Determination of Rifapentine and 25-O-desacetyl Rifapentine from 100 µl human breastmilk by LC-MS/MS using protein precipitation and solid phase extraction

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DECLARATION

I, Buyisile Mkhize, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ABSTRACT

There is currently no information available on the transfer of the second-line anti-TB drug, rifapentine and its metabolite, into breastmilk. The subsequent implications to the breastfed infant, as well as consequences of long-term exposure to potentially sub-therapeutic drug levels with regards to the development of drug resistant bacteria is therefore not known. A liquid chromatography method with detection by mass spectrometry (LC-MS/MS) is described for the quantification of rifapentine and its metabolite, 25-O-desacetyl rifapentine in human breastmilk, using rifampicin-d3 as an internal standard. An AB Sciex 4000 mass spectrometer at unit resolution in the multiple reaction monitoring (MRM) mode was used to monitor the transition of the protonated precursor ions m/z 877.5, m/z 835.4 and m/z 827.4 to the product ions m/z 151.1, m/z 453.2 and m/z 151.200 for rifapentine, 25-O-desacetyl rifapentine and rifampicin-d3, respectively. Ions were produced using Electro spray ionisation (ESI) in the positive ionisation mode. An Agilent Poroshell 120 EC-C18 (4.6 x 50 mm, 2.7 µm) column was used for chromatographic separation using an isocratic method of acetonitrile containing 0.1% formic acid and water containing 10% methanol and 0.1% formic acid (55:45, v/v), at a flow rate of 450 µl per minute. The retention times for rifapentine, 25-O-desacetyl rifapentine and rifampicin-d3 were ~2.67, ~1.88 and ~1.96 minutes, respectively.

The method was developed and validated according to FDA guidelines. The extraction method consisted of a combination of protein precipitation and C18 solid phase extraction. Rifapentine and 25-O-desacetyl rifapentine showed no significant carry over on the Agilent autosampler. The method was reproducible when analysed with human breastmilk from six different sources from Western Cape Maternity Breastmilk Bank.

Rifapentine mean extraction yield was 84.2% (%CV = 1.7) and that of 25-O-desacetyl rifapentine was 71.1% (%CV = 10.8). Rifapentine had a mean process efficiency of 80.4% (%CV = 4.7) and that of 25-O-desacetyl rifapentine was 95.7% (%CV = 5.7). Intra- and inter day validations over 3 days were performed. The calibration curves fit a quadratic regression with 1/x weighting over a concentration range of 2 – 2000 ng/ml for both rifapentine and 25-O-desacetyl rifapentine based on the analyte/internal standard peak area ratios, the accuracy ranged from 92.9% to 105.5% for both rifapentine and 25-O-desacetyl rifapentine standards. The Quality Controls accuracy ranged from 97.4% to 106.0% for both rifapentine and 25-O-desacetyl rifapentine. Stock solutions were shown to be stable for 69 days at -80°C.

Rifapentine and 25-O-desacetyl rifapentine were stable in human breastmilk for up to 72 hours at approximately -80°C and -20°C, on benchtop for ~4.5 hours on ice and after three freeze-thaw cycles. Rifapentine and 25-O-desacetyl rifapentine were shown to be stable on the autosampler over a period of approximately 48 hours after which the entire batch could be reinjected. Autosampler stability revealed a decrease in peak area ratios, indicating that a partial batch cannot be reinjected after 48 hours in case of instrument failure.

This method will be utilized in the analysis of patient samples from a clinical study in South Africa in breastfeeding women with tuberculosis.

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- To develop a suitable extraction method of rifapentine and 25-O-desacetyl rifapentine from breastmilk 14
- To develop a robust LC-MS/MS method for rifapentine and 25-O-desacetyl rifapentine quantitation and validate according to FDA guidelines 14

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I. LIST OF ABBREVIATIONS

% CV Percentage Coefficient of Variation. Used to express Precision of an analytical procedure

% Difference (% Bias) The difference between analyte concentration against reference concentration expressed as a percentage

% Difference (% Bias) = (Found value – reference value / reference value) x 100

% Accuracy (% Nominal) Analyte concentration against the nominal concentration expressed as a percentage

% Accuracy (% Nominal) = (Found value / Nominal value) x 100

~	Approximately
°	Degrees
µg	Microgram
µl	Microliter
ACN	Acetonitrile
C	Celsius
Conc.	Concentration
FDA	Food and Drug Administration
g	Gram
HPLC	High Performance Liquid Chromatography
ISTD	Internal Standard
LC-MS/MS	Liquid Chromatography with Tandem Mass Spectrometry
LLE	Liquid-liquid extraction
LLOQ	Lowest Limit of Quantification
MeOH	Methanol

min	Minutes
mg	Milligram
ml	Millilitre
N_2	Nitrogen
n	Number of determinants
n/a	Not applicable
ng	Nanogram
PHREE	Phospholipid Removal Columns
QC	Quality Control
rcf	Relative centrifugal force
rpm	Revolutions per minute
sec	Seconds
SOP	Standard Operating Procedure
SPE	Solid Phase Extraction
STDEV	Standard Deviation
SS	Stock Solution
STD	Calibration Standard
ULOQ	Upper Limit of Quantification
v/v	Volume per volume
WS	Working Solution

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1 Introduction

1.1 Tuberculosis

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* which predominantly affects the lungs, but can also affect the kidneys, brain and spine. There are two forms, namely latent and active TB. In latent TB, the immune system supresses the TB bacteria, but if the immune system is compromised TB can become active. Active TB makes a person sick and can spread through the air when a person coughs or sneezes. It is estimated that 2 billion people in the world have latent TB infection, therefore they harbour *M. tuberculosis* in a nonreplicating dormant stage in their tissues with 10% of people reactivating to active TB at some point in their life (Zumla, et al., 2015). The World Health Organization (WHO) reported that in 2017 an estimated 10.0 million people developed TB; 5.8 million were men, 3.2 million were women and 1.0 million were children (WHO, 2018). India, Indonesia, China, Philippines, Nigeria, Pakistan and South Africa accounted for 27%, 8%, 9%, 6%, 4%, 5% and 3%, respectively, of the TB cases (WHO, 2018). Of these cases, 9% were of Human Immunodeficiency Virus (HIV) co-infected individuals, of which 72% were in Africa (WHO, 2018). WHO Europe and America region were reported to each have 3% of the global cases (WHO, 2018). WHO reported that approximately 920 000 individuals infected with HIV developed TB and approximately 300 000 individuals died from HIV-associated TB (WHO, 2018). However, concurrent use of antiretroviral (ARV) therapy and anti-TB drugs have significantly reduced the mortality rate of HIV/TB patients. Concurrent treatment of HIV and TB can be challenging due to potentially underlying patient comorbidities, toxicity and resistance, drug-related factors such as adherence to therapy and complex drug interactions (Regazzi, et al., 2014). HIV infected individuals are thought to be at a higher risk of developing drug-resistant TB (Suchindran, et al., 2009).

Multidrug resistant TB (MDR-TB) is characterised by resistance to at least two of the first-line anti-TB drugs while extensively drug resistant TB (XDR-TB) is characterized by resistance to at least four anti-TB drugs; these may include isoniazid, rifampin and any fluoroquinolones (Zumla, et al., 2015). Resistance can arise due to lack of treatment compliance by TB patients and sub-therapeutic drug levels, to mention a few. Surveillance data from the WHO reports that there are 4.1% new rifampicin-resistant or MDR-TB cases while 19% are from cases of individuals who have previously been treated with TB (WHO, 2017). WHO estimated 558 000 individuals developed resistance to rifampicin and 82% were diagnosed with MDR-TB (WHO, 2018). An estimated 8.5% of TB cases were reported to be of XDR-TB (WHO, 2018).

1.2 Tuberculosis treatment

Treatment of active TB requires combination therapy comprising of two months of isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol (EMB) followed by four months of RIF and INH (Iacobino, et al., 2017). The recommended dosage for these first-line oral anti-TB drugs is 5 mg/kg once daily for INH, 10 mg/kg once daily for RIF, 25 mg/kg once daily for PZA and 15-25 mg/kg once daily for EMB (Zumla, et al., 2015). These first-line anti-TB drugs are used for the treatment of active TB based on their ability to target the bacterial cell and inhibit its growth. The mechanism of action for rifampicin is not clearly understood. It has been shown to inhibit the *M. tuberculosis* cellular process by binding to the bacterial β-subunit responsible for DNA-dependent RNA polymerase, resulting in transcriptional interference which subsequently results in cell death (Nusrath & Hanna, 2017). Pyrazinamide is a pro-drug that is converted in the human body to pyrazinoic acid which enters the bacterial cell via passive diffusion causing cytoplasmic acidification, resulting in the inhibition of bacterial enzymes and membrane transport (Nusrath & Hanna, 2017). INH is a pro-drug that is acetylated by N-acetyl transferase to N-acetyl INH. It enters the bacterial cell via passive diffusion and inhibits the synthesis of the bacterial nucleic acids and cell wall lipids using isonicotinic-acetyl-nicotinamide-adenine-dinucleotide (INH-NAPD) and adenosine diphosphoribose (NAD) adducts (Bardou, et al., 1998) (Timmins & Deretic, 2006). The mechanism of action of EMB is to inhibit the arabinosyl transferases responsible for the bacterial cell wall biosynthesis thus permitting the drug to freely penetrate the bacterial cell to kill the bacteria (Goude, et al., 2009).

Latent TB requires nine months of INH treatment or three months of rifapentine once weekly together with INH, three to four months of INH and RIF or three to four months of RIF only (Dutta & Karakousis, 2014) (Zumla, et al., 2015). To ensure compliance from TB patients, directly observed therapy (DOT) is applied where a health professional typically observes the patient taking the respective anti-TB drugs as scheduled and document the observation, allowing the patients to be monitored and data to be generated (Karumbi & Garner, 2015). The effectiveness of DOT in reducing treatment failure observed in some studies (Tian, et al., 2014) has not been replicated in others (Pasipanodya & Gumbo, 2013).

MDR-TB requires treatment with injectables for at least 8 months followed by 12-18 months of continuation phase (Zumla, et al., 2015). MDR-TB drugs are chosen based on drug-susceptibility and the injectables kanamycin and capreomycin are drugs to consider, together with the fluoroquinolones such as levofloxacin and moxifloxacin (Zumla, et al., 2015). Other anti-TB drugs such as cycloserine and ethionamide, together with PZA and EMB are considered in incorporating second-line anti-TB drugs in treatment regimens for MDR-TB (Zumla, et al., 2015). XDR-TB treatment is usually chosen according to bacterial susceptibility testing results (Heyckendorf, et al., 2014). XDR-TB requires

intensive treatment using a minimum of six active anti-TB drugs followed by four anti-TB drugs in the continuation phase (Zumla, et al., 2015). Treatment for MDR and XDR-TB can include the use of bedaquiline, linezolid, clofazimine, amoxicillin, imipenem, high-dose INH, thioacetazone and clarithromycin (WHO, 2014).

The current struggle for TB treatment is limited access to rapid and affordable diagnostics, long duration of treatment, high costs and lack of compliance which often leads to drug resistance. The WHO has set a target to end the TB epidemic by 2035 and to achieve this they aim to expand the scope and reach of interventions, produce health and development policies and systems, and pursue innovative scientific knowledge (WHO, 2018). New anti-TB drugs have been developed to aid in reducing the TB epidemic and treatment duration, such as, pretomanid and bedaquiline (Zumla, et al., 2015). Some drugs such as rifapentine, linezolid, moxifloxacin, meropenem and clofazimine (initially approved for treatment of leprosy) have been repurposed to treat TB as they have shown potential in clinical studies (Zumla, et al., 2015). Host-directed therapy (HDT) is being investigated as part of TB treatment and it is intended to target immune response with the purpose of controlling *M. tuberculosis* replication (Zumla, et al., 2015). A weakened immune system fails to eliminate TB infection, therefore HDT provides new ways of effectively eliminating TB infection while attempting to keep the immune system strengthened. HDT strategies aim to manage MDR- and XDR-TB cases, reduce inflammation and tissue damage related to TB infection and treat patients with comorbid or chronic diseases such as HIV and diabetes (Kolloli & Subbian, 2017). The Figure below shows various ways in which *M. tuberculosis* can be targeted by HDT strategies.

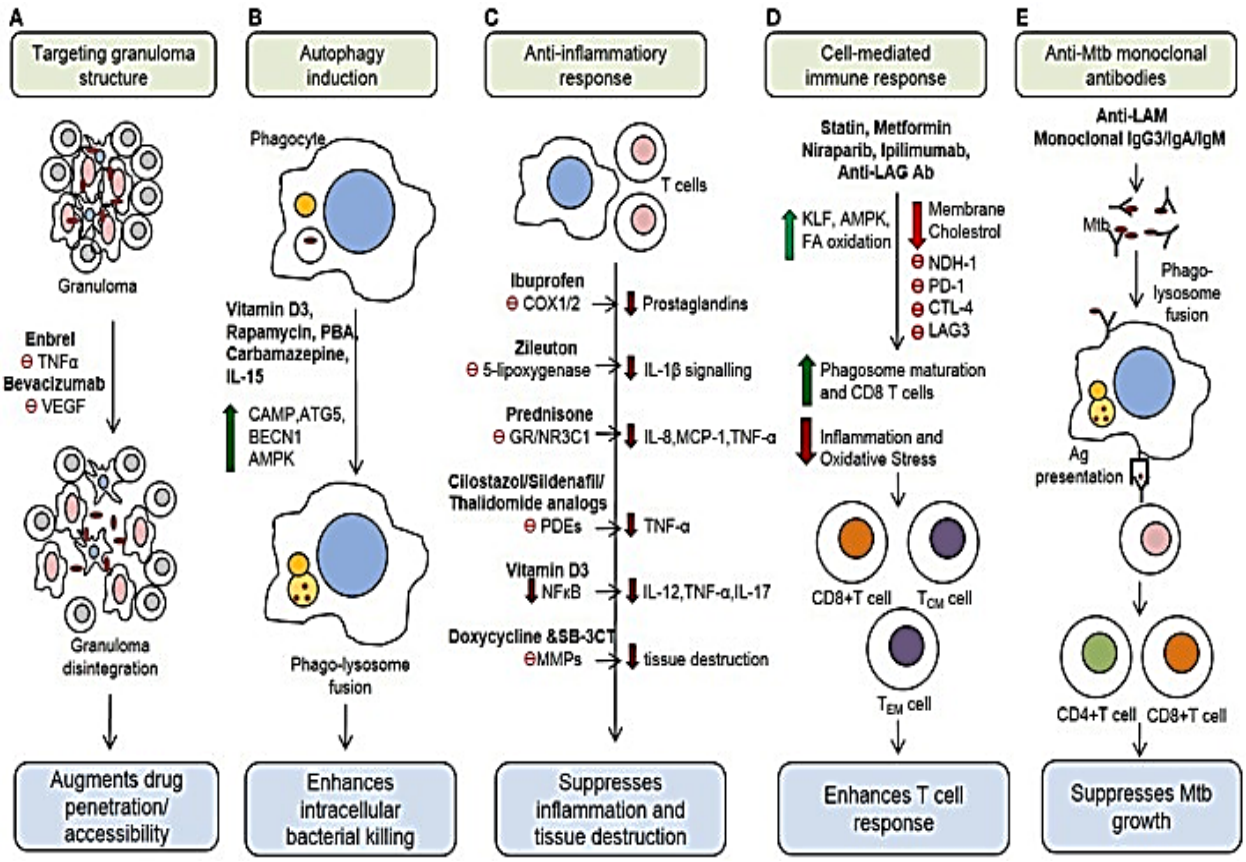


Figure 1.1: Host-directed therapy against M. tuberculosis

(**A**) HDT drugs change the integrity of granuloma and enhance drug accessibility. (**B**) Some HDT drugs increase production of antimicrobial peptides, reactive oxygen and induce autophagy in infected cells. (**C**) HDT drugs prevent proinflammatory responses that decrease inflammation and tissue damage during active stage of TB infection. (**D**) HDT agents control cell-mediated immune responses including antigen-specific T cell responses. (**E**) Monoclonal antibody administration and other emerging HDT concepts for TB treatment. Abbreviations: VEGF, vascular endothelial growth factor; PBA, phenylbutyrate; CAMP, cathelicidin antimicrobial peptide; ATG5, autophagy-related protein 5; BECN1, beclin-1; AMPK, AMP-activated protein kinase; COX1/2, cyclooxygenase-1/2; GR, glucocorticoid receptor; PDE, phosphodiesterases; MMPs, matrix metalloproteinases; KLF, Kruppel-like factor; PD-1, programmed cell death 1 receptor; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; LAG3, lymphocyte activation gene 3; LAM, Lipoarabinomannan (Kolloli & Subbian, 2017).

1.3 Rifapentine and 25-O-desacetyl Rifapentine

Rifamycins consist of rifampin, rifapentine and rifabutin (Chaisson, 2003) (Drew, 2018). They are deacetylated to 25-deacetyl rifamycins by human arylacetamide deacetylase ((Nakajima, et al., 2011). They are mainly used for the treatment of TB infection and in some cases invasive staphylococcal infections (Forrest & Tamura, 2010). Rifamycins are used in combination therapy in TB treatment to

prevent development of resistance due to mutations in the *rpoB* gene (Drew, 2018). Rifamycins are inducers of cytochrome P450 enzymes such as CYP3A4, which result in a decrease in bioavailability and increase in the clearance of co-administered drugs (Drew, 2018) (Forrest & Tamura, 2010). Rifamycin adverse effects have been reported to include hematologic, central nervous system, gastrointestinal, dermatological, hepatitis and production of orange-red discoloured body fluids (Martinez, et al., 1999). Rifapentine will be discussed further as this was the drug studied for this project.

Rifapentine is a derivative of rifamycin used for the treatment of pulmonary TB (Zurlinden, et al., 2016). It induces three cytochrome P450 enzymes, namely; CYP3A4, CYP2C8 and CYP2C9 isozymes. Concomitant treatment with rifapentine can result in reduced serum concentrations of drugs that are metabolized by the CYP enzymes (Munsiff, et al., 2006). Rifapentine has a long half-life of 13.2 to 14.1 hours and a minimal inhibitory concentration (MIC) of approximately 0.02 mg/ml (Zurlinden, et al., 2016). Rifapentine works by inhibiting bacterial DNA-dependent RNA polymerase (Wehrli, 1983). It is metabolized mainly by the liver via hydrolysis and deacetylation to 25-O-desacetyl rifapentine and is excreted predominantly (70%) by biliary excretion (Reith, et al., 1998) (Munsiff, et al., 2006). Absorption of rifapentine improves when administered with food, which helps those patients with gastrointestinal problems, but decreases INH peak concentrations by 20 - 50% during combination therapy (Mannisto, et al., 1982) (Peloquin, et al., 1999) (Zent & Smith, 1995). Treatment of active TB with rifapentine requires 600 mg orally twice weekly for two months and once weekly during the continuation phase of four months (FDA, 2010). A study by Jindani et al. investigating high-dose rifapentine with moxifloxacin for pulmonary tuberculosis reported that rifapentine and moxifloxacin administered once weekly during the continuation phase of six months was noninferior to the standard daily administration (Jindani, et al., 2014). For latent TB, the FDA approved 10 mg/kg orally once or twice weekly and rifapentine has been combined with INH for three months once weekly (Nguta, et al., 2015). Rifapentine should be considered a first line TB drug under special conditions and the United States National TB treatment guidelines recommends rifapentine for the treatment of TB in selected patients (Blumberg, et al., 2003). Rifapentine combined with INH once a week in the continuation phase can be used to treat HIV-seronegative patients with noncavitary, drug-susceptible pulmonary tuberculosis who have negative sputum smears upon completion of the initial phase of treatment (Blumberg, et al., 2003). Treatment of HIV infected patients with rifapentine has been associated with rifamycin resistance (CDC, 2002) (Vernon, et al., 1999). Rifapentine can also be prescribed to patients who are resistant to streptomycin and/or ethambutol (CDC, 2002) (Vernon, et al., 1999).

In healthy volunteers both rifapentine and its metabolite are highly protein bound, namely 98% and 93%, respectively (Burman, et al., 2001) (Egelund, et al., 2014). The free fraction is pharmacologically active and can be established by determining the total concentration compared to the fraction not bound to plasma proteins (Egelund, et al., 2014). In a study consisting of predominantly males (10 mg/kg of body weight/dose), rifapentine free fraction was reported in Africans to be 1.39% while in non-Africans it was 0.59% (Egelund, et al., 2014). Free rifapentine maximum concentration (Cmax) was reported to be 0.14 µg/ml in Africans and 0.10 µg/ml in non-Africans (Egelund, et al., 2014). 25-O-desacetyl rifapentine free fraction was reported in Africans to be 6.23% while in non-Africans it was 2.44% (Egelund, et al., 2014). Free 25-O-desacetyl rifapentine Cmax was reported to be 0.42 µg/ml in Africans and 0.28 µg/ml in non-Africans (Egelund, et al., 2014). Total rifapentine mean Cmax was found to increase with dose; 12.2 µg/ml at 600 mg, 14.6 µg/ml at 900 mg and 18.6 µg/ml at 1200 mg (Wiener, et al., 2004). High rifapentine free fraction has been noted in patients with reduced body mass index (BMI), albumin concentrations and total concentration of rifapentine (Egelund, et al., 2014). Rifapentine area under the curve (AUC) was associated with sex, race, dose and plasma albumin concentrations using multivariate regression analyses (Wiener, et al., 2004). With increasing dosages of 600, 900 and 1200 mg the AUC increased to 1.39 and 1.61-fold while the half-life ranged from 14.4 to 16.4 hours (Wiener, et al., 2004). Wiener et al. reported the AUC and Cmax to be lower in non-Africans compared to Africans and in males compared to females which corresponded with what the Egelund et al. study found (Wiener, et al., 2004) (Egelund, et al., 2014). Increasing of dosages has been associated with decreased bioavailability, and it has been suggested this may be due to the ability of the drug to be saturated when transported across the gut wall, dose-dependent increase in hepatic or pre-systemic clearance, sampling error, and the solubility and dissolution of drugs co-administered with rifapentine (Dooley, et al., 2012).

The structure of rifapentine and its metabolite are shown in Figure 1.2 below, where a cyclopentane group can be noted. The molecular weight of rifapentine ($C_{47}H_{64}N_4O_{12}$) is 877.045 g/mol and for its metabolite ($C_{45}H_{62}N_4O_{11}$) is 835.008 g/mol.

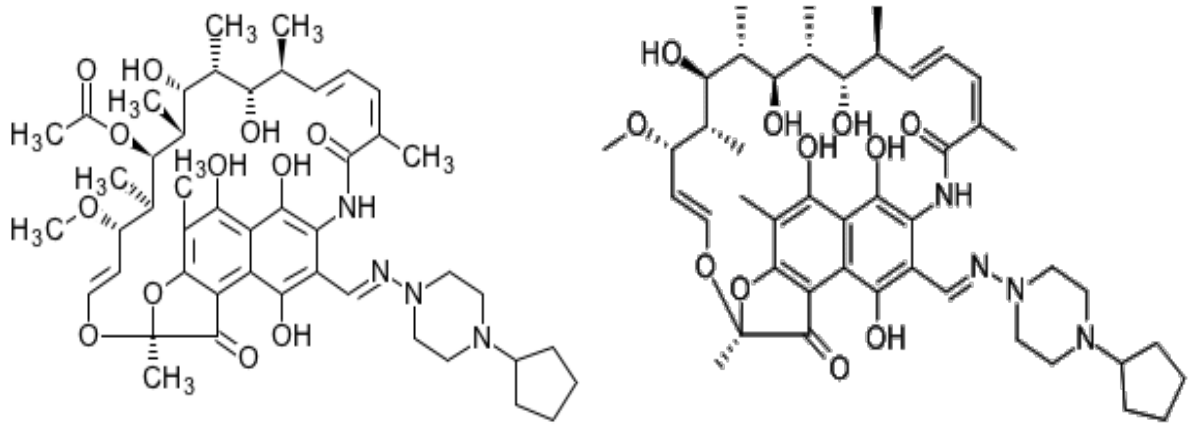


Figure 1.2:The structure of rifapentine and 25-O-desacetyl rifapentine

Figure 1.3 below shows a schematic of the proposed disposition of orally administered Rifapentine based on a study conducted by Reith et al. 1998. It also shows the 3-formyl derivatives of rifapentine and its metabolite formed by non-enzymatic hydrolysis. The study indicates that following absorption of rifapentine from the gastrointestinal tract it can be metabolized not only in the liver but in other tissues including the blood. Rifapentine is mainly eliminated by biliary excretion, as indicated in the diagram, and to a lesser extent in the urine.

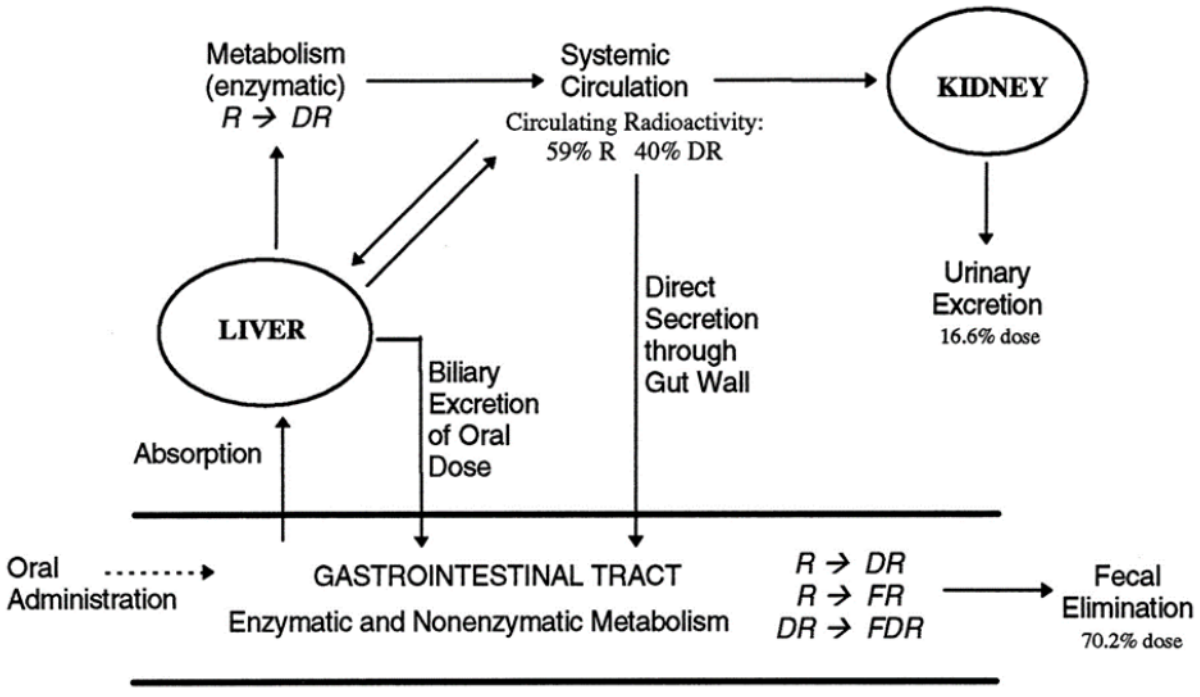


Figure 1.3: Schematic of proposed disposition of orally administered rifapentine

Abbreviations: R, rifapentine; DR, 25-O-desacetyl-rifapentine; FR, 3-formyl-rifapentine; FDR, 3-formyl-25-O-desacetyl rifapentine (Reith, et al., 1998).

1.4 Transfer of drugs into breastmilk

Breastmilk contains many biologically active components which have an essential role in infant growth and health (Villasenor, et al., 2014). Breastmilk also contains significant amounts of fats and proteins (Rezk, et al., 2007). Health benefits of breastfeeding to the infant include reduced risk of sudden infant death syndrome, type II diabetes and allergies, to mention a few (Ito & Lee, 2003). A mothers' benefit from breastfeeding include an increased bond with the infant, increase in maternal levels of oxytocin and results in decreased postpartum bleeding and quicker uterine involution (Ito & Lee, 2003). When drugs enter breastmilk, they mostly exist based on maternal plasma level, therefore, when drug plasma levels decrease in the plasma the breastmilk levels also decrease (Ito, 2000). Protein binding also plays a significant role in the transfer of drugs into breastmilk. Most drugs circulate in the maternal plasma bound to the proteins, the most predominant of which is albumin. The unbound drug fraction diffuses into the breastmilk while the bound fraction remains in the maternal plasma (Ito, 2000). Drugs with long half-lives or which have active metabolites able to penetrate breastmilk could result in greater potential exposure to the infant (Ito & Koren, 1994). It has been recommended that drugs suitable for administration to breastfeeding women include those with low bioavailability and low absorption (Ito & Koren, 1994). However, not all drugs transferred into breastmilk are transferred in clinically significant amounts and the drug may therefore not pose a risk to the infant (Hale, 2004). Therefore, physicians consider several factors before prescribing a drug to a breastfeeding mother, which include: the need for the drug by the mother, potential effects of the drug on milk production, amount of the drug excreted into the breastmilk, the extent of oral absorption by the breastfeeding infant and any potential adverse effects on the breastfeeding infant (Hale, 2004). The infant's age is also a contributing factor as adverse effects associated with drug exposure through breastfeeding typically occur in neonates younger than 2 months and rarely in infants older than 6 months (Chaves & Lamounier, 2004). A ratio is used to determine the drug concentration in breastmilk and is calculated by comparing it to the drug concentration in maternal plasma. This is called the milk-to-plasma drug concentration (Ito & Lee, 2003). The effect of the drug is determined by the amount of milk the infant ingests and the therapeutic dose of the drug (Horn, 2015). The ratio varies over time and therefore a time averaged ratio is used to represent the drug's kinetics unless the concentration in breastmilk is known to be similar to that in maternal plasma (Sachs, 2013) (Anderson, et al., 2003). If the relative infant dose (RID) is below 10% of the therapeutic dose it is considered safe unless the drug or its active metabolite accumulates (Ito, 2000). The RID is directly proportional to the milk-to-plasma ratio and inversely proportional to the drug clearance rate by the infant (Ito & Koren, 1994). Therefore, drugs with reduced clearance rate tend to result in high levels of exposure and drugs with increased clearance rate tend to result in low levels of exposure regardless of the milk-to-plasma ratio being

high (Horn, 2015). The ratio is calculated from an equation that takes into consideration the drug's physical and chemical characteristics (Begg & Atkinson, 1992). The ratio does not provide information on the absolute amount of drug transferred to the milk (Ito, 2000).

The equation is expressed as follows:

RID = A × [(milk-to-plasma ratio)/CLI] × 100, where A is the average milk intake per kg per minute, CLI is the rate of drug cleared by the infant (ml/kg/minute).

There is currently limited data available on the transfer of anti-TB drugs into breastmilk and the subsequent effect on a breastfeeding infant. To date, the only drugs for which safety has been shown during breastfeeding are isoniazid, rifampicin, streptomycin, kanamycin and cycloserine, the latter four drugs having minimal passage into breastmilk (Gupta, et al., 2016). For rifampicin, a related rifamycin antibiotic, breastmilk concentrations have been shown to be very low. Peak levels of 0.05% of rifampicin levels observed in plasma are found in breastmilk with standard dosing for TB treatment (CDC, 2002). No data is available for the second-line anti-TB drug, rifapentine.

1.5 Liquid chromatography tandem mass spectrometry

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) is a combination of High-Performance Liquid Chromatography (HPLC) for separation capacity and Mass Spectrometry for the detection capacity, making LC-MS/MS highly sensitive and specific. The separation in the HPLC column is either on normal or reverse phase. In normal phase the mobile phase is more hydrophobic while the stationary phase is more polar. The opposite is true for the reverse phase. LC-MS/MS requires several essentials, namely an autosampler to introduce the sample into the mobile phase flow which is usually a combination of water and an organic solvent and carries the sample into the column. Degassers remove bubbles from the mobile phase and pumps deliver the mobile phase at a constant flow rate. A column is used for chromatographic separation and the retention of the analyte will depend on its affinity to the stationary phase. Separated species are sprayed into the Atmospheric Pressure Ion Source (API) where ions are formed in the gas phase and the mass analyser (i.e. Quadrupole, Ion trap, Time of Flight and Magnetic sector) sorts them according to their mass to charge ratio and the detector amplifies the signal from the ions of interest and gives rise to a chromatogram displaying the response against time.

The diagram below from Abdelrahman et al. displays a typical LC-MS/MS system (Abdelrahman, et al., 2018). Following analyte extraction, the eluent is injected into the HPLC column and sprayed into the Mass Spectrometer. Ions are produced (i.e. via Electrospray Ionization process which will be discussed later) and enter the mass analyser (i.e. Quadrupole) where Quadrupole 1 (Q1) scans precursor ions,

in Quadrupole 2 (Q2) (also known as collision cell) the ions undergo fragmentation and in Quadrupole 3 (Q3) product ions are identified and scanned. The detector amplifies the signal produced by the product ions and generates a chromatogram. The data is processed, analysed and reported.

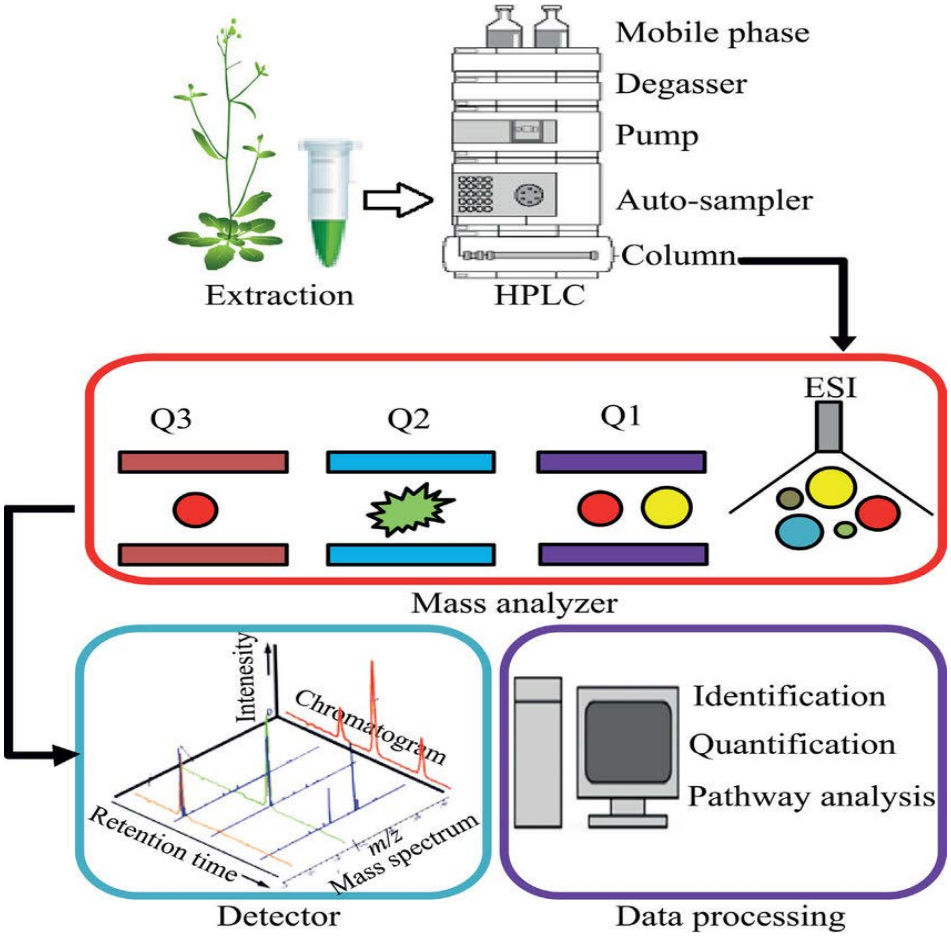


Figure 1.4: Schematic displaying a typical Liquid Chromatography Tandem Mass Spectrometer system

Ionization sources that are generally used are, Atmospheric Pressure Chemical Ionization (APCI), Electrospray Ionization (ESI) and Matrix-assisted Laser Desorption/Ionization (MALDI). For this project, ESI was utilized and its principle will therefore be discussed. In ESI, an electrical field is applied to the HPLC eluent, charged droplets are produced at the capillary tip, the charged droplets are desolvated by using heat and nebulising gas (i.e. nitrogen) to form coulombic fission and ions are produced in the gas phase (Ho, et al., 2003). To ensure efficient charged droplet formation, liquid surface tension, eluent flow rate and electrolyte concentration need to be maintained accordingly (Konermann, et al., 2012). ESI produces multiple charged species which is why it can be used for analysing large molecules.

Below is a diagram from Chem LibreTexts displaying how ions are formed in electrospray ionization (LibreTexts, 2017). The HPLC eluent is sprayed through the capillary tip (spraying nozzle) and the coulombic forces are increased due to the repulsion of anions or cations. As the number of anions or

cations increase, they cause the eluent meniscus to change shape thus forming a Taylor cone. The Taylor cone releases charged droplets containing the analyte of interest. The droplets are desolvated, reducing the solvent radius but not its charge. The Rayleigh limit is achieved when the droplet surface tension reaches equilibrium with the electrostatic repulsive forces. This causes the surface charges to come closer together as the droplet is further desolvated. The droplet undergoes coulombic fission (explosion) due to the surface charges stress and the charged analyte of interest enters the sample cone for further analysis in the Mass Spectrometer.

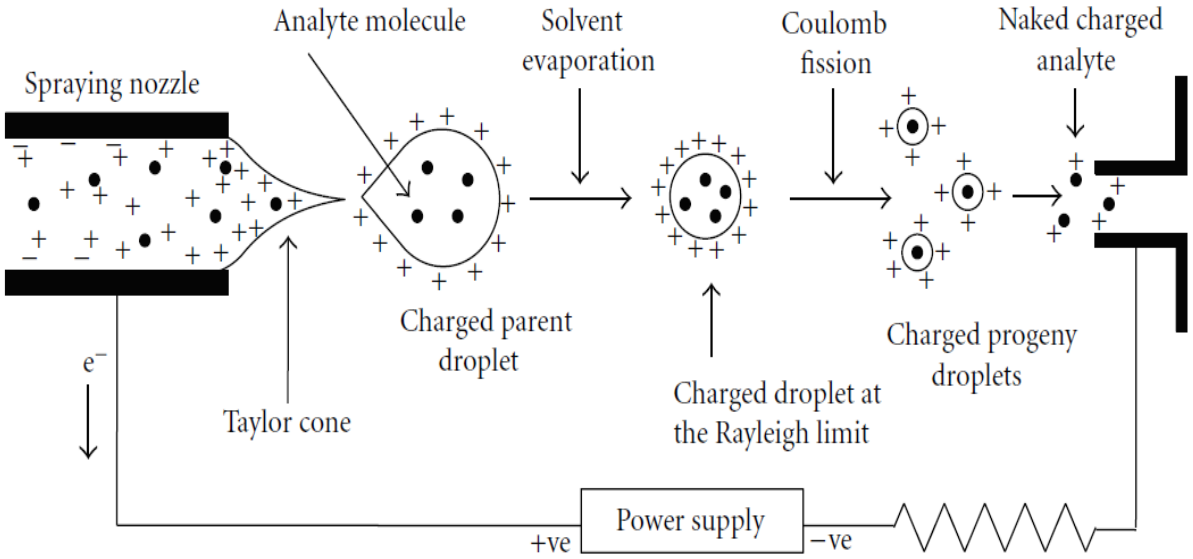


Figure 1.5: Schematic representation of the Electrospray Ionization process

1.6 Quantification methods for Rifapentine

Bioanalytical assays have been published for the quantification of rifapentine and its metabolite, mainly in human plasma, by means of liquid chromatography-tandem mass spectrometry (LC-MS/MS). Winchester et al. developed a multiplex method for the quantification of the rifamycin antibiotics, rifabutin, rifampin and, rifapentine, as well as their metabolites, in human plasma by means of LC-MS/MS (Winchester, et al., 2015). The authors used protein precipitation with trifluoroacetic acid (TFA): acetonitrile (0.1:99.9, v/v) as the extraction method and an ACE®C18 (3 x 100 mm) column was used for chromatographic separation with water: acetonitrile (55:45, v/v) with 0.5% formic acid as the mobile phase (Winchester, et al., 2015). Parsons et al. developed and validated a bioanalytical method to quantify rifapentine from dried blood spot samples using LC-MS/MS analysis (Parsons, et al., 2014). To extract the analyte, they applied a protein precipitation method with 90:10 methanol: 50 mM ammonium formate buffer (v/v) containing 0.50 mg/ml ascorbic acid as the extraction solvent (Parsons, et al., 2014).

1.7 Drug analysis in breastmilk

There have been bioanalytical assays developed and validated on ARV drugs in dried breastmilk spots using LC-MS/MS. To prepare dried breastmilk spots (DBMS) 30µl of human breastmilk were spotted onto Whatman 903 Protein Saver Cards (Olagunju, et al., 2015) (Olagunju, et al., 2015) (Waitt, et al., 2017). DBMS is advantageous since lower volumes of breastmilk are used and requires simple sample storage (i.e. room temperature) and transport (Olagunju, et al., 2015). However, the disadvantage of DBMS is that consistency of the method is subject to accurate pipetting of the breastmilk and spotting it which may be difficult as breastmilk is white, and the spotting cards are usually also white (Waitt, et al., 2017).

ARV drugs have also been quantified in the liquid form of the breastmilk. Rezk et al. used 200 µl of breastmilk to extract ARV drugs lamivudine, zidovudine, stavudine, nevirapine, nelfinavir and lopinavir (Rezk, et al., 2007) (Rezk, et al., 2008). Rezk et al. encountered problems such as ion suppression and enhancement due to matrix effects, carry-over and a limited calibration range with using breastmilk in liquid form (Rezk, et al., 2008) (Rezk, et al., 2007). To overcome problems due to lipids and protein in the samples, extensive sample clean-up was applied by adding a greater volume (125 µl) of penta-fluoropropionic acid to dissociate fat globules (Rezk, et al., 2008). It was difficult to eliminate the fats as the lipids and proteins are closely linked in breastmilk (Rezk, et al., 2008). A clean sample enables reproducibility and prevents column degradation (Rezk, et al., 2007). Rezk et al. used two methods, a combination of protein precipitation with SPE and liquid-liquid extraction with SPE which allowed the accurate quantification of the ARV drugs (Rezk, et al., 2007) (Rezk, et al., 2008). In solid phase extraction the authors found that increasing the percentage of the organic solvent (90% MeOH) to elute the analytes was sufficient and it enabled an increase in extraction efficiency (Rezk, et al., 2008). Having a wide calibration range (10-10 000 ng/ml) enabled accurate quantification of the analytes (Rezk, et al., 2008). For optimal extraction results, Rezk et al. recommend a wide calibration range for the method and for the calibration standards and quality controls to be prepared using intermediate stock solutions and not serial dilutions (Rezk, et al., 2008). Spiking the ARV drugs in breastmilk homogenized at room temperature and mixing well by gentle agitation enabled optimal extraction results (Rezk, et al., 2008). Lastly, constant mixing of samples during aliquoting ensured equilibration of the analytes and the lipids at different concentration levels used for the calibration range (Rezk, et al., 2008). Comparing analysis of drugs in DBMS, skim milk and whole breastmilk, whole breastmilk analysis is of clinical significance for accurate pharmacokinetic and pharmacodynamic predictions as the drugs partition through the various components of breastmilk (i.e. lipids) and the infants ingest whole breastmilk (Rezk, et al., 2008).

No validated LC-MS/MS methods have been published for the quantification of rifapentine and 25-O-desacetyl rifapentine in human breastmilk.

2 Project Rationale, Aim and Objectives

2.1 Rationale

There is no data on the use of Rifapentine in breastfeeding women, its transfer into breastmilk, the effect on the infant, if the concentration levels are of clinical significance to the breastfed infant and the effects of long-term exposure to sub-therapeutic drug levels and the potential development of drug resistance. This prevents health professionals from making evidence-based recommendations on the optimal TB drug regimens for breastfeeding mothers. The assay will be used to analyse clinical samples and data generated will be used to interpret and evaluate clinically significant amounts (if any) of rifapentine transferred into breastmilk. Developing a sensitive and robust LC-MS/MS method that will be compatible with complex biological fluids such as breastmilk is of importance to the study to attain reliable data.

2.2 Aim

To develop and validate a bioanalytical assay for the quantitation of the anti-tuberculosis drug, rifapentine and 25-O-desacetyl rifapentine, in human breastmilk.

2.3 Objectives

- To develop a suitable extraction method of rifapentine and 25-O-desacetyl rifapentine from breastmilk
- To develop a robust LC-MS/MS method for rifapentine and 25-O-desacetyl rifapentine quantitation and validate according to FDA guidelines

3 Assay development

3.1 Method development

Developing a sensitive and robust extraction and LC-MS/MS method is of utmost importance to obtain reliable data. Effective sample pre-treatment is therefore required before the sample is injected onto the LC-MS/MS to remove endogenous components that may interfere with the ionization process of the analyte of interest, result in HPLC and autosampler blockages or contaminate the instrument over time due to residual proteins or salts. It is also important to ensure that the extraction solvent used is suitable for efficient analyte extraction. The most commonly used sample preparation methods are, protein precipitation, Liquid-liquid extraction (LLE) and Solid phase extraction (SPE).

3.2 Mass spectrometer optimisation

The source parameters of an API 3000 mass spectrometer were optimized for rifapentine and its metabolite, which included nebulising gas, turbo gas, curtain gas, collision gas, source temperature, ion spray voltage by means of flow injection analysis.

3.3 Chromatography development

The analytes (rifapentine and 25-O-desacetyl rifapentine) and potential internal standards (rifampicin-d3, 25-desacetyl rifampicin-d3 and rifaximin) were infused on an API 3000 (AB Sciex) at a concentration of 200 ng/ml for the analytes and 80 ng/ml for the internal standards. For chromatographic separation, a Poroshell C18 column (4.6 x 50 mm, 2.7 µm) was used and the mobile phase applied was acetonitrile: 0.1% formic acid in water: methanol (60: 30: 10, v/v/v) with a flow rate of 300 µl/min. The initial analytical run time was 10 minutes but was later reduced to 3 minutes by decreasing the scan time and increasing the flow rate to 450 µl/min. Figure 3.1 shows a chromatogram of the analyte, metabolite and three potential internal standards with a flow rate of 300 µl/min and an analytical run of 10 minutes while Figure 3.2 shows a chromatogram of the analyte, metabolite and one internal standard (chosen for providing optimal quantification of rifapentine and its metabolite) with a flow rate 450 µl/min with an analytical run of 3 minutes.

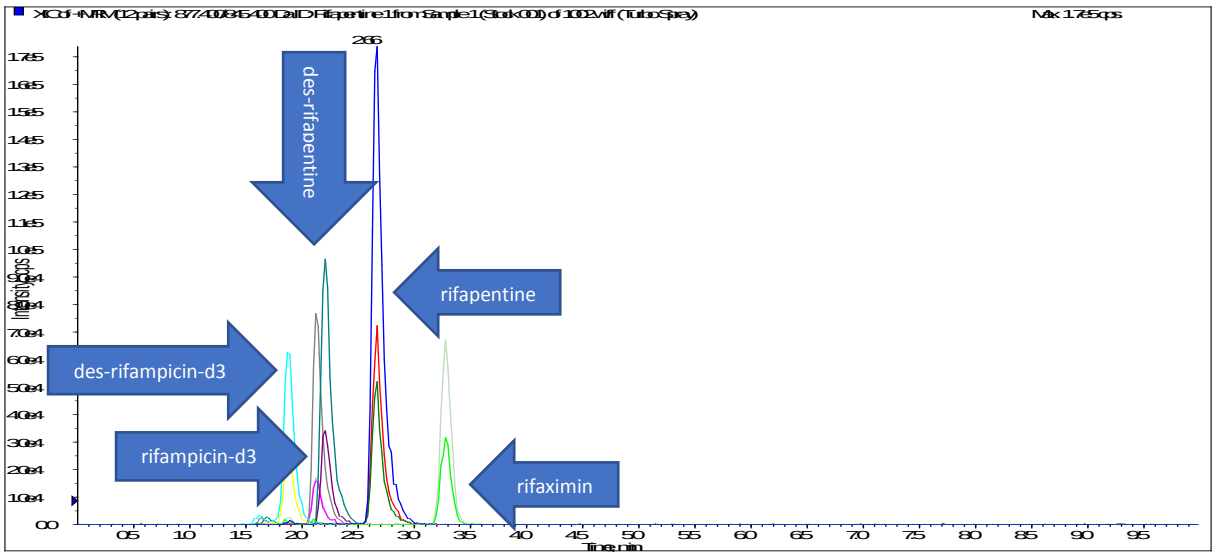


Figure 3. 1: Representative initial chromatogram of the analytes and internal standards

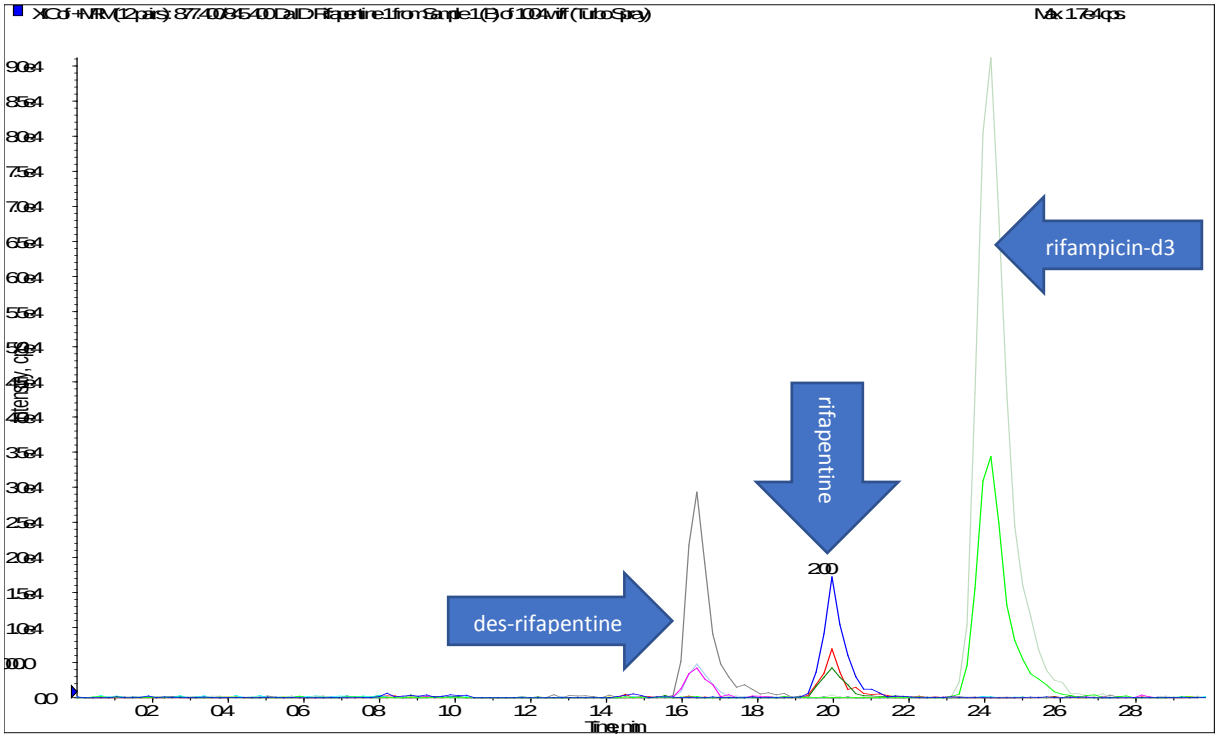


Figure 3. 2: Representative chromatogram of the analytes and internal standard – reduced run time

3.4 Extraction development

3.4.1 Protein precipitation and PHREE extraction

Protein precipitation is a simple and quick method aimed at removing proteins from the sample through the addition of a solvent, leaving denatured proteins to pellet at the bottom of the tube. This can be achieved by lowering the sample pH with the addition of an acid or by adding an organic solvent

(for example methanol) to the sample which results in the proteins precipitating out of solution, usually followed by centrifugation at a high speed.

The first extraction method performed was protein precipitation. Published methods of rifapentine and 25-O-desacetyl rifapentine in human plasma mainly used this form of sample preparation (Winchester, et al., 2015). Various breastmilk volumes, namely 50, 100 and 200 µl were investigated to determine the optimal volume from which to extract the analyte and metabolite. This was performed in triplicate per volume using 400 µl of acetonitrile as a precipitation solvent. Figure 3.3 below shows volume optimization of breastmilk.

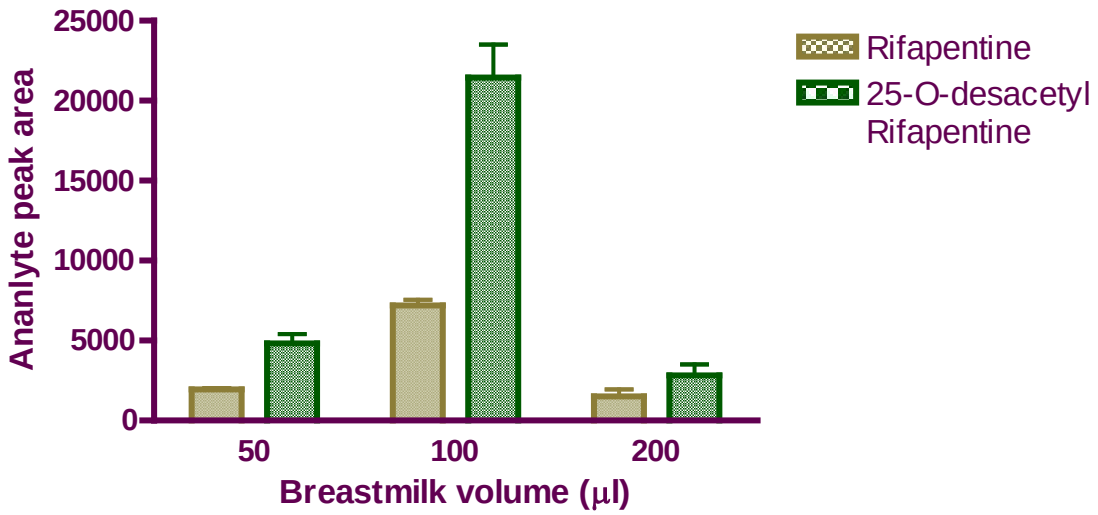


Figure 3. 3: Breastmilk volume optimization

A volume of 100 µl of breastmilk was shown to achieve the highest recovery of rifapentine and 25-O-desacetyl rifapentine. Rezk et al. found 200 µl of breastmilk to be sufficient in extracting the ARV drugs, as the large volume enabled the analytes to equilibrate well in the presence of endogenous components (Rezk, et al., 2007) (Rezk, et al., 2008). In this project a volume of 200 µl resulted in poor recovery of the analytes, possibly due to an abundance of endogenous matrix components that may have led to ion suppression and/or matrix effects (Rezk, et al., 2008). A volume of 50 µl was also not sufficient for the extraction of adequate quantities of rifapentine and its metabolite for the purposes of assay development.

Different solvents were optimized to determine which was best suited for absolute recovery of rifapentine and 25-O-desacetyl rifapentine with the protein precipitation method. The following solvents were optimized: acetonitrile, methanol, a combination of acetonitrile and methanol (50:50, v/v), acetonitrile with water (95:5, v/v) containing 1 mg/ml ascorbic acid and methanol with water (95:5, v/v) containing 1 mg/ml ascorbic acid. Figure 3.4 below show that acetonitrile with water (95:5,

v/v) and 1 mg/ml ascorbic acid was best suited to extract both rifapentine and 25-O-desacetyl rifapentine as this solvent combination resulted in the greatest recovery of the analyte and metabolite.

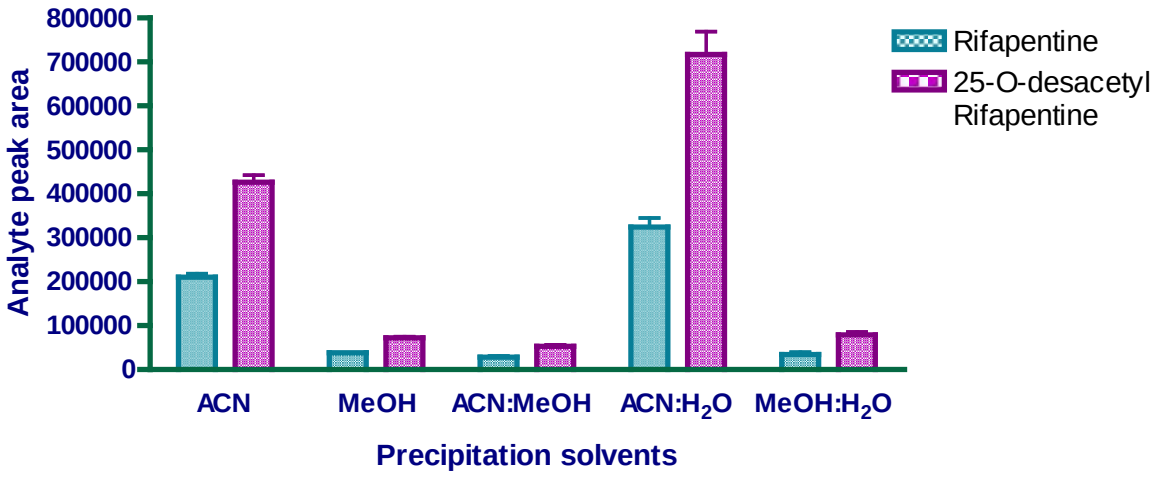


Figure 3. 4: Precipitation solvent optimization for rifapentine and 25-O-desacetyl rifapentine.

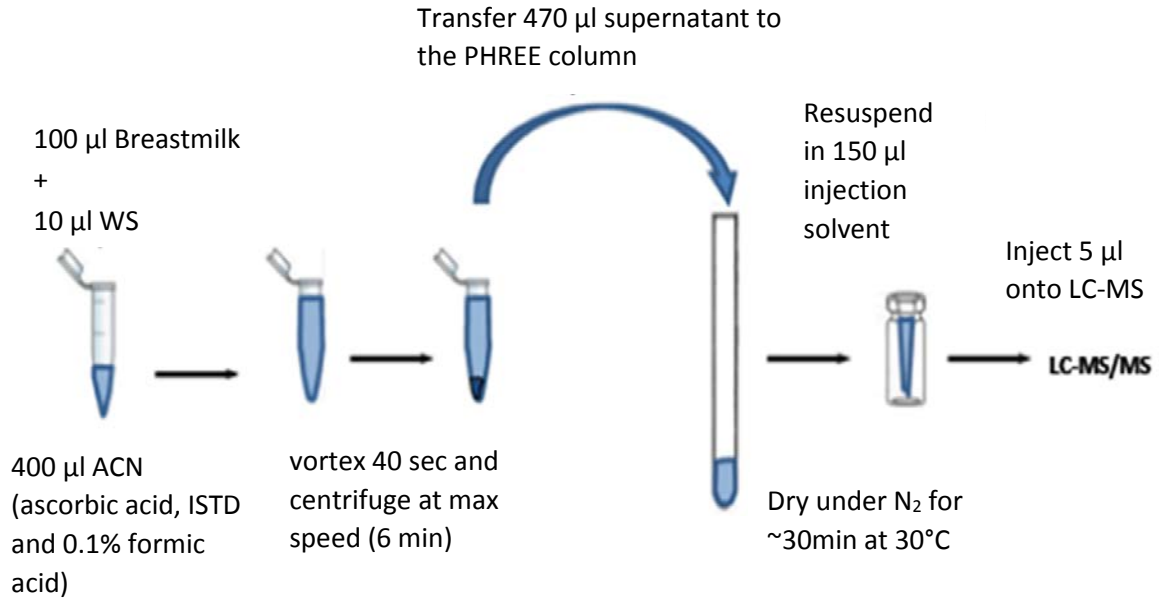
Abbreviations: ACN - acetonitrile; MeOH - methanol; ACN: MeOH -acetonitrile and methanol (50:50, v/v); ACN: H_2O – acetonitrile and water (95:5, v/v) with 1 mg/ml ascorbic acid; MeOH: H_2O – methanol and water (95:5, v/v) with 1 mg/ml ascorbic acid

Based on the data obtained above, the initial protein precipitation method used 100 µl of breastmilk spiked with rifapentine and 25-O-desacetyl rifapentine to which 400 µl precipitation reagent (acetonitrile: water (95:5, v/v) with 1mg/ml ascorbic acid) was added. The sample was vortexed for 1 minute, centrifuged at 13 000 g for 5 minutes, after which 450 µl of supernatant was transferred into a borosilicate tube. The sample was concentrated under nitrogen at 40°C for approximately 20 minutes after which it was reconstituted in 200 µl injection solvent containing acetonitrile: 0.1% formic acid in water: methanol (60: 30: 10, v/v/v) and vortexed for 30 seconds. The sample was transferred into a 96-well plate and 5 µl was injected onto LC-MS/MS for analysis.

Despite accurate calibration curves obtained using this protein precipitation method, residual fatty components were observed in the samples, making them incompatible with LC-MS/MS. This was similar to what Rezk et al. found when quantitating ARV drugs in breastmilk (Rezk, et al., 2007) (Rezk, et al., 2008). Therefore, further sample clean-up had to be applied. This led to the second extraction method investigated, namely protein precipitation with phospholipid removal columns (PHREE).

PHREE (Phenomenex, USA) were incorporated in the protein precipitation method to eliminate excess proteins, lipids and phospholipids that could cause ion suppression or enhancement. As suggested by

the manufacturer's instructions and with additional adaptations, for this method, 400 µl acetonitrile containing 0.1% formic acid and ascorbic acid (1 mg/ml) was used as the precipitating reagent which contained the investigational internal standard (rifampicin-d3) at 250 ng/ml. After concentration under nitrogen, the sample was reconstituted in 150 µl injection solvent (acetonitrile: 0.1% formic acid in water: methanol (60: 30: 10, v/v/v). The method followed is indicated below in Figure 3.5.



Abbreviations: WS: Working solution; ACN: Acetonitrile: max speed = 20238 g; N_2: nitrogen; LC-MS: liquid chromatography tandem mass spectrometry; ISTD: internal standard

Figure 3. 5: Protein precipitation technique with PHREE columns

Despite accurate calibration curves, sample clean-up appeared to be insufficient as matrix effects (data shown below in Table 3.4 1 and 3.4 2) were evident even though the sample appeared to be cleaner upon visual inspection. Matrix effects are caused by the presence of an unintended analyte or any other interfering compound in a biological sample that may interfere with the ionization process. The acceptance criteria for the matrix effects regression slope determined using an isotopically labelled analogue of the analyte as internal standard should not be more than 5%, while when using an internal standard that is not an-isotopically labelled analogue of the analyte the acceptance criteria for the regression slope should not be more than 10%. Matrix effects testing was used to test if the method was robust and reproducible.

Table 3.4 1: Matrix effects for rifapentine using protein precipitation with PHREE columns

Table A: The peak areas for the analyte and internal standard at high, medium and low concentrations

	High Concentration		Medium Concentration		Low Concentration	
	Analyte Peak Area	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**
Matrix 1	35300000	4830000	22000000	5280000	729000	7050000
Matrix 2	35100000	6390000	21600000	6710000	666000	6440000
Matrix 3	35800000	5000000	22700000	5310000	747000	6050000
Matrix 4	25900000	3180000	14700000	3270000	518000	3900000
Matrix 5	27000000	6540000	15000000	6810000	545000	7440000
Matrix 6	29300000	4020000	16700000	4810000	575000	5020000

Table B: The peak area ratios and regression slope for each matrix

	High Conc.	**Medium Conc.**	**Low Conc.**	**Area Ratio**
	1600 ng/ml	**800 ng/ml**	**10.0 ng/ml**	**v Conc.**
	Peak Area Ratio	**Peak Area Ratio**	**Peak Area Ratio**	**Regression Slope**
Matrix 1	7.31	4.17	0.103	0.00453
Matrix 2	5.49	3.22	0.103	0.00339
Matrix 3	7.16	4.27	0.124	0.00442
Matrix 4	8.14	4.50	0.133	0.00504
Matrix 5	4.13	2.20	0.0733	0.00255
Matrix 6	7.29	3.47	0.115	0.00451
Average	**6.59**	**3.64**	**0.109**	**0.00407**
STDEV	**1.48**	**0.858**	**0.0207**	**0.000921**
% CV	**22.5**	**23.6**	**19.1**	**22.6**

Abbreviation: conc.: concentration; stdev: standard deviation

Table 3.4 2: Matrix effects for 25-O-desacetyl rifapentine using protein precipitation with PHREE columns

Table A: The peak areas for the analyte and internal standard at high, medium and low concentrations

	High Concentration		Medium Concentration		Low Concentration	
	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area
Matrix 1	14400000	748000	8090000	765000	243000	887000
Matrix 2	19700000	776000	12000000	774000	213000	769000
Matrix 3	13600000	534000	7910000	559000	173000	616000
Matrix 4	8460000	219000	4810000	225000	110000	27800
Matrix 5	15500000	786000	9630000	755000	182000	795000
Matrix 6	9910000	577000	6690000	690000	127000	683000

Table B: The peak area ratios and regression slope for each matrix

	High Conc. 1600 ng/ml	Medium Conc. 800 ng/ml	Low Conc. 10.0 ng/ml	Area Ratio v Conc.
	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Regression Slope
Matrix 1	19.3	10.6	0.274	0.00119
Matrix 2	25.4	15.5	0.277	0.0158
Matrix 3	25.5	14.2	0.280	0.0158
Matrix 4	38.6	21.4	3.95	0.0218
Matrix 5	19.7	12.8	0.228	0.0123
Matrix 6	17.2	9.70	0.185	0.0107
Average	**24.3**	**14.0**	**0.867**	**0.0147**
STDEV	**7.81**	**4.21**	**1.51**	**0.00407**
% CV	**32.2**	**30.0**	**174.6**	**27.6**

Abbreviation: conc.: concentration; stdev: standard deviation

The regression slopes for rifapentine and 25-O-desacetyl rifapentine were 22.6% and 27.6%, respectively, and therefore did not meet the acceptance criteria for matrix effects when using an internal standard that is not an isotopically labelled analogue of the analytes. These results indicated that matrix effects influenced the precision and accuracy of the assay. Liquid-liquid extraction was

therefore considered to achieve a cleaner sample with fewer endogenous interfering matrix components.

3.4.2 Liquid-liquid extraction

Liquid-liquid extraction (LLE) involves adding an organic (immiscible) solvent to an aqueous solvent which results in two immiscible layers. The aqueous and organic phase solvents were optimized to determine which solvent would result in better recovery of the analytes. The aqueous phase solvents optimized were ammonium acetate (100 mM, pH 4.81, pH adjusted by adding acetic acid), water, water with ascorbic acid (1 mg/ml) and ammonium bicarbonate (100 mM, pH 9.19). The organic phase solvents optimized were ethyl acetate, hexane and a combination of ethyl acetate and hexane (50:50, v/v). Figure 3.6 below show the optimization of the aqueous phase solvents for rifapentine and 25-O-desacetyl rifapentine.

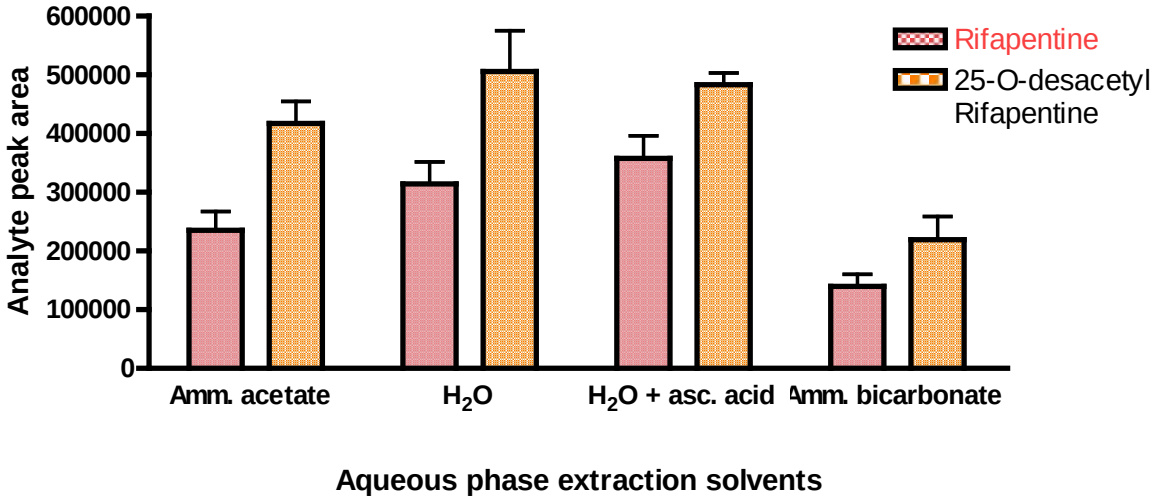


Figure 3. 6: Aqueous phase extraction solvents optimized for rifapentine and 25-O-desacetyl rifapentine.

Abbreviations: Amm. acetate – Ammonium acetate (100 mM) (pH 4.81); H_2O - water; H_20 + asc. acid – water with 1 mg/ml ascorbic acid; Amm. bicarbonate – Ammonium bicarbonate (100 mM) (pH 9.19)

Water with ascorbic acid provided better recovery of rifapentine while water alone provided better recovery of 25-O-desacetyl rifapentine. However, water with ascorbic acid was chosen as the aqueous phase because ascorbic acid in general provides protection by preventing oxidation (Bao, et al., 2008) and this combination showed a low coefficient of variation (%CV) for both rifapentine and 25-O-desacetyl rifapentine in comparison to the water without ascorbic acid.

Figure 3.7 below shows optimization of the organic phase solvents for Rifapentine and 25-O-desacetyl Rifapentine.

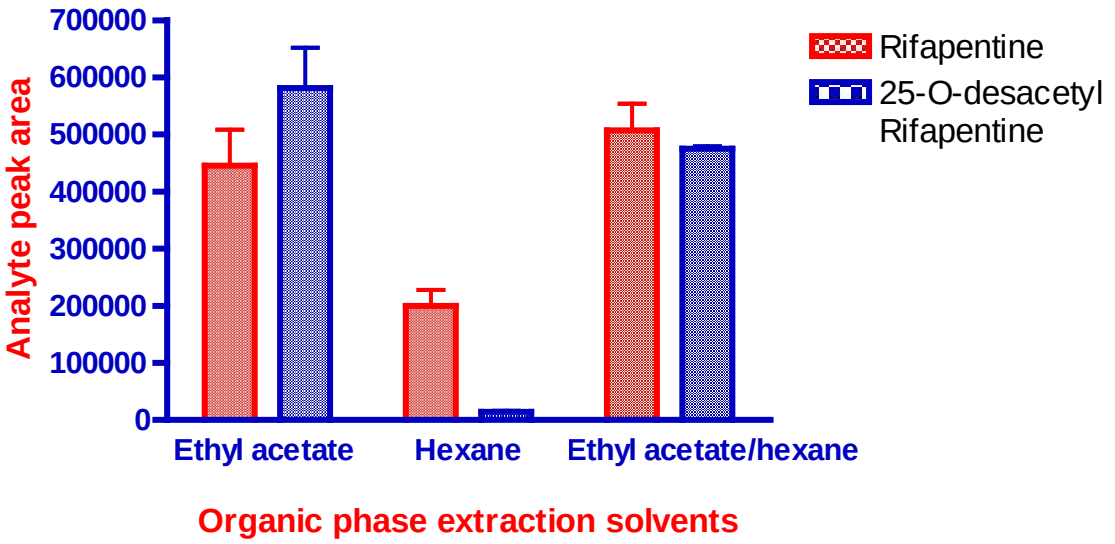


Figure 3. 7: Organic phase extraction solvents optimized for rifapentine and 25-O-desacetyl rifapentine.

Figure legend: Ethyl acetate/hexane – (50:50, v/v)

A combination of ethyl acetate and hexane (50:50, v/v) provided better recovery of rifapentine while ethyl acetate alone provided better recovery of 25-O-desacetyl rifapentine. However, a combination of ethyl acetate and hexane (50:50 v/v) was chosen as the organic phase as this combination showed consistency with a low percentage coefficient of variation (% CV) for both rifapentine and 25-O-desacetyl rifapentine compared to ethyl acetate alone.

The liquid-liquid extraction method was therefore finalized as follows: 100 µl of breastmilk spiked with rifapentine and 25-O-desacetyl rifapentine; 100 µl of aqueous solvent (water with 1 mg/ml ascorbic acid); 750 µl of organic solvent (ethyl acetate and hexane, 50:50, v/v) containing investigational internal standard at 250 ng/ml (rifampicin-d3); vortex for 1 minute; centrifuge at 13 000 g for 5 minutes; freeze samples in a freeze-plate at approximately 30°C; pour off supernatant into borosilicate tubes; concentrate under nitrogen at 40°C; reconstitute in 200 µl injection solvent (acetonitrile: 0.1% formic acid in water: methanol (60: 30: 10, v/v/v); vortex for 30 seconds; transfer 200 µl into a 96-microwell plate and inject 5 µl onto LC-MS/MS for analysis.

The fatty components of the breastmilk resulted in the occasional sample-specific formation of emulsions. Furthermore, for samples in which two definitive immiscible layers formed, residual fatty substances were observed after sample concentration under nitrogen. Despite the liquid-liquid-extraction resulting in cleaner samples than protein precipitation, matrix effects experiments (data

shown below in Table 3.4.3 and 3.4.4) indicated interference as a result of endogenous sample components.

Table 3.4 3: Matrix effects for rifapentine using Liquid-liquid extraction

Table A: The peak areas for the analyte and internal standard at high, medium and low concentrations

	High Concentration		Medium Concentration		Low Concentration	
	Analyte Peak Area	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**
Matrix 1	40300000	4430000	25300000	4720000	923000	6620000
Matrix 2	39100000	5910000	25800000	6090000	862000	6550000
Matrix 3	40000000	4790000	26600000	5440000	923000	6280000
Matrix 4	31300000	3100000	18300000	3460000	681000	4260000
Matrix 5	32000000	5540000	19200000	6840000	677000	7170000
Matrix 6	35800000	4410000	21800000	4850000	781000	5120000

Table B: The peak area ratios and regression slope for each matrix

	High Conc.	**Medium Conc.**	**Low Conc.**	**Area Ratio**
	1600 ng/ml	**800 ng/ml**	**10.0 ng/ml**	**v Conc.**
	Peak Area Ratio	**Peak Area Ratio**	**Peak Area Ratio**	**Regression Slope**
Matrix 1	9.10	5.36	0.139	0.00563
Matrix 2	6.62	4.24	0.131	0.00408
Matrix 3	8.35	4.89	0.147	0.00516
Matrix 4	10.1	5.89	0.159	0.00625
Matrix 5	5.78	2.81	0.0944	0.00357
Matrix 6	8.12	4.49	0.153	0.00501
Average	**8.01**	**4.51**	**0.137**	**0.00495**
STDEV	**1.59**	**0.943**	**0.0233**	**0.000986**
% CV	**19.8**	**20.9**	**16.9**	**19.9**

Abbreviation: conc.: concentration; stdev: standard deviation

Table 3.4 4: Matrix effects for 25-O-desacetyl rifapentine using Liquid-liquid extraction

Table A: The peak areas for the analyte and internal standard at high, medium and low concentrations

	High Concentration		Medium Concentration		Low Concentration	
	Analyte Peak Area	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**
Matrix 1	2250000	120000	1080000	122000	35000	125000
Matrix 2	2740000	115000	1830000	102000	33700	153000
Matrix 3	2170000	80900	1610000	73800	30900	112000
Matrix 4	1400000	36800	751000	30400	21000	32800
Matrix 5	2670000	101000	1430000	96500	29700	136000
Matrix 6	1710000	71800	1120000	81900	21400	105000

Table B: The peak area ratios and regression slope for each matrix

	High Conc.	**Medium Conc.**	**Low Conc.**	**Area Ratio**
	1600 ng/ml	**800 ng/ml**	**10.0 ng/ml**	**v Conc.**
	Peak Area Ratio	**Peak Area Ratio**	**Peak Area Ratio**	**Regression Slope**
Matrix 1	18.8	8.85	0.280	0.0116
Matrix 2	23.8	17.9	0.220	0.0148
Matrix 3	26.8	21.8	0.275	0.0166
Matrix 4	38.0	24.7	0.640	0.0235
Matrix 5	26.4	14.8	0.218	0.0164
Matrix 6	23.8	13.7	0.203	0.0148
Average	**26.3**	**17.0**	**0.306**	**0.0163**
STDEV	**6.44**	**5.76**	**0.166**	**0.00395**
% CV	**24.5**	**33.9**	**54.4**	**24.2**

Abbreviation: conc.: concentration; stdev: standard deviation

The regression slopes of rifapentine and 25-O-desacetyl rifapentine were 19.9% and 24.2%, respectively. They therefore did not meet the acceptance criteria for matrix effects using an internal standard that is not an isotopically labelled analogue of the analytes. This indicates that matrix effects influenced the precision and accuracy of the assay. For this reason, solid phase extraction was considered for sample clean-up. Furthermore, an API 4000 mass spectrometer (AB Sciex) was subsequently used for analysis due to instrument availability.

3.4.3 Mass spectrometer re-optimisation

Parameters of an API 4000 mass spectrometer were optimized for rifapentine and its metabolite, which included nebulising gas, turbo gas, curtain gas, collision gas, source temperature, ion spray voltage and flow injection analysis.

3.4.4 Chromatography re-development

Rifapentine and 25-O-desacetyl rifapentine were infused onto an API 4000 Mass Spectrometer at a concentration of 100 ng/ml. Stock solutions were prepared at 1 mg/ml for the analytes and internal standard. In order to overcome the matrix effects observed for the protein precipitation and liquid-liquid extraction, modifications were made to the mobile phase composition. Mobile phase A consisted of water: methanol (90:10, v/v) containing 0.1% formic acid and mobile phase B consisted of acetonitrile with 0.1 % formic acid. The analytical run time (Poroshell C18 column; 4.6 x 50 mm, 2.7 µm) was 4 minutes with a flow rate of 450 µl/min and an isocratic mobile phase composition of 45% mobile phase A and 55% mobile phase B. Figure 3.8 below shows a representative chromatogram. At this stage, rifampicin-d3 was chosen as the most suitable internal standard in the absence of a stable isotopically labelled analogue for rifapentine and its metabolite. Dooley et al. and Parsons et al. used rifampicin-d3 as an internal standard to quantitate and validate rifapentine and its metabolite, and found that the internal standard compensated well during extraction and on-instrument (Parsons, et al., 2014) (Dooley, et al., 2012). Chromatography from the Parsons et al. study was similar to the chromatography displayed below where 25-O-desacetyl rifapentine eluted first followed by rifampicin-d3 and lastly rifapentine in an analytical run time of 4 minutes (Parsons, et al., 2014).

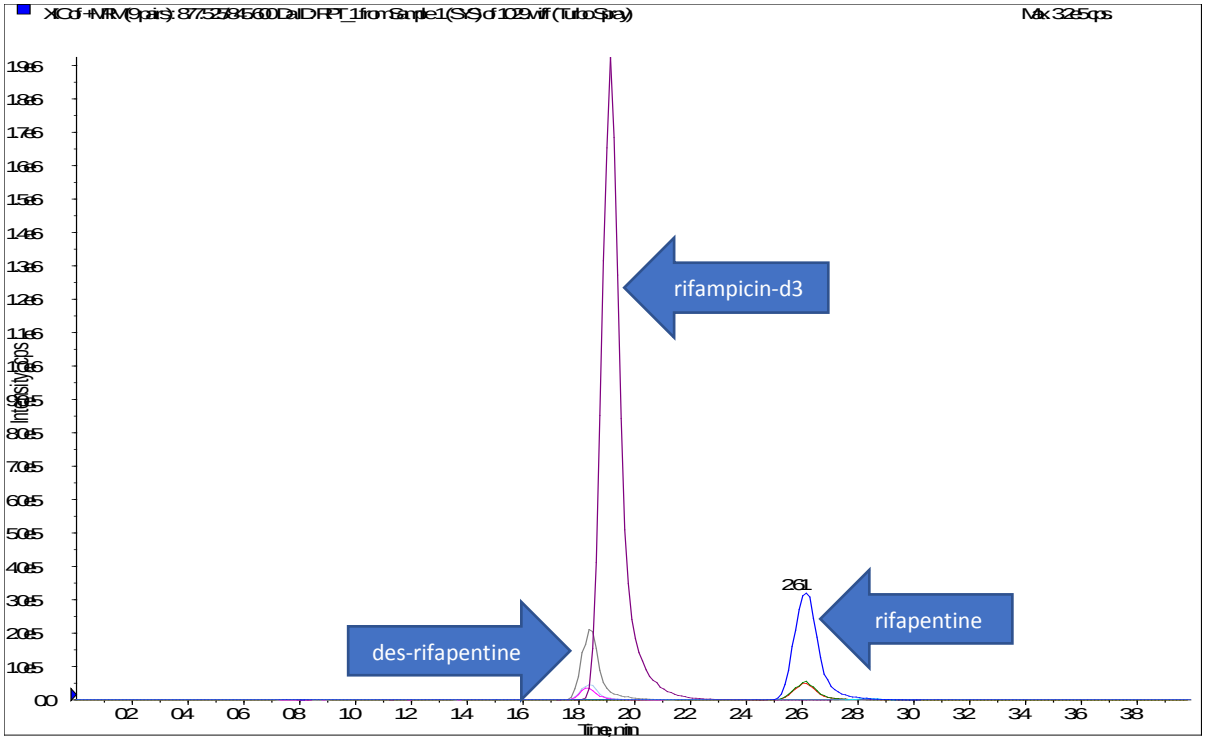


Figure 3. 8: Representative chromatogram for rifapentine and 25-0-desacetyl rifapentine

3.4.5 Solid phase extraction development

C18 SPE Vac cartridges (Waters Sep-Pak, 50 mg Silica) were used for Solid phase extraction (SPE). SPE provides effective sample clean up, is sensitive and selective, but it is time consuming. To determine which solvent would be appropriate for column conditioning, washing and eluting of rifapentine and 25-O-desacetyl rifapentine, solvents were optimized, acetonitrile and methanol. Methanol was found to be the appropriate solvent for rifapentine and 25-O-desacetyl rifapentine. A published method describing SPE sample clean-up of rifampicin and levofloxacin in catheter segments from a mouse model of a device-related infection in which rifapentine was used as an internal standard was used as a guide to ensure effective sample clean-up (Bao, et al., 2008).

The wash step was optimized to determine how many wash steps and what percentage of the organic solvent (methanol) can be applied to achieve effective elution of the analytes (Figure 3.9). Briefly, after each step of the SPE process, the resulting eluent was collected, evaporated to dryness, reconstituted, samples added to a 96 microwell plate and loaded into the sampler and quantified by means of LC-MS/MS.

Figure 3.9 below depicts wash step optimization (one wash for each tube) for rifapentine and 25-O-desacetyl rifapentine. The column was conditioned with 1 ml methanol and equilibrated with 1 ml water before loading the sample, which had undergone prior protein precipitation and supernatant dilution.

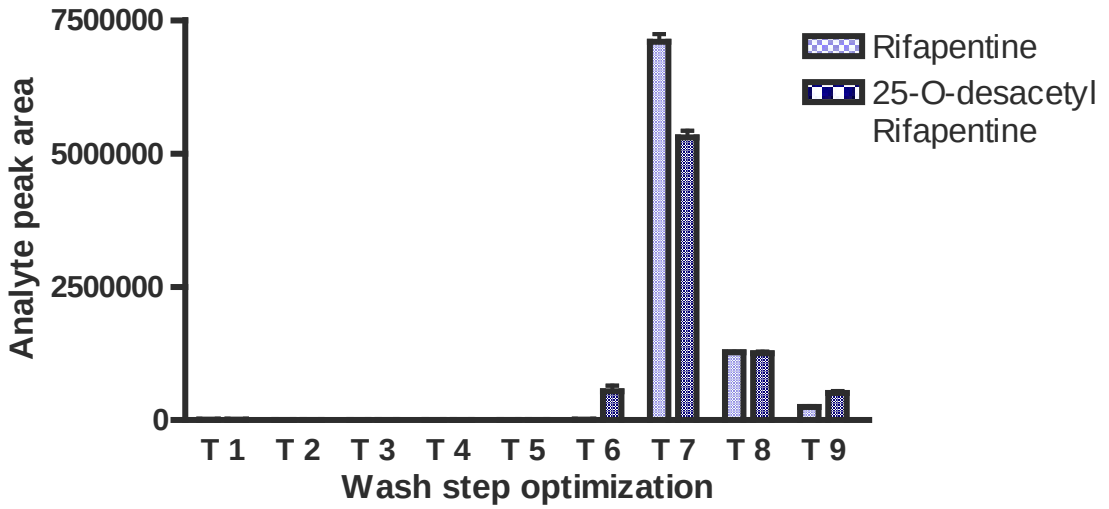


Figure 3. 9: Wash step optimization for rifapentine and 25-O-desacetyl rifapentine.

Abbreviations: T 1 -Tube 1, 900 µl of sample loaded; T 2- Tube 2, 1 ml H_2O/MeOH (90:10, v/v); T 3 - Tube 3, 1 ml H_2O/MeOH (80:20, v/v); T 4 - Tube 4, 1 ml H_2O/MeOH (70:30, v/v); T 5 - Tube 5, 1 ml H_2O/MeOH (60:40, v/v); T 6 - Tube 6, 1 ml H_2O/MeOH (50:50, v/v); T 7 - Tube 7, 1 ml H_2O/MeOH (30:70, v/v); T 8 - Tube 8, 1 ml H_2O/MeOH (10:90, v/v); T 9 - Tube 9, 1 ml methanol (100%)

The graph above indicates that when water: methanol 50:50 (v/v) is applied the analytes begin to elute from the SPE columns. This indicated that the wash step could contain a maximum of 40% methanol in order to remove endogenous matrix components. Optimizing the wash step also helped to determine the quantity and nature of the wash steps required to eliminate matrix effects, which were checked each time a wash step was optimized (data shown below in Table 3.4 5 and 3.4 6). There were two wash steps (wash 1: 90% water with 10% methanol; wash 2: 60% water with 40% methanol) implemented using solid phase extraction for the matrix effects results depicted below for both rifapentine and 25-O-desacetyl rifapentine.

Table 3.4 5: Matrix effects for rifapentine using Solid Phase Extraction

Table A: The peak areas for the analyte and internal standard at high, medium and low concentrations

	High Concentration		Medium Concentration		Low Concentration	
	Analyte Peak Area	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**
Matrix 1	32100000	5130000	17600000	4870000	175000	5720000
Matrix 2	31800000	4690000	16300000	5210000	175000	5710000
Matrix 3	29100000	3740000	16800000	3930000	184000	4390000
Matrix 4	36000000	3520000	20800000	3460000	170000	2690000
Matrix 5	35500000	6530000	19900000	6570000	206000	8090000
Matrix 6	18600000	3700000	10500000	4050000	140000	5150000

Table B: The peak area ratios and regression slope for each matrix

	High Conc.	**Medium Conc.**	**Low Conc.**	**Area Ratio**
	1600 ng/ml	**800 ng/ml**	**10.0 ng/ml**	**v Conc.**
	Peak Area Ratio	**Peak Area Ratio**	**Peak Area Ratio**	**Regression Slope**
Matrix 1	6.26	3.61	0.0305	0.00391
Matrix 2	6.78	3.13	0.0306	0.00424
Matrix 3	7.78	4.27	0.0419	0.00486
Matrix 4	10.2	6.01	0.0632	0.00639
Matrix 5	5.44	3.03	0.0254	0.00340
Matrix 6	5.03	2.59	0.0271	0.00314
Average	**6.92**	**3.8**	**0.0365**	**0.00432**
STDEV	**1.89**	**1.24**	**0.0143**	**0.00118**
% CV	**27.4**	**32.8**	**39.1**	**27.3**

Abbreviation: conc.: concentration; stdev: standard deviation

Table 3.4 6: Matrix effects for 25-O-desacetyl rifapentine using Solid Phase Extraction

Table A: The peak areas for the analyte and internal standard at high, medium and low concentrations

	High Concentration		Medium Concentration		Low Concentration	
	Analyte Peak Area	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**
Matrix 1	21800000	1170000	9700000	932000	154000	815000
Matrix 2	20800000	1270000	12100000	982000	135000	913000
Matrix 3	15800000	820000	7480000	672000	91400	803000
Matrix 4	14800000	454000	6260000	362000	44100	354000
Matrix 5	26400000	986000	14100000	885000	192000	780000
Matrix 6	15900000	803000	8630000	862000	133000	872000

Table B: The peak area ratios and regression slope for each matrix

	High Conc.	**Medium Conc.**	**Low Conc.**	**Area Ratio**
	1600 ng/ml	**800 ng/ml**	**10.0 ng/ml**	**v Conc.**
	Peak Area Ratio	**Peak Area Ratio**	**Peak Area Ratio**	**Regression Slope**
Matrix 1	18.6	10.4	0.188	0.0116
Matrix 2	16.4	12.3	0.147	0.0102
Matrix 3	19.3	11.1	0.113	0.0120
Matrix 4	32.6	17.3	0.124	0.0204
Matrix 5	26.8	15.9	0.246	0.0167
Matrix 6	19.8	10.0	0.152	0.0124
Average	**22.2**	**12.8**	**0.162**	**0.0139**
STDEV	**6.16**	**3.05**	**0.0486**	**0.00388**
% CV	**27.7**	**23.7**	**30.0**	**27.9**

Abbreviation: conc.: concentration; stdev: standard deviation

Matrix effects influenced the precision and accuracy of the assay for rifapentine and 25-O-desacetyl rifapentine as the regression slopes were 27.3% and 27.9%, respectively. This did not meet the acceptance criteria for an internal standard that is not an isotopically labelled analogue of the analyte.

Various publications have reported instability of rifamycins (Parsons, et al., 2014) (Fox, et al., 2011) (Dooley, et al., 2012) (Winchester, et al., 2015) (Melo, et al., 2011). Adding ascorbic acid during extraction helps to protect the analytes as it acts as an antioxidant (He, et al., 1996). Ascorbic acid

concentrations were investigated to determine the optimal concentrations that would protect rifapentine and its metabolite from oxidation. Figure 3.10 shows different ascorbic acid concentrations for rifapentine and 25-O-desacetyl rifapentine that were added with the precipitation reagent.

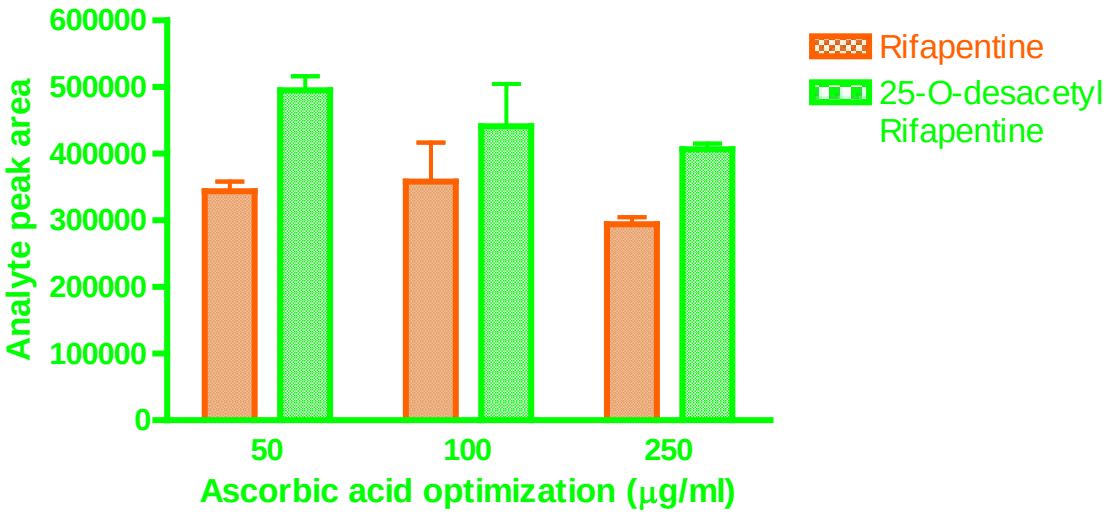


Figure 3. 10: Optimization of ascorbic acid concentration for rifapentine and 25-O-desacetyl rifapentine.

At 100 µg/ml the optimal effect of ascorbic acid is observed for rifapentine while 50 µg/ml is observed for 25-O-desacetyl rifapentine. An ascorbic acid concentration of 50 µg/ml provided consistency and a low percentage coefficient of variation (%CV) for both rifapentine and 25-O-desacetyl rifapentine, and the analyte peak areas for rifapentine were similar at both concentrations. Therefore, 50 µg/ml was the concentration that was chosen.

The solid phase extraction method initially applied was as follows for sample preparation: 100 µl breastmilk; 10 µl working solution containing rifapentine and 25-O-desacetyl rifapentine; 200 µl methanol containing 250 ng/ml internal standard (Rifampicin-d3); vortex 30 seconds; sonicate 5 minutes; vortex 10 seconds; centrifuge at 13 000 g for 5 minutes; transfer 250 µl supernatant into a 2 ml tube and add 1.75 ml water containing 50 µg/ml ascorbic acid; vortex 10 seconds. Following sample preparation, the column was conditioned with 1 ml methanol, equilibrated with 1 ml water; 2 ml of the sample was loaded; 2 wash steps were applied - 1 ml of water and methanol (90:10, v/v) and 1 ml of water and methanol (70:30, v/v); the sample was eluted using 500 µl methanol containing 0.1 % formic acid. The samples were concentrated under nitrogen at 40°C, reconstituted in 150 µl of injection solvent (acetonitrile: 0.1% formic acid in water: methanol (60: 30: 10, v/v/v) and 5 µl was injected into the LC-MS/MS for analysis.

This method produced cleaner samples compared to protein precipitation and liquid-liquid extraction methods, as expected. However, matrix effects were still evident (data shown below in Table 3.4 7 and 3.4 8).

Table 3.4 7: Matrix effects of rifapentine using Solid Phase Extraction

Table A: The peak areas for the analyte and internal standard at high, medium and low concentrations

	High Concentration		**Medium Concentration**		**Low Concentration**	
	Analyte Peak Area	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**
Matrix 1	39100000	26200000	25900000	26000000	385000	26900000
Matrix 2	39700000	27300000	26800000	27900000	378000	25800000
Matrix 3	39800000	27700000	27000000	28300000	378000	26000000
Matrix 4	42600000	29400000	28500000	28500000	363000	25600000
Matrix 5	42400000	29300000	27500000	28700000	381000	26200000
Matrix 6	29800000	14800000	18400000	15100000	273000	15300000

Table B: The peak area ratios and regression slope for each matrix

	High Conc.	**Medium Conc.**	**Low Conc.**	**Area Ratio**
	1600 ng/ml	**800 ng/ml**	**10.0 ng/ml**	**v Conc.**
	Peak Area Ratio	**Peak Area Ratio**	**Peak Area Ratio**	**Regression Slope**
Matrix 1	1.49	0.996	0.0143	0.000928
Matrix 2	1.45	0.961	0.0146	0.000904
Matrix 3	1.44	0.954	0.0145	0.000893
Matrix 4	1.45	1.00	0.0141	0.000901
Matrix 5	1.45	0.958	0.0145	0.000900
Matrix 6	2.01	1.21	0.0178	0.00125
Average	**1.55**	**1.01**	**0.0150**	**0.000964**
STDEV	**0.228**	**0.101**	**0.00139**	**0.000142**
% CV	**14.7**	**10.0**	**9.3**	**14.8**

Abbreviation: conc.: concentration; stdev: standard deviation

Table 3.4 8: Matrix effects of 25-O-desacetyl rifapentine using Solid Phase Extraction

Table A: The peak areas for the analyte and internal standard at high, medium and low concentrations

	High Concentration		Medium Concentration		Low Concentration	
	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area
Matrix 1	25000000	26200000	15600000	26000000	207000	26900000
Matrix 2	25300000	27300000	15400000	27900000	181000	25800000
Matrix 3	24200000	27700000	15900000	28300000	184000	26000000
Matrix 4	26900000	29400000	16600000	28500000	187000	25600000
Matrix 5	27900000	29300000	15700000	28700000	190000	26200000
Matrix 6	17000000	14800000	9420000	15100000	128000	15300000

Table B: The peak area ratios and regression slope for each matrix

	High Conc.	Medium Conc.	Low Conc.	Area Ratio
	1600 ng/ml	800 ng/ml	10.0 ng/ml	v Conc.
	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Regression Slope
Matrix 1	0.954	0.600	0.00770	0.000595
Matrix 2	0.927	0.552	0.00702	0.000578
Matrix 3	0.874	0.562	0.00708	0.000545
Matrix 4	0.915	0.582	0.00730	0.000571
Matrix 5	0.952	0.547	0.00725	0.000594
Matrix 6	1.149	0.624	0.00837	0.000717
Average	**0.962**	**0.578**	**0.00745**	**0.000600**
STDEV	**0.0962**	**0.0300**	**0.000507**	**0.0000603**
% CV	**10.0**	**5.2**	**6.8**	**10.0**

Abbreviation: conc.: concentration; stdev: standard deviation

Matrix effects influenced the precision and accuracy of the assay for rifapentine as the regression slope was 14.8%, indicating it did not meet the acceptance criteria when using an internal standard that is not an isotopically labelled analogue of the analyte. The regression slope for 25-O-desacetyl rifapentine met the acceptance criteria when using an internal standard that is not an isotopically

labelled analogue of the analyte. However, since rifapentine failed to meet the acceptance criteria it necessitated further method development. For the results described above, two wash steps were incorporated, namely 1ml of water methanol 90:10 (v/v) and 1 ml of water methanol 70:30 (v/v). Therefore, the method was scrutinized and further optimized. The final method incorporated the two wash steps described above, but each was performed twice to remove interfering endogenous matrix components.

The sample concentration step under a gentle, continuous stream of nitrogen was also optimized at different temperatures to determine the temperature best suited to concentrate the SPE eluent. Figure 3.11 below indicates the optimization of the concentration step for rifapentine and 25-O-desacetyl rifapentine.

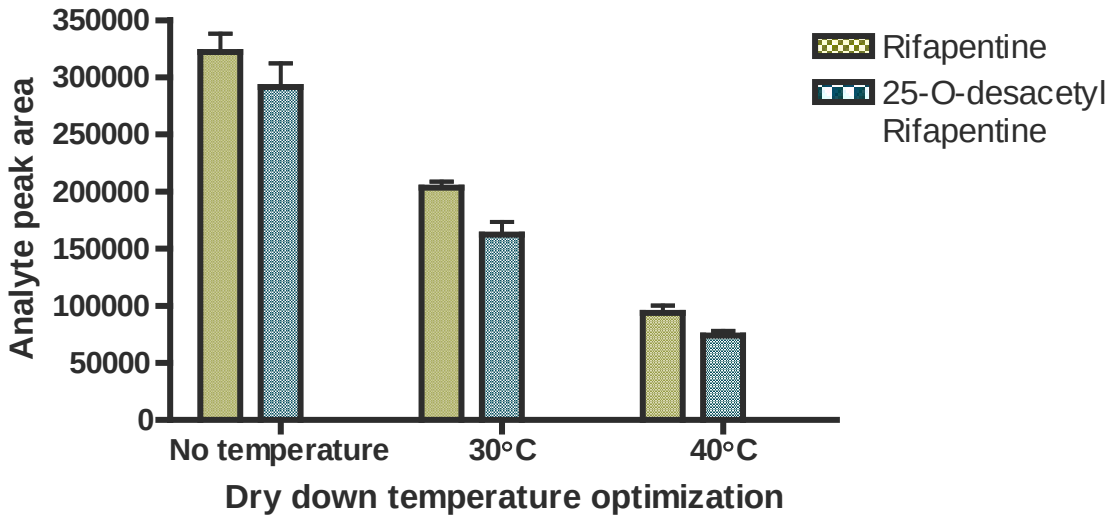


Figure 3. 11: Sample concentration temperature optimization for rifapentine and 25-O-desacetyl rifapentine.

As depicted on the graph above, applying increasing temperatures of 30°C and 40°C resulted in the degradation of the analytes regardless of the presence of ascorbic acid in the samples. However, a sample concentration temperature of 30°C was chosen as a compromise although it was clear that when no temperature was applied to the heating block there was better recovery of the analytes. This was done because the effect of the nitrogen being applied without temperature on the heating block resulted in the heating block becoming cold. The samples therefore took a long time to dry (approximately an hour and 30 minutes) while at 30°C it took approximately 30 minutes. The minimum temperature to which the heating block could be set was 30°C as per instrument design.

4 Final Assay

4.1.1 Reagents and Chemicals

Rifapentine (98.7% purity) was purchased from Sanofi (Bridgewater, New Jersey, United States) while 25-O-desacetyl rifapentine (95.84% purity) and rifampicin-d3 (98% purity) was purchased from Toronto Research Chemicals (North York, Ontario, Canada). Methanol and ascorbic acid were purchased from Sigma-Aldrich (Darmstadt, Germany) while 2-Isopropanol and formic acid were purchased from Merck (Darmstadt, Germany). Acetonitrile was purchased from Honeywell (Pittsburgh, USA). LC-MS/MS grade Millipore water was produced in-house (Merck-Millipore, Germany).

4.1.2 HPLC

An Agilent Poroshell 120 EC-C18 column was used for chromatographic separation (4.6 x 50 mm, 2.7 µm), the column temperature was set at approximately 30°C. An Agilent 1200 binary pump was used. An isocratic mobile phase of acetonitrile containing 0.1% formic acid (B) and water containing 10% methanol and 0.1% formic acid (A) (55:45, v/v) was used at a flow rate of 450 µl/min and the run time was 4 minutes. An Agilent 1200 Autosampler set at approximately 8°C was used and a 5 µl injection volume was used to introduce the sample from a 96-well plate into the mass spectrometer.

4.1.3 Mass Spectrometry

An API4000 mass spectrometer with multiple reaction monitoring (MRM) in unit resolution (pause time 5 msec) was used and ions are produced using an ESI source in the positive ionisation mode. The nebuliser gas (Gas 1) (arbitrary unit) was set at 50 and turbo gas (Gas 2) (arbitrary unit) at 40. The curtain gas (arbitrary unit) was set at 30 and the collision gas (arbitrary unit) at 5. The source temperature was set at 350°C and the ion spray voltage at 5000 V. Flow injection analysis (FIA) was used to optimise the source settings.

4.1.4 Quantitation parameters

Analyst 1.6.2 software (Analyst Classic quantitation algorithm) was used to quantify rifapentine, 25-O-desacetyl rifapentine and rifampicin-d3. The bunching factor for rifapentine, 25-O-desacetyl rifapentine and rifampicin-d3 was 1 while the smoothing factor for both rifapentine and rifampicin-d3 was 3 and for 25-O-desacetyl rifapentine it was 5. Rifapentine elutes at approximately 2.70 minutes, 25-O-desacetyl rifapentine at approximately 1.90 minutes and rifampicin-d3 at approximately 2.17 minutes. The calibration curve fits a quadratic (weighted by 1/x) concentration regression over the range of 2 - 2000 ng/ml for both rifapentine and the metabolite based on the analyte/ISTD peak area ratios. Rifampicin-d3 internal standard was used to quantitate both rifapentine and its metabolite.

Table 4. 1: MS/MS settings

	Rifapentine	25-O-desacetyl Rifapentine	Rifampicin-d3
Protonated molecular ion mass (m/z) $[M+H]^+$	877.5	835.4	827.4
Product ion mass (m/z) Quantifier	845.6	803.5	795.6
Product ion mass (m/z) Qualifier	151.1	453.2	n/a
Dwell time (ms)	80	80	80
De-clustering potential (V)	96	66	96
Entrance potential (V)	10	10	10
Collision energy (eV)	31	21	27
Collision cell exit potential (V)	42	44	12

Quantifier: most abundant product ions that are used to quantify an analyte peak. Qualifier: less abundant product ions used in monitoring an analyte of interest.

4.2 Preparation of stock solutions, working solutions, calibration standards and quality controls

4.2.1 Stock solution preparation (rifapentine and 25-O-desacetyl rifapentine)

Stock solutions were prepared at 1 mg/ml in methanol (SS1 and SS1M). The weighed mass of the analytes was adjusted for purity. Stock solutions were stored at approximately -80°C until required. Stock solutions were used to prepare working solutions which were spiked into blank biological matrix. Stock solutions were tested for accuracy using HPLC UV.

Table 4.2 1: Representative preparation of rifapentine stock solution (SS1)

Solvent used	Volume of solvent (ml)	Weighed mass of analyte (mg)	Adjusted* mass of analyte (mg)	SS1 concentration (µg/ml)
Methanol	1.234	1.250	1.234	1000

** Reason for Adjustment (e.g. purity, salt, hydrate): Purity*
** Calculation: 1.25 x 0.987*

Table 4.2 2: Representative preparation of 25-O-desacetyl rifapentine stock solution (SS1M)

Solvent used	Volume of solvent (ml)	Weighed mass of analyte (mg)	Adjusted* mass of analyte (mg)	SS1M concentration (µg/ml)
Methanol	0.786	0.820	0.786	1000

** Reason for Adjustment (e.g. purity, salt, hydrate): Purity*
** Calculation: 0.820 x 0.9584*

Table 4.2 3: Preparation of rifampicin-d3 stock solution (ISS1)

Solvent used	Volume of solvent (ml)	Weighed mass of analyte (mg)	Adjusted* mass of analyte (mg)	ISS1 concentration (µg/ml)
Methanol	0.500	0.500	0.500	1000

** Reason for Adjustment (e.g. purity, salt, hydrate): None, volume of solvent was added directly to the vial*

4.2.2 Calibration standards preparation

The 1mg/ml stocks of rifapentine and 25-O-desacetyl rifapentine are diluted 1:1 with methanol to a volume of 500 µg/ml. Working solutions (WS) are prepared volumetrically in MeOH: H_2O (70:30, v/v). A volume of 48 µl of a 500 µg/ml stock of rifapentine and 25-O-desacetyl rifapentine are added to 995 µl of blank solvent for WS1. Serial dilutions are then performed as shown in Table 4.2.4 below to obtain WS2 – WS11. Aliquots of 35 µl of each working solution were stored in 1.5 ml microcentrifuge tubes at approximately -80°C. When extracting calibration standards, 10 µl of WS were spiked into 100 µl of blank breastmilk to obtain the concentrations of the calibration range depicted in Table 4.2.4. Calibration standards were spiked with WS on the day of extraction. For optimal extraction results, Rezk et al. recommend that the calibration standards and quality controls be prepared using intermediate stock solutions and not serial dilutions (Rezk, et al., 2008).

4.2.3 Quality controls preparation

Working solutions (WS) for QC samples are prepared volumetrically in MeOH: H_2O (70:30, v/v), using the same methodology used to prepare working solutions for the calibration standards. To obtain WS Dilute, 148 µl of a 500 µg/ml stock of rifapentine and 148 µl of 25-O-desacetyl rifapentine are added to 1386 µl of blank solvent and serially diluted to obtain WS_H, WS_M, WS_SYS, WS_L, WS_LLOQ1 and WS_LLOQ2, as depicted on Table 4.2.5. For the validation, the LLOQ was evaluated at two concentrations, namely 2 and 4 ng/ml. The assay performed well, and the API 4000 was sufficiently sensitive to accurately and reproducibly measure the analytes across a range of 2 to 2000 ng/ml during the inter- and intra-day validations. Aliquots of 35 µl of QC working solutions (WS) were stored in 1.5 ml microcentrifuge tubes at approximately -80°C. During an extraction 10 µl of the working solutions of QC H, M, L and LLOQ were spiked into 100 µl of blank breastmilk to obtain the concentrations of the calibration range depicted on Table 4.2.5. Quality Control were spiked with WS on the day of extraction due to the analytes precipitating out of solution when pre-spiked and stored at -80°C. Working solution stability was tested on LC-MS/MS.

Table 4.2 4: Preparation of Working Solutions and Calibration Standards for rifapentine and 25-O-desacetyl rifapentine

Working Solution (WS)	Blank solvent volume (µl)	Spiking solution	Spiking solution volume (µl)	WS Solution concentration (ng/ml)	Volume of breastmilk (µl)	Volume WS spiked (µl)	STD	RPT+dRPT Final concentration (ng/ml)
WS1	995	SS1 + SS1M	48 x 2	21998	100	10.0	**STD1-ULOQ**	2000
WS2	200	WS1	600	16499	100	10.0	**STD 2**	1500
WS3	400	WS2	400	8250	100	10.0	**STD 3**	750
WS4	400	WS3	400	4125	100	10.0	**STD 4**	375
WS5	434	WS4	400	1980	100	10.0	**STD 5**	180
WS6	475	WS5	380	880	100	10.0	**STD 6**	80.0
WS7	514	WS6	400	385	100	10.0	**STD 7**	35.0
WS8	426	WS7	450	198	100	10.0	**STD 8**	18.0
WS9	500	WS8	401	88.0	100	10.0	**STD 9**	8.00
WS10	400	WS9	400	44.0	100	10.0	**STD 10**	4.00
WS11	200	WS10	200	22.0	100	10.0	**STD 11-LLOQ**	2.00

Abbreviation: WS – Working Solution; STD – Standard; ULOQ – Upper limit of quantification; LLOQ – lowest limit of quantification

Table 4.2 5: Preparation of rifapentine and 25-desacetyl rifapentine Quality Control Working Solutions

Working Solution (WS)	Blank solvent volume (µl)	Spiking solution	Spiking solution volume (µl)	WS Solution concentration (ng/ml)	Volume of breastmilk (µl)	Volume WS spiked (µl)	STD	RPT+dRPT Final concentration (ng/ml)
SS1; SS1M	**150**	**SS1; SS1M**	**150**	**n/a**	**n/a**	**n/a**	**n/a**	**n/a**
WS_ Dil	1386	**SS1 + SS1M**	148 x 2	43995	100	10.0	**DIL**	4000
WS_ H	1050	**WS_ DIL**	700	17598	100	10.0	**H**	1600
WS_ M	700	**WS_ H**	700	8799	100	10.0	**M**	800
WS_ SYS	1350	**WS_ M**	150	880	100	10.0	**SYS**	80.0
WS_ L	1500	**WS_ SYS**	100	55.0	100	10.0	**L**	5.00
WS_ LLOQ 1	150	**WS_ L**	600	44.0	100	10.0	**LLOQ 1**	4.00
WS_ LLOQ 2	250	**WS_ LLOQ 1**	250	22.0	100	10.0	**LLOQ 2**	2.00

Abbreviation: Dil – Dilution; H – high; M – Medium; SYS – system suitability sample; L – low; LLOQ – lowest limit of quantification

4.3 Extraction method

This is therefore the finalized extraction method below which is a combination of protein and solid phase extraction. This combination of extraction methods allowed for sufficient sample clean up before it is added to the SPE C18 columns.

Sample preparation requires thawing blank breastmilk samples and vortexing for 1 minute. Working solutions for calibration standards and quality controls, as well as internal standard, were sonicated on ice (as it was observed that the analytes degrade at room temperature) for five minutes and vortexed for 30 seconds.

Protein precipitation was performed on ice. A volume of 100 µl of blank breastmilk was aliquoted into 1.5 ml microcentrifuge tubes and 10 µl of appropriate working solution for calibration standards and quality controls was added into the 100 µl blank breastmilk. The blank and double blank samples receive 10 µl of blank solvent (MeOH: H_2O, 70:30, v/v). To the 1.5 ml tubes, 250 µl of precipitation reagent (MeOH) containing Rifampicin-d3 (internal standard) at 250 ng/ml was added. The tubes were vortexed at maximum speed for 30 seconds, centrifuged for five minutes at 20238 g (maximum speed) and 300 µl of supernatant transferred into 2 ml microcentrifuge tubes. A volume of 1.5 ml of water was added and tubes vortexed for 10 seconds.

SPE was performed at room temperature. A volume of 1 ml methanol was used to condition the C18 SPE Vac cartridges (Waters Sep-Pak, 50 mg Silica). The cartridges were equilibrated with 1 ml water. The sample was loaded in two 1 ml steps and eluted under positive pressure between the addition steps. The cartridges were washed twice with 1 ml H_2O: MeOH (90:10, v/v). The cartridges undergo a second wash step, twice, with 1 ml H_2O: MeOH (80:20, v/v). To elute the analytes, 500 µl of elution solvent (MeOH with 0.1 % formic acid and 50 µg/ml ascorbic acid) was added and eluted under positive pressure, collecting eluent in a glass borosilicate tube. The elution step was repeated, and the eluent collected into the same borosilicate tube. The sample was concentrated under nitrogen at approximately 30°C for approximately 30 minutes. To reconstitute, 200 µl of injection solvent containing mobile phase A and B (50:50, v/v) containing 20 µg/ml ascorbic acid was used and the sample was vortexed for 30 seconds. Samples were added to a 96 microwell plate and loaded into the sampler. Mobile Phase A contained H_2O: MeOH: formic acid (90:10:0.1, v/v/v) and Mobile Phase B contained acetonitrile with 0.1% formic acid. For analysis 5 µl was injected into the LC-MS/MS instrument.

5 Assay validation

A bioanalytical assay is validated to determine if it is robust, reproducible and reliable for the quantification of the analyte in a biological matrix. Validation experiments conducted should include, but not limited to, matrix effects, recovery, process efficiency, sensitivity and specificity, carry-over, cross-talk, stability and re-injection reproducibility (FDA, 2018).

5.1.1 Inter- and intra-day validation

Inter-day validation evaluates accuracy and precision over at least 2 days, while an intraday validation is evaluated by analysing accuracy and precision within a single run. Each validation consists of calibration standards in duplicate and quality control samples (QC's) in sextuplicate, covering the entire calibration range for the analytes. Precision of inter and intraday validations should be less than 15% for standards and QC samples at high, medium and low levels and less than 20% for the Lowest Limit of Quantification (LLOQ). At least 75% of standards and 67% of quality controls should meet the acceptance criteria of nominal concentration 85-115% and 80-120% for LLOQ. Furthermore, 50% of QC's at each level should pass. Accuracy is described as the closeness to a determined value of the nominal concentration of the analyte while precision (Coefficient of Variation = %CV) is described as the closeness of more than one measurement to the analyte of interest (FDA, 2018).

5.1.2 Stability assessments

Stability was assessed under different conditions to determine if the analyte is affected by the conditions. The stability of stock and working solutions is evaluated to determine stability during storage and during expected duration on-bench. High and low QC samples are used to assess matrix stability and are spiked in blank matrix at the respective concentration (in sextuplicate). Analysis of the stability QC samples is done against a valid calibration curve from calibration standards spiked on the day of extraction and at each level the mean concentration should be within 15% of the nominal concentration. Freeze-thaw, matrix stability, stock and working solution stability, benchtop, reinjection reproducibility and autosampler stability is determined.

5.1.2.1 Stock solution accuracy and stability

Stock solution accuracy and stability was assessed to ensure the integrity of stock solutions prepared by different analysts and to determine if temperature and duration of storage have an influence on the stock solution stability. The analytes and internal standard of interest were assessed for stability at approximately -80°C for 69 days and at room temperature for approximately four hours. The percentage difference for stock solution accuracy should not be more than 5% between stock solutions and should not have a percentage CV above 15%. The acceptance criteria for stock solution stability is that the accuracy of the test solutions should be within 10% of the reference stock. Stock

solution accuracy and stability was tested by means of HPLC (Thermo Scientific) using the HPLC method described in section 4.2.1 above with UV detection at 336 nm.

5.1.2.2 Working Solution stability

Working solution stability was assessed at high and low concentrations to determine if there are any stability issues during storage at approximately -80°C or at room temperature for approximately four hours that may be concentration dependent. The acceptance criteria for working solution stability was the same as for stock solution stability described in Section 4.2 above.

5.1.2.3 Matrix stability

This evaluation should include at least the length of time from when the first sample was drawn during the clinical phase to the date of the final sample analysis during the analytical phase of a study. High and low QC samples were placed at -20°C and -80°C and matrix stability was determined for approximately 3 days. The percentage CV and accuracy should be within 15% of the nominal concentration.

5.1.2.4 Fresh versus Frozen stability

Fresh versus frozen stability was assessed by preparing a fresh set of calibration standards in breastmilk. These are tested against quality controls stored at approximately -80°C for 2 days. The fresh calibration standards are used to quantify the frozen quality controls. Accuracy and precision are the same as for the inter- and intra- validations described previously.

5.1.2.5 Freeze and Thaw stability

The freeze and thaw stability was evaluated to mimic the intended sample handling conditions to be used during sample analysis. Freeze-thaw stability was tested after 3 cycles. High and low QC samples were frozen for approximately 23 hours and thawed for approximately one hour on ice. Percentage difference should be within 15% of the nominal concentration and precision of the measured values should be less than 15%.

5.1.2.6 Benchtop stability

High and low QCs were frozen at approximately -80°C and placed at room temperature for approximately 4.5 hours on ice until extraction. These samples were analysed against a valid calibration curve consisting of freshly prepared standards. Percentage difference should be within 15% of the nominal concentration and precision of the measured values should be less than 15%.

5.1.2.7 Reinjection reproducibility

Reinjection reproducibility evaluation was performed to determine whether the analytical run can be reanalysed by reinjection in case of instrument interruptions. Reinjection reproducibility

demonstrates that following a batch injection and then reinjection, the results should not be significantly different and should not generate a significantly different calibration curve. The autosampler was set at approximately 8°C. Reinjection reproducibility was evaluated against the same calibration standards and quality controls originally injected within approximately 48 hours. The acceptance criteria for standards and QC's are the same as those described for the intra- and inter-day validation batches.

5.1.2.8 Autosampler stability

To evaluate autosampler stability, the same batch used for the reinjection evaluation is used to analyse the peak area ratios of the high and low quality control samples to compare the percentage difference to that of the first injection.

5.1.3 Specificity

The bioanalytical method is tested to determine if it is specific for the analyte of interest in the presence of endogenous components in the sample. Six different blank sources of breastmilk were extracted without an internal standard at the upper limit of quantitation (ULOQ).

5.1.4 Sensitivity

The bioanalytical method is tested to determine if it is sensitive enough to detect the LLOQ in the presence of endogenous components in the sample. Six different blank sources of breastmilk were spiked and extracted at LLOQ (2 ng/ml) without an internal standard. The signal-to-noise ratio for the analyte of interest at LLOQ should be greater than 5 times the response when compared to the response of the blank at the retention time of the analyte.

5.1.5 Cross-talk

Cross-talk must be assessed for the parent analyte, its metabolite and the respective internal standard used. Theoretical upper limit of quantification (ULOQ, 2000 ng/ml) and LLOQ (2 ng/ml) concentrations of the analyte of interest are assessed and injected in sextuplet. A peak area of less than 20% of the LLOQ is acceptable for the analyte of interest when injecting the ULOQ of another analyte or working concentration of the internal standard. To classify as cross-talk, the analytes should have the same retention time.

5.1.6 Carry-over

Carry-over is determined by the presence of an unwanted analyte signal in a blank sample following the injection of a sample with a high analyte concentration. Carry-over in a blank sample should be less than 20% of the LLOQ and less than 5% of the internal standard.

5.1.7 Recovery

Recovery determines the ability of the assay to efficiently extract the analyte of interest. Recovery does not need to be 100% but must be consistent and reproducible. Recovery samples (extracted with internal standard spiked normally) are compared to blank extracted samples from 6 different matrices and reconstituted with injection solution containing the analytes at theoretical high, medium and low concentrations and the internal standard at the working concentration of the method. Quality controls prepared in 6 different matrices are each spiked at high, medium and low concentration levels and extracted. Precision (%CV) of the mean recovery should be less than 15% while the reproducibility within each concentration level should also be less than 15%.

5.1.8 Matrix effects

Matrix effects are characterized by the presence of endogenous components in a biological sample that may interfere with the ionization process (can either enhance or supress the ionization process). The Matuszewski et al. method was used as a guideline when matrix effects was tested (Matuszewski, et al., 1998). The strategies of Trufelli et al. and Panuwet et al. were followed to eliminate matrix effects, namely to ensure the appropriate extraction methods were applied to remove sample interferences, relevant mass spectrometry and chromatographic settings to ensure retention of the analytes without ion suppression or enhancement signals, using the appropriate calibration range with the appropriate stable isotopically labelled analogue (Trufelli, et al., 2011) (Panuwet, et al., 2016). To determine matrix effects blank matrix from at least six different sources are extracted without an internal standard (in triplicate). Each extracted matrix was then reconstituted with injection solution spiked at theoretical high, medium and low concentration levels. The slope variability (%CV) should not be more than 5% for each matrix at high, medium and low concentrations and the precision at each level should be less than 15%.

5.1.9 Process efficiency

Process efficiency compares the combined effect of instrument response and matrix effects of an extracted sample with the response of an unextracted sample. Quality controls prepared in 6 different matrices are each spiked at high, medium and low concentration levels and extracted. This was compared to injection solvent spiked at theoretical high, medium and low analyte concentrations. Process efficiency precision and the reproducibility at each concentration level should be less than 15%. Furthermore, the mean process efficiency across the high, medium and low concentration range should have a precision of less than 15%.

6 Results and Discussion

A bioanalytical assay is validated to ensure that it is reproducible, accurate, precise, sensitive, specific and determines stability of the analyte of interest during an extraction and subsequent storage conditions (FDA, 2018). Accuracy and precision of intra- and inter-day validation over a calibration range of 2 to 2000 ng/ml was evaluated over three consecutive days. A full set of working solution calibration standards and quality controls were prepared and frozen at approximately -80°C. For each run the appropriate WS aliquots were thawed and used to spike blank breastmilk. A run consisted of calibration standards in duplicate and quality controls in sextuplet to obtain a calibration curve. A simple model for the calibration curve describing the relationship between the concentration and response was applied using suitable weighting and statistical tests for goodness of fit. A calibration curve ranging from LLOQ to ULOQ must consist of at least 6-8 calibration levels. The regression model (quadratic weighted by 1/x) selected for validation was used for quantifying study samples.

The calibration curve should fit a measured plot of response against nominal concentrations of the calibration standards accordingly in such that an r^2 fit parameter is as close to one as possible. To consider the method validated, intra- and inter-day percentage accuracy and precision should be between 85-115%, except for the LLOQ that must fall between 80-120%. Each calibration standard (in duplicate) should meet the stated criteria, however, only a single calibration standard is permitted to fail at each concentration. The failed calibration standard should be excluded from the calibration curve, but not any failed quality controls, and the calibration range must not be affected by the exclusion of either an LLOQ or ULOQ.

6.1 Validation 1

Intra-day accuracy and precision is determined by evaluating the calibration standards in duplicate and 6 quality control replicates at each level to produce one calibration curve. The assay is evaluated by calculating the regression equation and creating a calibration curve based on peak area ratios of analyte to internal standard, as displayed on the tables and figures below.

6.1.1 Validation 1 – rifapentine

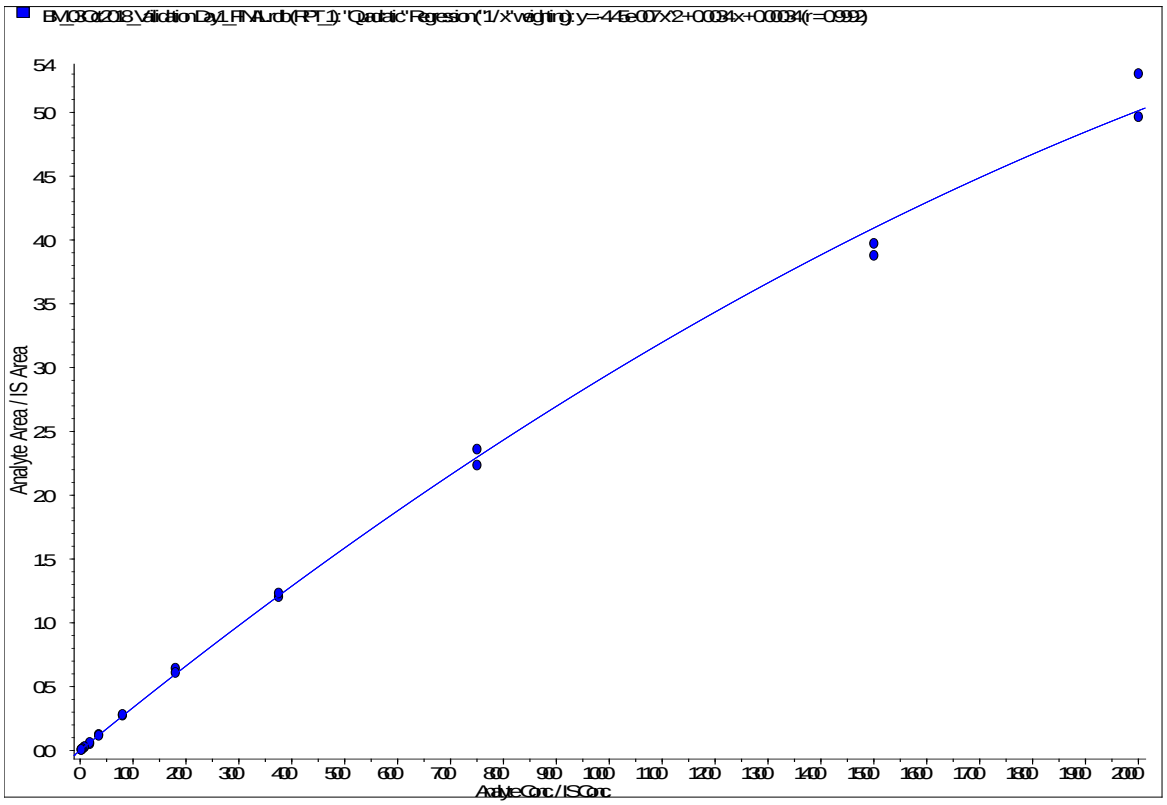


Figure 6. 1: Representative calibration curve of rifapentine - Validation 1

The regression equation used was Quadratic (weighted 1/x concentration, $f(x) = a + bx + cx^2$, as indicated in Table 6.1. Accuracy and precision results for calibration standards and quality controls are summarized for rifapentine Validation 1, as indicated in Table 6.2 and 6.3 respectively.

Table 6. 1: Regression equation for rifapentine - Validation 1

Validation Batch	Quadratic Calibration Curve Parameters			
	A	b	C	r
1	-0.000000445	0.0034	0.00034	0.9992

Table 6. 2: Rifapentine calibration standards accuracy and precision - Validation 1

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
S11	2.00	1.93	0.0182	0.9	96.7	2 of 2
S10	4.00	3.91	0.0416	1.1	97.6	2 of 2
S9	8.00	8.20	0.148	1.8	102.5	2 of 2
S8	18.0	16.8	2.04	12.2	93.2	2 of 2
S7	35.0	35.8	1.49	4.2	102.4	2 of 2
S6	80.0	82.9	1.24	1.5	103.6	2 of 2
S5	180	189	7.37	3.9	105.2	2 of 2
S4	375	377	6.46	1.7	100.7	2 of 2
S3	750	750	32.4	4.3	100.1	2 of 2
S2	1500	1420	31.1	2.2	94.7	2 of 2
S1	2000	2080	155	7.4	104.0	2 of 2

Abbreviation: Conc. – concentration; std – standard; Dev – deviation

Table 6. 3: Summary of rifapentine intra-validation quality controls - Validation 1

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
QC LLOQ	2.00	1.94	0.113	5.8	96.9	6 of 6
QC L	5.00	4.83	0.218	4.5	96.6	6 of 6
QC M	800	795	27.7	3.5	99.4	6 of 6
QC H	1600	1598	50.6	3.2	99.9	6 of 6

Abbreviation: Conc. – concentration; std - standard; Dev – deviation

6.1.2 Validation 1 – 25-O-desacetyl rifapentine

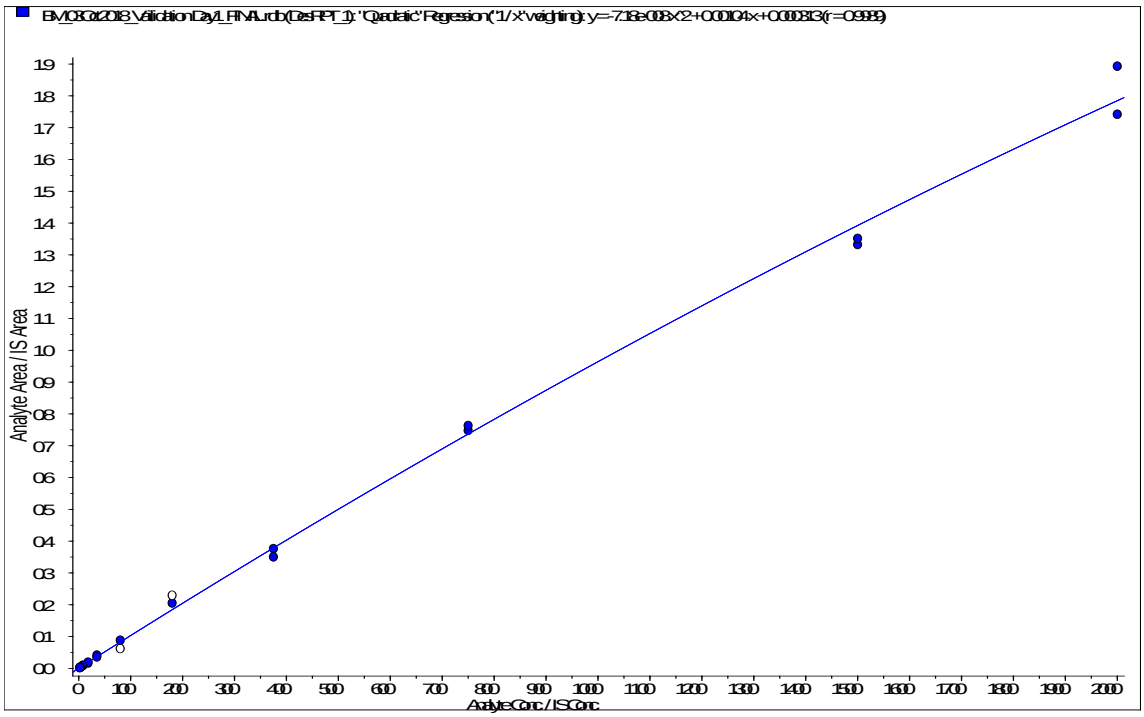


Figure 6. 2: Representative calibration curve for 25-O-desacetyl rifapentine - Validation 1

The regression equation used was Quadratic (weighted 1/x concentration, $f(x) = a + bx + cx^2$, as indicated in Table 6.4. Accuracy and precision results for Calibration Standards and Quality Controls are summarized for 25-O-desacetyl rifapentine Validation 1, as indicated in Table 6.5 and 6.6 respectively.

Table 6. 4: Regression equation for 25-O-desacetyl rifapentine - Validation 1

Validation Batch	**Quadratic Calibration Curve Parameters**			
	A	**b**	**C**	**r**
1	-0.0000000718	0.00104	0.000313	0.9989

Table 6. 5: 25-O-desacetyl rifapentine calibration standards accuracy and precision - Validation 1

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
S11	2.00	1.94	0.045	2.3	97.1	2 of 2
S10	4.00	3.59	0.190	5.3	89.7	2 of 2
S9	8.00	8.55	0.478	5.6	106.8	2 of 2
S8	18.0	17.0	1.71	10.1	94.7	2 of 2
S7	35.0	37.1	3.91	10.5	106.0	2 of 2
S6	80.0	85.4	N/A	N/A	106.7	1 of 2
S5	180	201	N/A	N/A	111.5	1 of 2
S4	375	359	18.5	5.2	95.8	2 of 2
S3	750	770	11.6	1.5	102.7	2 of 2
S2	1500	1439	16.3	1.1	95.9	2 of 2
S1	2000	2045	144	7.0	102.2	2 of 2

Abbreviation: Conc. – concentration; std - standard; Dev – deviation

One of the standards 5 and 6 failed to meet the acceptance criteria, therefore were excluded.

Table 6. 6: Summary of 25-O-desacetyl rifapentine intra-validation quality controls - Validation 1

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
QC LLOQ	2.00	1.98	0.186	9.4	90.1	6 of 6
QC L	5.00	4.92	0.368	7.5	90.9	6 of 6
QC M	800	805	72.4	9.0	96.7	6 of 6
QC H	1600	1704	140	8.2	106.5	6 of 6

Abbreviation: Conc. – concentration; std - standard; Dev – deviation

6.2 Validation 2

Inter-batch accuracy and precision is evaluated by assessing two separate batches consecutively. The calibration curve consists of duplicate calibration standards and quality controls in sextuplet.

6.2.1 Validation 2 – rifapentine

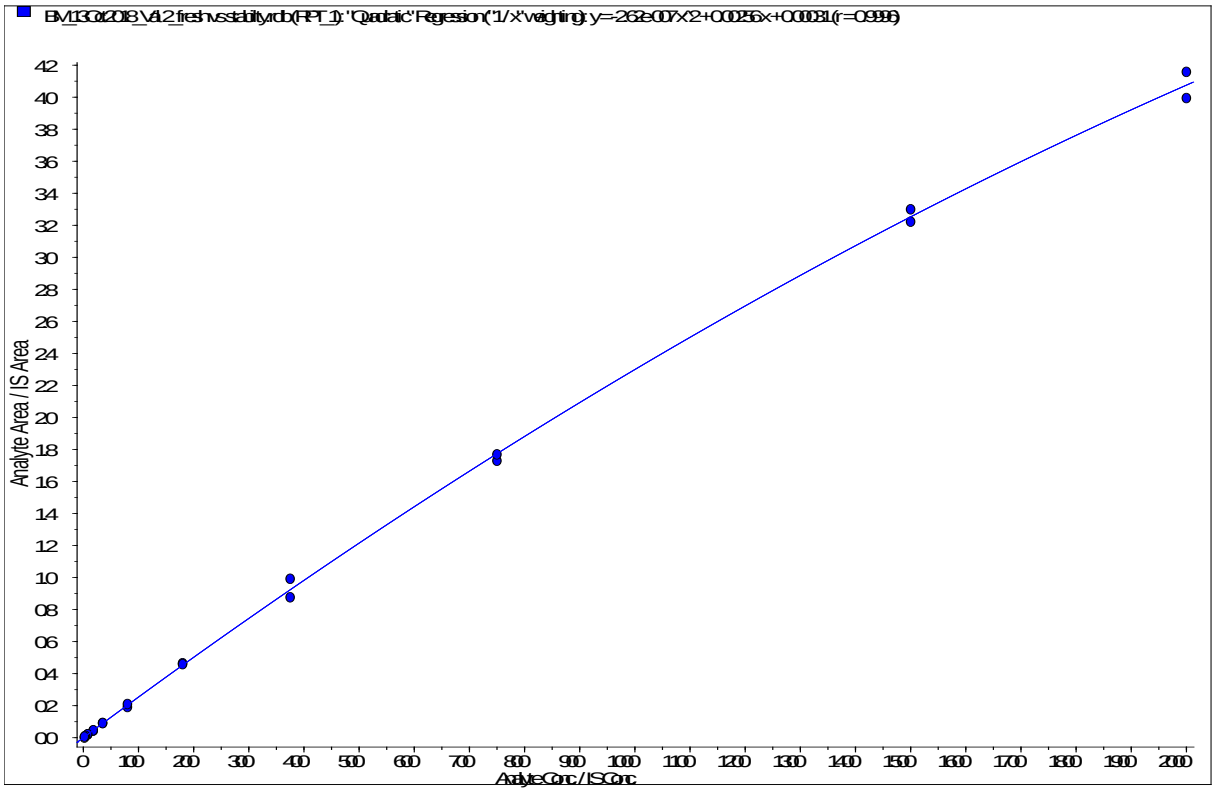


Figure 6. 3: Representative calibration curve for rifapentine - Validation 2

This validation batch was used to evaluate stability samples against a calibration curve. The regression equation used was Quadratic (weighted 1/x concentration, $f(x) = a + bx + cx^2$, as indicated in Table 6.7. Accuracy and precision results for calibration standards and quality controls are summarized for rifapentine Validation 2, as indicated in Table 6.8 and 6.9 respectively.

Table 6. 7: Regression equation for rifapentine Validation 2

Validation Batch	Quadratic Calibration Curve Parameters			
	A	b	C	r
2	-0.000000262	0.00256	0.00031	0.9996

Table 6. 8: rifapentine calibration standards accuracy and precision – Validation 2

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
S11	2.00	2.13	N/A	N/A	106.4	1 of 2
S10	4.00	3.91	0.0699	1.8	97.7	2 of 2
S9	8.00	8.02	0.133	1.7	100.3	2 of 2
S8	18.0	17.4	0.623	3.6	96.8	2 of 2
S7	35.0	35.6	0.188	0.5	101.6	2 of 2
S6	80.0	78.8	5.26	6.7	98.4	2 of 2
S5	180	184	1.70	0.9	102.0	2 of 2
S4	375	379	34.6	9.1	101.2	2 of 2
S3	750	739	12.9	1.8	98.5	2 of 2
S2	1500	1505	30.8	2.1	100.3	2 of 2
S1	2000	2001	76.3	3.8	100.0	2 of 2

Abbreviation: Conc. – concentration; std - standard; Dev – deviation

One of standard 11 failed to meet the acceptance criteria, therefore was excluded.

Table 6. 9: Summary of rifapentine inter-validation quality controls – Validation 2

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
QC LLOQ	2.00	2.18	0.149	6.8	109.1	6 of 6
QC L	5.00	4.90	0.111	2.3	98.0	6 of 6
QC M	800	816	32.2	3.9	102.0	6 of 6
QC H	1600	1606	63.9	4.0	100.3	6 of 6

Abbreviation: Conc. – concentration; std - standard; Dev – deviation

6.2.2 Validation 2 – 25-O-desacetyl rifapentine

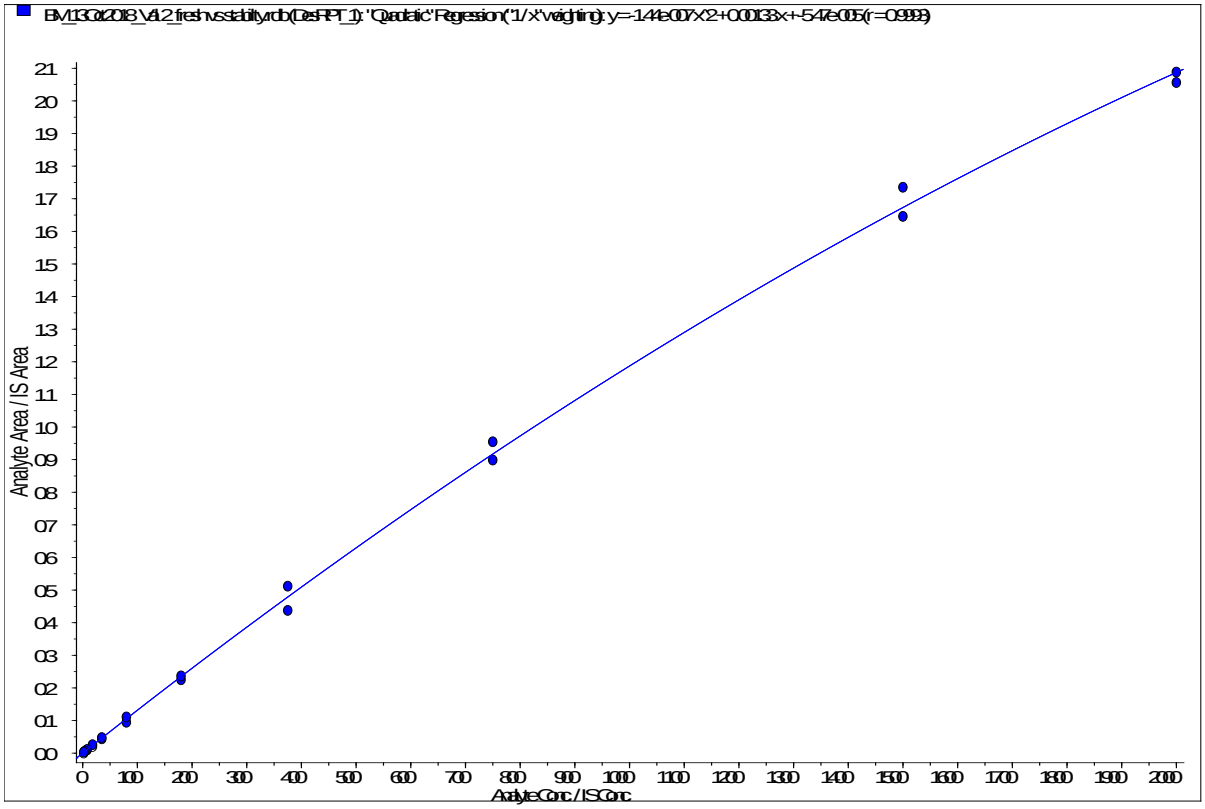


Figure 6. 4: Representative calibration curve for 25-O-desacetyl rifapentine - Validation 2

This validation batch was used to evaluate stability samples against a fresh curve. The regression equation used was Quadratic (weighted 1/x concentration, $f(x) = a + bx + cx^2$, as indicated in Table 6.10. Accuracy and precision results for calibration standards and quality controls are summarized for 25-O-desacetyl rifapentine Validation 2, as indicated in Table 6.11 and 6.12 respectively.

Table 6. 10: Regression equation for 25-O-desacetyl rifapentine Validation 2

Validation Batch	Quadratic Calibration Curve Parameters			
	A	b	C	r
2	-0.000000144	0.00133	-0.0000547	0.9993

Table 6. 11: 25-O-desacetyl rifapentine calibration standards accuracy and precision - Validation 2

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
S11	2.00	2.23	N/A	N/A	111.3	1 of 2
S10	4.00	4.00	0.433	10.8	99.9	2 of 2
S9	8.00	7.71	0.401	5.2	96.4	2 of 2
S8	18.0	19.4	N/A	N/A	107.8	1 of 2
S7	35.0	34.4	2.03	5.9	98.3	2 of 2
S6	80.0	77.8	8.75	11.2	97.2	2 of 2
S5	180	177	6.52	3.7	98.2	2 of 2
S4	375	372	42.9	11.6	99.1	2 of 2
S3	750	758	35.6	4.7	101.1	2 of 2
S2	1500	1520	70.6	4.6	101.3	2 of 2
S1	2000	1980	29.6	1.5	99.0	2 of 2

Abbreviation: Conc. – concentration; std – standard; Dev – deviation

One of the standards 8 and 11 failed to meet the acceptance criteria, therefore were excluded.

Table 6. 12: Summary of 25-O-desacetyl rifapentine inter-validation quality controls - Validation 2

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
QC LLOQ	2.00	2.40	0.604	25.1	120.2	6 of 6
QC L	5.00	4.83	0.459	9.5	96.6	6 of 6
QC M	800	857	56.5	6.6	107.2	6 of 6
QC H	1600	1702	90.0	5.3	106.4	6 of 6

Abbreviation: Conc. – concentration; std - standard; Dev – deviation

QC LLOQ failed, it yielded a percentage accuracy above the 120.0 % acceptance criteria as one of the QC samples failed.

6.3 Validation 3

Inter-batch validation is performed to assess the accuracy and precision within a batch.

6.3.1 Validation 3 – Rifapentine

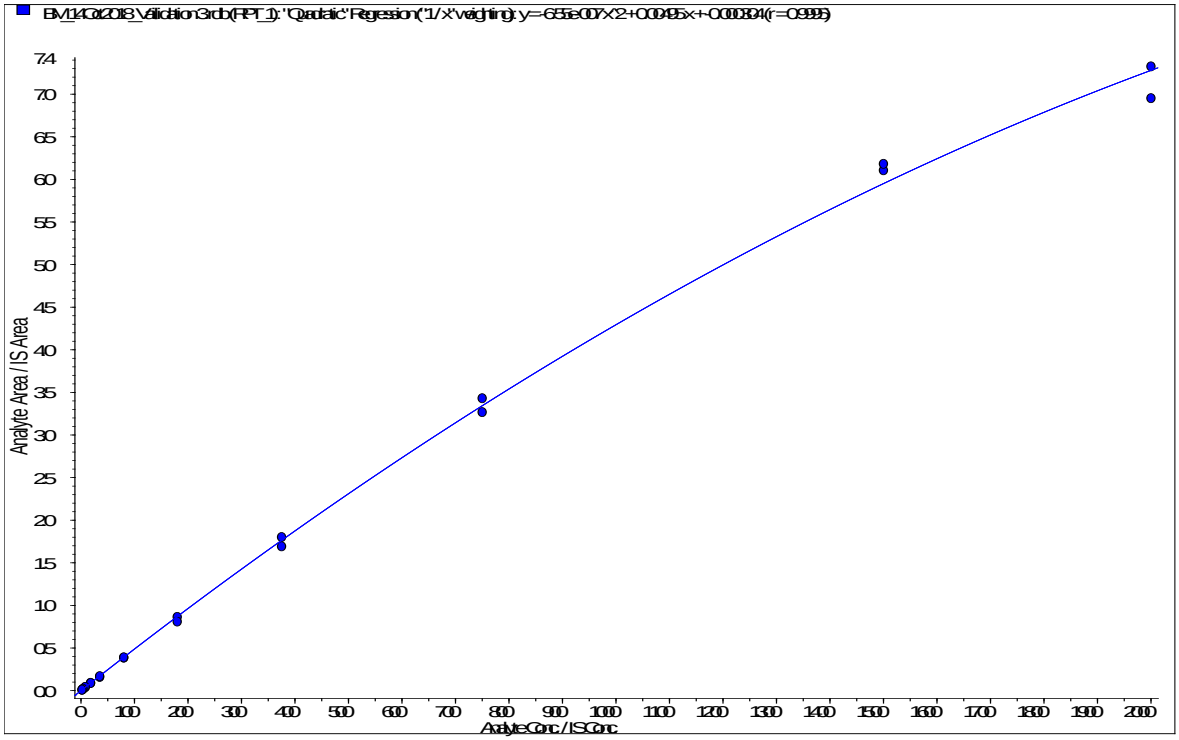


Figure 6. 5: Representative calibration curve for rifapentine - Validation 3

The regression equation used was Quadratic (weighted 1/x concentration, $f(x) = a + bx + cx^2$, as indicated in Table 6.13. Accuracy and Precision results for calibration standards and quality controls are summarized for rifapentine Validation 3, as indicated in Table 6.14 and 6.15 respectively.

Table 6. 13: Regression equation for rifapentine Validation 3

Validation Batch	Quadratic Calibration Curve Parameters			
	A	b	C	r
3	-0.000000655	0.00495	-0.000304	0.9995

Table 6. 14: rifapentine calibration standards accuracy and precision - Validation 3

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
S11	2.00	2.02	0.161	8.0	100.9	2 of 2
S10	4.00	4.11	0.227	5.5	102.8	2 of 2
S9	8.00	8.35	0.481	5.8	104.4	2 of 2
S8	18.0	1.1	0.383	2.1	100.7	2 of 2
S7	35.0	33.4	1.33	4.0	95.3	2 of 2
S6	80.0	79.2	0.593	0.7	99.0	2 of 2
S5	180	173	8.16	4.7	96.2	2 of 2
S4	375	371	17.3	4.7	99.0	2 of 2
S3	750	751	29.1	3.9	100.2	2 of 2
S2	1500	1566	18.4	1.2	104.4	2 of 2
S1	2000	1942	110	5.6	97.1	2 of 2

Abbreviation: Conc. – concentration; std - standard; Dev – deviation

Table 6. 15: Summary of rifapentine inter-validation quality controls - Validation 3

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
QC LLOQ	2.00	1.94	0.0910	4.7	96.8	6 of 6
QC L	5.00	4.87	0.0949	1.9	97.4	6 of 6
QC M	800	812	20.5	2.5	101.5	6 of 6
QC H	1600	1585	28.2	1.8	99.1	6 of 6

Abbreviation: Conc. – concentration; std - standard; Dev – deviation

6.3.2 Validation 3 – 25-O-desacetyl rifapentine

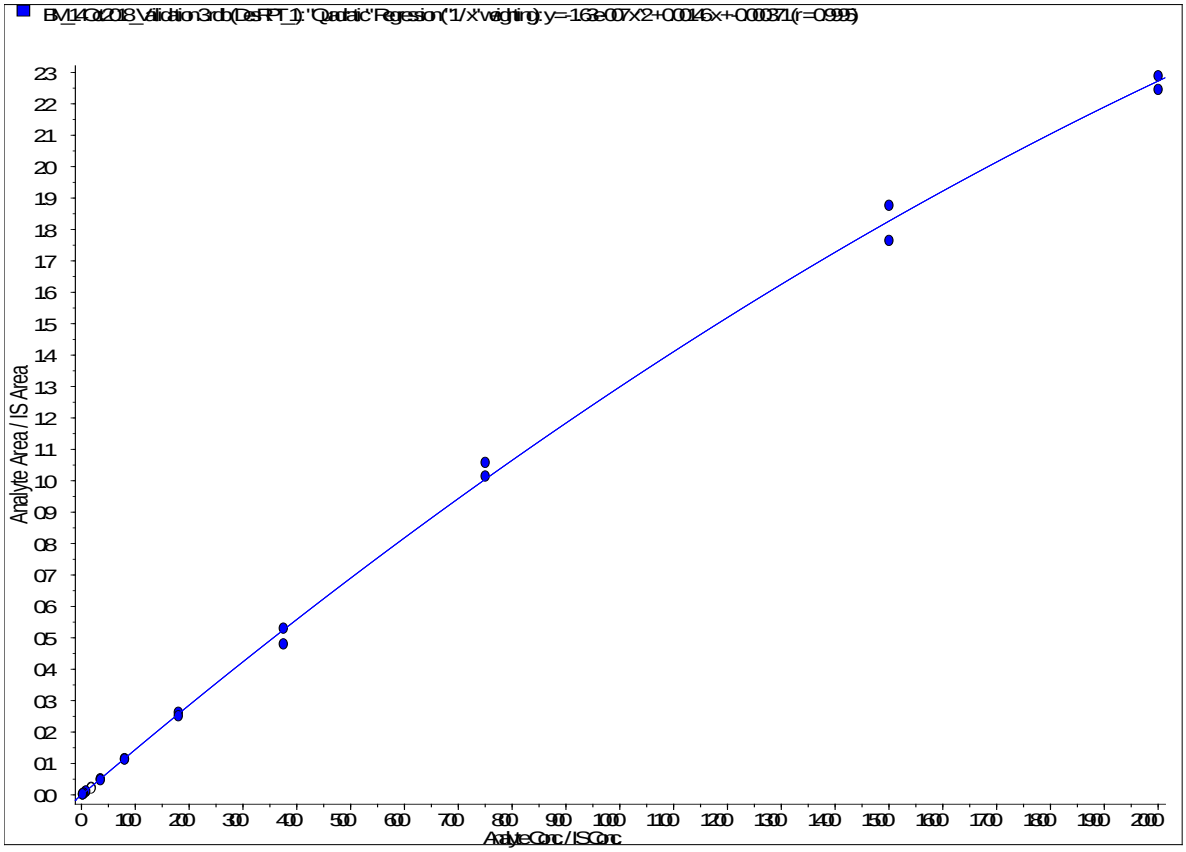


Figure 6. 6: Representative calibration curve for 25-O-desacetyl rifapentine -Validation day 3

The regression equation used was Quadratic (weighted 1/x concentration, $f(x) = a + bx + cx^2$, as indicated in Table 6.16. Accuracy and precision results for calibration standards and quality controls are summarized for 25-O-desacetyl rifapentine Validation 3, as indicated in Table 6.17 and 6.18 respectively.

Table 6. 16: Regression equation for 25-O-desacetyl rifapentine Validation 3

Validation Batch	Quadratic Calibration Curve Parameters			
	A	b	C	r
3	-0.000000163	0.00146	-0.000371	0.9995

Table 6. 17: 25-O-desacetyl rifapentine calibration standards accuracy and precision - Validation 3

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
S11	2.00	2.10	0.281	13.4	105.1	2 of 2
S10	4.00	4.06	0.208	5.1	101.5	2 of 2
S9	8.00	8.12	0.507	6.2	101.5	2 of 2
S8	18.0	16.6	N/A	N/A	92.2	1 of 2
S7	35.0	34.3	1.47	4.3	98.0	2 of 2
S6	80.0	79.2	0.853	1.1	99.0	2 of 2
S5	180	180	5.65	3.1	99.9	2 of 2
S4	375	361	26.3	7.3	96.1	2 of 2
S3	750	777	25.4	3.3	103.5	2 of 2
S2	1500	1495	81.2	5.4	99.7	2 of 2
S1	2000	1995	37.7	1.9	99.7	2 of 2

Abbreviation: Conc. – concentration; std - standard; Dev – deviation

One of standard 8 failed to meet the acceptance criteria and was excluded.

Table 6. 18: Summary of 25-O-desacetyl rifapentine inter- validation quality controls - Validation day 3

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
QC LLOQ	2.00	2.17	0.180	8.3	108.7	6 of 6
QC L	5.00	4.83	0.423	8.7	96.5	6 of 6
QC M	800	817	61.1	7.5	102.2	6 of 6
QC H	1600	1718	136	7.9	107.4	6 of 6

Abbreviation: Conc. – concentration; std - standard; Dev – deviation

6.4 Validation summary

Complete accuracy and precision of the assay is evaluated by calculating the accuracy and precision statistics over inter- and intra- validation batches (3 in total). Accuracy is expressed as the percentage of the nominal concentration of the analyte (% Accuracy) while precision is expressed as the

coefficient of variation (% CV) of the analyte of interest. Summary of the combined regression, calibration standards and quality control results (all 3 validations) of rifapentine and 25-O-desacetyl rifapentine are indicated in the tables below.

6.4.1 Validation Summary: rifapentine

Table 6. 19: Complete summary of calibration curve parameters: Validation 1-3 - rifapentine

Validation Batch	Quadratic Calibration Curve Parameters			
	A	b	C	r
1	-0.000000445	0.0034	0.00034	0.9992
2	-0.000000262	0.00256	0.00031	0.9996
3	-0.000000655	0.00495	-0.000304	0.9995

Table 6. 20: Complete summary of calibration standard accuracy and precision: Validation 1-3 - rifapentine

Day	STD	1	2	3	4	5	6	7	8	9	10	11
		2000	**1500**	**750**	**375**	**180**	**80.0**	**35.0**	**18.0**	**8.00**	**4.00**	**2.00**
		ng/ml										
	Replicate	**Observed concentration (ng/ml)**										
1	1	2190	1400	727	373	194	82.0	36.9	15.3	8.10	3.93	1.92
	2	1970	1440	773	382	184	83.8	34.8	18.2	8.31	3.88	1.95
2	1	2050	1530	729	355	185	75.0	35.7	17.0	7.93	3.86	*No peak*
	2	1950	1480	748	404	182	82.5	35.4	17.9	8.12	3.96	2.13
3	1	1860	1550	772	359	179	78.8	32.4	18.4	8.01	4.27	1.90
	2	2020	1580	731	383	167	79.6	34.3	17.9	8.69	3.95	2.13
n		**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**5**
Average		**2007**	**1497**	**747**	**376**	**182**	**80.3**	**34.9**	**17.5**	**8.19**	**3.98**	**2.01**
STDEV		**111**	**68.9**	**21.4**	**17.9**	**8.84**	**3.19**	**1.52**	**1.16**	**0.275**	**0.150**	**0.115**
% CV		**5.5**	**4.6**	**2.9**	**4.8**	**4.9**	**4.0**	**4.3**	**6.6**	**3.4**	**3.8**	**5.7**
% Accuracy		**100.3**	**99.8**	**99.6**	**100.3**	**101.0**	**100.4**	**99.8**	**96.9**	**102.4**	**99.4**	**100.3**

Statistics calculated using Analyst® 1.6.2 software. Italics: Failed acceptance criteria but not a statistical outlier. No peak: wrong concentration of the analyte added, thus did not produce a peak. STDEV: Standard deviation; STD: Standard

Table 6. 21: Complete quality control accuracy and precision estimation: rifapentine

Day	Quality controls	QC LLOQ	QC Low	QC Medium	QC High
		2.00	**5.00**	**800**	**1600**
		ng/ml			
	Replicate	**Observed concentration (ng/ml)**			
1	1	1.94	4.94	795	1660
	2	1.85	4.47	755	1600
	3	1.86	4.93	787	1560
	4	1.83	4.66	816	1580
	5	2.08	4.98	835	1530
	6	2.07	5.01	783	1650
2	1	2.18	4.98	817	1530
	2	2.47	4.86	857	1670
	3	2.06	4.70	837	1620
	4	2.14	4.89	815	1660
	5	2.07	4.98	810	1620
	6	2.17	4.99	761	1530
3	1	2.09	4.93	812	1590
	2	1.96	4.82	845	1620
	3	1.87	4.87	797	1600
	4	1.97	4.89	824	1550
	5	1.86	5.00	806	1590
	6	1.86	4.72	787	1550
n		**18**	**18**	**18**	**18**
Average		**2.02**	**4.87**	**808**	**1595**
STDEV		**0.164**	**0.145**	**27.3**	**46.8**
% CV		**8.1**	**3.0**	**3.4**	**2.9**
% Accuracy		**100.9**	**97.4**	**101.0**	**99.7**

Statistics calculated using Analyst® 1.6.2 software. Italics: Failed acceptance criteria but not a statistical outlier. Data included. STDEV: Standard deviation

6.4.2 Validation Summary: 25-O-desacetyl rifapentine

Table 6. 22: Complete summary of calibration curve parameters: Validation 1-3: 25-O-desacetyl rifapentine

Validation Batch	Quadratic Calibration Curve Parameters			
	A	b	C	r
1	-0.0000000718	0.00104	0.000313	0.9989
2	-0.000000144	0.00133	-0.0000547	0.9993
3	-0.000000163	0.00146	-0.000371	0.9995

Table 6. 23: Complete summary of calibration standard accuracy and precision: Validation 1-3 – 25-O-descetyl rifapentine

Day	STD	1	2	3	4	5	6	7	8	9	10	11
		2000	**1500**	**750**	**375**	**180**	**80.0**	**35.0**	**18.0**	**8.00**	**4.00**	**2.00**
		ng/ml										
	Replicate	**Observed concentration (ng/ml)**										
1	1	1940	1430	762	372	201	*[59.9]*	39.9	15.8	8.88	3.72	1.91
	2	2150	1450	779	346	*[225]*	85.4	34.3	18.3	8.21	3.45	1.97
2	1	2000	1470	733	341	172	71.6	33.0	*[15.1]*	7.42	3.69	*No peak*
	2	1960	1570	784	402	181	84.0	35.8	19.4	7.99	4.30	2.23
3	1	2020	1440	794	379	184	79.8	35.3	16.6	7.76	4.21	1.90
	2	1970	1550	759	342	176	78.6	33.2	*[15.1]*	8.47	3.91	2.30
n		**6**	**6**	**6**	**6**	**5**	**5**	**6**	**4**	**6**	**6**	**5**
Average		**2007**	**1485**	**769**	**364**	**183**	**79.9**	**35.3**	**17.5**	**8.12**	**3.88**	**2.06**
STDEV		**75.8**	**59.9**	**21.9**	**24.8**	**11.2**	**5.42**	**2.53**	**1.6**	**0.519**	**0.326**	**0.189**
% CV		**3.8**	**4.0**	**2.8**	**6.8**	**6.1**	**6.8**	**7.2**	**9.3**	**6.4**	**8.4**	**9.2**
% Accuracy		**100.3**	**99.0**	**102.5**	**97.0**	**101.6**	**9599.9**	**100.7**	**97.4**	**101.5**	**97.0**	**103.1**

Statistics calculated using Analyst® 1.6.2 software. Italics: Failed acceptance criteria but not a statistical outlier. Data included. No peak: wrong concentration of the analyte added, thus did not produce a peak. STDEV: Standard deviation; STD: Standard. Bracketed concentrations indicate failed standards that were excluded data from collated stats.

Table 6. 24: Complete quality control accuracy and precision estimation: 25-O-desacetyl rifapentine

Day	Quality controls	QC LLOQ	QC Low	QC Medium	QC High
		2.00	**5.00**	**800**	**1600**
		ng/ml			
	Replicate	**Observed concentration (ng/ml)**			
1	1	*1.41*	4.71	739	1790
	2	1.99	3.76	729	1730
	3	*1.48*	5.26	615	1500
	4	1.79	4.50	817	1810
	5	1.90	5.19	840	1560
	6	2.23	3.86	902	1840
2	1	1.86	4.86	825	1610
	2	*3.58*	4.07	796	1780
	3	2.07	4.68	884	1620
	4	2.22	5.48	932	1800
	5	2.37	5.00	902	1640
	6	2.33	4.90	804	1770
3	1	*2.47*	4.89	858	1700
	2	2.00	5.43	858	1580
	3	2.23	4.17	815	1740
	4	1.99	5.02	733	1560
	5	2.24	4.84	883	1930
	6	2.12	4.61	756	1800
n		**14**	**18**	**18**	**18**
Average		**2.10**	**4.74**	**816**	**1709**
STDEV		**0.180**	**0.503**	**79.1**	**118**
% CV		**8.6**	**10.6**	**9.7**	**6.9**
% Accuracy		**104.8**	**94.7**	**102.0**	**106.8**

Statistics calculated using Analyst® 1.6.2 software. Italics: Failed acceptance criteria but not a statistical outlier. Data included. STDEV: Standard deviation

Results from validation 1-3 above indicate that rifapentine and 25-O-desacetyl rifapentine over a calibration range of 2 - 2000 ng/ml were successfully validated. The LLOQ for rifapentine and 25-O-desacetyl rifapentine was set at the concentration of the lowest validated standard, namely 2 ng/ml. The results indicate the method is accurate and precise over a calibration range of 2 - 2000 ng/ml based on analyte/internal standard peak area ratios with a quadratic calibration curve (weighting 1/x) for both rifapentine and 25-O-desacetyl rifapentine. Parsons et al. had a validated calibration curve range of 50 – 80 000 ng/ml for both rifapentine and its metabolite from dried blood spots, while the Dooley et al. calibration curve range was 10 – 50 000 ng/ml for rifapentine and 70 – 35 000 ng/ml for the metabolite from plasma (Dooley, et al., 2012) (Parsons, et al., 2014). Rezk et al. had a calibration curve ranging from 10 – 20 000 ng/ml for the quantitation of ARV drugs (Rezk, et al., 2007) (Rezk, et al., 2008). The validated calibration curve for this project covers a broad range and has a very low LLOQ which enables the analysis of patient samples with low rifapentine concentrations.

6.5 Stability assessment

Stability assessments were performed to ensure that rifapentine and 25-O-desacetyl rifapentine are not affected by the assay procedure or related conditions. The assay of study samples can be performed within the indicated criteria as established in the Tables below for both rifapentine and 25-O-desacetyl rifapentine.

6.5.1 Stock solution accuracy and stability

Stock solutions SS1 for rifapentine and SS1M for 25-O-desacetyl rifapentine were prepared in methanol and stored at approximately -80°C for 69 days. Stability was assessed by storing an aliquot at room temperature (approximately 4 hours), 4°C (approximately 24 hours), -20°C (approximately 24 hours) and compared to a reference stock stored at -80°C and a freshly prepared stock. The assessment was performed after 24 hours. Below are the results of rifapentine and 25-O-desacetyl rifapentine stock solution stability assessment. Stock solutions were tested on the spectrophotometer using the absorbance of 336 nm for both rifapentine and 25-O-desacetyl rifapentine.

Table 6. 25: Stock solution stability of rifapentine in methanol

	Fresh	-80°C	-20°C	4°C	Room temp
Absorbance 1	0.230	0.250	0.256	0.249	0.273
Absorbance 2	0.236	0.245	0.252	0.242	0.261
Absorbance 3	0.241	0.235	0.246	0.244	0.259
Average	0.236	0.243	0.251	0.245	0.264
STDEV	0.00551	0.00764	0.0050	0.0036	0.0076
% CV	2.3	3.1	2.0	1.5	2.9
% Difference	**Ref**	**3.3**	**6.6**	**4.0**	**12.2**
% Difference	**-3.2**	**Ref**	**3.3**	**0.7**	**8.6**

Abbreviation: STDEV: standard deviation; Ref: reference

Table 6. 26: Stock solution stability of 25-O-desacetyl rifapentine in methanol

	Fresh	-80°C	-20°C	4°C	Room temp
Absorbance 1	0.257	0.277	0.302	0.270	0.306
Absorbance 2	0.264	0.283	0.306	0.307	0.307
Absorbance 3	0.268	0.279	0.299	0.303	0.307
Average	0.266	0.280	0.302	0.293	0.307
STDEV	0.00283	0.00306	0.0035	0.0203	0.0006
% CV	1.1	1.1	1.2	6.9	0.2
% Difference	**Ref**	**5.1**	**13.7**	**10.3**	**15.3**
% Difference	**-4.9**	**Ref**	**8.1**	**4.9**	**9.7**

Abbreviation: STDEV: standard deviation; Ref: reference

Rifapentine stock solutions were stable when stored for 69 days at approximately -80°C, and for 24 hours at approximately -20°C and 4°C, as can be seen by comparing the absorbances at these conditions to the freshly prepared stock. However, rifapentine did not appear to be stable when stored for 4 hours on ice at room temperature when compared to the freshly prepared stock. However, when compared to the aliquot frozen at approximately -80°C, the sample stored at room temperature on ice met the criteria for stability at less than 10% difference to the reference stock. 25-O-desacetyl rifapentine stocks were stable at approximately -80°C for 69 days. However, a relatively large percentage difference between the freshly prepared stock and the -80°C stock was evident and the remaining stability conditions do not meet the acceptance criteria when using the freshly prepared stock as the reference. When using the -80°C stock solution as the reference, however, the analyte appears to meet the criteria for stability for 4 hours at room temperature on ice and for 24 hours at

4°C and -20°C. To confirm these results, stability should be evaluated at room temperature for 2 to 3 hours. Parsons and Dooley et al. prepared stock solutions in dimethyl sulfoxide (DMSO) and stored the stocks in the dark at -80°C until use whereas for this project stock solutions were prepared in methanol (Parsons, et al., 2014) (Dooley, et al., 2012). Several authors have also mentioned potential rifamycin instability in ambient light, storing stock solutions in the dark until use and protecting dried blood spots (DBS) from light using aluminium foil (Parsons, et al., 2014).

6.5.2 Working solution stability

Working solutions (refer to table 4.2 4 for preparation details) for rifapentine and 25-O-desacetyl rifapentine were prepared in methanol and water (70:30, v/v) and stored at approximately -80°C. Working solutions were used to spike calibration standards and quality controls at each concentration level in breastmilk. Stability was assessed by preparing fresh working stocks and storing aliquots of high and low quality controls working solutions (1 at each level) at room temperature (on ice for approximately 4 hours), 4°C (approximately 24 hours), -20°C (approximately 24 hours) and compared to a reference stock stored at -80°C by means of LC-MS/MS. Below are the results of rifapentine and 25-O-desacetyl rifapentine working solution stability assessment.

Table 6. 27: Working solution stability of rifapentine at Low concentration level

	Fresh Low	-80°C Low	-20°C Low	4°C Low	RT Low
Peak Area 1	67800	61600	73600	74000	69800
Peak Area 2	71900	60700	75000	74200	71500
Peak Area 3	68700	60200	73800	71200	68900
Peak Area 4	68300	59800	72500	69000	67200
Peak Area 5	69500	61400	72000	72700	67400
Peak Area 6	66900	59800	69100	72700	70000
Average	68850	60583	72667	72300	69133
STDEV	1729	786	2039	1945	1649
% CV	**2.5**	**1.3**	**2.8**	**2.7**	**2.4**
% Difference	**Ref**	**-12.0**	**5.5**	**5.0**	**0.4**

Abbreviation: STDEV: standard deviation; Ref: reference; RT: room temperature

Table 6. 28: Working solution stability of rifapentine at High concentration level

	Fresh High	-80°C High	-20°C High	4°C High	RT High
Peak Area 1	21500000	22100000	21500000	24000000	16200000
Peak Area 2	21100000	21700000	21700000	24300000	16100000
Peak Area 3	21200000	22200000	21700000	24100000	16100000
Peak Area 4	21400000	22100000	21600000	24400000	15800000
Peak Area 5	21300000	22000000	21900000	24100000	16000000
Peak Area 6	21400000	22100000	21700000	24100000	16000000
Average	21316667	22033333	21683333	24166667	16033333
STDEV	147196	175119	132916	150555	136626
% CV	**0.7**	**0.8**	**0.6**	**0.6**	**0.9**
% Difference	**Ref**	**3.4**	**1.7**	**13.4**	**-24.8**

Abbreviation: STDEV: standard deviation; Ref: reference; RT: room temperature

Table 6. 29: Working solution stability of 25-O-desacetyl rifapentine at Low concentration level

	Fresh Low	-80°C Low	-20°C Low	4°C Low	RT Low
Peak Area 1	15700	14100	15700	15900	16500
Peak Area 2	16400	13000	16100	16300	14500
Peak Area 3	16200	12400	16000	16000	14600
Peak Area 4	16400	14100	15500	15300	14100
Peak Area 5	16200	13100	16700	15200	14400
Peak Area 6	16000	13000	15800	14400	15400
Average	16150	13283	15967	15517	14917
STDEV	266	679	418	691	889
% CV	**1.6**	**5.1**	**2.6**	**4.5**	**6.0**
% Difference	**Ref**	**-17.8**	**-1.1**	**-3.9**	**-7.6**

Abbreviation: STDEV: standard deviation; Ref: reference; RT: room temperature

Table 6. 30: Working solution stability of 25-O-desacetyl rifapentine at High concentration level

	Fresh High	-80°C High	-20°C High	4°C High	RT High
Peak Area 1	5240000	5510000	5310000	6210000	3810000
Peak Area 2	5330000	5260000	5210000	5590000	3750000
Peak Area 3	5730000	5000000	5130000	5720000	3820000
Peak Area 4	5400000	5140000	5250000	6120000	3540000
Peak Area 5	5820000	5270000	5070000	5800000	3770000
Peak Area 6	5490000	5820000	5420000	5790000	3760000
Average	5501667	5333333	5231667	5871667	3741667
STDEV	228859	291868	125605	240950	102648
% CV	**4.2**	**5.5**	**2.4**	**4.1**	**2.7**
% Difference	**Ref**	**-3.1**	**-4.9**	**6.7**	**-32.0**

Abbreviation: STDEV: standard deviation; Ref: reference

The percentage difference for stability of the working solutions should not be higher than 10%. A high % CV (>15%) may indicate the working solution is not stable at the representative temperature condition. The results of the working stock stability experiments yielded conflicting results. Rifapentine in the low and high working stock solutions was stable at -20°C when compared to a freshly prepared stock. However, at -80°C within the low WS, it resulted in a greater than 10% difference compared to the fresh stock. This does not correspond with the high concentration WS, where rifapentine was shown to be stable at -80°C. Furthermore, rifapentine in the low WS stock was stable at 4°C and on ice at room temperature, although within the high WS concentration, under those conditions it was shown to result in a greater than 10% difference compared to the fresh stock. Similarly, the low WS also failed for the metabolite at -80°C, suggesting that there may have been an on-bench error affecting the accuracy of this sample. The low WS of the metabolite was also stable at room temperature on-ice, at 4°C and at -20°C. Within the high WS stock, 25-O-desacetyl rifapentine was stable at 4°C, -20°C and -80°C, but not at room temperature on-ice. Parsons et al. and Dooley et al. found that the analytes were light sensitive hence the working solutions were stored in the dark (Dooley, et al., 2012) (Parsons, et al., 2014). The above working solution stability results indicate inconsistency and instability of the analytes. Parson et al. and Dooley et al. used DMSO in preparing the working solutions and protecting the analytes from light, however, working solution stability data was not shown (Dooley, et al., 2012) (Parsons, et al., 2014). The working solution stability test for this project failed possibly due to on-bench error rather than instability of the analytes under the tested conditions. This experiment should be repeated and would include room temperature stability for

approximately 2 hours, the maximum intended time that the working solutions would be kept at room temperature during spiking of breastmilk for calibration standards and QC preparation.

6.6 Matrix stability

This evaluation should include at least the length of time from when the first sample was drawn during the clinical phase to the date of the final sample analysis during the analytical phase of a study. Quality control samples for rifapentine and 25-O-desacetyl rifapentine were prepared in breastmilk and stored at approximately -80°C and -20°C for approximately 72 hours. These quality control samples were analysed against a freshly prepared calibration curve during validation 2 and compared to nominal concentrations to assess long term stability in matrix at approximately -80°C and -20°C.

Table 6. 31: Storage stability in matrix at approximately -80°C for rifapentine

	High Concentration		**Low Concentration**	
	Nominal concentration (ng/ml)	**Observed concentration (ng/ml)**	**Nominal concentration (ng/ml)**	**Observed concentration (ng/ml)**
Sample 1	1600	1610	5.00	4.18
Sample 2		1690		4.66
Sample 3		1440		5.01
Sample 4		1550		5.17
Sample 5		1620		5.49
Sample 6		1560		4.91
	Average	1578	**Average**	4.90
	STDEV	84.2	**STDEV**	0.449
	% CV	5.3	**% CV**	9.2
	% Difference	-1.4	**% Difference**	-1.9

Abbreviation: STDEV: standard deviation

Table 6. 32: Storage stability in matrix at approximately -80°C for 25-O-desacetyl rifapentine

	High Concentration		Low Concentration	
	Nominal concentration (ng/ml)	**Observed concentration (ng/ml)**	**Nominal concentration (ng/ml)**	**Observed concentration (ng/ml)**
Sample 1	1600	1650	5.00	4.23
Sample 2		1760		4.55
Sample 3		1610		4.67
Sample 4		1650		5.15
Sample 5		1660		5.78
Sample 6		1750		4.99
	Average	1680	**Average**	4.90
	STDEV	60.7	**STDEV**	0.542
	% CV	3.6	**% CV**	11.1
	% Difference	5.0	**% Difference**	-2.1

Abbreviation: STDEV: standard deviation

Table 6. 33: Storage stability in matrix at approximately -20°C for rifapentine

	High Concentration		Low Concentration	
	Nominal concentration (ng/ml)	**Observed concentration (ng/ml)**	**Nominal concentration (ng/ml)**	**Observed concentration (ng/ml)**
Sample 1	1600	1690	5.00	4.77
Sample 2		1480		4.89
Sample 3		1540		4.89
Sample 4		1720		4.84
Sample 5		1590		4.91
Sample 6		1730		4.70
	Average	1625	**Average**	4.83
	STDEV	103.7	**STDEV**	0.083
	% CV	6.4	**% CV**	1.7
	% Difference	1.6	**% Difference**	-3.3

Abbreviation: STDEV: standard deviation

Table 6. 34: Storage stability in matrix at approximately -20°C for 25-O-desacetyl rifapentine

	High Concentration		Low Concentration	
	Nominal concentration (ng/ml)	**Observed concentration (ng/ml)**	**Nominal concentration (ng/ml)**	**Observed concentration (ng/ml)**
Sample 1	1600	1790	5.00	5.13
Sample 2		1510		4.83
Sample 3		1580		4.75
Sample 4		1810		4.77
Sample 5		1790		5.13
Sample 6		1840		5.03
	Average	1720	**Average**	4.94
	STDEV	139	**STDEV**	0.177
	% CV	8.1	**% CV**	3.6
	% Difference	7.5	**% Difference**	-1.2

Abbreviation: STDEV: standard deviation

The precision and accuracy, as well as the percentage difference compared to the nominal concentration, for both rifapentine and 25-O-desacetyl rifapentine are within 15%, which indicates that the analytes are stable when stored at approximately -80°C and -20°C for approximately 72 hours. Parsons et al. reported that the different QC levels were stable in plasma samples for one day unprotected from light and two days when protected from light, however, samples were unstable in long term stability tests (data was not shown) (Parsons, et al., 2014). Further testing will be performed during sample analysis to establish longer term storage stability in matrix.

6.7 Fresh versus Frozen stability

To determine the "fresh" versus "frozen" effect, a fresh set of calibration standards are prepared in breastmilk and tested against stored (at approximately -80°C) QC samples. The fresh calibration standards are used to quantify the QC's.

Table 6. 35: Calibration standards accuracy and precision – "Fresh" versus "Frozen" stability of rifapentine

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
S11	2.00	2.13	N/A	N/A	106.4	1 of 2
S10	4.00	3.91	0.0699	1.8	97.7	2 of 2
S9	8.00	8.02	0.133	1.7	100.3	2 of 2
S8	18.0	17.4	0.623	3.6	96.8	2 of 2
S7	35.0	35.6	0.188	0.5	101.6	2 of 2
S6	80.0	78.8	5.26	6.7	98.4	2 of 2
S5	180	184	1.70	0.9	102.0	2 of 2
S4	375	379	34.6	9.1	101.2	2 of 2
S3	750	739	12.9	1.8	98.5	2 of 2
S2	1500	1505	30.8	2.1	100.3	2 of 2
S1	2000	2001	76.3	3.8	100.0	2 of 2

Abbreviation: Std Dev: standard deviation

Table 6. 36: Quality control accuracy and precision – "Fresh" versus "Frozen" stability of rifapentine

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
QC LLOQ	2.00	2.18	0.149	6.8	107.4	6 of 6
QC L	5.00	4.90	0.111	2.3	98.1	6 of 6
QC M	800	816	32.2	3.9	100.8	6 of 6
QC H	1600	1606	63.9	4.0	98.7	6 of 6

Abbreviation: Std Dev: standard deviation

Table 6. 37: Calibration standards accuracy and precision – "Fresh" versus "Frozen" stability of 25-O-desacetyl rifapentine

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
S11	2.00	2.23	N/A	N/A	111.3	1 of 2
S10	4.00	4.00	0.433	10.8	99.9	2 of 2
S9	8.00	7.71	0.401	5.2	96.4	2 of 2
S8	18.0	19.4	N/A	N/A	107.8	1 of 2
S7	35.0	34.4	2.03	5.9	98.3	2 of 2
S6	80.0	77.8	8.75	11.2	97.2	2 of 2
S5	180	177	6.52	3.7	98.2	2 of 2
S4	375	372	42.9	11.6	99.1	2 of 2
S3	750	758	35.6	4.7	101.1	2 of 2
S2	1500	1520	70.6	4.6	101.3	2 of 2
S1	2000	1980	29.6	1.5	99.0	2 of 2

Abbreviation: Std Dev: standard deviation

Table 6. 38: Quality control accuracy and precision – "Fresh" versus "Frozen" stability of 25-O-desacetyl rifapentine

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
QC LLOQ	2.00	2.40	0.604	25.1	107.4	6 of 6
QC L	5.00	4.83	0.459	9.5	98.1	6 of 6
QC M	800	857	56.5	6.6	100.8	6 of 6
QC H	1600	1702	90.0	5.3	98.7	6 of 6

Abbreviation: Std Dev: standard deviation

The acceptance criteria for standards and QC samples are the same as for the intra- and inter-day validation batches. The quality controls precision and accuracy for both rifapentine and 25-O-desacetyl rifapentine are within 15% except for QC LLOQ for 25-O-desacetyl Rifapentine that has a % CV of 25.1 due to two QC's that failed. This indicates that the analyte stability is not influenced by sample freezing.

6.8 Freeze and Thaw stability

The freeze and thaw stability are evaluated to mimic the intended sample handling conditions to be used during sample analysis. To determine freeze-thaw stability, low and high quality controls in breastmilk are frozen at approximately -80°C and put through 3 consecutive freeze and thaw cycles. Sample aliquots were prepared and frozen for at least 24 hours prior to starting the experiment. Each sample was thawed sufficiently (approximately 1 hour) for each cycle at room temperature (on ice) followed by approximately 23 hours freezing time. The samples were analysed against a valid calibration curve and compared to the nominal concentrations of the high and low quality controls. The measured concentrations and calculated differences after 3 cycles are presented in Table 6.39 and 6.40.

Table 6. 39: Freeze and thaw stability of rifapentine

	High Concentration		Low Concentration	
	Nominal QC High ng/ml	**Observed F/T High ng/ml**	**Nominal QC Low ng/ml**	**Observed F/T Low ng/ml**
Sample 1	1600	1560	5.00	4.58
Sample 2		1570		4.49
Sample 3		1540		4.69
Sample 4		1450		4.70
Sample 5		1540		4.83
Sample 6		1530		5.44
	Average	1532	**Average**	4.79
	STDEV	42.6	**STDEV**	0.339
	% CV	2.8	**% CV**	7.1
	% Difference	-4.3	**% Difference**	-4.2

Abbreviation: Std Dev: standard deviation; F/T: freeze and thaw

Table 6. 40: Freeze and thaw stability of 25-O-desacetyl rifapentine

	High Concentration		Low Concentration	
	Nominal QC High ng/ml	**Observed F/T High ng/ml**	**Nominal QC Low ng/ml**	**Observed F/T Low ng/ml**
Sample 1	1600	1770	5.00	4.95
Sample 2		1580		5.09
Sample 3		1720		4.44
Sample 4		1550		5.32
Sample 5		1670		5.07
Sample 6		1570		5.89
	Average	1643	**Average**	5.13
	STDEV	90.3	**STDEV**	0.475
	% CV	5.5	**% CV**	9.3
	% Difference	2.7	**% Difference**	2.5

Abbreviation: Std Dev: standard deviation; F/T: freeze and thaw

A high precision and percentage difference (> 15%) of the measured values may indicate freeze-thaw instability. The results indicated in the tables above show that rifapentine and 25-O-desacetyl rifapentine are stable in breastmilk for at least 3 freeze-thaw cycles. Rifapentine and its metabolite in human plasma were reported to be stable for approximately 29 hours at room temperature for at least 4 freeze-thaw cycles (UCT, 2015). Winchester et al. reported stability of the analytes for three freeze-thaw cycles in plasma for approximately six hours at room temperature and approximately 12 hours at -80°C (Winchester, et al., 2015). Others have also reported at least three freeze-thaw cycles of rifapentine and rifampicin (Fox, et al., 2011) (Parsons, et al., 2014).

6.9 Benchtop stability

To determine benchtop stability, low and high quality controls were prepared and placed at room temperature (on ice) for approximately 4.5 hours (longer than the maximum anticipated time that future study samples will be left thawed until extraction). The samples were analysed against a valid calibration curve. The measured concentrations were compared to the nominal concentrations and the results are presented in Table 6.41 and 6.42.

Table 6. 41: Benchtop stability of rifapentine for approximately 4.5 hours

	High Concentration		**Low Concentration**	
	Nominal QC High ng/ml	**Observed BT QC High ng/ml**	**Nominal QC Low ng/ml**	**Observed BT QC Low ng/ml**
Sample 1	1600	1500	5.00	4.15
Sample 2		1580		5.14
Sample 3		1510		5.19
Sample 4		1570		4.01
Sample 5		1520		4.83
Sample 6		1670		4.81
	Average	1558	**Average**	4.69
	STDEV	64	**STDEV**	0.498
	% CV	4.1	**% CV**	10.6
	% Difference	-2.6	**% Difference**	-6.2

Abbreviation: Std Dev: standard deviation; BT: benchtop

Table 6. 42: Benchtop stability of 25-O-desacetyl rifapentine for approximately 4.5 hours

	High Concentration		Low Concentration	
	Nominal QC High ng/ml	**Observed BT QC High ng/ml**	**Nominal QC Low ng/ml**	**Observed BT QC Low ng/ml**
Sample 1	1600	1590	5.00	4.82
Sample 2		1550		5.27
Sample 3		1640		5.22
Sample 4		1680		4.71
Sample 5		1620		5.26
Sample 6		1920		5.50
	Average	1667	**Average**	5.13
	STDEV	132	**STDEV**	0.301
	% CV	7.9	**% CV**	5.9
	% Difference	4.2	**% Difference**	2.6

Abbreviation: Std Dev: standard deviation; BT: benchtop

The precision and percentage difference of rifapentine and 25-O-desacetyl rifapentine compared to the nominal concentrations are reported to be within 15%. Therefore, stability for both the analyte and metabolite in breastmilk are indicated for at least 4.5 hours at room temperature (on ice). Winchester et al. reported that after 24 hours there were negligible changes in the rifamycin concentrations when benchtop stability was investigated in ambient light conditions, but they still met the acceptance criteria (Winchester, et al., 2015).

6.10 Reinjection reproducibility

Following the injection of a validation batch consisting of duplicate calibration standards and quality controls in six-fold at high, medium, low and LLOQ concentrations, the extracted samples (in a 96-microwell plate) remain in the autosampler at approximately 8°C for approximately 48 hours. The analytical run is reinjected in its entirety after approximately 48 hours. The reinjection reproducibility for approximately 48 hours is presented in the Tables below.

Table 6. 43: Calibration standards accuracy and precision – Reinjection reproducibility for rifapentine reinjected after ~ 48 hours

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
S11	2.00	1.90	N/A	N/A	95.1	1 of 2
S10	4.00	4.03	0.176	4.4	100.8	2 of 2
S9	8.00	8.27	0.115	1.4	103.3	2 of 2
S8	18.0	17.4	1.84	10.6	96.6	2 of 2
S7	35.0	33.9	1.12	3.3	96.9	2 of 2
S6	80.0	80.8	0.296	0.4	101.1	2 of 2
S5	180	187	5.69	3.0	104.0	2 of 2
S4	375	386	2.18	0.6	103.0	2 of 2
S3	750	721	105	14.5	96.1	2 of 2
S2	1500	1509	26.2	1.7	100.6	2 of 2
S1	2000	2009	179	8.9	100.4	2 of 2

Abbreviation: Std Dev: standard deviation; Conc.: concentration

Table 6. 44: Quality control accuracy and precision – Reinjection reproducibility for rifapentine reinjected after ~ 48 hours

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
QC LLOQ	2.00	1.66	0.136	8.2	82.9	6 of 6
QC L	5.00	4.82	0.503	10.4	96.4	6 of 6
QC M	800	814	86.2	10.6	101.8	6 of 6
QC H	1600	1768	97.4	5.5	110.5	6 of 6

Abbreviation: Std Dev: standard deviation; Conc.: concentration

Table 6. 45: Calibration standards accuracy and precision – Reinjection reproducibility for 25-O-desacetyl rifapentine reinjected after ~ 48 hours

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
S11	2.00	1.85	0.0974	5.3	92.5	2 of 2
S10	4.00	3.72	N/A	N/A	92.9	1 of 2
S9	8.00	8.40	0.478	5.7	105.0	2 of 2
S8	18.0	17.9	0.967	5.4	99.6	2 of 2
S7	35.0	34.3	1.49	4.3	97.9	2 of 2
S6	80.0	81.4	11.3	13.8	101.8	2 of 2
S5	180	196	7.89	4.0	108.8	2 of 2
S4	375	375	18.2	4.9	100.1	2 of 2
S3	750	751	0.609	0.1	100.2	2 of 2
S2	1500	1414	63.4	4.5	94.3	2 of 2
S1	2000	2073	60.4	2.9	103.7	2 of 2

Abbreviation: Std Dev: standard deviation; Conc.: concentration

Table 6. 46: Quality control accuracy and precision – Reinjection reproducibility for 25-O-desacetyl rifapentine reinjected after ~ 48 hours

Sample ID	Nominal Conc. ng/ml	Mean Observed Conc. ng/ml	Std Dev	% CV	% Accuracy	n
QC LLOQ	2.00	1.98	0.134	6.7	99.0	6 of 6
QC L	5.00	4.84	0.786	16.2	96.8	6 of 6
QC M	800	797	48.7	6.1	99.6	6 of 6
QC H	1600	1857	97.1	5.2	116.1	6 of 6

Abbreviation: Std Dev: standard deviation; Conc.: concentration

The precision and accuracy of the standards and QC's for rifapentine and 25-O-desacetyl rifapentine are reported to be within 15 % except for one QC High failure that resulted in a percentage accuracy of 116.1% and one QC Low failure with a percentage accuracy of 16.2%. The QCs still meet the criteria as 50% at each QC level passed and 67% of the total QCs in the batch passed. This indicates that an

entire batch may be injected within approximately 48 hours with the autosampler set at approximately 8°C. Reinjection stability will also be tested in future after 24 hours on-instrument.

6.11 Autosampler stability

To evaluate autosampler stability the same batch used for the reinjection evaluation after approximately 48 hours on-instrument (reinjection reproducibility), was used to analyse peak area ratios of the high and low QC samples to assess the percentage difference when compared to the first injection (Validation 1). This offers an estimation of absolute autosampler stability over approximately 48 hours, to determine whether the batch may be partially reinjected or should be restarted in its entirety. Below are Tables indicating autosampler stability for rifapentine and 25-O-desacetyl rifapentine.

Table 6. 47: Autosampler stability for extracted samples: High concentration for rifapentine

High Concentration			
Validation 1, Batch Start	Peak area	ISTD peak area	Ratio
Injection 1	34000000	7700000	4.42
Injection 2	32800000	7630000	4.30
Injection 3	33300000	7890000	4.22
Injection 4	34200000	8040000	4.25
Injection 5	33300000	8010000	4.16
Injection 6	34700000	7900000	4.39
Average	**33716667**	**7861667**	**4.29**
STDEV	**702614**	**164853**	**0.100**
% CV	**2.1**	**2.1**	**2.3**
Validation 1 Re-injection End	Peak area	ISTD peak area	Ratio
Injection 7	27200000	7420000	3.67
Injection 8	26300000	7290000	3.61
Injection 9	24200000	7330000	3.30
Injection 10	26800000	7390000	3.63
Injection 11	27200000	7460000	3.65
Injection 12	27100000	7290000	3.72
Average	**26466667**	**7363333**	**3.59**
STDEV	**1162182**	**70899**	**0.148**
% CV	**4.4**	**1.0**	**4.1**
% Difference after Validation 1 Re-injection		**-16.2**	

Abbreviation: ISTD: internal standard; STDEV: standard deviation

Table 6. 48: Autosampler stability for extracted samples: Low concentration for rifapentine

Low Concentration			
Validation 1, Batch Start	Peak area	ISTD peak area	Ratio
Injection 1	130000	7590000	0.0171
Injection 2	114000	7370000	0.0155
Injection 3	127000	7460000	0.0170
Injection 4	120000	7440000	0.0161
Injection 5	130000	7540000	0.0172
Injection 6	131000	7540000	0.0174
Average	**125333**	**7490000**	**0.0167**
STDEV	**6861**	**80994**	**0.000758**
% CV	**5.5**	**1.1**	**4.5**
Validation 1 Re-injection	Peak area	ISTD peak area	Ratio
Injection 7	102000	7430000	0.0137
Injection 8	83600	7240000	0.0115
Injection 9	94600	7280000	0.0130
Injection 10	92200	7040000	0.0131
Injection 11	106000	7120000	0.0149
Injection 12	105000	6960000	0.0151
Average	**97233**	**7178333**	**0.0136**
STDEV	**8695**	**171862**	**0.00132**
% CV	**8.9**	**2.4**	**9.7**
% Difference after Validation 1 Re-injection		**-19.0**	

Abbreviation: ISTD: internal standard; STDEV: standard deviation

Table 6. 49: Autosampler stability for extracted samples: High concentration for 25-O-desacetyl rifapentine

High Concentration			
Validation 1, Batch Start	Peak area	ISTD peak area	Ratio
Injection 1	12500000	7700000	1.62
Injection 2	12000000	7630000	1.57
Injection 3	11000000	7890000	1.39
Injection 4	13200000	8040000	1.64
Injection 5	11500000	8010000	1.44
Injection 6	13100000	7900000	1.66
Average	**12216667**	**7861667**	**1.55**
STDEV	**879583**	**164853**	**0.112**
% CV	**7.2**	**2.1**	**7.2**
Validation 1 Re-injection End	Peak area	ISTD peak area	Ratio
Injection 7	9520000	7420000	1.28
Injection 8	9280000	7290000	1.27
Injection 9	9050000	7330000	1.23
Injection 10	10200000	7390000	1.38
Injection 11	9320000	7460000	1.25
Injection 12	9650000	7290000	1.32
Average	**9503333**	**7363333**	**1.29**
STDEV	**399032**	**70899**	**0.0535**
% CV	**4.2**	**1.0**	**4.1**
% Difference after Validation 1 Re-injection		**-17.0**	

Abbreviation: ISTD: internal standard; STDEV: standard deviation

Table 6. 50: Autosampler stability for extracted samples: Low concentration for 25-O-desacetyl rifapentine

Low Concentration			
Validation 1, Batch Start	Peak area	ISTD peak area	Ratio
Injection 1	39400	7590000	0.00519
Injection 2	31000	7370000	0.00421
Injection 3	43000	7460000	0.00576
Injection 4	37000	7440000	0.00497
Injection 5	42900	7540000	0.00569
Injection 6	32500	7540000	0.00431
Average	**37633**	**7490000**	**0.00502**
STDEV	**5107**	**80994**	**0.000663**
% CV	**13.6**	**1.1**	**13.2**
Validation 1 Re-injection	Peak area	ISTD peak area	Ratio
Injection 7	38300	7430000	0.00515
Injection 8	26000	7240000	0.00359
Injection 9	29300	7280000	0.00402
Injection 10	25100	7040000	0.00357
Injection 11	34200	7120000	0.00480
Injection 12	31800	6960000	0.00457
Average	**30783**	**7178333**	**0.00428**
STDEV	**5032**	**171862**	**0.000659**
% CV	**16.3**	**2.4**	**15.4**
% Difference after Validation 1 Re-injection		**-14.7**	

Abbreviation: ISTD: internal standard; STDEV: standard deviation

The tables above indicate that for the high and low QC sample peak area ratios of rifapentine, the percentage difference between the first and second injections are greater than 15% with values of -16.2 and -19% respectively. Similarly, the ratios for the high concentration of the metabolite show a similar trend with a value of -17%. Although the low concentration of the metabolite percentage difference is less than 15%, it is very close to the cut-off criteria, with a value of -14.7%. It appears that even with the addition of ascorbic acid to the injection solvent, rifapentine and its metabolite still degrade over time on-instrument, as indicated by the decrease in peak areas of the analytes and

internal standard, leading to a decrease in the ratio. This shows that a partial batch cannot be reinjected after 48 hours on-instrument at approximately 8°C, and that the batch should alternatively be reinjected in its entirety. Dooley et al. used foil to cover the autosampler and protect samples from light, which could have helped in providing stability of the analytes (Dooley, et al., 2012).

6.12 Specificity

Very high specificity of LC-MS/MS precludes the detection of any analytes that do not possess the capability to produce the specific parent ion followed by formation of the specific product ion produced and monitored in the mass spectrometer. Representative chromatograms of rifapentine and 25-O-desacetyl rifapentine ULOQ are presented in Figures 6.7 to 6.8.

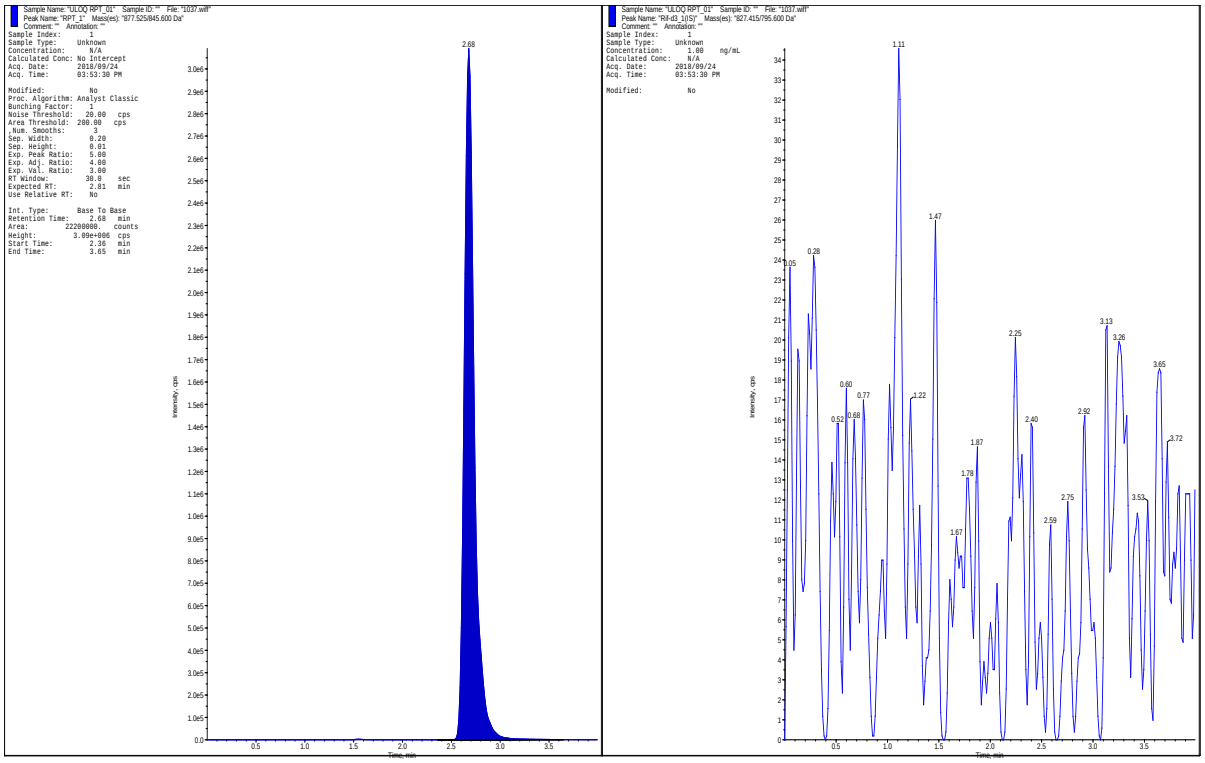


Figure 6. 7: Representative chromatogram of ULOQ at 2000 ng/ml for rifapentine

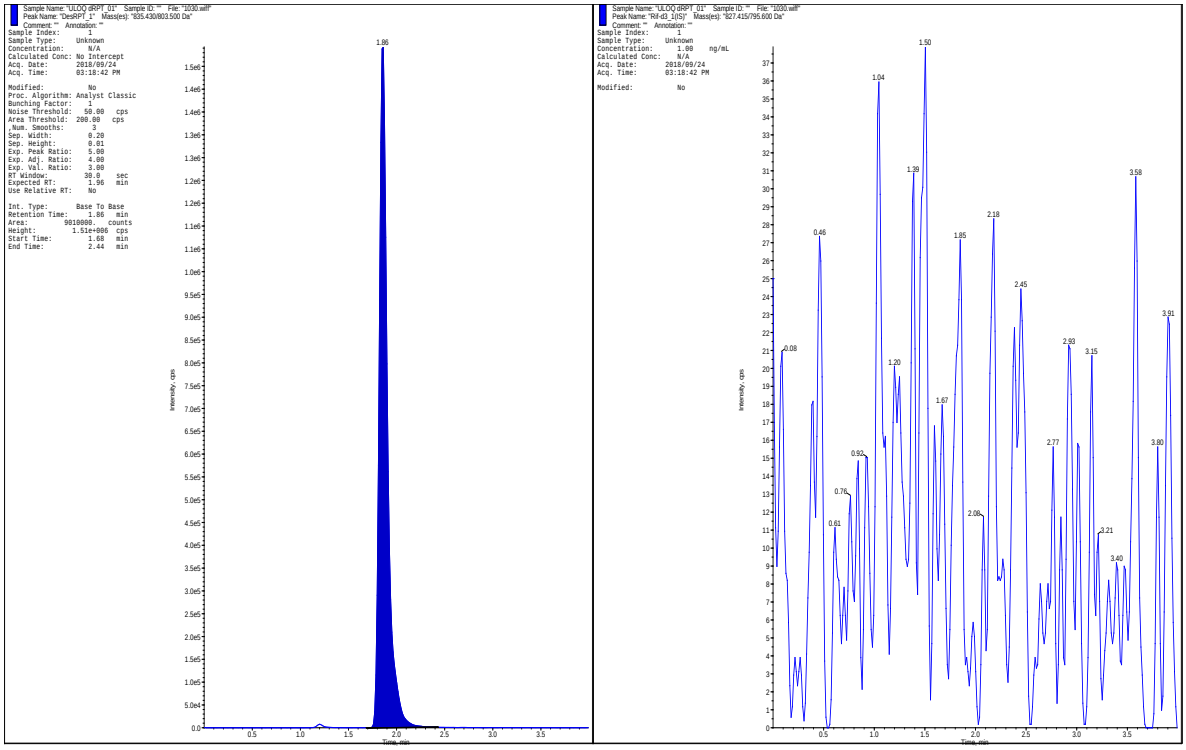


Figure 6. 8: Representative chromatogram of ULOQ at 2000 ng/ml for 25-O-desacetyl rifapentine

The chromatograms above indicate that the method is specific for rifapentine and its metabolite with no interfering peaks. This corresponded with literature reports of methods specific for the rifamycins (Fox, et al., 2011) (Winchester, et al., 2015).

6.13 Sensitivity

The LLOQ of this method is 2 ng/ml. Representative chromatograms of the LLOQ for both rifapentine and 25-O-desacetyl rifapentine including signal to noise (S/N) ratios are presented in Figure 6.9.

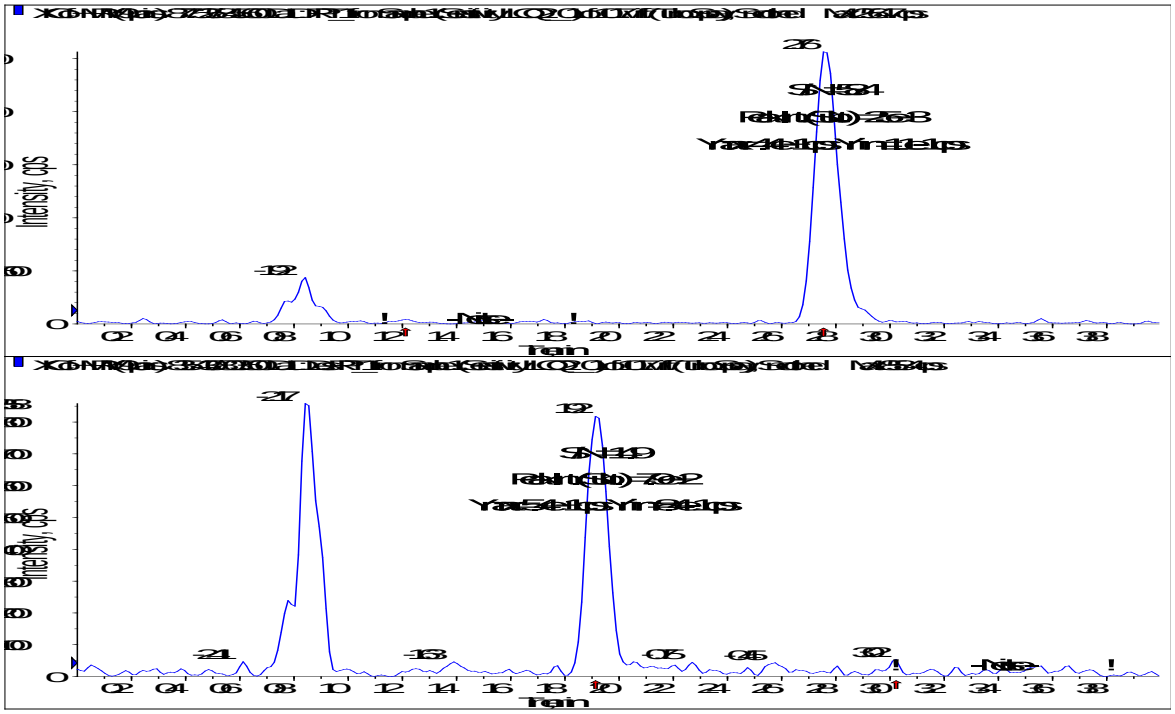


Figure 6. 9: Chromatogram of the S/N ratio for rifapentine and 25-O-desacetyl rifapentine, respectively: LLOQ at 2 ng/ml

The signal-to-noise ratio for the analyte of interest at LLOQ should be greater than 5 times the response when compared to the response of the blank at the retention time of the analyte. Interfering peaks at the retention time of interest should be < 20% of the LLOQ and should not be > 5% of the internal standard mean response. The above representative chromatograms for rifapentine and 25-O-desacetyl rifapentine at LLOQ of 2 ng/ml, show acceptable intensities with the signal to noise ratios of 58.4 and 14.9 rifapentine and 25-O-desacetyl rifapentine, respectively.

6.14 Cross-talk

Cross-talk must be assessed for the respective analyte, its metabolite and the internal standard used. ULOQ and LLOQ concentrations of rifapentine, 25-O-desacetyl rifapentine and rifampicin-d3 were assessed and injected at least 6 times (Matuszewski, et al., 1998).

Table 6.51 below presents the cross-talk peak areas for rifapentine and 25-O-desacetyl rifapentine.

Table 6. 51: Cross-talk peak areas for rifapentine and 25-O-desacetyl rifapentine

	Average analyte peak areas		
Injection	**Rifapentine**	**25-O-desacetyl rifapentine**	**Rifampicin-d3**
Rifapentine LLOQ	58567	0	0
Rifapentine ULLOQ	21733333	0	0
25-desRifapentine LLOQ	0	25650	0
25-desRifapentine ULLOQ	1001	9111667	0
Rifampicin-d3	0	0	10340000

** Cross-talk calculation: (1001/58567) *100 = 1.71%*

A peak area of less than 20% is acceptable for the analyte of interest when the ULOQ of the other analyte or internal standard is injected. In this method, no cross-talk was observed between rifapentine, 25-O-desacetyl rifapentine and rifampicin-d3 (internal standard).

6.15 Carry-over

A double blank sample (without analyte and internal standard) was positioned in the injection sequence immediately after the highest calibration standard in order to evaluate the possibility of carry-over effects. Chromatograms of double blank samples are presented in Figures 6.10 and 6.11.

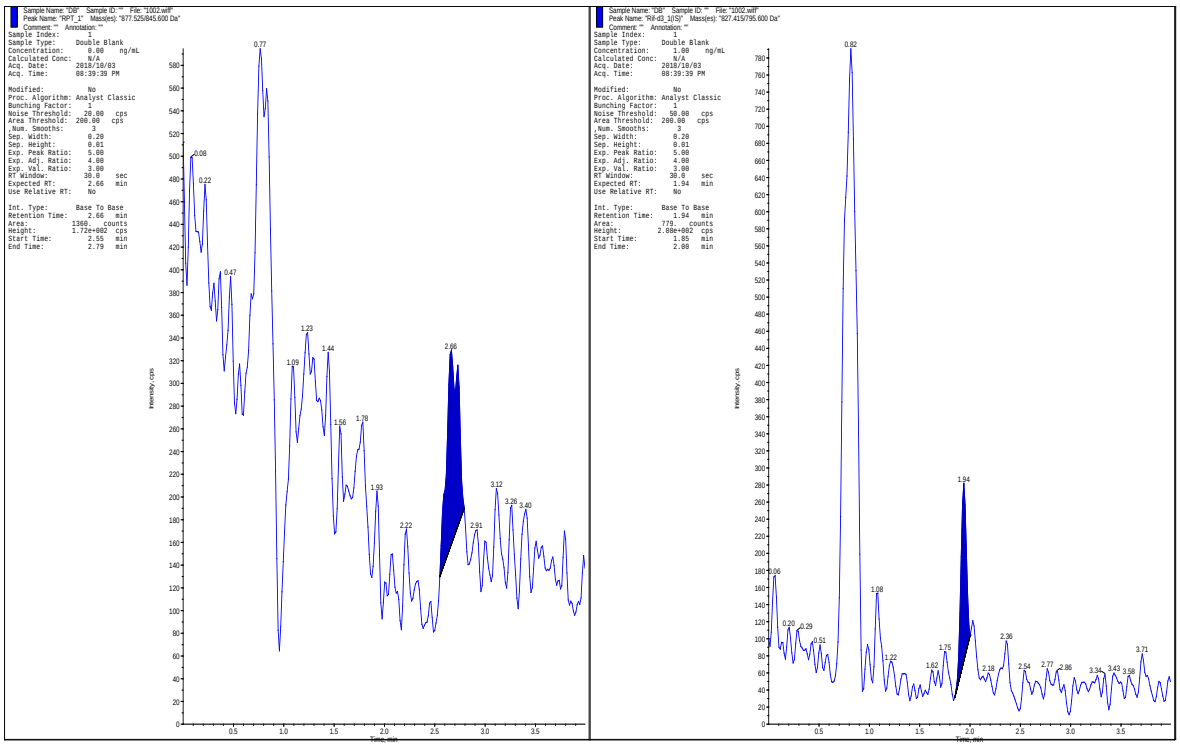


Figure 6. 10: Chromatogram of a double blank breastmilk sample – rifapentine

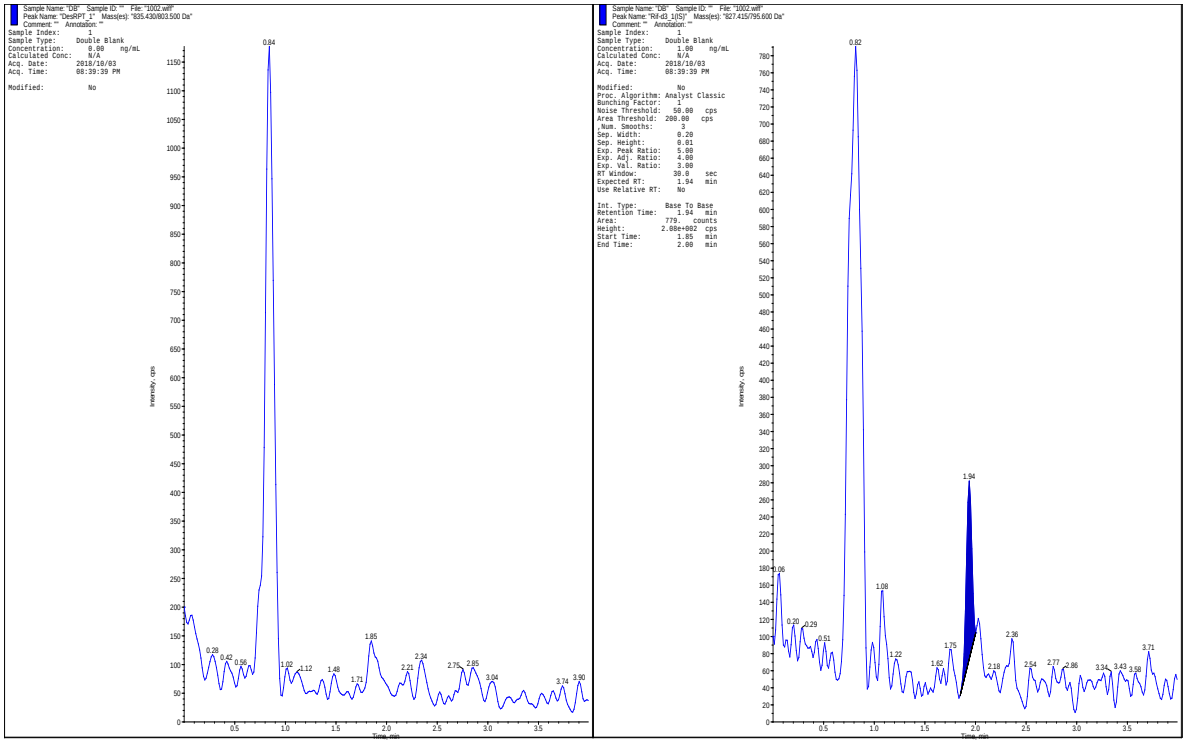


Figure 6. 11: Chromatogram of a double blank breastmilk sample - 25-O-desacetyl rifapentine

A blank sample (without analyte) was also included to determine the possibility of contamination of the analyte by the internal standard without an additional carry-over effect. Chromatograms of blank samples are presented in Figures 6.12 and 6.13.

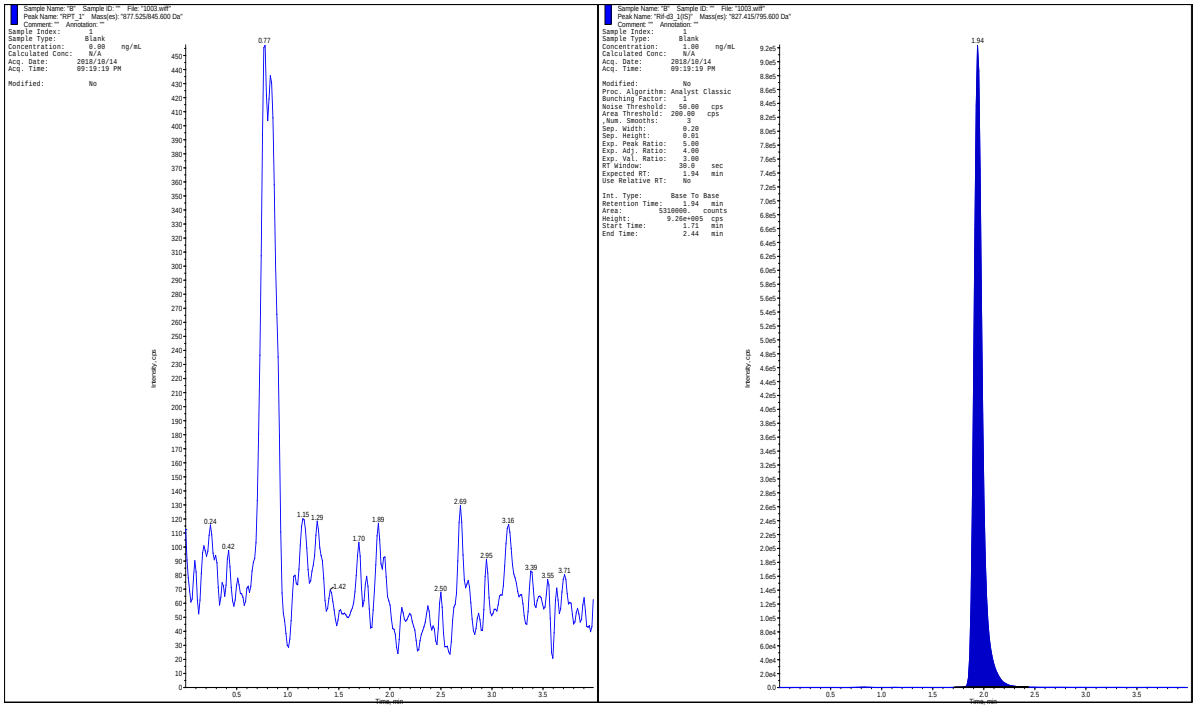


Figure 6. 12: Chromatogram of a blank breastmilk sample - rifapentine

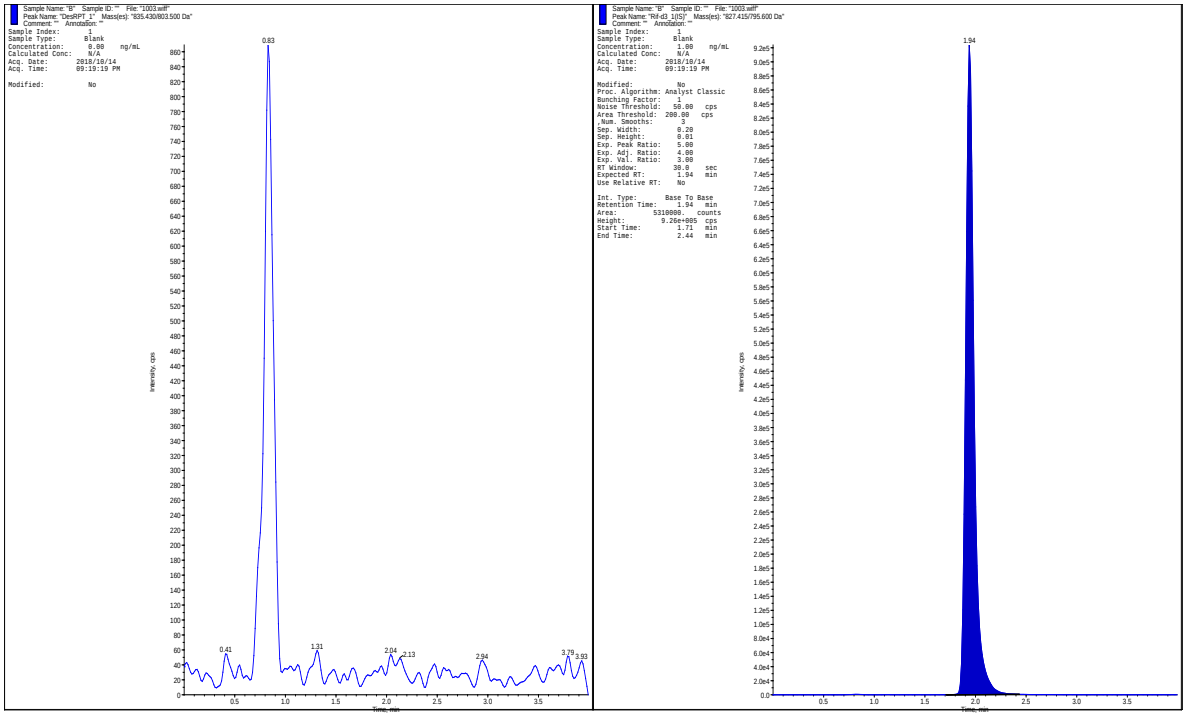


Figure 6. 13: Chromatogram of a blank breastmilk sample - 25-O-desacetyl rifapentine

An observed analyte peak in the double blank should be less than 20% of the peak area obtained at the LLOQ. An observed internal standard peak should not be above 5% of the peak observed for the internal standard at the working concentration.

An observed analyte peak when internal standard is present at the working concentration should not be greater than 20% of the peak area obtained at the LLOQ. The chromatograms shown above indicate that no significant carryover or contamination was observed in the double blank or blank samples for rifapentine and 25-O-desacetyl rifapentine.

6.16 Recovery

Extraction recovery relates to the extraction efficiency of the analytical process within limits of variability. It is determined by comparing the analytical response of blank matrix spiked with rifapentine and 25-O-desacetyl rifapentine and extracted with the response of the blank matrix first extracted and then spiked with rifapentine and 25-O-desaectyl rifapentine (theoretical, represents 100% recovery). No recovery of the internal standard is calculated. For the extracted test samples, six quality controls at each concentration level (low, medium and high) are extracted as per analytical method from six different sources of matrix. For the theoretical samples, blank matrix from six different sources are extracted and the samples are reconstituted in injection solvent spiked at theoretical high, medium and low concentrations.

Peak areas observed after extraction are compared to the theoretical peak areas and expressed as percentage recovery. Tables 6.52 and 6.53 present recovery results for rifapentine and 25-O-desacetyl rifapentine.

Table 6. 52: Recovery of rifapentine

	High Concentration (1600 ng/ml)		**Medium Concentration (800 ng/ml)**		**Low Concentration (5.00 ng/ml)**	
	Recovery Peak Area	**Theoretical Peak Area**	**Recovery Peak Area**	**Theoretical Peak Area**	**Recovery Peak Area**	**Theoretical Peak Area**
Sample 1	24100000	31600000	13400000	16400000	93500	103000
Sample 2	22800000	28700000	11700000	15300000	102000	101000
Sample 3	24900000	27800000	13800000	15500000	87000	100000
Sample 4	24500000	27200000	13700000	15300000	88100	102000
Sample 5	22600000	28000000	12900000	14400000	74300	116000
Sample 6	24800000	27500000	13900000	15700000	83800	116000
Average	**23950000**	**28466667**	**13233333**	**15433333**	**88117**	**106333**
STDEV	**1009455**	**1616993**	**833467**	**650128**	**9301**	**7554**
% CV	**4.2**	**5.7**	**6.3**	**4.2**	**10.6**	**7.1**
% Recovery		**84.1**		**85.7**		**82.9**
				Average % Recovery		**84.2**
				Average % CV		**1.7**

Table 6. 53: Recovery of 25-O-desacetyl rifapentine

	High Concentration (1600 ng/ml)		Medium Concentration (800 ng/ml)		Low Concentration (5.0 ng/ml)	
	Recovery Peak Area	**Theoretical Peak Area**	**Recovery Peak Area**	**Theoretical Peak Area**	**Recovery Peak Area**	**Theoretical Peak Area**
Sample 1	7470000	11300000	3500000	5310000	40400	**[30500]*
Sample 2	5530000	9620000	3070000	4360000	44800	39700
Sample 3	7070000	8100000	3650000	4580000	42600	43900
Sample 4	6890000	8270000	3870000	4580000	37700	41400
Sample 5	6000000	9360000	3730000	4400000	28800	45700
Sample 6	6390000	9800000	3560000	5070000	42100	58000
Average	6558333	9408333	3563333	4716667	39400	45740
STDEV	721177	1165031	274493	385158	5709	7229
% CV	11.0	12.4	7.7	8.2	14.5	15.8
% Recovery		69.7		75.5		86.1
				Average % Recovery		77.1
				Average % CV		10.8

**Statistical Outlier*

The mean recovery of a quantitative drug assay method is required to be consistent and the precision of the measured recovery expressed as percentage coefficient of variation should not be greater than 15% for any concentration of the analyte that is determined. Recovery reproducibility between concentration levels should not be greater than 15%.

The mean recovery of rifapentine and 25-O-desacetyl rifapentine from breastmilk over the calibration range is 84.2 % with a CV (%) of 1.7 and 77.1 % with a CV (%) of 10.8, respectively. The mean recovery was within acceptable limits. For the metabolite, the precision at the low concentration for the theoretical sample is 15.8%, above the acceptance criteria of 15%. Furthermore, one sample has been excluded as an outlier. This experiment should be repeated to obtain better consistency for the sample subset. Previous publications have shown recovery of both rifapentine and its metabolite to be higher in plasma compared to whole blood DBS. The recovery of both rifapentine and its metabolite ranged from 94-98% and 96-100% in plasma, respectively (Parsons, et al., 2014). Whole blood DBS recovery of rifapentine and its metabolite ranged from 42-64% and 56-70%, respectively (Parsons, et al., 2014). Recovery of rifapentine and its metabolite in human plasma was above 86% with protein precipitation

(Winchester, et al., 2015), whereas in the current project the combination of both protein precipitation and SPE enabled high recovery of analytes from breastmilk.

6.17 Matrix effects

Matrix effects relates to the presence of endogenous components in a biological sample being studied. Matrix effects are important in LC-MS/MS analysis and may become evident once unknown clinical samples are analysed. The presence of endogenous components may affect ionization of an analyte or internal standard. Having an appropriate internal standard that adequately follows the analyte path can, in some instances, minimise the effect of endogenous matrix components. Appropriate steps should be taken to minimize the influence of endogenous matrix components. The Matuszewski method attempts to quantify the effect across the calibration range of the assay using different matrix sources (Matuszewski, et al., 1998). Six different blank sources of breastmilk were extracted (without internal standard). Each individual extracted matrix sample was reconstituted in injection solvent spiked at high, medium and low concentrations (any calculations for dilutions were considered) and one at one concentration of the internal standard. It is recommended that a stable isotopically labelled internal standard is used in quantitative assays (Matuszewski, et al., 1998). Disadvantages of using a stable isotopically labelled internal standard includes inadequate isotopic purity and in some cases the lack of sufficient stability of the isotopically labelled internal standard which leads to contamination of an unlabelled material and isotopic exchange during extraction (Matuszewski, et al., 1998). Dooley et al. used rifampicin-d3 as an internal standard due to unavailability of a stable isotopically labelled analogue for rifapentine and its metabolite, for this project rifampicin-d3 was also used as an internal standard due to unavailability of a stable isotopically labelled analogue (Dooley, et al., 2012). The acceptance criteria for the regression slope determined using an isotopically labelled analogue of the analyte as internal standard should not be more than 5%, while when using a non-isotopically labelled analogue of the analyte the acceptance criteria for the regression slope should not be more than 10%.

Matrix effects results are presented in Table 6.54 and 6.55 and the overall % CV of the regression slopes calculated.

Table 6. 54: Matrix effect for rifapentine extracted from breastmilk

Table A: The peak areas for the analyte and internal standard at high, medium and low concentrations

	High Concentration		Medium Concentration		Low Concentration	
	Analyte Peak Area	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**
Matrix 1	31600000	4150000	16400000	3700000	103000	3530000
Matrix 2	28700000	3930000	15300000	3350000	105000	3770000
Matrix 3	27800000	3800000	15500000	3440000	116000	3760000
Matrix 4	27200000	3670000	15300000	3520000	112000	3750000
Matrix 5	28000000	3780000	14400000	3420000	115000	4140000
Matrix 6	27500000	3600000	15700000	3540000	122000	4250000

Table B: The peak area ratios and subsequent regression slope for each matrix

	High Conc. 1600 ng/ml Peak Area Ratio	**Medium Conc. 800 ng/ml Peak Area Ratio**	**Low Conc. 5.00 ng/ml Peak Area Ratio**	**Area Ratio v Conc. Regression Slope**
Matrix 1	7.61	4.43	0.0292	0.00475
Matrix 2	7.30	4.57	0.0279	0.00456
Matrix 3	7.32	4.51	0.0309	0.00457
Matrix 4	7.41	4.35	0.0299	0.00463
Matrix 5	7.41	4.21	0.0278	0.00463
Matrix 6	7.64	4.44	0.0287	0.00477
Average	7.45	4.42	0.0290	0.00465
STDEV	0.145	0.125	0.00119	0.0000914
% CV	2.0	2.8	4.1	2.0

Table 6. 55: Matrix effect for 25-O-desacetyl rifapentine extracted from breastmilk

Table A: The peak areas for the analyte and internal standard at high, medium and low concentrations

	High Concentration		Medium Concentration		Low Concentration	
	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area
Matrix 1	11300000	4150000	5310000	3700000	22200	3530000
Matrix 2	9620000	3930000	4360000	3350000	29500	3770000
Matrix 3	8100000	3800000	4580000	3440000	27400	3760000
Matrix 4	8270000	3670000	4580000	3520000	27500	3750000
Matrix 5	9360000	3780000	4400000	3420000	31000	4140000
Matrix 6	9800000	3600000	5070000	3540000	29800	4250000

Table B: The peak area ratios and subsequent regression slope for each matrix

	High Conc. 1600 ng/ml Peak Area Ratio	Medium Conc. 800 ng/ml Peak Area Ratio	Low Conc. 5.00 ng/ml Peak Area Ratio	Area Ratio v Conc. Regression Slope
Matrix 1	2.72	1.44	0.00629	0.00170
Matrix 2	2.45	1.30	0.00782	0.00153
Matrix 3	2.13	1.33	0.00729	0.00133
Matrix 4	2.25	1.30	0.00733	0.00141
Matrix 5	2.48	1.29	0.00749	0.00155
Matrix 6	2.72	1.43	0.00701	0.00170
Average	2.46	1.35	0.00721	0.00154
STDEV	0.240	0.0680	0.000522	0.000151
% CV	9.8	5.0	7.2	9.8

High precision of the measured values may indicate a matrix effect across the 6 different matrix sources. For methods not utilizing stable isotopically labelled analogues of the analytes as internal standards, the acceptance criteria for the regression slope is that the precision across 6 different matrices should be less than 10%. Parsons et al. reported matrix effects to be higher in plasma compared to whole blood DBS (Parsons, et al., 2014). The matrix factor for whole blood DBS of rifapentine and its metabolite ranged from 77-90% and 78-92%, respectively (Parsons, et al., 2014).

Plasma matrix factor of rifapentine and its metabolite ranged from 92-99% and 92-101%, respectively (Parsons, et al., 2014). The precision of the regression slope for rifapentine and 25-O-desacetyl rifapentine are reported to be within 10 % in this study, indicating that matrix effects do not adversely influence the precision and accuracy of the assay.

6.18 Process efficiency

Process efficiency is determined by comparing the instrument response of an extracted sample with the response of an unextracted sample. Quality controls from six different matrix sources are each spiked at high, medium and low analyte concentrations. These are compared to injection solvent spiked at the theoretical high, medium and low analyte concentrations. Process efficiency precision and the reproducibility at each concentration level should not be greater than 15%.

Table 6. 56: Process efficiency of rifapentine

	High Concentration 1600 ng/ml		**Medium Concentration 800 ng/ml**		**Low Concentration 5.00 ng/ml**	
	Recovery Peak Area	**Process efficiency Peak Area**	**Recovery Peak Area**	**Process efficiency Peak Area**	**Recovery Peak Area**	**Process efficiency Peak Area**
Sample 1	24100000	27000000	13400000	14400000	93500	115000
Sample 2	22800000	31100000	11700000	15000000	102000	113000
Sample 3	24900000	30900000	13800000	15700000	87000	117000
Sample 4	24500000	30400000	13700000	15300000	88100	111000
Sample 5	22600000	32100000	12900000	16800000	74300	113000
Sample 6	24800000	30200000	13900000	16500000	83800	113000
Average	23950000	30283333	13233333	15616667	88117	113667
STDEV	1009455	1740594	833467	910860	9301	2066
% CV	4.2	5.7	6.3	5.8	10.6	1.8
% PE		79.1		84.7		77.5
				Average % PE		80.4
				Average % CV		4.7

Table 6. 57: Process efficiency of 25-O-desacetyl rifapentine

Table A: The analyte and internal standards at the high, medium and low concentrations

	High Concentration 1600 ng/ml		**Medium Concentration 800 ng/ml**		**Low Concentration 5.00 ng/ml**	
	Analyte Peak Area	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**
Recovery	7470000	7510000	3500000	8390000	40400	7210000
	5530000	6800000	3070000	6210000	44800	7620000
	7060000	7640000	3650000	7620000	42600	7610000
	6890000	8090000	3870000	7550000	37700	6050000
	6000000	7110000	3720000	7220000	28800	5370000
	6390000	7370000	3560000	7180000	42100	7540000
	Analyte Peak Area	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**
Process Efficiency	8250000	9430000	3760000	7920000	51700	7410000
	11600000	10900000	3870000	8800000	34400	5920000
	9600000	11000000	4270000	9450000	37000	5980000
	11100000	11400000	4220000	9310000	32100	5530000
	11900000	11400000	5440000	10100000	33300	5580000
	10700000	11200000	5110000	10200000	32900	5660000

Table B: The analyte to peak area ratios

	High Concentration 1600 ng/ml		Medium Concentration 800 ng/ml		Low Concentration 5.00 ng/ml	
	Recovery Peak Area Ratio	Process Efficiency Peak Area Ratio	Recovery Peak Area Ratio	Process Efficiency Peak Area Ratio	Recovery Peak Area Ratio	Process Efficiency Peak Area Ratio
Sample 1	0.995	0.875	0.417	0.475	0.00560	0.00698
Sample 2	0.813	1.06	0.494	0.440	0.00588	0.00581
Sample 3	0.924	0.873	0.479	0.452	0.00560	0.00619
Sample 4	0.852	0.974	0.513	0.453	0.00623	0.00580
Sample 5	0.844	1.04	0.515	0.539	0.00536	0.00597
Sample 6	0.867	0.955	0.496	0.501	0.00558	0.00581
Average	0.882	0.964	0.486	0.477	0.00571	0.00609
STDEV	0.0660	0.0811	0.0361	0.0373	0.000304	0.000458
% CV	7.5	8.4	7.4	7.8	5.3	7.5
% PE		91.5		101.9		93.7
				Average % PE		95.7
				Average % CV		5.7

Process efficiency evaluates the combined contribution of matrix effects and instrument response to the robustness of the method. The mean process efficiency of rifapentine and 25-O-desacetyl rifapentine from breastmilk over the tested range is 80.4% with a CV (%) of 4.7 and 95.7% with a CV (%) of 5.7, respectively. Instrument drift was noted over the duration of analysis, therefore, for 25-O-desacetyl rifapentine the analyte to internal standard ratio was used to compensate for any variation. The mean process efficiency was within acceptable limits. Parsons et al. reported high process efficiency in plasma compared to whole blood DBS (Parsons, et al., 2014). Plasma process efficiency ranged from 91-93% and 92-97% for Rifapentine and its metabolite, respectively (Parsons, et al., 2014). Process efficiency in whole blood DBS ranged from 33-58% and 44-65% for rifapentine and its metabolite, respectively (Parsons, et al., 2014).

7 Conclusion and Future work

Several extraction methods were evaluated, but persistent residual fatty components within the sample resulted in matrix effects, as observed by Rezk et al. (Rezk, et al., 2007) (Rezk, et al., 2008). The finalized method combining protein precipitation and solid phase extraction enabled extensive sample clean-up to remove fatty components and have acceptable matrix effects. Overall matrix effects from six different sources of human breastmilk for both rifapentine and its metabolite was 2.0% and 9.8%, respectively.

Addition of ascorbic acid enabled the stability of rifapentine and its metabolite during extraction, which was similar to that observed by Parsons et al. and Winchester et al. (Parsons, et al., 2014) (Winchester, et al., 2015).

Rifapentine mean extraction yield was 84.2% (%CV = 1.7) and that of 25-O-desacetyl rifapentine was 71.1% (%CV = 10.8), however, precision for the metabolite at QC low does not meet the acceptance criteria, therefore, this experiment should be repeated in future work. The extraction method using 100 µl of breastmilk and protein precipitation followed by solid phase extraction results in good recovery of the analytes. Rifapentine had a mean process efficiency of 80.4% (%CV = 4.7) and that of 25-O-desacetyl rifapentine was 95.7% (%CV = 5.7), indicating that the method is robust.

The finalized extraction method was validated according to FDA guidelines on an API 4000 using an Agilent Poroshell 120 EC-C18 (4.6 x 50 mm, 2.7 µm) for chromatographic separation with an isocratic method of acetonitrile containing 0.1% formic acid and water containing 10% methanol and 0.1% formic acid (55:45, v/v) and a flowrate of 450 µl/ml. Rifampicin-d3 was used as an internal standard due to the unavailability of a stable isotopically labelled analogue of the analytes, as has similarly been reported by Bao et al. (Bao, et al., 2008).

Intra- and inter- day validations over 3 days were successfully performed. The calibration curves fit a quadratic regression with 1/x weighting over a concentration range of 2 - 2000 ng/ml for both rifapentine and 25-O-desacetyl rifapentine based on the analyte/internal standard peak area ratios. The broad calibration range chosen using free fraction information as a guideline for rifapentine and 25-O-desacetyl rifapentine was validated as there is no information on the transfer of rifapentine into breastmilk and expected levels are unknown at this stage (Egelund, et al., 2014). The overall accuracy ranged from 96.9% to 102.4% and 92.9% to 105.5% for rifapentine and 25-O-desacetyl rifapentine calibration standards, respectively. The Quality Controls accuracy ranged from 97.4% to 101.0% and 99.1% to 106.0% for rifapentine and 25-O-desacetyl rifapentine, respectively. Other reported

analytical method validations for rifapentine are from total plasma and whole blood/DBS, which require a higher concentration range (Winchester, et al., 2015) (Parsons, et al., 2014).

The method was shown to be specific and sensitive for rifapentine and 25-O-desacetyl rifapentine with no interfering peaks in the breastmilk samples. This was due to extensive sample clean-up that was effective for the method. There was also no significant carry-over on the Agilent autosampler observed for either rifapentine or 25-O-desacetyl rifapentine.

Rifapentine and 25-O-desacetyl rifapentine were stable in human breastmilk for up to 72 hours at approximately -80°C and -20°C, on benchtop for ~4.5 hours on ice and after three freeze-thaw cycles. Parsons et al. and Winchester et al. similarly reported that three freeze-thaw cycles did not affect the stability of the analytes in plasma and whole blood DBS (Parsons, et al., 2014) (Winchester, et al., 2015). Furthermore, it has been shown that concentrations of the analytes were not greatly affected after being at room temperature for at least 24 hours (Winchester, et al., 2015).

The tested conditions were evaluated to mimic the handling of clinical samples during collection, transport, storage and analysis. Stored quality controls were analysed against a freshly prepared calibration curve. Fresh versus frozen experiments showed that freezing had no impact on stability of the analytes. Rifapentine and 25-O-desacetyl rifapentine were shown to be stable on-instrument over period of approximately 48 hours after which the entire batch could be reinjected. Autosampler stability revealed a decrease in peak area ratios, indicating that a partial batch cannot be reinjected after 48 hours in case of instrument failure. Future work should establish the absolute autosampler stability after 24 hours

Stock solutions for Rifapentine and 25-O-desacetyl rifapentine were shown to be stable in methanol for up to 69 days at approximately -80°C, approximately 24 hours at -20°C (except for metabolite) and 4°C. However, at room temperature for approximately 4 hours rifapentine and 25-O-desacetyl rifapentine were not stable. Future work will be to repeat on-bench stock solution test for 2-3 hours.

Working solution stability yielded conflicting results and needs to be repeated in future as the results did not make scientific sense. Rifapentine at the low and high working stock solutions was stable at approximately -20°C. At approximately -80°C rifapentine was unstable at the low WS but was stable at -80°C of the high WS. Rifapentine at the low WS was stable at approximately 4°C and on ice at room temperature but was unstable under these conditions. At high WS stock, 25-O-desacetyl rifapentine was stable at 4°C, -20°C and -80°C but not at room temperature on-ice. Low WS stock of the metabolite was stable at room temperature on-ice, at 4°C and at -20°C, but failed at -80°C.

In summary the method is well suited for the analysis of rifapentine and 25-O-desaceetyl rifapentine in human breastmilk. This method will be utilized in the analysis of patient samples from a clinical study in South Africa in breastfeeding women with tuberculosis, it will assist in providing clinicians with information on the safety of putting breastfeeding women on drug regimens including rifapentine.

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