
Accuracy	158.02	-0.65	-33.74	286.99	83.39
Precision	18.99	16.92	32.90	66.49	90.02
Within-run 200 ng/ml 7 calibrator's					
	Day 1	Day 2	Day 3	Day 4	Between Run
Accuracy	-6.98	-14.30	-4.88	33.38	-2.85
Precision	12.75	9.71	18.67	27.17	27.27
Within-run 800 ng/ml 7 calibrator's					
	Day 1	Day 2	Day 3	Day 4	Between Run
Accuracy	-0.26	-4.84	-8.40	-6.32	-4.50
Precision	4.92	4.96	3.62	14.49	5.60
Within-run 10000 ng/ml 7 calibrator's					
	Day 1	Day 2	Day 3	Day 4	Between Run
Accuracy	-3.51	7.24	-3.25	17.17	2.38
Precision	6.37	6.63	10.00	16.51	13.26

Table 9: Matrix affects for Rat plasma, C-150 is calibrator C at 150 ng/mL and H-15000 is calibrator H at 15,000 ng/mL
Matrix effects for rat plasma was validated as it was between .85 and 1.15 indicating that matrix effects will not inhibit our analysis.

Calibrator	MF for analyte	MF IS	MF Normalized	Average Matrix Factor
C-150	0.929	0.826	0.853	0.866
C-150	1.036	1.413	0.828	
C-150	1.353	1.331	0.918	
H-15000	0.743	1.025	0.850	0.863
H-15000	0.987	0.870	0.797	
H-15000	0.621	0.946	0.943	

Table 10: This table shows the percent recovery obtained at C and H calibrator levels. Recovery was consistent across all three samples for both C and H calibrator levels, this will support validation of the method. This was determined by looking at the normalized recovery in comparison to each other.

Calibrator-amount ng/mL	% Recovery analyte	% Recovery IS	% Recovery Normalized	Average % Recovery
C-150	26.33	49.30	53.42	54.14
C-150	33.39	64.95	51.41	
C-150	33.09	57.46	57.58	
H-15000	31.04	25.97	119.54	118.20
H-15000	27.15	22.15	122.58	
H-15000	35.44	31.51	112.48	

Table 11: Freeze thaw stability for Rat plasma
Freeze thaw stability data for rat plasma indicates that samples will lose enough analyte over multiple freeze thaw cycles to no longer be valid for analysis. This was determined by looking at the % difference from composite which would need to be less than 15% difference.

Rat Plasma Freeze Thaw Stability				
	QCL	QCM	QCH	QCVH
Prepared Concentration	15	200.00	800.00	12000.00
Mean Measured Concentration	7.231	174.72	697.73	12080.50
Composite Concentration	11.116	187.36	748.87	12040.25
Freeze Thaw 3 Cycle Composite Value	0.353	154.09	699.85	9770.32
Freeze Thaw 3 Cycle % Difference from Composite	-96.825	-17.76	-6.55	-18.85
Freeze Thaw 6 Cycle Composite Value	1.557	161.72	772.07	9382.98
Freeze Thaw 6 Cycle % Difference from Composite	-85.989	-13.69	3.10	-22.07

Table 12: Freeze Thaw Stability Studies for Rabbit plasma .
Freeze thaw stability data for rabbit plasma indicates that samples will lose enough analyte over 6 freeze thaw cycles to no longer be valid for analysis. However this data indicates that 3 freeze thaw cycles will not impact analyte levels enough to impact analysis. This was determined by looking at the % difference from composite which would need to be less than 15% difference.

Rabbit Plasma Freeze Thaw Stability				
	QCL	QCM	QCH	QCVH
Prepared Concentration	15.00	200.00	800.00	10000.00
Mean Measured Concentration	27.51	194.29	749.29	10238.47
Composite Concentration	21.26	197.15	774.65	10119.24
Freeze Thaw 3 Cycle Composite Value	23.05	168.99	768.43	10821.46
Freeze Thaw 3 Cycle % Difference from average	8.45	-14.28	-0.80	6.94
Freeze Thaw 6 Cycle Composite Value	15.57	162.35	747.06	11943.55
Freeze Thaw 6 Cycle % Difference from average	-26.75	-17.65	-3.56	18.03

Table 13: **Rat Plasma Benchtop Stability**
Bench top stability data for rat plasma indicates that samples will lose enough analyte over 24 hours at room temp that they would no longer be valid for analysis due to loss of analyte. This was determined by looking at the % difference from composite which would need to be less than 15% difference.

Rat Plasma Bench Top Stability				
	QCL	QCM	QCH	QCVH
Prepared Concentration	15	200.00	800.00	12000.00
Mean Measured Concentration	7.231	174.72	697.73	12080.50
Composite Concentration	11.116	187.36	748.87	12040.25
Bench Top Composite Value	3.3682	149.55	669.20	8226.80
Bench Top % Difference from average	-69.699	-20.18	-10.64	-31.67

Table 14: Bench top stability for rabbit plasma after 24 hours
Bench top stability data for Rabbit plasma indicates that samples will lose enough analyte over 24 hours at room temp that they would no longer be valid for analysis due to loss of analyte. This was determined by looking at the % difference from composite which would need to be less than 15% difference.

Rabbit Plasma Bench Top Stability				
	QCL	QCM	QCH	QCVH
Prepared Concentration	15.00	200.00	800.00	10000.00
Mean Measured Concentration	27.51	194.29	749.29	10238.47
Composite Concentration	21.26	197.15	774.65	10119.24
Bench Top Composite Value	29.19	151.11	780.40	13611.00
Bench Top % Difference from average	37.33	-23.35	0.74	34.51