Development and validation of a liquid chromatography mass spectrometry (LC/MS/MS) assay for the determination of plasma 4β-hydroxycholesterol in HIV-infected children in Africa

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DECLARATION

I, Precious Ngwalero , hereby declare that the work, on which this thesis is based, is my own 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

4β-hydroxycholesterol (4β-OHC) is a metabolite of cholesterol formed by Cytochrome (CYP) 3A4/5/7 enzymes. It has recently been proposed as an endogenous biomarker for CYP3A4/5/7 activity. This may be useful in prediction of drug-drug interactions and other metabolic processes affected by regulators of CYP3A activity. The aim of this study was to develop and validate an LC/MS/MS assay for the determination of 4β-OHC in human plasma and use 4β-OHC as a biomarker of CYP3A4/5/7 metabolism in HIV-infected children with and without treatment in Africa.

Determination of 4β-OHC from plasma was performed by saponification and derivatisation reaction processes followed by high performance liquid chromatography with MS/MS detection on an AB Sciex Qtrap 5500 mass spectrometer. Since 4β-OHC is an endogenous metabolite in human plasma, a stable isotope labeled (SIL) analogue, 4β-OHC-D7, was used as a surrogate analyte for the preparation of calibration standards and quality controls. A second SIL analogue, 4β-OHC-D4 was used as the internal standard. The transitions of the protonated derivatised products were monitored at m/z 613, 620 and 617 to the product ions m/z 490, 497 and 494 for 4β-OHC, 4β-OHC-D7 and 4β-OHC-D4 respectively. The calibration curve fitted a quadratic (weighted by 1/concentration^2) regression over the range 2-500 ng/ml. Validation accuracy and precision statistics summary for three consecutive runs were between 98.9% and 103%, and 3.5% and 12% respectively of all quality controls. The assay's recovery, selectivity and analyte stability were established.

The validated assay was successfully applied on clinical samples, where 4β-OHC was used as a biomarker to investigate the levels of CYP3A induction in HIV-infected children with and without treatment containing non-nucleoside reverse transcriptase inhibitors (NNRI). It was found that plasma 4β-OHC concentrations at baseline were significantly lower in children belonging to the naïve group compared to nevirapine (NVP) and efavirenz (EFV) groups. When NVP and EFV groups were compared at non-baseline treatment weeks, the median 4β-OHC concentrations were significantly higher in EFV group than the NVP group. Regarding the effect of time on treatment, a significant increase in 4β-OHC concentrations was observed from baseline to each of the non-baseline weeks in naïve group. Conversely, in the NVP group, there was a significant decrease in 4β-OHC

concentrations from baseline to each of the non-baseline weeks. Time did not show any significant effect on 4β-OHC concentrations in EFV group. Furthermore, at baseline, age, sex and weight did not affect 4β-OHC concentrations in all the three groups.

This study has provided a method that would be utilised to determine plasma 4β-OHC concentrations using relatively small volumes – typical of samples taken from children. The results of this study suggest that children on antiretroviral therapy (ART) are at risk of effects of CYP3A induction, as indicated by the increase of 4β-OHC concentrations in the NVP and EFV groups. Additionally, prolonged use of the ART may activate some nuclear receptors that regulate CYP3A enzyme activity thereby negatively affecting, for example, the regulation of lipid and glucose metabolism. The developed method may therefore be useful in predicting drug-drug interactions in the context of multiple therapy and may also be used in predicting other metabolic processes affected by regulators of CYP3A activity. Further prospective studies with larger sample sizes are required to confirm and build on the evidence shown in this study.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACN	Acetonitrile
AIDS	Acquired immune deficiency syndrome
ALQ	Above limit of quantification
APCI	Atmospheric pressure chemical ionisation
API	Atmospheric pressure ionisation
APPI	Atmospheric pressure photo-ionisation
AOAC	Association Of Analytical Communities
ART	Antiretroviral therapy
BHT	Butylated hydroxytoluene
CAR	Constitutive Androstane receptor
CD4+	Cluster of differentiation 4
CAD	Collision gas
CHAPAS	Children with HIV in Africa-Pharmacokinetics and Acceptability/Adherence of simple antiretroviral regimes
Conc.	Concentration
CUR	Curtain gas
CV	Coefficient of variation
CYP3A	Cytochrome P450 3A
DMSO	Dimethylsulfoxide
DPE	Dipicolinyl ester
DMAP	4-Dimethylaminopyridine
EDTA	Ethylenediaminetetra acetic acid
EFV	Efavirenz
EI	Electron impact ionisation
EMEA	European Medicines Agency
ESI	Electrospray ionisation
Et_3N	Triethylamine
FA	Formic Acid
FDA	Food and Drug Administration
FIA	Flow injection analysis
FTS	Freeze and thaw stability
G	Relative centrifugal force
GC	Gas chromatography

HIV	Human Immunodeficiency Virus
HLB	Hydrophilic-Lipophilic-Balanced
HPLC	High-performance liquid chromatography
HREC	Human research ethics committee
H_2O	Water
ISTD	Internal standard
IQR	Interquartile range
KOH	Potassium hydroxide
LC	Liquid chromatography
LC/MS	Liquid chromatography mass spectrometry
LC/MS/MS	Liquid chromatography tandem mass spectrometry
LLE	Liquid-liquid extraction
LOD	Lower limit of detection
LLOQ	Lower limit of quantification
LTS	Long-term stability
LXRα	Liver X receptor alpha
m/z	Mass-to-charge ratio
MALDI	Matrix assisted laser desorption ionisation
MEOH	Methanol
MF	Matrix factor
MNBA	2-methyl-6-nitrobenzoic anhydride
M	Molar concentration
mg	Milligram
ml	Milliliters
MNR-ESD	Maximum normed residual/extreme studentised deviate test
MRM	Multiple reaction monitoring
MS	Mass spectrometer
MS/MS	Tandem mass spectrometer
ng	Nanogram
NC	Nebuliser current
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitors
NVP	Nevirapine
OIS	On-instrument Stability
PXR	Pregnane X receptor
PFP	Pentafluorophenyl

pH	Power of Hydrogen
PI	Protease inhibitor
QC	Calibration quality controls
Q	Quadrupole
REF	Reference
SB	Stable bond
SIL	Stable isotope labelled
S/N	Signal-to-Noise ratio
SPE	Solid phase extraction
SS	Stock solution
STD	Standard
ST DEV	Standard Deviation
SYS	System performance verification standard
UK	United Kingdom
UNAIDS	Joint United Nations programme on HIV/AIDS
ULOQ	Upper limit of quantification
USA	United States of America
V/V	Volume-to-volume ratio
W/V	Weight-to-volume ratio
WHO	World Health Organisation
˚C	Degree Celsius
µA	Microampere
%	Percentage
µg	Microgram
µl	Microlitres
C	Carbon
D	Deuterium
H	Hydrogen
K	Potassium
O	Oxygen
Sqrt	Square root
T	Thymus

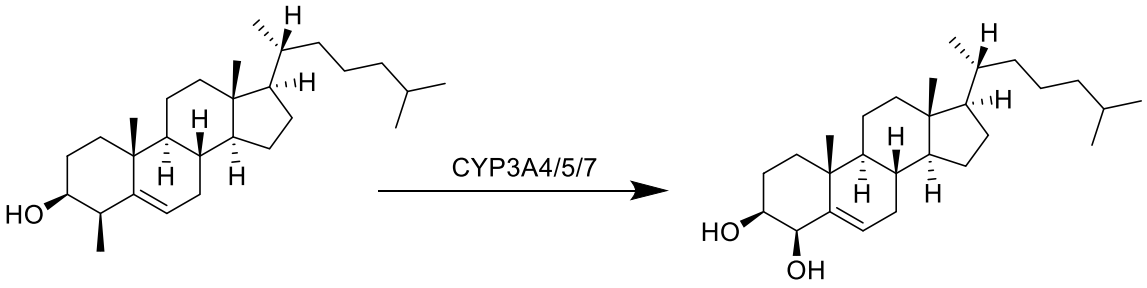
LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: LITERATURE REVIEW

1.1 4β-hydroxycholesterol

4β-hydroxycholesterol (4β-OHC) is a metabolite present in plasma (Breuer, 1995). It is produced from the enzymatic conversion of cholesterol by members of the cytochrome P450 3A (CYP3A) subfamily – such as CYP3A4/5/7 (Diczfalusy 2011; Bodin *et al.,* 2002; Bodin *et al.,* 2001) as shown in Figure 1.1, below. Recently, 4β-OHC attracted interest in biomedical research. It has been proposed that plasma levels of 4β-OHC may indicate CYP3A4/5/7 enzyme activity – the main drug-metabolising enzyme. In this way 4β-OHC could be used as an endogenous biomarker to predict CYP3A-mediated drug-drug interactions (Diczfalusy *et al.,* 2011; Diczfalusy *et al.,* 2008a; Kanebratt *et al.,* 2008). The CYP3A enzyme is regulated by nuclear receptors such as the Pregnane X receptor (PXR) and Constitutive Androstane receptor (CAR) (Moreau *et al.,* 2008). It has also been known that therapy with agents activating these nuclear receptors may lead to metabolic complications (Moreau *et al.,* 2008; Kodama *et al.,* 2004). 4β-OHC may therefore also be used as a potential marker of these metabolic complications.



Cholesterol 4β-hydroxycholesterol

Figure 1.1 Enzymatic conversion of cholesterol to 4β-hydroxycholesterol

In order to detect and measure the concentration of metabolites such as 4β-OHC and other chemical compounds in biological matrices, appropriate bioanalytical methods need to be developed and validated.

1.2 Bioanalytical methods

These are methods that have been developed and validated for the determination of chemical compounds such as drugs and metabolites in biological matrices like blood, plasma and urine (Huang *et al.,* 2014; Galteau & Shamsa, 2003; Bodin *et al.,* 2001). Such

bioanalytical methods play significant roles in evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic and toxicokinetic studies (Bressolle, Bromet-Petit & Audran, 1996; Björkman, 2006). These studies normally support most of the regulatory filings (Sonawane *et al.,* 2014; Ludwig, 2010).

Bioanalytical method strategies mainly involve method development, validation and sample analysis (method application). Method development and validation processes are often closely tied, and determine what conditions are adequate and/or ideal for the sample analysis required (Tiwari & Tiwari, 2010). However, before starting the method-development process, there is always a need for a literature review to establish what is already known about the bioanalytical methodology. Mass spectrometry coupled with chromatographic separations is the most commonly used bioanalytical instrumentation for detection and quantification of biological components like 4β-OHC in plasma (Zhao *et al.,* 2012; Goodenough *et al.,* 2011; Diczfalusy, 2004).

1.2.1 Mass spectrometry application

Mass spectrometry (MS) is an analytical tool that operates by ionising chemical compounds to generate charged molecules or molecule fragments and measures their mass-to-charge ratios (Arpino, 1992). MS has both qualitative and quantitative uses, with increasing scientific application. These uses include determining masses of unknown particles, elemental composition of a sample or molecule, and elucidating the chemical structures of unknown molecules. MS consists of three modules (Flanagan *et al.,* 2008):

a. Ionisation sources like atmospheric pressure chemical ionisation (APCI), electrospray ionisation (ESI), atmospheric pressure photo-ionisation (APPI) and matrix assisted laser desorption ionisation (MALDI).
b. Mass analysers like quadrupoles, time of flight, and ion traps.
c. Detectors such as electron multiplier and Faraday cup.

The power of MS can be increased dramatically by linking mass analysers in series. Tandem mass spectrometry (also known as MS/MS or MS^2) involves two stages of mass analysis, in order to examine selectively the fragmentation of particular ions in a mixture of different ions (McNaught, 1997; de Hoffmann, 1996) – as shown in Figure 1.2.

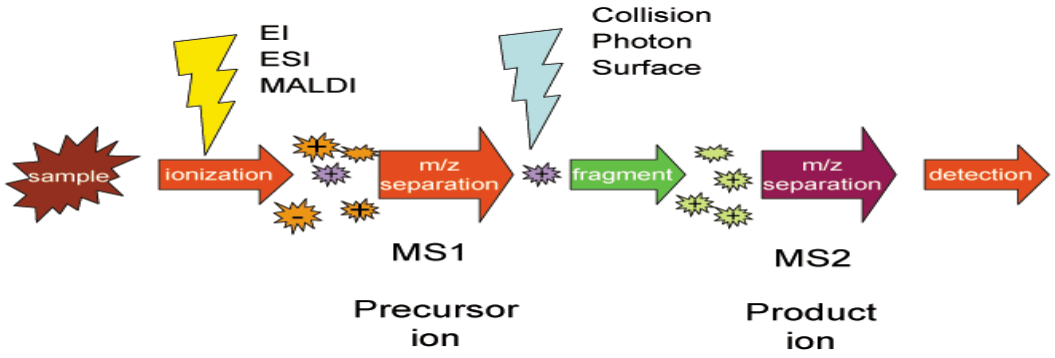


Figure 1.2. Schematic diagram of tandem mass spectrometry modules
(source: http://en.wikipedia.org/wiki/Tandem_mass_spectrometry)

In a typical MS/MS procedure, a sample is loaded onto the MS/MS instrument, and it undergoes vaporisation. The components of the sample get ionised by one of a variety of methods – for instance by impacting them with an electron beam, which results in the formation of charged particles (ions). The first mass analyser (Q1) is set to accelerate ions of a selected mass to charge ratio (m/z) into the collision cell (Q2) – where they are fragmented by high-energy collisions with gas such as helium. The selected ions which pass through from Q1 are referred to as the precursor ions. The ions formed from the fragmentation of the precursor ion are called product ions. In such cases the fragmentation produced is usually fairly reproducible. The second mass analyser (Q3) is used to scan the product ions, or selectively allow one or more such ions through to the detector, where they are detected and converted to a digital output. Results are therefore displayed as spectra of the relative abundance of detected ions, by the mass-to-charge ratio (Flanagan *et al.,* 2008; de Hoffmann, 1996).

The identification of unknown atoms or molecules in the sample can be done by correlating known masses to the identified masses, or through a characteristic fragmentation pattern (Flanagan *et al.,* 2008). An important enhancement to the mass-resolving and mass-determining capabilities of a tandem mass spectrometer is by coupling it with chromatographic separation techniques like gas and liquid chromatography (van de Merbel *et al.,* 2011; Bodin *et al.,* 2001).

1.2.2 Gas chromatography

This is a chemical-analysis instrument for separating analytes in a complex sample. It uses an inert carrier gas like helium or an unreactive gas such as nitrogen, as a mobile phase. This mobile phase carries chemical constituents of a sample and flow-through the column

(stationary phase). The gas stream passes at relatively constant rate but the analytes being carried separate based on their individual interaction with the stationary phase.

As the chemicals exit the end of the column, they are detected and identified electronically. Carrier gas-flow rate, column length and temperature are some of the factors that affect the retention time of the analyte. Gas chromatography (GC) is the most commonly used chromatography technique for separating and analysing compounds that can be vaporised without decomposition (Flanagan *et al.,* 2008; Venn, 2008).

1.2.3 Liquid chromatography

Unlike GC that uses inert gas as a mobile phase in separation, identification and quantification of a component (referred to as the analyte) in a complex sample, liquid chromatography (LC) uses liquid solvent as a mobile phase – which flows through the stationary phase. The stationary phase consists of a solid adsorbent material filled in a column. LC is described as two types: high and low pressure. High pressure is distinguished from the low pressure in that it typically relies on the force of gravity to pass the mobile phase through the column, while high pressure uses pumps to pass a pressurised liquid solvent containing the sample mixture through a column. During separation of the components in the column, each component in the sample interacts slightly differently with the adsorbent material in the column, causing different retention effects as they flow out to the detector. The detector then generates a signal for quantitative analysis of the component in the sample mixture (Flanagan *et al.,* 2008; Venn, 2008). LC has different types of separation techniques, depending on mobile and stationary phase composition. These separation techniques include the normal-phase, reversed phase, bioaffinity and size-exclusion techniques (Flanagan *et al.,* 2008). Reverse-phase LC is the dominant separation technique for most pharmaceutical applications (>95%) (Chandrul & Srivastava, 2010). Reverse-phase separation uses aqueous or moderately polar solvents like methanol and acetonitrile as a mobile phase, and a non-polar stationary phase which comprises a silica surface modified with non-polar functional groups e.g. C18, phenyl (Flanagan *et al.,* 2008; McDonald *et al.,* 2007).

LC coupled to tandem mass spectrometry (MS/MS) is the most advanced and widely used technique that combines the physical separation capabilities of LC with the mass analysis capabilities of tandem mass spectrometry (Bansal & DeStefano, 2007). LC/MS/MS has

replaced gas chromatography mass spectrometry systems in most laboratories, used for the detection and quantification of 4β-OHC, as it was shown to be more selective (can interface to MS-ESI, APCI, APPI), sensitive and reproducible (Byers *et al.,* 2014; Van de Merbel *et al.,* 2011; Honda *et al.,* 2009).

1.3 Bioanalytical method development

The bioanalytical method development, is the process of creating a procedure to enable a compound of interest to be identified and quantified in a biological matrix (Naudiyal, Kumar & Kothiyal, 2014). The method development process is specific and unique for each compound (Shah *et al.,* 2000). During this process, the analysts involved should be aware of the conditions that need to be considered, for the compound to be analysed. These conditions include: nature of the compound, biological matrix, suitable internal/external standard (STD), stock solution stability, preparation of calibration STDs and quality control (QC) samples (i.e. for endogenous compound analysis), concentrations levels, cost of the analysis, speed of the analysis, quantitative or qualitative measurement, necessary equipment and regulatory requirements to be met (i.e. clinical sample analysis) (Naudiyal, Kumar & Kothiyal, 2014). Analysts may find the information from the literature on similar compounds or from past experience with similar compounds (Chandrul & Srivastava, 2010). In this study, therefore, the LC/MS/MS method development process was mostly based on past experience with similar endogenous compounds and LC/MS/MS published methods which were developed to determine 4β-OHC and other oxysterols in plasma and tissues.

The method development process is divided into sample extraction, separation, detection and evaluation of the results (Sharma, 2011; Chandramouli, Kumar & Bibhishan, 2010). Each step in the process requires evaluation to determine the extent to which environmental, matrix, or procedural variables could affect the estimation of analyte in the matrix – from the time of collection of the samples to the time of analysis (European Medicines Agency, 2012; Sharma, 2011). Subsequently, a method that has been developed is assessed based on selectivity, accuracy, precision, recovery, sensitivity, reproducibility and stability of the analyte in spiked biological matrices (European Medicines Agency, 2012; Guidance for industry, 2001).

1.3.1 Conditions to be considered when developing a bioanalytical method

The physical and chemical properties of a compound need to be considered before developing a method, so as to be aware of the analyte's chemical structure, molecular weight, solubility and stock solution stability. On the other hand, the biological matrix from which the compound is to be assessed needs to be considered, as some of the matrices are scarce. Furthermore, when developing bioanalytical methods for endogenous compounds such as oxysterols, the choice of matrix for the preparation of calibration STDs and QC samples need to be considered; such as the use of surrogate matrix or surrogate analyte. QC samples are used to ensure that a test is valid and results are reliable.

For consideration of STDs in bioanalytical methods, there are two types of STDs that are commonly used: calibration and internal STDs. Calibration STDs are used to plot the calibration curve during quantification. They contain a known amount of the analyte in a certain range and are run with unknown samples. They contain series of an increasing amount of the analyte in a certain range and are run as a batch in addition to the unknown samples. The calibration STDs, QCs and unknown samples are always supposed to be treated the same. If this does not occur, the accuracy and precision of the procedure may be affected (Harvey, 2000).

An internal standard (ISTD) is a compound added in a fixed amount of a known concentration to every sample to be analysed (except to the double blank sample). It is used to normalise the response through determination of the ratio of the analyte and ISTD peak areas (Harvey, 2000; Choi, Gusev & Hercules, 1999). Even though the analyte and ISTD response may vary over time, their ratio practically remains the same. ISTDs are commonly used – especially when many sample preparation steps are required before the sample can be injected for analysis. They are often useful as qualitative markers, mostly in manual sample preparation to correct pipetting, extraction errors and to monitor detector stability (Lowes *et al.,* 2011). The best ISTD is an isotopically labelled version of the molecule that needs to be analysed. An isotopically labelled ISTD needs to have almost similar extraction recovery, chromatographic retention time and ionisation response to the analyte in mass spectrometry (Lowes *et al.,* 2011; Chan *et al.,* 2004). Goodenough *et al*. (2011) supported the use of stable isotope labelled ISTD for the determination of 4β-OHC in human plasma.

Choice of an appropriate detector is also essential. The selection of a detector usually depends on the physical and chemical properties of the compound of interest, assay requirements, and the availability of the instrument. Thus the most sensitive and selective detector needs to be used.

1.3.2 Biological sample collection and spiked sample extraction

Biological matrices commonly used to detect analytes of interest include blood, plasma and urine. Human blood is usually collected by vein puncture, and is withdrawn into tubes with anticoagulants such as EDTA and heparin. Plasma is obtained by blood centrifugation (Kirthi *et al.,* 2014; Venn, 2008). There are different methods to introduce the analyte into blank plasma when preparing calibration STD and QCs. Common methods include dissolving the solute directly in plasma or dissolving the analyte in a suitable solvent (water or organic solvent) and spiking the plasma with the solution.

Once the sample containing the analyte is collected, the next step is sample extraction. The sample extraction process is the exchange of the analyte from biological matrix into a suitable solvent, and it helps to clean and/or concentrate the sample before analysis. Some of the biological interferences that are removed during extraction are proteins, salts and other endogenous macro/micro components (Wells, 2003). The most commonly used procedures in biological sample preparations are protein precipitation, liquid-liquid extraction (LLE) and solid-phase extraction (SPE).

1.3.2.1 Protein precipitation

Protein precipitation is commonly used in routine analysis to remove proteins. Precipitation can be induced by the addition of an organic modifier, a salt, or by changing the pH which influences the solubility of the proteins (Venn, 2008). The samples are vortex mixed, centrifuged and the supernatant can either be injected into a chromatographic system or evaporated to dryness and thereafter reconstituted in a suitable injection solvent. Protein precipitation is simple and less time consuming compared to SPE and LLE. However, the sample clean-up is non-selective and often contains small molecules. The risk could be that endogenous compounds or other drugs interfere in the chromatographic system. The protein precipitation technique is often combined with SPE to produce a clean extract (Venn 2000; Thurman & Mills, 1998).

1.3.2.2 Liquid-liquid extraction

Liquid-liquid extraction depends on the analyte partitioning between an aqueous phase (normally the biofluid sample) and a water immiscible organic solvent. The degree of partitioning is influenced by the lipophilicity of the analyte, pH of the aqueous phase, the nature of the organic solvent, and the relative volumes of the two phases (Welankiwar *et al.,* 2014; Venn, 2008). With LLE, analytes can either be underivatised or derivatised with chemical reagents. The derivatisation process helps to enhance ionisation of some of the compounds like oxysterol so as to attain better sensitivity and selectivity. The underivatisation method has been used for determination of 4β-OHC and other oxysterols in plasma (Byers *et al.,* 2014; Helmschrodt *et al.,* 2013). The underivatisation approach has some limitations: low sensitivity (requires large plasma sample volume) or the analyses performed in non-plasma matrices which make direct sensitivity comparisons to plasma difficult. Derivative formation (derivatisation) has also been reported for 4β-OHC determination with the advantage of using relatively small sample volume to attain the required sensitivity (Huang *et al.,* 2014; Xu *et al.,* 2013; Goodenough *et al.,* 2011). The use of a small plasma volume is important – especially when blood samples are obtained from a paediatric population and measurements of two or more variables need to be obtained from the same sample.

1.3.2.3 Solid-phase extraction

As the name suggests, SPE is performed by retaining the analyte from the biological matrix onto a solid support (sorbent). It consists of four steps: conditioning, sample loading, washing, and elution. Due to different choices of sorbents, SPE is a fairly costly, but powerful technique with low solvent consumption and a higher selectivity than LLE (Venn, 2008; Wells, 2003). Similar to the underivatised LLE, it also has the limitation of a large sample volume to obtain the required sensitivity (van de Merbel *et al.,* 2011).

1.3.3 Method development on LC/MS/MS

Some important steps need to be followed when developing a method on LC/MS/MS. These include analyte tuning for the MS- by infusion of analyte/s and ISTD solutions, chromatographic and extraction development.

A. Infusion of analyte/s and internal standard

Depending on the solubility of the analyte, primary stock solutions of the analyte and ISTD are prepared in a suitable solvent such as ethanol and DMSO. A secondary stock solution containing relatively low concentration (i.e 10 μg/ml) of the analyte is prepared. During infusion, a relatively low concentration of the analyte is formed by preparing a solution using solvents – that can easily evaporate and enhance formation of ions. The selected solution is infused into the mass spectrometer separately using a syringe pump. Thereafter, a spectrum of each analyte is obtained depending on its mass to charge (m/z) ratio, from which a method for quantitative analysis is set up. Solvents commonly used for infusion are acetonitrile/methanol with low concentrations of weak and volatile acids such as formic and acetic acid. However, for analytes that cannot be injected directly, derivatisation is done prior to infusion (Naudiyal, Kumar & Kothiyal, 2014; Kang, 2012; Whitmire *et al.,* 2011).

B. Chromatography development

During chromatography development, choice of column, mobile phase and injection solution need to be considered, in order to achieve better peak resolution and shape, high sensitivity, and selectivity of the analyte:

- *Choice of column* – Choice of column determines the retention time of the analyte and peak shape (coupled with other factors like mobile-phase composition, column oven temperature and pH). Analyte molecules that interact strongly with the stationery phase will move slowly through the column and elute late, while analyte molecules that interact less strongly will move rapidly through the column and elute early (Naudiyal, Kumar & Kothiyal, 2014; Chandrul & Srivastava, 2010).
- *Mobile-phase selection* – This mainly depends on whether the sample contains ionic or ionisable compounds, in order to find a suitable composition. The composition during separation either remains constant with time (isocratic) or is changed with time (gradient). Its characteristics, such as solvents' composition, their proportion, pH value, and flow rate/s, have a profound effect on separation of sample components (Whitmire *et al.,* 2011; Chandrul & Srivastava, 2010).
- *Injection solution selection* – By the end of the extraction, the type of mixture/single solvent used to inject the extracts into the chromatographic system need to be

determined. Factors such as injection volume and the effect of change in pH of the injection solution, should also be taken into consideration.

Subsequently a thorough investigation of the peak shape, resolution and retention time of the chromatogram is done when the choice of mobile phase, injection solution, injection volume, column, column oven temperature and flow rate/s, are finalised.

C. Flow-injection analysis (FIA)

When a satisfactory chromatography method has been achieved, the method is optimised using flow-injection optimisation in tuning the ion source parameters of the mass spectrometer (e.g. the curtain gas, nebuliser gas, turbo gas, ion spray voltage, and source temperature), and from the compound parameters mainly the declustering potential is optimised (Naudiyal, Kumar & Kothiyal, 2014; Whitmire *et al.,* 2011).

Therefore, using the optimised method, instrument response repeatability, dilution sequence, and sensitivity tests using stock solutions are performed.

D. Extraction development

When the suitable extraction method has been identified after a thorough literature survey, preliminary recovery assessment, repeatability, matrix effect, sensitivity and dilution sequence are evaluated in a biological matrix in order to assess the robustness of the extraction method.

1.4 Method validation

Bioanalytical method validation is an important process needed to demonstrate that the analytical method developed is suitable for its intended purpose. This assures that the quantification of an analyte in a biological matrix is reproducible, reliable and suitable for the application (European Medicines Agency, 2012; Guidance for industry, 2001 Kollipara *et al.,* 2011). Shah *et al.* (2007) also stated that all bioanalytical methods must be validated if the results are to be used to support registration of a new drug or reformulation of an existing one. General and specific standard operating procedures and good record keeping are important to a successfully validated method. Full, partial and cross validation of bioanalytical methods define and characterise different types and levels of method validation (European Medicines Agency, 2012; Guidance for industry, 2001).

Firstly, full validation is used during development and implementation of a novel bioanalytical method, analysis of a new drug entity, and revisions to an existing method that add metabolite quantification. Secondly, partial validation evaluates modifications of already validated bioanalytical methods. Some of the typical bioanalytical methods described in this category include: biological method transfers between laboratories or analysts, changes in analytical methodology, changes in sample processing procedures, modifications to accommodate limited sample volume like in paediatric studies, and change in matrix within species. Lastly, in cross validation, parameters are compared when two or more bioanalytical methods are used to generate data within the same study or across different studies. An original validated bioanalytical method becomes a reference while a revised bioanalytical method becomes a comparator.

According to the Food and Drug administration (FDA), Guidance for industry (2001) and European Medicines Agency 2012 (EMA) guidelines on bioanalytical method validation, the fundamental parameters requiring validation consist of selectivity, accuracy and precision, recovery, sensitivity, linearity, reproducibility, matrix effect (for LC-MS/MS only), dilution integrity, carry-over, and stability. They are therefore described below:

1.4.1 Selectivity/specificity

The terms selectivity and specificity are often used interchangeably. Selectivity/Specificity generally refers to the ability of the bioanalytical method to measure and differentiate the analytes in the presence of other endogenous components present in the sample (Causon, 1997). These could include metabolites, impurities, degradants, or matrix components. (Rozet *et al.,* 2011). Selectivity and specificity ensure the reliability of the measurements in the presence of endogenous components in the matrix or sample.

Evidence needs to be provided that the component quantified is the intended analyte. Analysis of blank samples of the appropriate biological matrix, such as plasma and urine, should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should be ensured at the lower limit of quantification (LLOQ). If the method is intended to quantify more than one analyte, each analyte needs to be tested and it needs to be ensured that there is no interference. Selectivity acceptance criteria indicate that there should be no endogenous peaks within a 10% window of the retention time of the analyte and the ISTD. If any peak is present at the retention time of the analyte,

its response should be ≤ 20% of the response of an extracted sample at LLOQ, and the response of an endogenous peak at the retention time of ISTD should be ≤ 5% of the response of the ISTD at the concentration to be used in the study. Specificity can also be established by comparing retention time of the analyte in at least one reference solution, with its retention time in extracted matrix samples.

1.4.2 Accuracy, precision and recovery

The accuracy of an analytical method is described as the closeness of mean test results obtained by the method, to the true value (concentration) of the analyte (Murugan *et al.,* 2013; Singh *et al.,* 2008). It is expressed, in percentage, as depicted in the formula below:

$$\% \, Nominal = \frac{Mean \, concentration}{Nominal \, concentration} \, X \, 100$$

Accuracy is determined by replicate analysis of samples spiked with known amounts of analyte (i.e. QCs). It should be measured using a minimum of five determinations per concentration level, at a minimum of three concentrations in the range of the expected study sample concentrations. The low QC (within three times the LLOQ), Medium QC (around 50% of the calibration curve range) and high QC (at least 75% of the upper calibration curve range) are recommended. During accuracy measurement, the mean value should be within 15% of the nominal value, except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the nominal value serves as the measure of accuracy.

The precision of an analytical method is a measure of random error and is described as the closeness of repeated individual measures of an analyte, when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix (Tiwari & Tiwari, 2010; Hartmann, Massart & McDowall, 1994). Precision measurement should also be done at low, medium and high QC concentration levels, with the coefficient of variation (CV) not exceeding 15% – except for the LLOQ, where it should not exceed 20%. Precision is calculated as:

$$\% \, CV = \frac{Standard \, deviation}{Mean} \, X \, 100$$

Accuracy and precision are further subdivided into within-run and between-run. Within-run describes measurement of accuracy and precision by analysing in a single run, while between-run describes the measurement of accuracy and precision from at least three runs analysed, on at least two different days.

The recovery of the analyte is determined by the comparison of response of blank matrix spiked with the analyte before extraction with the response of blank matrix spiked with the analyte after extraction (representing 100%). Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. However, absolute recoveries cannot be determined if the sample preparation involves derivatisation, because derivatives are not usually available as reference substances (Singh *et al.,* 2008). Recovery does not need to be 100%, but the extent of recovery of an analyte and of the ISTD should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three different concentration levels (low, medium, and high), with theoretical samples that represent 100% recovery. Recovery is calculated as:

$$\% \text{ Recovery} = \frac{\text{Mean response of the test samples}}{\text{Mean response of the theoretical samples}} X\ 100$$

1.4.3 Sensitivity

Sensitivity is measured using the LLOQ of the calibration STD curve which can be quantitatively determined with acceptable accuracy and precision (Murugan *et al.,* 2013; Causon, 1997). The analyte's response at the LLOQ should be at least five times the signal of the blank extract at the analytes retention time – signal to noise ratio (S/N). The limit of detection (LOD) is defined as the lowest concentration that can be distinguished from the background noise – with no guarantee about the bias or imprecision of the result by an assay (Pandey *et al.,* 2010; Singh *et al.,* 2008). This is usually set as the concentration that produces a signal-to-noise ratio >3:1 (Shrivastava & Gupta, 2011).

1.4.4 Linearity and range/calibration curve

A calibration STD curve is the relationship between known concentrations of the analyte and instrument response. This describes a method's ability (within a given range) to obtain results which are either directly or mathematically proportional to the concentration of the

analyte, within a given range. The relationship between responses should be continuous, reproducible, and evaluated over a specified period of time.

It is recommended that a calibration curve be prepared in the same biological matrix as the samples in the intended study, by spiking the matrix with known concentrations of the analyte. In rare cases where matrices could be difficult to obtain – for instance with cerebrospinal fluid, the use of surrogate matrices could be justified. Selection of calibration STDs should be based on the concentration range expected in a particular study of interest. A calibration curve should consist of: a blank sample (matrix sample processed without analyte or ISTD), a zero sample (matrix sample processed without analyte, but with an ISTD), and at least six non-zero samples (matrix samples processed with analyte and ISTD) covering the expected range – including LLOQ.

1.4.5 Reproducibility

Reproducibility of the method is assessed by replicate measurements using the assay, including QCs, and possibly incurred samples. Reinjection reproducibility should be evaluated to assess if an analytical run could be reanalysed in the case of instrument interruptions.

1.4.6 Dilution integrity

Dilution of samples should not affect the accuracy and precision. If possible, dilution integrity should be illustrated by spiking the matrix with an analyte concentration above the upper limit of quantification (ULOQ), and diluting this sample with blank matrix (at least five determinations per dilution factor). Accuracy and precision should be within the set criteria. Dilution integrity should cover the dilution applied to the study samples.

1.4.7 Matrix effects

In biological chemical analysis, matrix refers to the components of a sample, other than the analyte. Matrix effects are of particular importance when dealing with LC/MS/MS analyses, and may only become visible once unknown clinical samples are analysed. The presence of co-extracted matrix background components may have an effect on the analyte and ISTD ionisation. Hence, having a suitable ISTD can, in some cases, minimise the effect of matrix components. Matrix effects should be investigated using at least six lots of blank matrix from individual donors. The matrix effect can be assessed either by

calculating the ISTD normalised matrix factor or using the Matuszewski methods (Matuszewski, 2006; Matuszewski, Constanzer & Chavez-Eng, 2003). When using the Matuszewski methods, a minimum of six different blank sources of the appropriate biological matrix should be extracted. Each individual matrix needs to be spiked at low, medium and high concentration levels, and at one concentration of the ISTD. The slope variability (% CV) for the six different matrix sources should not be >5%.

1.4.8 Carry-over

Carry-over is the effect that a previous sample exerts on a subsequent sample/s – and is usually due to the retention of extraneous compounds on the analytical column. Carry-over should be addressed and minimised during method development, but it should be assessed during validation by including a blank sample after a high concentration sample or calibration STD at the ULOQ. Carry-over in the blank sample following the high concentration STD should not be greater than 20% of the LLOQ, and 5% for the ISTD. Carry-over is calculated as;

$$\%\ Carry\text{-}over = \frac{Peak\ area\ of\ analyte\ in\ blank\ sample\ after\ ULOQ}{Peak\ area\ of\ analyte\ in\ LLOQ} \ X\ 100$$

1.4.9 Stability

Chemical stability of an analyte in a given matrix is assessed in various ways under specific conditions – for given time intervals. The conditions used in stability experiments should reflect situations likely to be experienced during actual sample handling and analysis. “The condition under which the stability is determined is largely dependent on nature of the analyte, the biological matrix, and the anticipated time period of storage (before analysis)” (Sonawane *et al.,* 2014). Stability samples should be compared to freshly made calibrators and/or freshly made QCs. At least three replicates – at each of the low and high concentrations – should be assessed. Stability sample results should be within 15% of nominal concentrations and with precision <15%. Stability is calculated thus:

$$Stability = \frac{Mean\ response\ of\ stability\ samples}{Mean\ response\ of\ reference\ samples} \ X\ 100$$

Several stability conditions are recommended to be evaluated. These are:

- *Freeze and thaw stability* – During freeze-thaw stability evaluations, the freezing and thawing of stability samples should be like the intended sample handling conditions to be used during sample analysis, and should be assessed for a minimum of three freeze-thaw cycles.
- *Bench-top stability* – Bench-top stability experiments should be designed and conducted to cover the laboratory handling conditions expected for study samples.
- *Long-term stability* – The storage duration in a long-term stability assessment should be equal to or more than the time between the date of first sample collection and the last date of sample analysis.
- *Stock solution stability* – The stability of stock solution of the analyte and ISTD should be evaluated, and stability data on the stock solution should be generated to justify the duration of stock solution storage stability.
- *Processed sample stability* – The stability of processed samples, including the resident time in the auto sampler, should be determined.

1.5 Acceptance criteria of an analytical run

An analytical run consist of study samples, a minimum of six calibration STDs analysed in duplicate, at least three levels of QCs analysed in duplicate, zero sample and blank sample. QC samples are spread over the run to control the analysis appropriately.

According to FDA Guidance for industry (2001) and EMA (2012), guidelines on bioanalytical method validation criteria for acceptance or rejection of an analytical run should be well defined. The run can be acceptable, although a batch might have to be rejected, as criteria were not met. The following acceptance criteria need to be applied:

- At least 75% of the calibration STDs – with a minimum of six – must be within the acceptable accuracy and precision criteria. The calibration STD should be rejected if one of the calibration STDs does not meet the criteria, and the calibration curve without this calibration STD should be re-evaluated and regression analysis performed.
- If LLOQ is the rejected calibration STD, the LLOQ for this analytical run is the next lowest acceptable calibration STD of the calibration curve. If the highest calibration

STD is rejected, the next acceptable lower calibration STD of the calibration curve should be ULOQ for this analytical run. The revised calibration range must cover all QC samples (low, medium and high).

- With the QC samples at least 67% of the QC samples and at least 50% at each concentration level should comply with the acceptable accuracy and precision criteria. In situations where these acceptance criteria are not met, the analytical run should be rejected and the study samples re-extracted and analysed.

1.6 Method application

1.6.1 Effect of HIV drug treatment

In 2013, approximately 35 million people worldwide were living with HIV/AIDS (UNAIDS, 2014a). Of these, 3.2 million were children under the age of 15 and 91% of these children were in Sub-Saharan Africa (UNAIDS, 2014b). HIV initiates progressive destruction of CD4+ T lymphocytes. The rate of CD4+ T lymphocyte decline determines the rate of immunodeficiency, and the subsequent development of HIV-related opportunistic infections (Feinberg, 1996). Early in the epidemic, survival rates of less than 1 year were observed (Barry *et al.,* 1999). However, following a number of advances – including the availability of antiretroviral therapy (ART) and the demonstration that combination therapy is more effective than monotherapy, the situation has changed significantly over the past years (Hammer *et al.,* 1997). ART is a lifelong strategy that offers patients an almost normal longevity. Nevertheless, this lifelong ART treatment may predispose the patients concerned to metabolic side effects such as impaired glucose tolerance and dyslipidaemia (Reust, 2011). These metabolic complications increase the risk of non-communicable diseases such as Type II diabetes and cardiovascular diseases (Reust, 2011; Jain *et al.,* 2001).

The antiretroviral agents are categorised into protease inhibitors (PIs) such as ritonavir boosted lopinavir (the most widely used PI in children and adults in African countries like South Africa); nucleoside reverse transcriptase inhibitors (NRTI) such as stavudine and lamivudine, and non-nucleoside reverse transcriptase inhibitors (NNRTI) such as nevirapine (NVP) and efavirenz (EFV). PIs and NNRTIs, unlike NRTIs, are extensively metabolised by cytochrome P450 (CYP) isozymes present in the liver and intestines (Fellay *et al.,* 2005; Barry *et al.,* 1999). NVP and EFV are reported to activate nuclear

receptors (PXR and CAR) that regulate the expression and activity of drug-metabolising enzymes such as CYP3A. Conversely, ritonavir is known to be the most potent inhibitor of the CYP3A enzyme (Dooley, Flexner & Andrade, 2008; Faucette *et al.,* 2007; Eagling, Back & Barry, 1997). The effects of HIV drugs on the CYP3A enzyme represents a potential clinical problem, considering the polypharmacotherapy often received by HIV-positive patients (both adults and children) and hence the potential for drug-drug interactions.

Apart from regulating the CYP3A enzyme, these nuclear receptors also mediate the expression of genes that regulate some of the enzymes and transporters – such as glucose-6 phosphatase and fatty acid translocase CD36 involved in metabolic processes (Moreau *et al.,* 2008). Activation of these nuclear receptors by drugs such as ART may impair the metabolic processes regulated by these affected genes (Chang, 2009; Moreau *et al.,* 2008; Kodama *et al.,* 2004). In this way, long-term ART poses a risk for metabolic complications and hence the non-communicable diseases associated with it. To our knowledge, no study has been done in Africa to assess the metabolic activation of CYP3A4/5/7 activity amongst children with HIV in different treatment regimens – by using 4β-OHC as a biomarker.

1.6.2 Cytochrome P450 enzyme activity

The Cytochrome P450 (CYP 450) are a group of enzymes responsible for the metabolism of most therapeutic drugs and play important roles in bioavailability, elimination, and drug-drug interactions (Kanebratt *et al.,* 2008; Hasler *et al.,* 1999). Humans have several different types of CYP 450 enzymes, with a wide array of substrates and functions (Hasler *et al.,* 1999; Li, Kaminski & Rasmussen, 1995). These CYP450 enzymes are classified into different families and subfamilies based on the amino acid sequence similarity e.g. CYP1A, CYP2B and CYP3A (Kanebratt *et al.,* 2008). Members of the cytochrome CYP3A (CYP3A4/5/7) enzymes specifically CYP3A4 and to a lesser extent CYP3A5 and CYP3A7 account for half of the total P450 enzymes in an average human liver and intestine. CYP3A7 is the major foetal form and is rarely expressed in adults (Williams *et al.,* 2002; Li, Kaminski & Rasmussen, 1995). These CYP3A enzymes are involved in the metabolism of up to 50% of the drugs currently in use – such as in antibacterial, antiviral and anticonvulsant therapy (Gorski *et al.,* 2003; Guengerich, 1999; Guengerich 1995). Therefore, such treatment therapies may cause induction or inhibition of CYP3A enzyme activity (Josephson *et al.,* 2008; Bodin *et al.,* 2001). The induction of CYP3A enzyme

activity can, in clinical practice, lead to enhanced clearance of the drug itself or of co-administered CYP3A drug substrates, thereby resulting in loss of therapeutic effect. Conversely the inhibition of CYP3A enzyme activity can lead to side effects due to decreased clearance of CYP3A drug substrates (Moltke *et al.,* 2001; Evans & Relling, 1999; Li, Kaminski & Rasmussen, 1995). Among patients with HIV infection, the activity of the hepatic CYP3A enzyme is reported to vary 6 to 30-fold, due to factors like genetic variability, concomitant drug therapy, gender, and age (Wolbold *et al.,* 2003; Watkins, 1996; Hunt, Westerkam & Stave, 1992; Watkins, 1992; Watkins *et al.,* 1989).

The magnitude of human CYP3A enzyme expression has been shown to vary between birth and adulthood. Studies in neonates and children up to 10 years of age show that CYP3A7 is found in the foetal liver and develops postnatally – leading to the rise of CYP3A4 during the first weeks after parturition (Cresteil, 1998; Lacroix *et al.,* 1997). Lacroix *et al.* (1997) also indicated that expression of CYP3A4 has been shown to be extremely weak in the foetus and begins to rise after birth – reaching 30-40% of the adult level in one month. Other studies have supported this theory and have reported that *in vitro* CYP3A4 enzyme levels increase very gradually during the first six months after birth. Levels for the 5–15 year age group were lower: approximately 25% of adult levels (Stevens, 2006; Stevens *et al.,* 2003). Clinical and preclinical data suggest that these changes of CYP3A enzyme expression for children aged 5 to 15 years, may be associated with growth hormone or hormonal changes related to sexual maturation (Cheung *et al.,* 1996). Regardless of age, the ligand-activated nuclear receptors PXR and/or CAR, regulate the CYP3A expression (Goodwin *et al.,* 2002; Goodwin, Hodgson & Liddle, 1999).

1.6.3 Drug effects on PXR/CAR nuclear receptors

The ligand-activated receptors CAR/PXR are highly co-expressed in the liver and intestine, where CYP3A concentrations are high (Ihunnah, Jiang & Xie, 2011). Most CYP3A4 inducers currently identified – such as rifampicin and phenobarbitone are confirmed activators of PXR, but not CAR. In contrast, EFV and NVP were classified as weak PXR activators, but demonstrate their CYP3A4 inductive effects predominantly through CAR activation (Faucette *et al.,* 2007). The mechanism of CAR/PXR in regulating CYP3A involves CAR/PXR binding to response elements of the CYP3A gene that confers transactivation (Goodwin, Hodgson & Liddle, 1999; Waxman, 1999). Burk *et al.* (2002) showed that CAR and PXR bind with higher affinity to CYP3A4 than CYP3A7. The drug-

metabolism enhancing properties of CAR/PXR have led to an avoidance of its activators in drug development – to reduce drug-drug interactions via the up-regulation of hepatic CYP3A4 (Ihunnah, Jiang & Xie, 2011). CAR/PXR also been found to regulate the expression of other sets of genes involved in bioactivation, detoxification, physiological and pathophysiological processes like inflammation, oxidative stress, and lipid, cholesterol and glucose homeostasis – if optimally activated (Swales & Bishop-Bailey, 2012; Chang, 2009; Moreau *et al.,* 2008). It is important therefore to determine a biomarker of CYP3A activity, considering its importance in the prediction of drug-drug interaction and other metabolic complications associated with ART.

1.6.4 4β-OHC as an endogenous biomarker for CYP3A4/5/7 activity

A well characterised and reliable endogenous biomarker of CYP3A enzyme activity, has the potential to be used to assess drug-drug interactions and to be a potential marker of nuclear receptor activation. There are currently several markers used to monitor CYP3A activity. One approach involves dosing with a probe drug like midazolam or erythromycin (Kasichayanula *et al.,* 2014; Gorski *et al.,* 2003). However, such studies are complex and pose challenges in specific patient populations such as paediatrics, where the administration of probe drugs may be detrimental to patient safety. The other method is the use of endogenous biomarkers like urinary 6β-hydroxycortisol to cortisol ratio (Galteau & Shamsa, 2003) and 4β-OHC (Diczfalusy *et al.,* 2008b). 4β-OHC has attracted interest recently as an endogenous biomarker for CYP3A4/5 enzyme activity and as an indirect marker of CAR/PXR activation (Diczfalusy *et al.,* 2008b). This has been preferred because of its good correlation with other accepted markers for CYP3A4/5 activity (Bodin *et al.,* 2001). Furthermore, unlike 6β-hydroxycortisol, 4β-OHC has a long half-life and plasma levels that do not show substantial diurnal variations (Diczfalusy *et al.,* 2008b). The use of cholesterol to 4β-OHC ratio has also been reported as a biomarker in conditions where the cholesterol concentration is changing (Diczfalusy *et al.,* 2011). *In vitro* studies have shown that CYP3A7 also catalyses conversion of cholesterol to 4β-OHC to a small extent, but *in vivo* 4β hydroxylase activity is unknown (Bodin *et al.,* 2002). Breuer *et al.* (1996) reported that very little 4β-OHC is formed by the autoxidation of cholesterol, but this can still be avoided by using antioxidants during sample preparation (Breuer *et al.,* 1996). The physiological role of 4β-OHC is unknown *in vivo*, but *in vitro* experiments have shown that it activates the nuclear receptor, liver X receptor alpha (LXRα) (Janowski *et al.,*

1996). The LXRα receptor helps in the regulation of genes involved with lipid metabolism (Tremblay-Franco *et al.,* 2015). It is possible that the highly elevated levels of 4β-OHC in patients treated with CYP3A enzyme inducers, may affect transcription of genes responsive to LXRα (Repa *et al.,* 2000).

In normal individuals, the basal plasma concentrations of 4β-OHC have a wide range of observed values – ranging from 18 to 99 ng/ml (Wide *et al.,* 2008). The use of drugs that induce or inhibit CYP3A enzymes may lead to an increase or decrease in 4β-OHC levels (Goodenough *et al.,* 2011; Wide *et al.,* 2008). A study that used a 4β-OHC/cholesterol ratio as a marker for CYP3A induction in antiretroviral therapy-naive HIV patients, illustrates that EFV increased the median plasma 4β-OHC/cholesterol ratio at 4th, 16th and 48th weeks by 257%, 291% and 165% respectively compared with the baseline levels (Habtewold *et al.,* 2013). Assays to measure 4β-OHC use several mechanisms, such as GC/MS or LC/MS/MS.

1.6.5 4β-OHC measurement in LC/MS/MS

The analysis of 4β-OHC by GC/MS and LC/MS/MS has been reported in the literature. LC/MS/MS is the most suitable instrument for the determination of 4β-OHC in plasma samples.

The recently published LC/MS/MS methods by Huang *et al.* (2014), Xu *et al.* (2013), Van de Merbel *et al.* (2011), Goodenough *et al.* (2011) and Honda *et al.* (2009) used various MS ionisation sources and sample extraction procedures to attain their proposed sensitivities. Most of these reported methods were using an ESI source and involved the derivatisation of 4β-OHC into a Dipicolinyl ester to enhance the ionisation efficiency. However, they had limitations such as a long run-time which was not conducive for high sample throughput, and some used surrogate matrix for the preparation of calibration STDs and QCs. The use of the same matrix with analysed samples is preferred for clinical sample analysis.

Atmospheric pressure photo-ionisation was reported for the analysis of 4β-OHC in human plasma. Although this method had no requirement of derivatisation, it still required a large sample volume (400 µl) to reach the required sensitivity and post-column addition of

reagent to facilitate the photo-ionisation process (van de Merbel *et al.,* 2011). Preparation of calibration STDs and QCs for plasma 4β-OHC measurement have been very challenging to attain in both derivatised and underivatised methods, since different lots of blank plasma samples contain unknown levels of oxysterols, including 4β-OHC.

Different approaches have been reported for the preparation of calibration STDs and QCs when determining endogenous compounds in plasma. These approaches include delipidation of plasma using the modified Folch method (Iverson, Lang & Cooper, 2001), stripping plasma with activated charcoal (DeBarber *et al.,* 2008), the use of a surrogate matrix like water (Huang *et al.,* 2014), and the use of a stable isotope labelled (SIL) surrogate analyte (Goodenough *et al.,* 2011). However, delipidated and charcoal-stripped plasma still reportedly contained trace amounts of the endogenous oxysterols which impacted on the ability to quantify low ng/ml concentrations in plasma. For the support of regulated work such as clinical studies, use of SIL surrogate analyte with similar chemical characteristics to the analyte of interest, could be a better option.

In this study, an LC/MS/MS assay with ESI was developed and validated (according to FDA, guidance for industry (2001) and EMA (2012) guidelines on bioanalytical method validation) – for the determination of 4β-OHC levels in the plasma of HIV-infected children on different antiretroviral treatments in Africa. A SIL analogue, 4β-OHC-D7 (surrogate analyte), was used for the preparation of calibration STDs and QCs in K_3EDTA human blank plasma. The second SIL analogue, 4β-OHC-D4 was used as an ISTD.

CHAPTER 2: PROJECT AIMS AND OBJECTIVES

2.1 Aim

The main aim of this study was to develop and validate a liquid chromatography mass spectrometry (LC/MS/MS) assay for the measurement of 4β-OHC levels in plasma, and to use 4β-OHC as an endogenous biomarker of CYP3A4/5/7 metabolism in order to infer the levels of metabolic activation of the CYP3A4/5/7 enzyme among young HIV-infected children with and without treatment.

2.2 Specific objectives

2.1.1 To determine and compare 4β-OHC levels in plasma of:

- Antiretroviral naïve children starting NVP.
- Experienced children staying on NVP.
- Experienced children switched from NVP to EFV.

2.1.2 To describe the effect of age, weight, and sex on the 4β-OHC levels in each group, and the effect of time on 4β-OHC concentrations in the respective treatment groups.

CHAPTER 3. LC/MS/MS METHOD DEVELOPMENT OF UNDERIVATISED AND DERIVATISED ASSAYS

Bioanalytical method strategies mainly involve method development, validation and sample analysis. In this study, underivatised and derivatised methods for the determination of 4β-OHC in human plasma were developed and compared. This chapter describes the background information for the underivatised and derivatised methods and the processes through which the methods were developed.

3.1 Introduction

Several studies have developed methods for the determination of oxysterols such as 4β-OHC in various matrices like plasma and cell tissues, using LC/MS/MS. Most of these studies utilised various MS ionisation techniques, sample extraction procedures, and also had a range of sensitivities. It was observed that the absence of acid and basic functional groups in the oxysterols make them difficult to ionise and this results in poor sensitivity and specificity.

Some of the previous oxysterol methods demonstrated that in order to attain sufficient sensitivity of oxysterols like 4β-OHC without chemical modification (derivatisation), a relatively unselective mass transition (loss of water) has to be selected using an APCI or APPI source (Byers *et al.,* 2014; Van de Merbel *et al.,* 2011). Atmospheric pressure photo-ionisation has been demonstrated to be more than twice as sensitive as APCI (Karuna, von Eckardstein & Rentsch, 2009). The challenge with APPI is that it is complex, and requires post-column addition of a reagent such as toluene to enable the photo-ionisation process; hence, it is not readily available in most bioanalytical laboratories (Van de Merbel *et al.,* 2011). Advantages of the underivatisation method include its simplicity (short sample preparation period) which allows high-throughput sample analysis and cost effectiveness (no need for derivatisation reagents). Nevertheless, this method still has a sensitivity problem – especially when using matrices like plasma. Previous underivatisation methods reported the use of a large sample volume (not less than 400 µl of plasma) to reach required sensitivities. Large sample volumes are not ideal if multiple analysis is needed from the same sample (such as pharmacokinetic and 4β-OHC measurements) or if the method is to be used in the analysis of the small volumes available in paediatric studies.

Derivatisation of 4β-OHC into a Dipicolinyl ester (DPE) was reported to enhance the ionisation using an ESI source, and improved the sensitivity more than the underivatised method (Huang *et al.,* 2014; Goodenough *et al.,* 2011). Other studies also supported that ESI alone is not the best ionisation source for oxysterols like 4β-OHC, because of poor ionisation – and that derivatisation of 4β-OHC to DPE enhances the ionisation efficiency with a better signal and chromatography (Honda *et al.,* 2009). In this way, apart from high sensitivity, the derivatisation method can also be used with small sample volumes to attain the required sensitivity (Xu *et al.,* 2013). As mentioned earlier, small sample volumes have an advantage when analysing 4β-OHC or other oxysterols in paediatric studies, or when multiple analyses are required from the same sample volume. Limitations associated with the derivatisation process include a relatively longer sample preparation compared to the underivatised method, and the cost of derivatising reagents. The processes of 4β-OHC underivatised and derivatised method development are therefore described and compared below:

3.2 Experimental: the development of a bioanalytical method

3.2.1 Analytes and internal standard

Since 4β-OHC is an endogenous analyte in human plasma, a SIL analogue, 4β-OHC –D7, was used as a surrogate analyte for the preparation of calibration STDs and QC samples. A second SIL analogue, 4β-OHC –D4, was used as an ISTD. Figures 3.1, 3.2 and 3.3 are the structures and properties of 4β-OHC, 4β-OHC-D7, and 4β-OHC-D4 respectively:

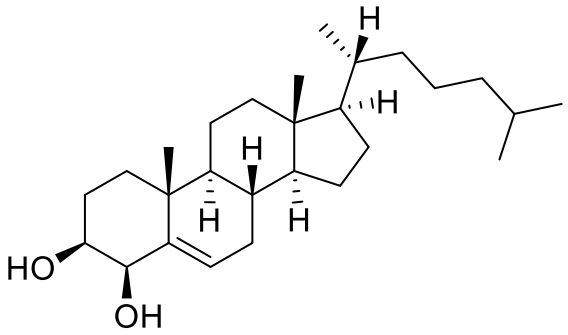


Figure 3.1: Chemical structure of the analyte 4β-OHC

Synonyms: (3β,4β)-Cholest-5-ene-3,4-diol; Cholest-5-ene-3β,4β-diol
Molecular Formula: $C_{27}H_{46}O_2$
Molecular Weight: 402.65
Exact Mass: 402.34

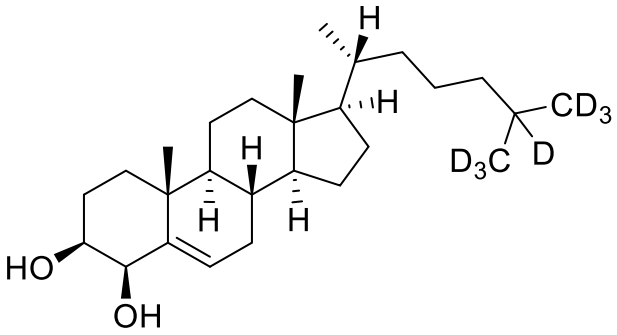


Figure 3.2 Chemical structure of surrogate analyte 4β-OHC-D7

Synonyms: (3β,4β)-Cholest-5-ene-3,4-diol-d7; Cholest-5-ene-3β,4β-diol-d7
Molecular Formula: $C_{27}H_{39}D_7O_2$
Molecular Weight: 409.70
Exact Mass: 409.39

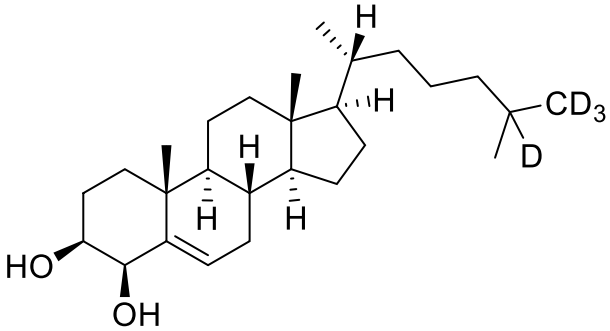


Figure 3.3 Chemical structure of ISTD 4β-OHC-D4

Synonyms: (3β,4β)-Cholest-5-ene-3,4-diol-d4; Cholest-5-ene-3β,4β-diol-d4
Molecular Formula: $C_{27}H_{42}D_4O_2$
Molecular Weight: 406.68
Exact Mass: 406.37

3.2.2 Preparation of stock solutions

The compounds; 4β-OHC, 4β-OHC-D7 and 4β-OHC-D4, were fairly soluble in DMSO – but highly soluble in ethanol. Primary stock solutions of these compounds (1 mg/ml) were prepared by dissolving 1 mg of each compound in 1 ml of ethanol, as shown in Tables 3.1 and 3.2 (below). Secondary stock solutions of these compounds were also prepared in ethanol (see Tables 3.1 and 3.2).

Table 3.1 Preparation of analytes primary (SS1) and secondary stock solutions (SS2-4)

Analyte	Solvent	Weighed Mass of analyte (mg)	Volume of solvent (ml)	SS1 Concentration (µg/ml)	SS2 Concentration (µg/ml)	SS3 Concentration (µg/ml)	SS4 concentration (µg/ml)
4β-OHC	Ethanol	1.00	1.00	1000	100	10.0	1.00
4β-OHC-D7	Ethanol	1.00	1.00	1000	100	10.0	1.00

Table 3.2 Preparation of ISTD primary (ISS1) and secondary stock solutions (ISS2-3)

Internal Standard	Solvent	Weighed Mass of Analyte (mg)	Volume of Solvent (ml)	ISS1 Concentration (µg/ml)	ISS2 Concentration (µg/ml)	ISS3 Concentration (µg/ml)
4β-OHC-D4	Ethanol	1.00	1.00	1000	100	10

A literature review was done to establish methods that have already been published for the determination of oxysterols like 4β-OHC in plasma. Underivatised and derivatised methods were identified as some of the suitable methods for the determination of oxysterols in human plasma. Derivatisation of oxysterols into Di/Picolinyl esters (Goodenough *et al.,* 2011), Girard P hydrazones (Griffiths *et al.,* 2006), Dimethylglycine (Jiang, Ory & Han, 2007) and Dansylated (Tang & Guengerich, 2010) derivatives, were some of the derivatisation methods found. These derivatives were mostly dependent on

several factors like sample volume, sensitivity requirements, reaction duration, type of instrument, and chemical reagents' availability. In this study, 4β-OHC underivatised and derivatised methods were therefore developed and compared, using LC/MS/MS.

3.2.3 Underivatised method

A procedure for the underivatised method is described below:

3.2.3.1 Mass spectrometry

From the secondary stock solutions prepared in Tables 3.1 and 3.2, infusion solutions of 4β-OHC, 4β-OHC-D7 and 4β-OHC-D4 were prepared in a mixture of acetonitrile and methanol, at a concentration of 1 µg/ml, and they were acidified with 0.1% formic acid. These solutions were infused at constant flow rates of 10 µl/min, using a Hamilton syringe (1.0 ml) to obtain the mass spectra of the protonated molecular ions and their fragments. An AB Sciex API 3200 LC/MS/MS was initially used, but the assay was later transferred to an AB Sciex Qtrap 5500 LC/MS/MS, in order to attain better sensitivity. The quantitation method was set at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the $[(M-H_2O) + H^+]$ ions at m/z 385, m/z 392 and m/z 389 – to the product ions of m/z 109, m/z 109 and m/z 109 for 4β-OHC, 4β-OHC-D7 and 4β-OHC-D4 respectively, as shown in Figures 3.4, 3.5 and 3.6 (below). The product ions selected were not the most abundant product ions on the spectra, but were selected during parameter optimisation because they showed high intensity and a continuous signal. These selected precursor and product ions are similar to other recent underivatised methods used (Byers *et al.,* 2014; Van de Merbel *et al.,* 2011).

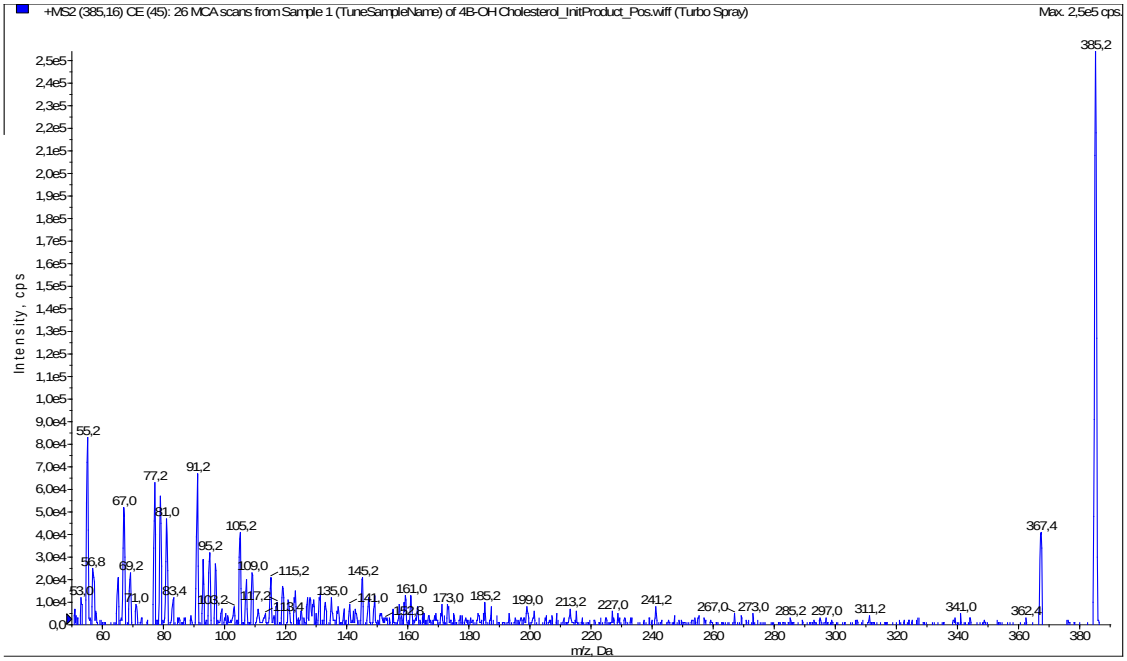


Figure 3.4 Initial product ion mass spectrum of 4β-OHC, showing the [(M-H_2O) + H^+] ions at m/z 385 and product ion 109.

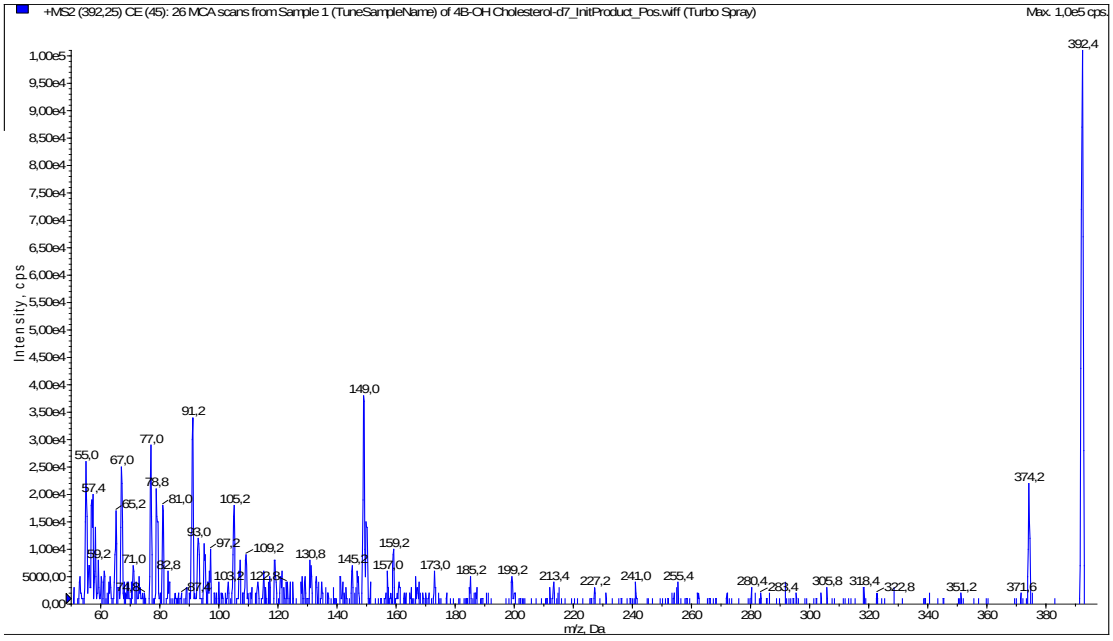


Figure 3.5 Initial product ion mass spectrum of 4β-OHC-D7, showing the [(M-H_2O) +H^+] ions at m/z 392 and product ion 109.

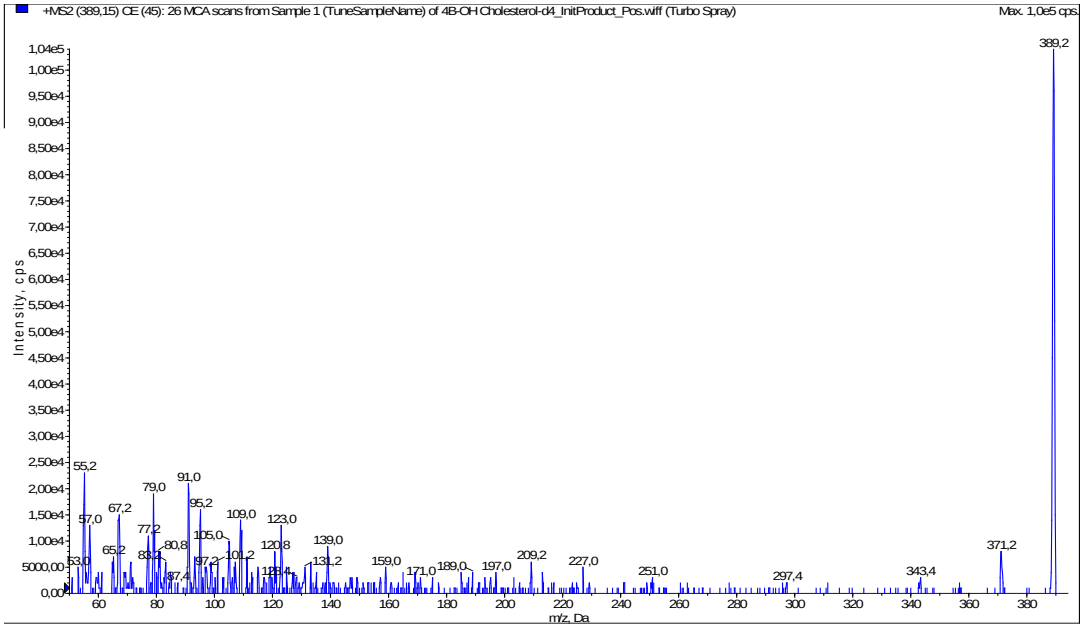


Figure 3.6 Initial product ion mass spectrum of 4β-OHC-D4, showing the [(M-H_2O) + H^+] ions at m/z 389 and product ion 109.

The LC/MS/MS system was interfaced with a DELL® Windows®XP computer running Analyst® software version 1.6.1. Analyst® software was used for chromatographic data acquisition, peak integration, and quantification.

3.2.3.2 Detection

Detection was done on an AB Sciex Qtrap 5500 mass spectrometer in the APCI mode. The APCI and mass spectrometer's settings are summarised in Tables 3.3, 3.4 and 3.5 respectively.

Table 3.3 APCI settings

Nebuliser gas (Gas 1) (arbitrary unit)	55
Turbo gas (Gas 2) (arbitrary unit)	55
CUR (curtain gas) (arbitrary unit)	30
CAD (collision gas) (arbitrary unit)	Medium
TEM (Source temperature) (°C)	500
NC (nebuliser current) µA	3

Table 3.4 MS/MS settings

	Underivatised 4β-OHC	**Underivatised 4β-OHC-D7**	**Underivatised 4β-OHC-D4**
Protonated precursor ion mass (m/z) [(M – H_2O) + H^+]	385.153	392.278	389.147
Product ion mass (m/z) Quantifier	109. 200	109.200	109.200
Dwell time (ms)	200	200	200
Declustering potential (V)	70	60	60
Entrance potential (V)	10	10	10
Collision energy (eV)	30	55	60
Collision cell exit potential (V)	20	22	20

Table 3.5 Scan description

Scan type	MRM
Polarity	Positive
Pause time (ms)	200

3.2.3.3 Chromatographic development

Different analytical columns were tested – initially using: (A) 0.1% formic acid in water, (B) 0.1% formic acid in acetonitrile as the mobile phase, and 0.1% formic acid + acetonitrile (90:10 v/v) as the injection solution. The mobile phase was delivered at a flow rate of 300 µl/min, using an Agilent 1260 series pump. The injection volume was 10 µl and the analytical column was kept at ~30^{o}C, with an Agilent 1260 series column compartment. Table 3.5 shows the analytical columns tested.

Table 3.6 Analytical columns tested

Manufacturer	Stationary phase	Particle size (µm)	Length (mm)	Diameter (mm)
Agilent	SB phenyl	1.8	100	2.1
Phenomenex	Gemini C6 phenyl	5	50	2.0
Phenomenex	Kinetex PFP	2.6	50	2.1

The best chromatographic conditions (with respect to the shape of the peak, the intensity, and the retention) were achieved with the Gemini C6 phenyl analytical column. The following compositions of mobile phase were tested on the Gemini C6 column – isocratically and using gradients:

1. (A) 0.1% formic acid in water; (B) 0.1% formic acid in methanol.
2. (A) 0.1% formic acid in water; (B) methanol: water: isopropanol (30:10:10 v/v/v).
3. (A) 0.1% formic acid in water; (B) acetonitrile.
4. (A) 0.1% formic acid in methanol: water (98:2 v/v); (B) 0.1% formic acid in acetonitrile: methanol (50:50 v/v).
5. (A) 0.1% formic acid in methanol: water (95:5 v/v); (B) 0.1% formic acid in acetonitrile: methanol: isopropanol (45:45:10 v/v/v).

Flow rates of 400, 300 and 200 µl/min were evaluated. The best result was obtained with (A) 0.1% formic acid in water; (B) 0.1% formic acid in methanol; (5:95 v/v) as mobile phase at a flow rate of 400 µl/min for 4β-OHC, 4β-OHC-D7, and 4β-OHC-D4.

The following injection solutions were tested at different injection volumes, using the above-mentioned chromatography system:

1. Acetonitrile.
2. Methanol.
3. 0.05% formic acid in acetonitrile.
4. 0.1% formic acid in acetonitrile.
5. 0.05% formic acid in methanol.
6. 0.1% formic acid in methanol.
7. methanol: acetonitrile (50:50 v/v).

8. methanol: water (50:50 v/v).
9. 0.1% formic acid in acetonitrile: methanol: water (50:30:20 v/v/v).
10. 0.05% formic acid in acetonitrile: methanol: water (50:30;20 v/v/v).
11. 0.1% formic acid in acetonitrile: methanol: water (40:50:10 v/v/v).

The best peak shapes and peak intensities were observed with 0.1% formic acid in acetonitrile: methanol: water (40:50:10) and 0.05% formic acid in methanol at 10 µl injection volume.

The optimal chromatographic system for all the analytes is summarised as follows:

- Analytical column: Gemini C6 Phenyl analytical column, kept at ~30^{o}C, with an Agilent 1260 series column compartment.
- Mobile phase: (A) 0.1% formic acid in water; (B) 0.1% formic acid in methanol; (5:95 v/v).
- Flow rate: Isocratic at 400 µl/min over 5 minutes.
- Injection solution and injection volume: The injection volume was 10 µl and the injection solution 0.1% formic acid in acetonitrile: methanol: water (40:50:10 v/v/v).

Flow injection analysis (FIA) was done to optimise the ion source parameters. The following assessments of the analytes in the optimised injection solution were performed:

- The repeatability of the instrument's response.
- The dilution sequence (linearity) within the expected concentration range.
- Sensitivity test at the lower limit of quantification (LLOQ).

The repeatability of the instrument's response for the surrogate analyte and ISTD ratio (n = 52) was consistent with a % CV (coefficient of variation) of 7. The regression of the dilution sequence in injection solvent for the concentration range 2 to 500 ng/ml was quadratic, with R^2 = 0.9973. (Shown in Appendix B; Table B1 and Figure B1 respectively).

The acceptable mean of the analyte's S/N response ratio at LLOQ should be more than 5. The S/N ratio of the surrogate analyte at 2 ng/ml was 28 and acceptable (Attached in Appendix B; Figure B2).

3.2.3.4 Extraction development

3.2.3.4.1 Biological matrix

Pooled K_3EDTA (anticoagulant) human blank plasma from the Western Province blood transfusion laboratory was used for the preparation of the calibration STDs and QC samples. During extraction, 4750 µl of human blank plasma was spiked with 250 µl of a 10 µg/ml secondary stock solution of 4β-OHC-D7 in ethanol (Table 3.1) – to obtain a plasma sample at a concentration of 500 ng/ml. Different preliminary extraction procedures like liquid-liquid, protein precipitation and solid phase were tested as summarised below:

a) Liquid-liquid extraction

1. Aliquot 200 µl of spiked plasma (using a yellow tip, wet-tip technique, placing the tip at 45° against the side of the tube).
2. Add 50 µl of ISTD (4β-OHC-D4; 100 ng/ml in water).
3. Add 10 µl antioxidant (BHT 5 µg/ml).
4. Add 875 µl 0.5M KOH in ethanol (cold saponification) for 3 hours.
5. Neutralise with 25 µl of 85% phosphoric acid + 1000 µl water (checked with pH meter).
6. Extract with hexane (2 x 3 ml).
7. Centrifuge at 2500 x g for 5 minutes.
8. Separate hexane phase and evaporate under nitrogen at 40^{o}C.
9. Reconstitute using 200 µl of 0.05% formic acid in methanol.
10. Vortex mix for 5 minutes.

b) Protein precipitation

1. Aliquot 50 µl of spiked plasma (using a yellow tip, wet-tip technique, placing the tip at 45° against the side of the tube).
2. Add 50 µl of ISTD (4β-OHC-D4; 100 ng/ml in water).
3. Add 1000 µl of methanol: isopropanol (50:50).
4. Vortex mix for 2 minutes.
5. Centrifuge at 2500 g for 5 minutes.
6. Transfer 900 µl of supernatant and evaporate under nitrogen at 40^{o}C.

7. Reconstitute with 100 µl of 0.05% formic acid in methanol.
8. Vortex mix for 5 minutes

c) Solid phase extraction

1. Aliquot 100 µl of spiked plasma (using a yellow tip, wet-tip technique, placing the tip at 45° against the side of the tube).
2. Add 50 µl of ISTD (4β-OHC-D4; 200 ng/ml in water).
3. Add 200 µl of fresh 2M sodium methoxide solution.
4. Mix and leave at ambient temperature (~20^{o}C) for 20 minutes.
5. Add 1000 µl of hexane.
6. Add 250 µl of water.
7. Vortex mix for 5 minutes.
8. Centrifuge at 2500 x g for 5 minutes at ~20^{o}C.
9. Freeze aqueous layer in a mixture of acetone and dry ice.
10. Transfer upper organic layer to a solid phase extraction cartridge (Oasis® HLB, 30 µm).
11. Condition and wash with 1 ml hexane.
12. Elute with 2 x 750 µl aliquots of a mixture of ethyl acetate and hexane (80:20 v/v).
13. Dry down under nitrogen at 40^{o}C.
14. Reconstitute with 100 µl of 0.1% formic acid in acetonitrile: methanol: water (40:50:10).
15. Vortex mix for 5 minutes.

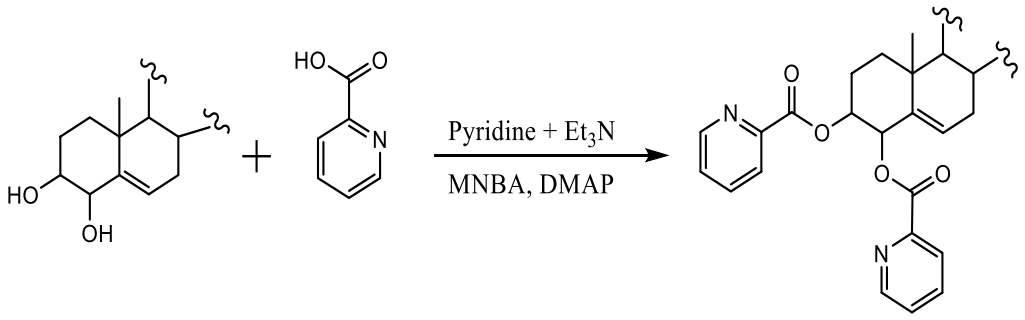
LLE and protein precipitation had very low intensity peaks for 4β-OHC, 4β-OHC-D7 and 4β-OHC-D4, and other coelution peaks of the endogenous components. SPE gave a better peak shape for 4β-OHC, 4β-OHC-D7 and 4β-OHC-D4 compared to others – but lacked sensitivity and specificity from other endogenous components in the blank human plasma. The underivatised method could possibly work better using an APPI source or a more sensitive mass spectrometer compared to the AB Sciex Qtrap 5500 mass spectrometer used in this study. The underivatisation method therefore lacked sensitivity and specificity and was not suitable for validation and clinical sample analysis.

3.2.4 Derivatised method

Since 4β-OHC has poor ionisation efficiency, the derivatisation of 4β-OHC to DPE enhanced the ionisation efficiency with a relatively short derivatisation time. The derivatised method procedure is therefore described below:

3.2.4.1 Mass spectrometry

Secondary stock solutions of 4β-OHC, 4β-OHC-D7 and 4β-OHC-D4 were prepared at a concentration of 500 ng/ml (see Tables 3.1 and 3.2). These stock solutions were derivatised with picolinic acid into DPE (see Figure 3.7). Thereafter, the samples were dried and reconstituted in acetonitrile and 0.1% formic acid (90:10 v/v). These solutions were used for mass spectrometer infusions.



4β-OHC Picolinic acid (Catalyst reagents) Dipicolinyl ester

Figure 3.7 Derivatisation reaction

Mass spectra of the protonated derivatised product molecular ions and their fragments were obtained by infusing the derivatised solutions into the mass spectrometer. The LC/MS/MS acquisition method was initially set up on an AB Sciex API 2000 LC/MS/MS, but was later transferred to an AB Sciex Qtrap 5500 LC/MS/MS to attain better sensitivity. The mass spectrometers were operated in the positive ionisation mode, detecting the derivatised products of 4β-OHC, 4β-OHC-D7 and 4β-OHC-D4 at unit resolution in multiple reaction monitoring (MRM) mode. The transition of the protonated derivatised product molecular ions at m/z 613, m/z 620 and m/z 617, to the product ions of m/z 490, m/z 497 and m/z 494 for 4β-OHC, 4β-OHC-D7 and 4β-OHC-D4, respectively, were followed. Their product ion mass spectra are presented in Figures 3.8, 3.9 and 3.10 respectively.

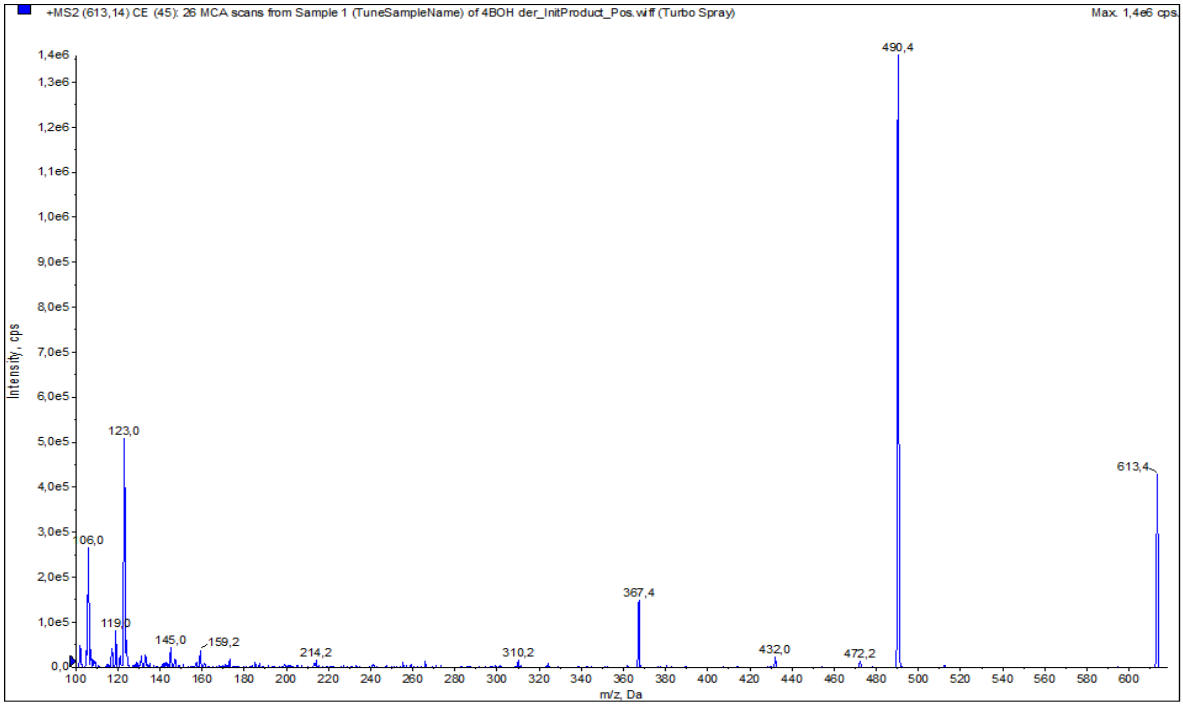


Figure 3.8 Initial product ion mass spectrum of the protonated derivatised 4β-OHC molecular ions at m/z 613 and product ions

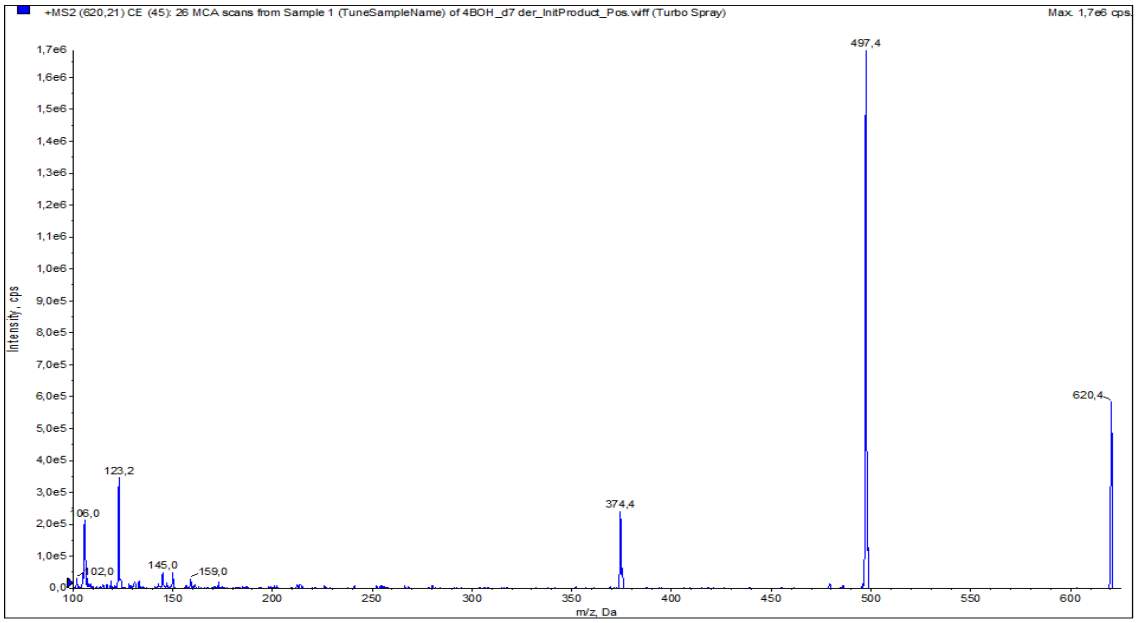


Figure 3.9 Initial product ion mass spectrum of protonated derivatised 4β-OHC-D7 molecular ions at m/z 620 and product ions

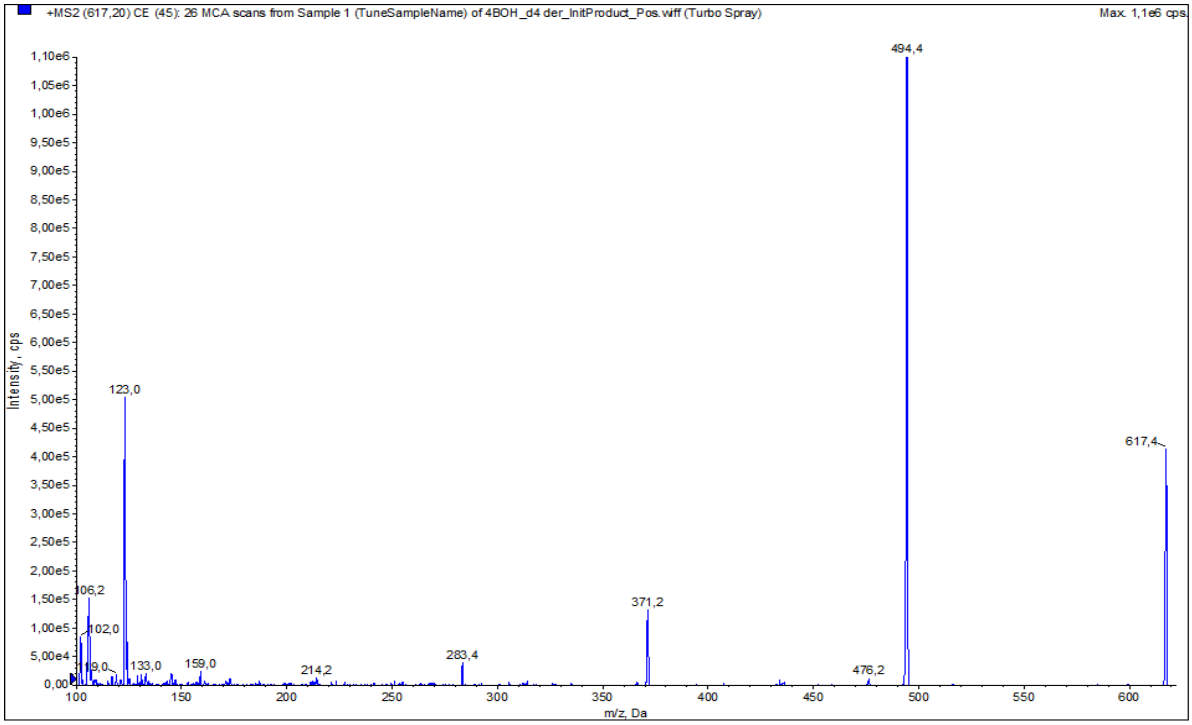


Figure 3.10 Initial product ion mass spectrum of protonated derivatised 4β-OHC-D4 molecular ions at m/z 617 and product ions

The LC-MS/MS system was interfaced with a DELL® Windows®XP computer running Analyst® software version 1.6.1. Analyst® software was used for chromatographic data acquisition, peak integration, and quantification.

3.2.4.2 Detection

Detection on an AB Sciex Qtrap 5500 mass spectrometer was done using an ESI source. Optimised ESI and MS/MS settings are summarised in Tables 3.7, 3.8 and 3.9, respectively.

Table 3.7 ESI settings

Nebuliser gas (Gas 1) (arbitrary unit)	35
Turbo gas (Gas 2) (arbitrary unit)	40
CUR (curtain gas) (arbitrary unit)	30
CAD (collision gas) (arbitrary unit)	Medium
TEM (source temperature) (°C)	300
IS (ion spray voltage) (V)	5500

Table 3.8 MS/MS settings

	Derivatised 4β-OHC	Derivatised 4β-OHC-D7	Derivatised 4β-OHC-D4
Protonated derivatised product molecular ion mass (m/z)	613.143	620.207	617.197
Product ion mass (m/z) Quantifier	490.300	497.300	494.200
Dwell time (ms)	200	200	200
Declustering potential (V)	45	50	40
Entrance potential (V)	10	10	10
Collision energy (eV)	15	15	15
Collision cell exit potential (V)	35	30	35

Table 3.9 Scan description

Scan type	MRM
Polarity	Positive
Pause time (ms)	200

3.2.4.3 Chromatographic development

Two analytical columns were tested, using (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol as the mobile phase, and acetonitrile and 0.1% formic acid in water (90:10 v/v) as the injection solution. The mobile phase was delivered at a flow rate of 300 µl/min using an Agilent 1260 series pump. The injection volume was 10 µl and the analytical column was kept at 30^{o}C, with an Agilent 1260 series column compartment. Table 3.10 shows the analytical columns tested.

Table 3.10 Analytical columns tested

Manufacturer	Stationary phase	Particle size (µm)	Length (mm)	Diameter (mm)
Agilent	SB phenyl	1.8	100	2.1
Phenomenex	Gemini C6 phenyl	5	50	2.0

The best chromatography system was achieved with the Agilent SB phenyl column. The following compositions of mobile phase were tested isocratically, using a binary pump on the Agilent column:

1. (A) 0.1% formic acid in methanol: water (98:2; v/v); (B) 0.1% formic acid in methanol: acetonitrile (50:50; v/v).
2. (A) 0.1% formic acid in water; (B) 0.1% formic acid in methanol.

Flow rates of 300 and 200 µl/min were evaluated. The best result was obtained with 100% (B) 0.1% formic acid in methanol-acetonitrile (50:50; v/v) as the mobile phase, at a flow rate of 200 µl/min. The following different injection solutions were tested at different injection volumes:

1. Acetonitrile.
2. Acetonitrile: water (90:10).
3. 0.05% formic acid in methanol.
4. 0.1% formic acid in acetonitrile.

Injection volumes of 10 µl and 2 µl were evaluated, and an injection volume of 2 µl and an injection solution of 0.05% formic acid in methanol gave the best peak shapes. Thus, the optimal chromatographic condition for all the analytes can be summarised as follows:

- The analytical column: Agilent SB phenyl 1.8 µm 2.1x100 mm analytical column, kept at 30^{o}C with an Agilent 1260 series column compartment.
- The mobile phase: 0.1% formic acid in methanol and acetonitrile (50:50; v/v) – which were delivered using an Agilent 1260 series pump at a flow rate of 200 µl/min over 5 minutes.
- The injection solution and injection volume: The injection volume was 2 µl and the

injection solution 0.05% formic acid in methanol.

FIA was done to optimise the ion source parameters. The retention time was 2.3 minutes for the derivatised 4β-OHC, 4β-OHC-D7, and 4β-OHC-D4 compounds.

Using the FIA optimised acquisition methods, assessments of analytes derivatised in pure solution, dried with nitrogen gas, and reconstituted with injection solution, were performed. These were the:

- Repeatability of the instrument's response;
- Dilution sequence (linearity) within the expected concentration range; and
- Sensitivity test, by assessing S/N at the expected LLOQ.

The repeatability of the instrument's response (i.e. the detector's response consistency) for the analytes and ISTD ratio (n = 117) was consistent with a % CV of 2.2 (attached in Appendix B; Table B3). The regression of the dilution sequence in injection solvent for the concentration range 2 to 500 ng/ml was quadratic, with R^2 = 0.9980 and S/N at 2 ng/ml was 10.8 and acceptable (Shown in Appendix B ; Figures B3 and B4 respectively).

3.2.4.4 Extraction development

3.2.4.4.1 Biological matrix

Pooled K_3EDTA human blank plasma from Western Province blood transfusion was also used for the preparation of the calibration STDs and QC samples. During extraction, 4900 µl of human blank plasma was spiked with 100 µl of a 10 µg/ml stock solution of 4β-OHC-D7, in ethanol, to obtain a plasma sample at 200 ng/ml concentration. It was extracted by using a preliminary extraction procedure, as outlined below:

1. Thaw spiked plasma samples at room temperature, and vortex mix briefly.
2. Aliquot 100 µl plasma into a clean, labelled 5 ml polypropylene tube (using a yellow tip, wet-tip technique, placing the tip at 45° against the side of the tube).
3. Add 100 µl of ISTD solution in water (4β-OHC-D4 200 ng/ml).
4. Vortex mix for 10 seconds.
5. Add 500 µl 1M ethanolic potassium hydroxide.
6. Vortex mix for 10 seconds.
7. Add 50 µl antioxidant solution (BHT 1 mg/ml).
8. Vortex mix for 10 seconds.

9. Leave in water bath at 37^{o}C for 1 hour.
10. Add 280 µl of water.
11. Add 1.5 ml of hexane.
12. Vortex mix for 1 minute.
13. Centrifuge at 1300 x g for 10 minutes at 4˚C.
14. Transfer 1.3 ml of the supernatant to a 5 ml polypropylene tube.
15. Evaporate to dryness under nitrogen at 40˚C.
16. Add 170 µl freshly prepared derivatising solution (see appendix A.2 for the preparation)
17. Vortex mix for 30 seconds.
18. Leave at room temperature for 30 minutes.
19. Add 1.5 ml of hexane.
20. Vortex mix for 1 minute.
21. Centrifuge at 1300 x g for 10 minutes at 4˚C.
22. Transfer 1.3 ml of the supernatant to a 5 ml polypropylene tube.
23. Evaporate to dryness under nitrogen at 40˚C.
24. Add 1.5 ml of hexane.
25. Vortex mix for 1 minute.
26. Centrifuge at 1300 x g for 10 minutes at 4˚C.
27. Transfer 1.3 ml of the supernatant to a 5 ml polypropylene tube.
28. Evaporate to dryness under nitrogen at 40˚C.
29. Reconstitute the residue with 400 µl of 0.05% formic acid in methanol solution.
30. Vortex mix for 1 minute.
31. Transfer the extract to a 96-well plate and inject 2 µl into the LC/MS/MS system.

Calibration STDs ranging from 2–500 ng/ml were prepared in human blank plasma volumetrically. The volume of 50 µl of a 10 µg/ml stock solution in ethanol of 4β-OHC-D7 was spiked into 950 µl of blank human plasma to obtain a 500 ng/ml sample. Thereafter, it was serially diluted (1:1) down to 2 ng/ml, with human blank plasma. Calibration STDs were extracted in duplicate, including two blanks (no surrogate analyte and no ISTD) and two zero samples (no surrogate analyte but with ISTD) to monitor the carry-over. The calibration curve was based on surrogate analyte/ISTD peak area ratios to calculate the regression equation. No carry-over was observed. The S/N at LLOQ was 10.2

(result attached in Appendix B Figure B6). The extraction consistency (n = 40) was satisfactory (Shown in Appendix B: Tables B4, B5). Derivatisation improved the ionisation efficiency and selectivity and thus achieved the required sensitivity and specificity.

3.2.5 Analyte and internal standard stability in stock solution

It is important to determine the stability of an analyte in stock solutions before validating the bioanalytical assay method – to ensure that the analytes are stable in the solvents that are being stored. Thus, the stability of the surrogate analyte and ISTD, in stock solution at room temperature and ~4^{o}C was performed before the method validation process. The remaining stability parameters were assessed during the process of method validation. Aliquots of stock solutions were stored at ~4^{o}C and room temperature for 14 days and compared to stock solutions stored at -80^{o}C (reference). The analytes were derivatised and analysed chromatographically in replicates of six, at low and high concentrations. A difference of more than 15% from the reference solution and a percentage CV greater than 15% of the measured values could indicate stock solution instability. As shown in Table 3.11 (below) the percentage difference and percentage CV were within the acceptable range and that indicates stability of the analyte in stock solution (ethanol) when stored for 14 days at different temperatures.

Table 3.11 Analyte stability in stock solution for 4β-OHC-D7 and 4β-OHC-D4 in ethanol (for 14 days)

Analyte		Mean analyte peak area (n=6)		
		Reference (-80 ºC)	(~4 ºC)	Room temperature
4β-OHC-D7	Peak area	44065000	44745000	45773333
	% difference	N/A	1.5	3.9
	% CV	0.8	0.8	0.7
4β-OHC-D4	Peak area	41141667	42708333	41473333
	% difference	N/A	3.8	0.8
	% CV	0.5	0.7	0.5

3.3 Discussion

3.3.1 Selection of surrogate analyte and internal standard

Since 4β-OHC is an endogenous metabolite, a SIL analogue, 4β-OHC-D7, was used as a surrogate analyte for preparation of calibration STDs and QCs in K_3EDTA human blank plasma. The second SIL analogue, 4β-OHC-D4, was used as an ISTD to compensate for the variability in the sample extraction process and fluctuations in mass spectrometry response (Lowes *et al.,* 2011). No peaks were observed for 4β-OHC-D7 and 4β-OHC-D4 in human blank plasma that was used to prepare STDs and QCs. This indicated there were no endogenous components in human blank plasma that would contribute to the overall signal of 4β-OHC-D7 and 4β-OHC-D4 when analysing clinical samples. The surrogate analyte approach, used matrix (human plasma) that was similar to the study samples. This supports the notion that when developing a method for regulated work such as clinical studies, it is ideal to prepare both calibration STDs and QCs in the same matrix as the clinical samples (European Medicines Agency, 2012; Guidance for industry, 2001).

In several studies the preparation of calibration STDs and QCs for the measurement of endogenous compounds (including 4β-OHC) in plasma have been very challenging to achieve, since different lots of blank plasma samples contain unknown levels of the endogenous compounds. Other studies tried to 'delipid' plasma using a Folch's method or by stripping using activated charcoal when preparing calibration STDs and QCs for measuring endogenous oxysterols (DeBarber *et al.,* 2008; Iverson, Lang & Cooper, 2001). However, delipidated plasma was found to contain traces of oxysterols, including 4β-OHC, which made their quantification in plasma samples difficult. Furthermore, other studies reported the use of surrogate matrix for preparation of both, or either one of, the calibration STDs and QCs (Huang *et al.,* 2014). Still, the use of surrogate matrix not only could make direct sensitivity comparisons to plasma inconclusive, but also could cause a significant bias in LC/MS/MS assays as a result of a difference in matrix effect, specificity, stability, and recovery. The approach of surrogate analyte mitigated the challenge posed by the endogenous compounds in human blank plasma, as others reported (Goodenough *et al.,* 2011) – and hence justifies the use in this study.

3.3.2 Alkaline hydrolysis (saponification)

The metabolite 4β-OHC exists as both free forms and esterified fatty esters in plasma (Diczfalusy *et al.,* 2011). Alkaline hydrolysis (saponification) was done to first cleave the hydroxycholesterol esters, in order to obtain free total 4β-OHC for accurate measurement in human plasma. As stated in most published methods (Huang *et al.,* 2014), in this study ethanolic potassium hydroxide and sodium methoxide were used for the saponification process in both the derivatisation and underivatisaton methods respectively. The antioxidant, BHT, was also used to eradicate any possible autoxidation of cholesterol to 4β-OHC during sample extraction (Breuer *et al.,* 1996). Following saponification processes, underivatisation and derivatisation processes were conducted. A more focused comparison of the individual methods is discussed below.

3.3.3 Underivatisation method

The method to determine underivatised 4β-OHC in human plasma was developed. The ionisation of the underivatised 4β-OHC was found to be poor in human blank plasma after solid phase extraction – compared to pure solutions. On the other hand, endogenous components in the human plasma were coeluting with the underivatised 4β-OHC. This indicated that the method lacked sensitivity and specificity in human plasma. This was not unexpected, as it is known that endogenous oxysterols – including 4β-OHC – have no acid and basic functional groups in their structures, which make their ionisation relatively difficult (Van de Merbel *et al.,* 2011). As other studies have noted, because of the absence of acid and basic functional groups in the endogenous oxysterols, a relatively unselective mass transition (loss of water) has to be selected using APCI or APPI, in order to attain sufficient sensitivity (Byers *et al.,* 2014). Atmospheric pressure photo-ionisation has been reported to be more than twice as sensitive as APCI for underivatised 4β-OHC, but this required post-column addition of reagent such as toluene to enable the photo-ionisation process. When the underivatised method was developed in this study, an APPI source was not available, and hence was not tested. The use of unselective mass transition (loss of water) selected in APCI in this present study, may explain the inadequate sensitivity.

Sample volume differences may also explain the difference of sensitivity of this method compared with other reported studies. Most of the reported underivatised methods were

using a large sample volume (400 µl) to attain their required sensitivities and we only used ≤ 200 µl of human plasma. The use of a large sample volume of more than 200 µl was not ideal in the present study due to the small sample volumes of paediatric clinical samples available for the assay to be applied to – once developed. Developing a method which uses small sample volumes is useful. It reduces the blood requirements, per patient, and also allows multiple analyses from the same patient sample – a common phenomenon in pharmacokinetic studies. Nevertheless, an underivatised method could have had the advantage of being less involving and less time-consuming if it were without the challenge of sensitivity and specificity. Ultimately, derivatisation of 4β-OHC into DPE was a viable option in this study.

3.3.4 Derivatisation method

Derivatisation of 4β-OHC (and 4β-OHC-D7 and 4β-OHC-D4) into their corresponding DPE was found to enhance the ionisation of 4β-OHC with an ESI source. The derivatisation method was more sensitive and specific, with good peak shapes and intensity. In this study, the DPE derivative was chosen among other derivatives because it is known to be more sensitive in plasma than other derivatives such as Dimethylglycine and Girard P hydrazones derivatives. (Huang *et al.,* 2014; Goodenough *et al.,* 2011; Honda *et al.,* 2009). The MRM transitions of the derivatised 4β-OHC, 4β-OHC-D7 and 4β-OHC-D7, in this study, were similar to most of those reported in the literature. The product ions were formed from the loss of picolinyl ester moiety, following collision-induced dissociation of the precursor ion (see Figure 3.11).

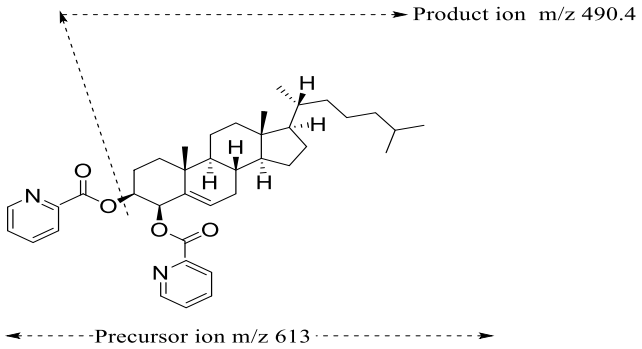


Figure 3.11 Chemical structure of 4β-OHC dipicolinyl ester, precursor and product ions used for the quantification.

3.3.5 Comparison with other studies

This derivatisation method developed in this study has been shown to be accurate, sensitive, specific, and suitable for analysis of a large number of samples – at a minimal run-time. Though the LLOQ (2 ng/ml) was attained at a relatively larger sample volume than recent LC/MS/MS derivatisation methods, this method has a short run-time of 5 minutes, simple mobile phase composition, and a small sample injection volume of 2 µl. One of the recent methods used small sample volumes, but had a long run-time and more solvents for mobile phase preparation (Goodenough *et al.,* 2011). Furthermore, others used a small sample volume, but had longer run-times, more solvents for the preparation of a mobile phase, and used a surrogate matrix rather than an authentic matrix (plasma) for preparation of calibration STDs and QCs (Huang *et al.,* 2014; Xu *et al.,* 2013) – which was not ideal for clinical sample analysis. Table 3.14 summarises the difference between this current method (highlighted) and the recent published LC/MS/MS methods that derivatised 4β-OHC to the corresponding DPE in plasma.

Table 3.12. Difference between current method and recently published LC-MS/MS derivatisation methods

Reference	Year	Concentration range (ng/ml)	Sample volume (µl)	Column	Mobile phase	Flow rate (ml/min)	Sample run time (min)	Matrix for preparation of STDs and QCs
Goodenough *et al.,*	2011	2-500	50	C18 (100 x 2.1 mm x 1.7 µm)	(Isocratic) (A) 0.1% FA in MEOH/H2O (98:2 v/v) (B) 0.1% FA in ACN/ MEOH (50:50 v/v)	0.5	11	Both in plasma (with surrogate analyte 4β-OHC-D7)
Xu *et al.,*	2013	5-500	5	Thermo hypersil gold (50 x 2.1 mm x1.9 µm)	(Gradient) (A) 0.1% FA in H2O (B) 0.1% FA in ACN	0.4	16	Both in 42% (w/v) human serum albumin, in phosphate-buffered saline
Huang *et al.,*	2014	5-500	50	Thermo hypersil gold (50 x 2.1mm x 1.9 um)	(Gradient) (A) 0.1% acetic acid in H2O (B) 0.1% acetic acid in ACN	0.4	12	Plasma for QC (4β-OHC analyte used) and water for STDs
Present		2-500	100	Agilent SB phenyl (100 mm x 2.1 x 1.8 µm)	(Isocratic) 100% - 0.1% FA in (ACN:MEOH) (50:50 v/v)	0.4	5	Both in plasma (surrogate analyte 4β-OHC-D7)

The assay's sensitivity requirements were based on reported data for endogenous levels of 4β-OHC in healthy human plasma – 18-99 ng/ml (Wide *et al.,* 2008). A concentration range of 2-500 ng/ml was achieved for quantification of 4β-OHC in blank human plasma, with an acceptable S/N ratio of 10.2 at LLOQ. The calibration curve was constructed based on the calculated ratio of peak areas of the derivatised products of 4β-OHC-D7 and the ISTD, 4β-OHC-D4. In this assay, 4β-OHC, 4β-OHC-D7 and 4β-OHC-D4, were shown to be stable in ethanol when stored at 4^{o}C and room temperature for 14 days. Stock solution stored at -80^{o}C for 24 hours was used as a reference, since freshly prepared stock solution was unavailable on the day this test was conducted. Other studies have also reported the stability of 4β-OHC at -20^{o}C in stock solution for 6 months (Byers *et al.,* 2014). Validation of the successfully developed derivatisation method is therefore described in the next chapter.

CHAPTER 4: LC/MS/MS METHOD VALIDATION

4.1 Introduction

In order to demonstrate that the LC/MS/MS method, that has been developed, is reliable and reproducible for clinical sample analysis, method validation needs to be performed. The derivatised method for measuring plasma 4β-OHC described in Chapter 3 was successfully validated according to the FDA 2001 and EMA 2012 guidelines. The complete method validation consisted of three validation batches – analysed on three different occasions. Fundamental validation parameters were also assessed, to ensure that the developed method is accurate, precise, and reproducible. The procedures followed during the validation process and results are described next.

4.2 Experimental: Validation of the bioanalytical method

4.2.1 Biological matrix

Calibration STDs and QC samples were prepared in a biological matrix (K_3EDTA blank human plasma) for the validation process. Before using the blank human plasma for the preparation of calibration STDs and QC samples, 12 lots of individual blank plasma sources were assessed for specificity and matrix effect.

4.2.1.1 Blank specificity

This was done to ensure there was no interfering peak at or near the retention time of the surrogate analyte 4β-OHC-D7, or ISTD 4β-OHC-D4, in the human blank plasma. The acceptance criteria for blank specificity are that the mean response of an interfering peak in a blank matrix at the retention time and mass transition of the analyte of interest should not be >20% of the mean response of the analyte at LLOQ and should not be >5% of the ISTD at the working concentration. Figure 4.1 (below) shows the chromatograms of the human blank plasma extract, which illustrate that the blank plasma was free of any interference from matrix components, and hence suitable for preparation of calibration STDs and QC samples.

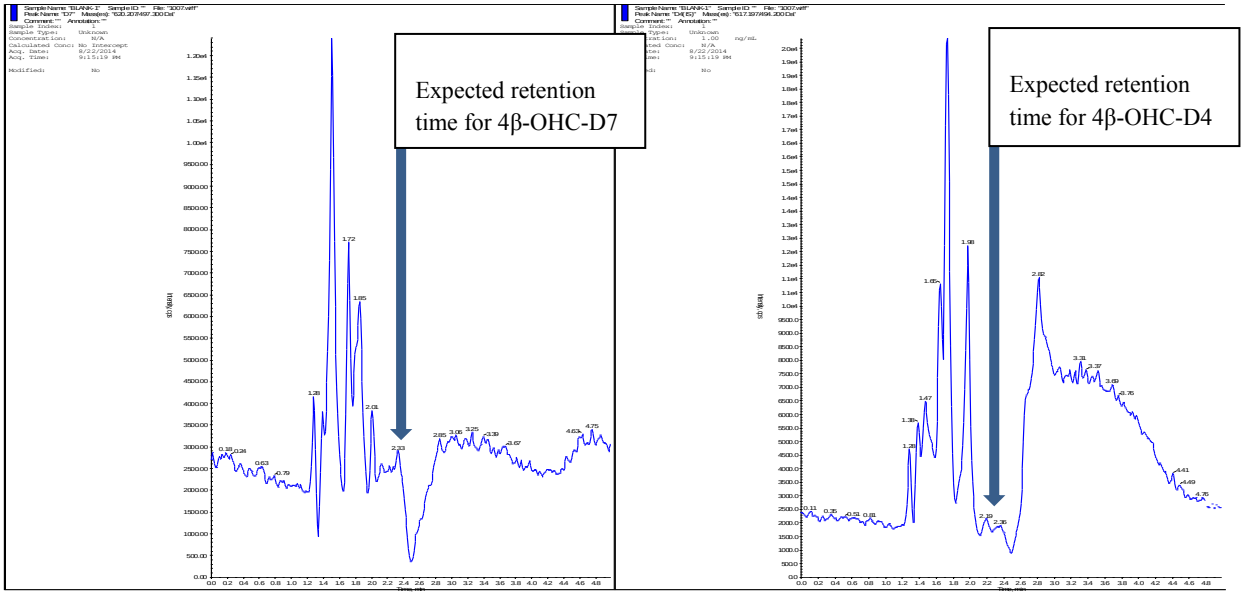


Figure 4.1: Representative chromatogram of human blank plasma extract

4.2.1.2 Matrix effect

The next step was to conduct matrix effect assessment at high and low concentrations, preceding the preparation of calibration STDs and QCs. This was done by assessing six lots of individual sources of matrix. The peak area ratios of the 4β-OHC-D7/4β-OHC-D4, for each level in each individual matrix source, were used to generate regressions. According to the guidelines (the 'Matuszewski method'), the slope variability (% CV) for the six different matrix sources should not be more than 5% to define the influence of the matrix effect. Table 4.1 presents the results of slope variability (% CV) for the six different human blank plasma samples – which was 1.0. This indicates there was no significant ionisation suppression or enhancement observed and that matrix effects do not adversely influence the precision of the assay.

Table 4.1 Matrix effect assessment for 4β-OHC-D7 in blank human plasma

	High Conc. 400 ng/ml Peak Area ratio	Low Conc. 3 ng/ml Peak Area ratio	Area Ratio v Conc. Regression Slope
K3EDTA 07/04/2014-17	1.14	0.0495	0.0028
K3EDTA 07/04/2014-23	1.16	0.0539	0.0028
K3EDTA 07/04/2014-20	1.17	0.0633	0.0028
K3EDTA 07/04/2014-16	1.15	0.0565	0.0028
K3EDTA 07/04/2014-25	1.17	0.0664	0.0028
K3EDTA 07/04/2014-19	1.18	0.0581	0.0028
Average	1.16	0.0580	0.0028
ST DEV	0.015	0.00617	0.00003
% CV	1.3	10.6	1.0

4.2.1.3 Preparation of calibration standards

Calibration STDs were prepared volumetrically in K_3EDTA human blank plasma at room temperature, by spiking 100.5 µl of SS2 (100 µg/ml of 4β-OHC-D7) into 20 ml of human blank plasma (STD 1) – which was then serially diluted with another human blank plasma to attain the desired concentrations of calibration STDs (STD 2- STD 9). Multiple 300 µl aliquots of each calibration STD were stored in individual 1.5 ml polypropylene tubes at approximately -80°C. Table 4.2 (below) shows how the calibrations STDs were prepared.

Table 4.2 Preparation of calibration STDs

Sample ID	Blank Plasma volume (ml)	Volume SS2 spiked (µl)	Dilution Source	Dilution Source Volume (ml)	Total Volume of dilution (ml)	4β-OHC-D7 Conc. (ng/ml)
STD 1 – ULOQ	20.0	100.5			20.1	500
STD 2	10.0		STD 1	10.0	20.0	250
STD 3	10.0		STD 2	10.0	20.0	125
STD 4	10.0		STD 3	10.0	20.0	62.5
STD 5	9.55		STD 4	10.0	19.5	32.0
STD 6	10.0		STD 5	10.0	20.0	16.0
STD 7	10.0		STD 6	10.0	20.0	7.99
STD 8	10.0		STD 7	10.0	20.0	4.00
STD 9 – LLOQ	10.0		STD 8	10.0	20.0	2.00

4.2.1.4 Preparation of quality control samples

Quality control samples were prepared volumetrically in K_3EDTA human blank plasma, at room temperature, using the same methodology used for the calibration STDs. A volume of 200.8 µl of the analyte SS2 (100 µg/ml of 4β-OHC-D7) was then spiked into 24.9 ml of human blank plasma to obtain QC 1 dilute. This QC 1 was serially diluted with human blank plasma to attain the desired concentrations (QC 2–QC 7). Multiple 300 µl aliquots of each QC were stored in individual 1.5 ml polypropylene tubes, and then stored at approximately -80°C. Table 4.3 (below) shows how the QCs were prepared.

Table 4.3 Preparation of QCs

Sample ID	Blank Plasma volume (ml)	Volume SS2 spiked (µl)	Dilution Source	Dilution Source Volume (ml)	Total Volume of dilution (ml)	4β-OHC-D7 Conc. (ng/ml)
QC 1 Dilute	24.9	200.8			25.1	800
QC 2	20.0		QC 1 Dilute	20.0	40.0	400
QC 3	20.0		QC 2	16.4	36.4	180
QC 4	20.0		QC 3	12.7	32.7	70.0
QC 5	20.0		QC 4	8.00	28.0	20.0
QC 6	20.0		QC 5	8.58	28.5	6.00
QC 7	14.0		QC 6	14.0	28.0	3.00

4.2.2 Validation results

In each batch assessed, validation was done by analysing plasma QCs at six concentration levels, (Q2-Q7) in replicates of six, in order to determine the accuracy and precision of the method. The QC values were calculated from a STD regression curve with nine concentration levels (2–500 ng/ml) in duplicate. A validation run sample batch list consisted of: system performance verification STDs (SYS), calibration STDs, blank samples (without surrogate analyte and ISTD), zero samples (without surrogate analyte, but with ISTD), and QCs. Table 4.4 represents a validation run sample batch list.

Table 4.4 Representation of a validation run sample batch list

Sample No.	Sample ID	Sample No.	Sample ID	Sample No.	Sample ID
1	SYS 1	26	QC 6	51	QC 3
2	SYS 2	27	QC 7	52	QC 4
3	SYS 3	28	STD 7	53	QC 5
4	SYS 4	29	STD 8	54	QC 6
5	SYS 5	30	STD 9	55	QC 7
6	STD 1	31	QC 2	56	STD 6
7	BLANK-1	32	QC 3	57	STD 7
8	ZERO-1	33	QC 4	58	STD 8
9	STD 2	34	QC 5	59	BLANK-5
10	STD 3	35	QC 6	60	QC 2
11	BLANK- 2	36	QC 7	61	QC 3
12	QC 2	37	STD 10	62	QC 4
13	QC 3	38	STD 1	63	QC 5
14	QC 4	39	STD 2	64	QC 6
15	QC 5	40	QC 2	65	QC 7
16	QC 6	41	QC 3	66	STD 9
17	QC 7	42	QC 4	67	STD 10
18	STD 4	43	QC 5	68	BLANK-6
19	STD 5	44	QC 6	69	ZERO
20	STD 6	45	QC 7	70	SYS 6
21	BLANK-3	46	STD 3	71	SYS 7
22	QC 2	47	STD 4	72	SYS 8
23	QC 3	48	STD 5	73	SYS 9
24	QC 4	49	BLANK-4	74	SYS 10
25	QC 5	50	QC 2		

The QC samples monitored over the three validation batches were performed on three different occasions to demonstrate intra-batch and inter-batch accuracy and precision. The calibration curve fitted a quadratic (weighted by 1/concentration^2) regression based on the 4β-OHC-D7/4β-OHC-D4 peak area ratio. Guidelines stipulate that for a valid method, the acceptance criteria for intra- and inter-batch accuracy is within 15% (i.e. % accuracy

should be 85% to 115%) over the entire calibration range and within 20% of nominal concentration at the LLOQ. The accepted intra- and inter-batch precision is less than or equal to 15 % (i.e. % CV≤ 15%) over the entire calibration range, and ≤ 20% at the lowest QC (LQC). Results for the intra-batch accuracy and precision for validation are given below.

4.2.2.1 Intra-batch accuracy and precision for validation batch 1

Figure 4.2 (below) depicts the calibration STD curve, which was quadratic $1/x^2$, and further to that, Tables 4.5 and 4.6 present the summary results of back-calculated calibration STD concentrations and QC samples, which were within the acceptable ranges for intra-batch accuracy and precision.

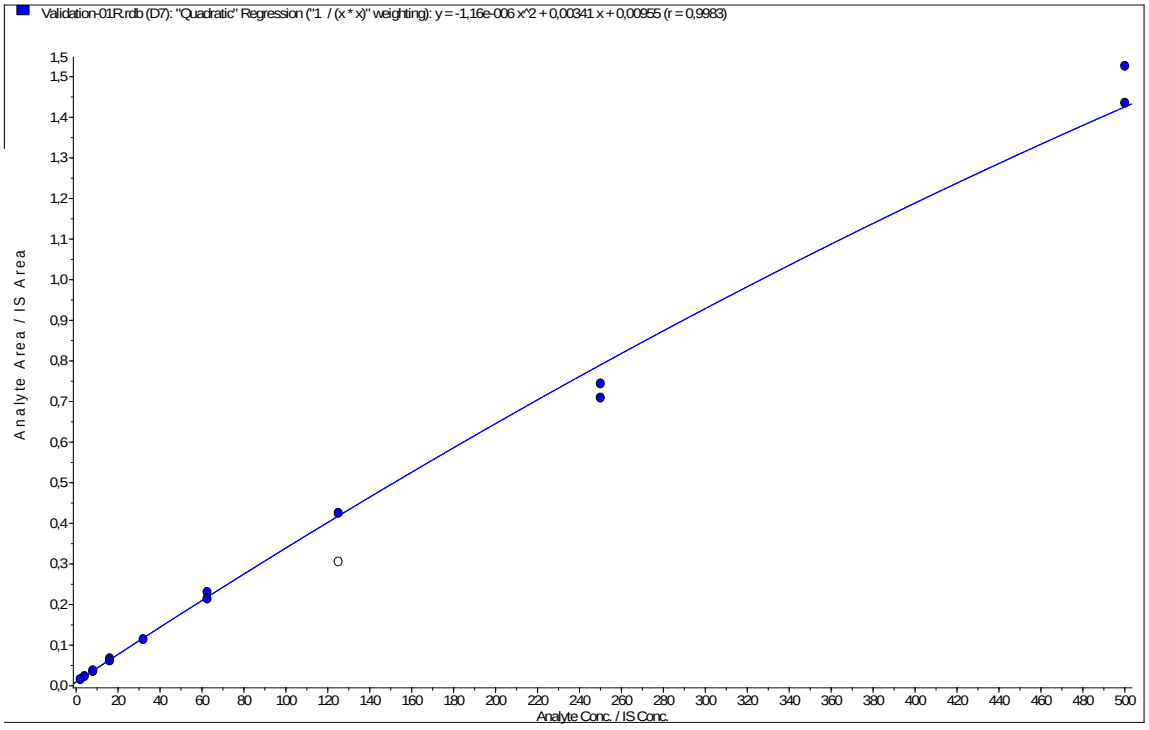


Figure 4.2: Calibration curve for validation batch 1 of 4β-OHC-D7

Table 4.5 Summary of back-calculated calibration STD concentrations of 4β-OHC-D7 for validation batch 1

Sample ID	Nominal Conc. (ng/ml)	Mean Observed Conc. (ng/ml)	Std Dev	% CV	% Accuracy	n
S9	2.00	1.95	N/A	N/A	97.5	*1 of 2
S8	4.00	4.14	N/A	N/A	103.5	*1 of 2
S7	7.99	8.04	0.417	5.2	100.6	2 of 2
S6	16.0	16.2	1.20	7.4	101.6	2 of 2
S5	32.0	31.1	N/A	N/A	97.2	*1 of 2
S4	62.5	63.9	3.61	5.6	102.2	2 of 2
S3	125	108	N/A	N/A	86.6	*1 of 2
S2	250	228	8.49	3.7	91.2	2 of 2
S1	500	526	28.9	5.5	105.1	2 of 2

* lost value due to sample aliquot error

Table 4.6 Summary of QC results of 4β-OHC-D7 for validation batch 1

Sample ID	Nominal Conc. (ng/ml)	Mean Observed Conc. (ng/ml)	Std Dev	% CV	% Accuracy	n
QC 7	3.00	3.34	0.451	13.5	111.4	*5 of 6
QC 6	6.00	5.70	0.841	14.7	95.1	6 of 6
QC 5	20.0	19.5	0.930	4.8	97.6	6 of 6
QC 4	70.0	68.4	2.21	3.2	97.6	6 of 6
QC 3	180	177	6.56	3.7	98.7	6 of 6
QC 2	400	411	33.2	8.1	102.8	6 of 6

* lost value due to sample aliquot error

4.2.2.2 Intra-batch accuracy and precision for validation batch 2

Figure 4.3 (below) indicates the calibration STD curve, which was quadratic ($1/x^2$), while Tables 4.7 and 4.8 (also below) show the summary results of back-calculated calibration STD concentrations and QC samples for batch 2 – which were within the acceptable ranges for intra-batch accuracy and precision.

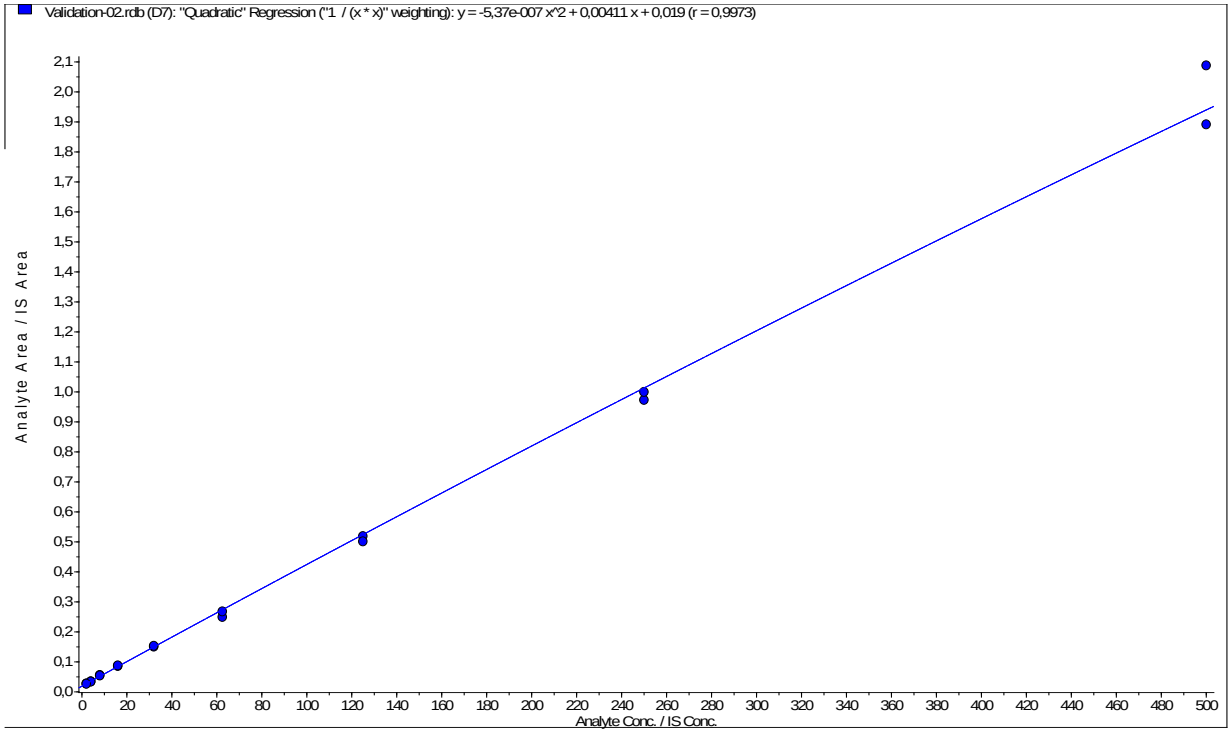


Figure 4.3: Calibration curve of 4β-OHC-D7 for validation batch 2

Table 4.7 Back-calculated calibration STD concentrations of 4β-OHC-D7 for validation batch 2

Sample ID	Nominal Conc. (ng/ml)	Mean Observed Conc. (ng/ml)	Std Dev	% CV	% Accuracy	n
S9	2.00	2.01	0.184	9.1	100.5	2 of 2
S8	4.00	3.74	0.177	4.7	93.6	2 of 2
S7	7.99	8.79	0.283	3.2	110.0	2 of 2
S6	16.0	16.5	0.354	2.1	103.4	2 of 2
S5	32.0	32.4	0.495	1.5	101.4	2 of 2
S4	62.5	58.8	3.18	5.4	94.0	2 of 2
S3	125	121	3.54	2.9	97.2	2 of 2
S2	250	243	4.24	1.7	97.2	2 of 2
S1	500	514	39.6	7.7	102.8	2 of 2

Table 4.8 Summary of QC results of 4β-OHC-D7 for validation batch 2

Sample ID	Nominal Conc. (ng/ml)	Mean Observed Conc. (ng/ml)	Std Dev	% CV	% Accuracy	n
QC 7	3.00	3.15	0.318	10.1	105.1	*5 of 6
QC 6	6.00	6.23	0.473	7.6	104.0	*5 of 6
QC 5	20.0	20.9	1.95	9.3	104.6	6 of 6
QC 4	70.0	70.7	3.12	4.4	100.9	6 of 6
QC 3	180	172	29.2	3.1	96.0	*5 of 6
QC 2	400	400	12.7	3.2	100.1	6 of 6

* lost value due to sample aliquot error

4.2.2.3 Intra-batch accuracy and precision for validation batch 3

Figure 4.4 (below) illustrates the calibration STD curve which was also quadratic ($1/x^2$), and Tables 4.9 and 4.10 (also below) show the summary results of back-calculated calibration STD concentrations and QC samples for batch 3 – which were within the acceptable ranges for intra-batch accuracy and precision.

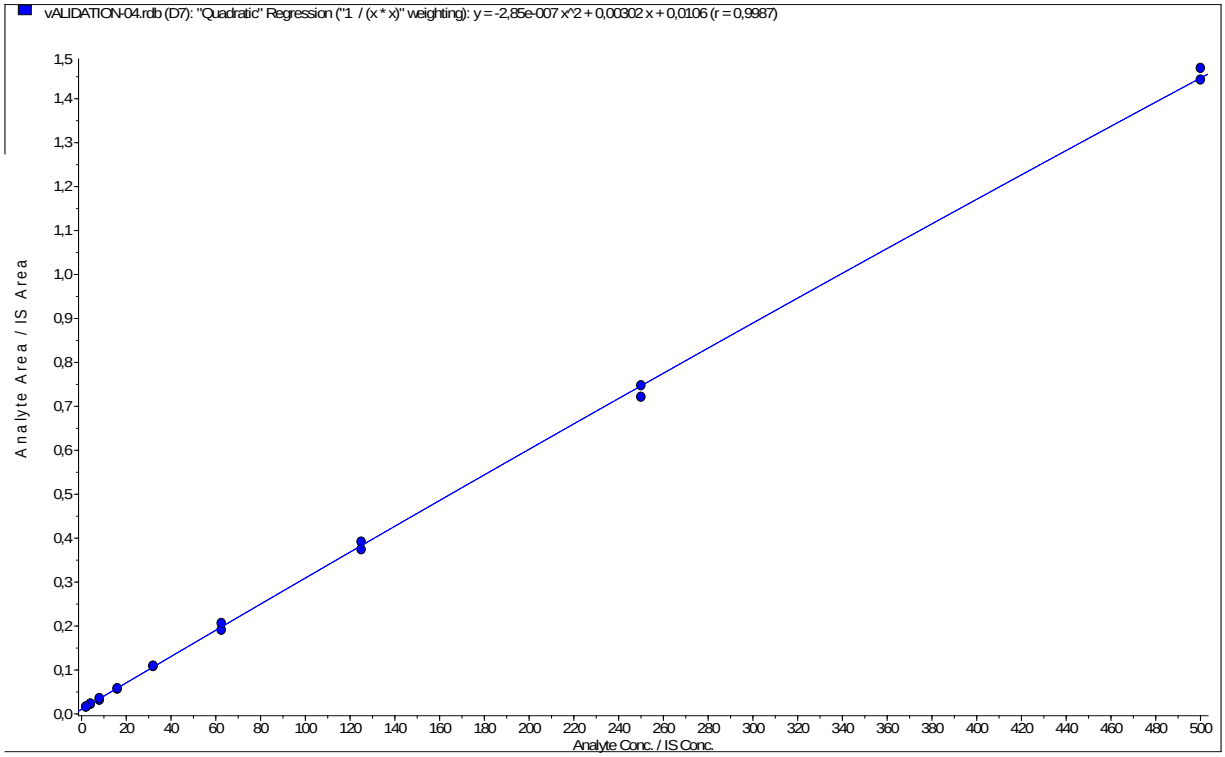


Figure 4.4 Calibration curve of 4β-OHC-D7 for validation batch 3

Table 4.9 Back-calculated calibration STD concentrations of 4β-OHC-D7 for validation batch 3

Sample ID	Nominal Conc. (ng/ml)	Mean Observed Conc. (ng/ml)	Std Dev	% CV	% Accuracy	n
S9	2.00	1.98	0.0541	2.7	98.9	2 of 2
S8	4.00	4.15	0.162	3.7	103.7	2 of 2
S7	7.99	7.75	0.966	12.5	97.9	2 of 2
S6	16.0	15.7	0.279	1.8	98.2	2 of 2
S5	32.0	32.8	0.255	0.8	102.5	2 of 2
S4	62.5	62.9	3.65	5.8	100.6	2 of 2
S3	125	125	4.22	3.4	100.0	2 of 2
S2	250	245	6.41	2.6	98.3	2 of 2
S1	500	504	6.85	1.4	100.7	2 of 2

Table 4.10 Summary of QC results of 4β-OHC-D7 for validation batch 3

Sample ID	Nominal Conc. (ng/ml)	Mean Observed Conc. (ng/ml)	Std Dev	% CV	% Accuracy	n
QC 7	3.00	2.89	0.259	8.9	96.6	6 of 6
QC 6	6.00	6.17	0.225	3.6	102.8	6 of 6
QC 5	20.0	20.2	0.745	3.7	100.9	6 of 6
QC 4	70.0	70.3	1.36	1.9	100.4	6 of 6
QC 3	180	183	4.73	2.6	101.9	6 of 6
QC 2	400	401	11.5	2.9	100.3	6 of 6

4.2.2.4 Inter-batch accuracy and precision

The inter-batch accuracy and precision of the assay procedure were assessed by calculating the accuracy and precision statistics for three consecutive batches. Tables 4.11 and 4.12, show the combined back-calculated calibration STD concentrations and QC samples for all the three batches – which were within the acceptable range for inter-batch accuracy and precision.

Table 4.11 Summary of back-calculated calibration STD concentrations results of 4β-OHC-D7 for three validation batches

Sample ID	Nominal Conc. (ng/ml)	Mean Observed Conc. (ng/ml)	Std Dev	% CV	% Accuracy	n
S9	2.00	1.98	0.09	5.0	99.3	*5 of 6
S8	4.00	3.99	0.24	6.3	99.7	*5 of 6
S7	7.99	8.19	0.68	8.3	102.5	6 of 6
S6	16.0	16.1	0.69	4.3	101.0	6 of 6
S5	32.0	32.3	0.76	2.4	101.0	*5 of 6
S4	62.5	61.8	3.62	5.8	98.9	6 of 6
S3	125	118	14.4	12.2	94.6	*5 of 6
S2	250	238	9.89	4.1	95.5	6 of 6
S1	500	514	24.2	4.7	102.9	6 of 6

* lost value due to sample aliquot error

Table 4.12 Summary of the combined QC results of 4β-OHC-D7 for the three validation batches

Sample ID	Nominal Conc. (ng/ml)	Mean Observed Conc. (ng/ml)	Std Dev	% CV	% Accuracy	n
QC 7	3.0	3.11	0.37	12	103	*16 of 18
QC 6	6.0	6.03	0.59	9.9	100	*17 of 18
QC 5	20.0	20.2	1.37	6.8	101	18 of 18
QC 4	70.0	69.8	2.43	3.5	99.7	18 of 18
QC 3	180	178	17.0	9.6	98.9	18 of 18
QC 2	400	404	20.8	5.2	101	18 of 18

* lost value due to sample aliquot error

4.2.3 Validation fundamental parameters

Fundamental validation parameters were successfully assessed, and are described below.

4.2.3.1 Stability assessment in matrix

a. Freeze-thaw stability

For the determination of freeze-thaw stability, high and low QCs were frozen at -80ºC, and then put through three consecutive freeze and thaw cycles. Sample aliquots were prepared

and stored frozen, for at least 24 hours – before starting this experiment. Each cycle consisted of unassisted thawing time at room temperature, followed by 12 to 24 hours' freezing time. These samples were analysed in one of the inter-day validation batches against a valid calibration curve and compared to controls from the batch, analysed at the same concentration. A % CV of more than 15% of the measured peak area ratio value at high and low concentration – indicates unacceptable freeze-thaw instability. High and low concentration samples that were assessed are presented in Tables 4.13 and 4.14 (below) respectively. Notably, the % CV of the surrogate analyte and ISTD were high due to the drop of instrument response – rather than surrogate analyte and ISTD instability. The ISTD compensated well for the decrease in instrument response. The results showed that the measured peak-area ratio values, at both high and low concentration levels, met the required acceptance criteria and that surrogate analyte and ISTD were stable over three freeze-thaw cycles.

Table 4.13 Freeze and thaw stability samples of 4β-OHC-D7 at high concentration

High Concentration (400 ng/ml)			
Sample	**Surrogate analyte peak area**	**ISTD peak area**	**Ratio**
QC 2	7751000	7631000	1.016
QC 2	6785000	6843000	0.992
QC 2	5443000	5057000	1.076
QC 2	5003000	4867000	1.028
QC 2	4204000	4099000	1.026
QC 2	*(2814000)	*(2680000)	* (1.050)
Average	5837200	5699400	1.027
ST DEV	1421230	1475275	0.031
% CV	24.3	25.8	3.0

* Statistical outlier (see Appendix C (Tables C1 and C2) for MNR outlier test)

Table 4.14 Freeze and thaw stability of 4β-OHC-D7 at low concentration

Low concentration (3 ng/ml)			
Sample	**Surrogate analyte peak area**	**ISTD peak area**	**Ratio**
QC 7	137600	7321000	0.019
QC 7	149800	7451000	0.020
QC 7	88230	4554000	0.019
QC 7	135100	6778000	0.010
QC 7	106200	5351000	0.010
QC 7	*(73100)	*(2359000)	*(0.031)
Average	123386	6291000	0.020
ST DEV	25338.9913	1279105	0.001
% CV	20.5	20.3	2.7

* Statistical outlier (see Appendix (Tables C3 and C4) for MNR outlier test)

b. Bench-top stability

In order to ascertain bench-top stability, high and low QCs were frozen at -80ºC, and subsequently left on the bench for approximately 15 hours. These samples were analysed against a valid calibration curve. A % CV of more than 15% of the measured peak-area ratio values, indicate bench-top instability. The calculated peak-area ratios for the high and low QCs are presented in Tables 4.15 and 4.16 (below) respectively. The results from these Tables show that the calculated % CV of the peak-area ratio was within the acceptable range, and that extracted samples containing the surrogate analyte and ISTD were stable on the bench for 15 hours.

Table 4.15 Bench-top stability of 4β-OHC-D7 high concentration

High Concentration (400 ng/ml)			
Sample	**Surrogate analyte peak area**	**ISTD peak area**	**Ratio**
QC 2	6989000	6886000	1.015
QC 2	6423000	6415000	1.001
QC 2	7489000	7292000	1.027
QC 2	5985000	5759000	1.039
QC 2	6025000	5851000	1.030
QC 2	*(3585000)	*(3494000)	1.026
Average	6582200	6440600	1.023
ST DEV	648119	658810	0.013
% CV	9.8	9.2	1.3

* Statistical outlier (see Appendix (Tables C5 and C6) for MNR outlier test)

Table 4.16 Bench-top stability of 4β-OHC-D7 low concentration

Low Concentration (3 ng/ml)			
Sample	**Surrogate analyte peak area**	**ISTD peak area**	**Ratio**
QC 7	149100	8019000	0.019
QC 7	117800	6185000	0.019
QC 7	134100	7329000	0.018
QC 7	125100	6179000	0.020
QC 7	125200	5842000	0.021
QC 7	*(85150)	* (3377000)	0.025
Average	130260	6710800	0.020
ST DEV	12012	922990	0.003
% CV	9.2	13.8	12.7

* Statistical outlier (see Appendix (Tables C7 and C8) for MNR outlier test)

c. <u>On-instrument stability</u>

On-instrument stability was assessed for the period of time that the extracted samples were expected to stay on-instrument during the batch run-time. Six QC samples from each of the high and low concentration levels – to be used as on-instrument stability (OIS) samples – were extracted and stored at 4^{o}C (Autosampler temperature) for 34 hours. Six QCs from each of the high and low concentration levels – to be used as on-instrument reference (OIS

ref) samples – were extracted and assayed together with the stored extracts. The peak areas of the surrogate analyte and the ISTD and the ratios of the OIS-samples and OIS-reference samples were compared. The high difference (more than 15%) between the mean ratios of the OIS stability samples and the OIS reference samples, and the more than 15% CV of the measured ratio values, could indicate on-instrument instability. The results of the peak-area ratios calculated were within the acceptable range – which shows that extracted samples were stable on-instrument for 34 hours. The results of high and low on-instrument reference samples are presented in Tables 4.17 -4.18 (below), and the results of high and low on-instrument stability samples are presented in Tables 4.19 -4.20 (below) respectively.

Table 4.17 On-instrument reference samples of 4β-OHC-D7 at high concentration

High Concentration (400 ng/ml)			
Reference sample	**Surrogate analyte Peak area**	**ISTD peak area**	**Ratio**
Injection 1	7372000	7201000	1.024
Injection 2	7274000	7219000	1.008
Injection 3	7574000	7275000	1.041
Injection 4	7529000	7266000	1.036
Injection 5	7007000	6990000	1.002
Injection 6	7382000	7049000	1.047
Average	7356333	7166667	1.026
ST DEV	203425	118809	0.018
% CV	2.8	1.7	1.8

Table 4.18 On-instrument reference samples of 4β-OHC-D7 at low concentration

Low Concentration (3 ng/ml)			
Reference sample	**Surrogate nalyte peak area**	**ISTD peak area**	**Ratio**
Injection 1	125000	6994000	0.018
Injection 2	136500	7200000	0.019
Injection 3	131700	7295000	0.018
Injection 4	130300	6891000	0.019
Injection 5	136900	7426000	0.018
Injection 6	125100	6908000	0.018
Average	130917	7119000	0.018
ST DEV	5231	220869	0.0005
% CV	4.0	3.1	2.5

Table 4.19 On-instrument stability samples of 4β-OHC-D7 at high concentration

High Concentration (400 ng/ml)			
Stability sample	**Surrogate analyte Peak area**	**ISTD peak area**	**Ratio**
Injection 1	4927000	4773000	1.032
Injection 2	4913000	4719000	1.041
Injection 3	4985000	4850000	1.027
Injection 4	5308000	5094000	1.042
Injection 5	5764000	5573000	1.034
Injection 6	5664000	5487000	1.032
Average	5260167	5082667	1.034
ST DEV	381172	370492	0.005
% CV	7.2	7.3	0.5
% Difference			0.8

Table 4.20 On-instrument stability samples of 4β-OHC-D7 at low concentration

Low Concentration (3 ng/ml)			
Stability sample	**Surrogate analyte Peak area**	**ISTD peak area**	**Ratio**
Injection 1	124200	6026000	0.021
Injection 2	105100	5645000	0.019
Injection 3	104700	5597000	0.019
Injection 4	111000	6081000	0.018
Injection 5	115500	6063000	0.019
Injection 6	112000	6061000	0.018
Average	112083	5912167	0.019
ST DEV	7252	226748	0.001
% CV	6.5	3.8	4.5
% Difference			0.1

4.2.3.2 Sensitivity

Sensitivity of this assay was assessed at 2 ng/ml, which was the LLOQ. The acceptable mean analyte S/N response at LLOQ, should be at least more than five times the response compared to the blank response at the retention time of interest. Figure 4.5 (below) shows the chromatogram of 4β-OHC-D7 at LLOQ, with the acceptable S/N ratio of 10.2.

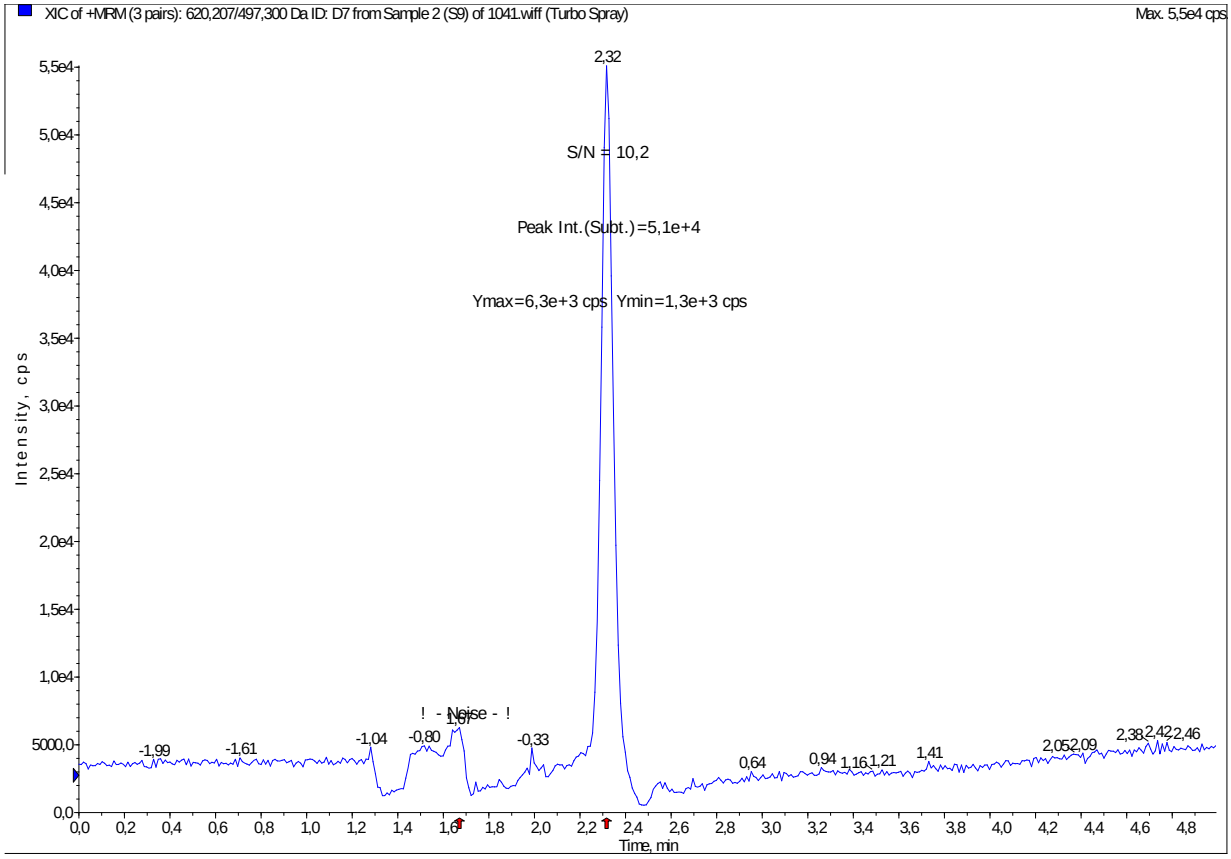


Figure 4.5 Signal to noise ratio of 4β-OHC-D7 at 2 ng/ml (LLOQ)

4.2.3.3 Carry-over

Carry-over was monitored in each core validation run, by placing six blank samples (without surrogate analyte and ISTD) in the injection sequence – in such a way that the highest calibration STDs were followed by a blank sample. Two zero samples (without surrogate analyte, but with ISTD) were also included in each run to determine the possible contamination of the surrogate analyte by the ISTD, without an additional carry-over effect. Representative blank and zero sample chromatograms are presented in Figures 4.6 and 4.7 (below) respectively.

To determine carry-over in each core validation run, six blank samples were positioned in the injection sequence, in such a way that the highest calibration STDs were followed by a blank sample. During carry-over assessment in blank samples, the peak observed for the analyte should not be more than 20% of the area of the peak obtained at the LLOQ. A peak observed for the ISTD should not be more than 5% of the peak observed for the ISTD at the working concentration. A chromatogram of a blank sample presented in Figure 4.6

(below) shows that there was no surrogate analyte and ISTD carry-over observed.

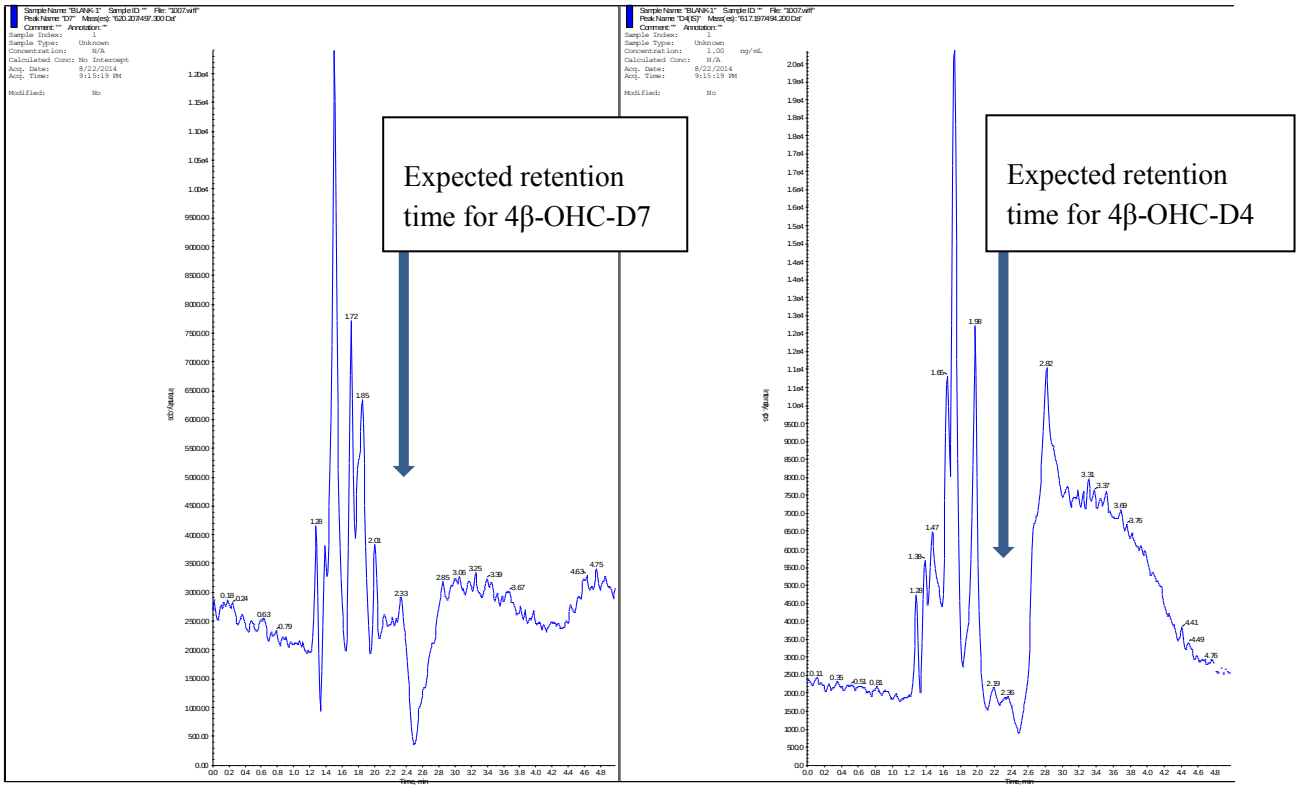


Figure 4.6 Chromatogram of a blank plasma sample

Two zero samples were also included to determine the possible contamination of the surrogate analyte by the ISTD, without an additional carry-over effect. The acceptance criteria state that a peak observed for the analyte when ISTD is present at the working concentration, should not be more than 20% of the area of the peak obtained at LLOQ. A chromatogram of a zero sample presented in Figure 4.8 and calculations shown in Table 4.21 illustrate that there was no significant contamination of the surrogate analyte by the ISTD.

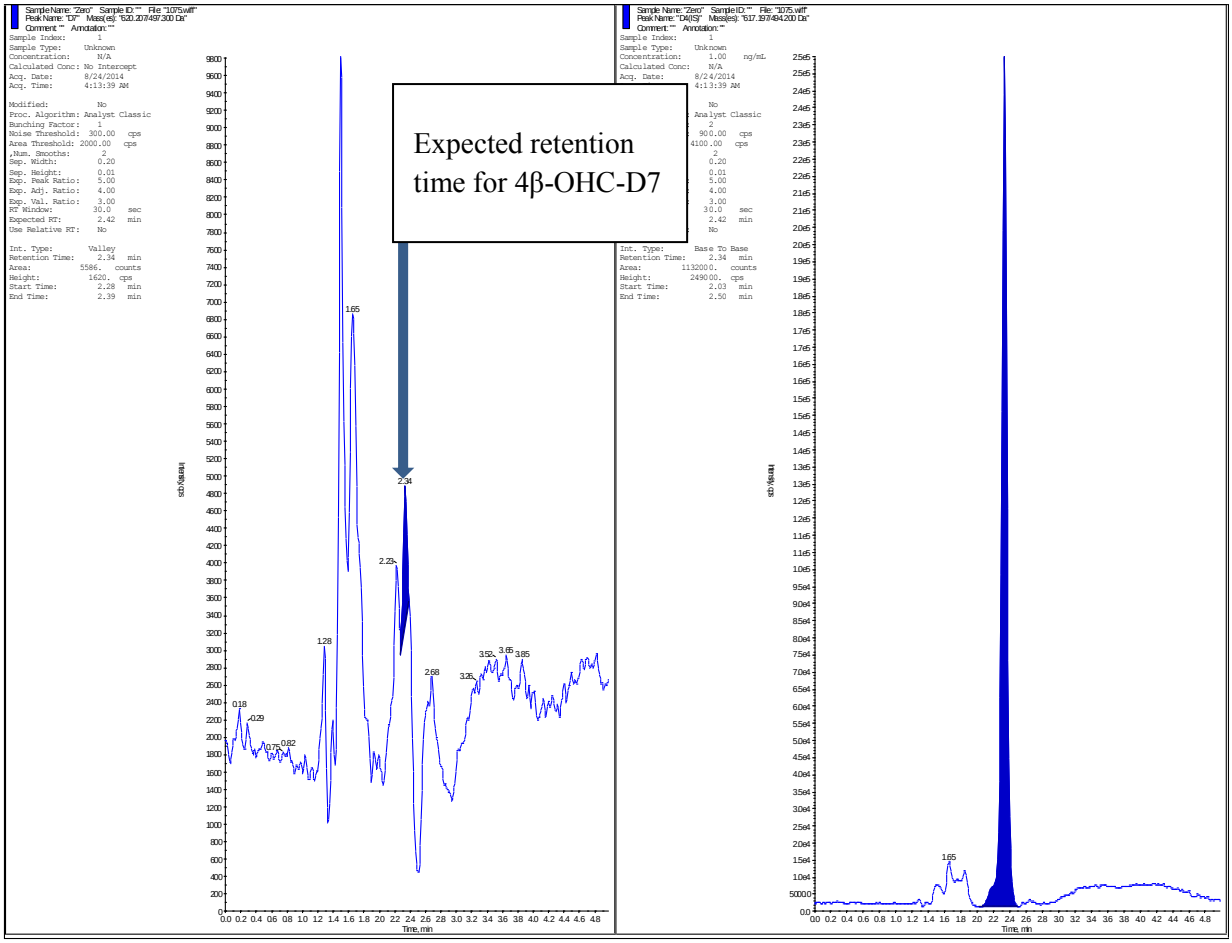


Figure 4.7 Chromatogram of a zero plasma sample

Table 4.21 Calculated percentage of surrogate analyte in a zero sample and at LLOQ

	Analyte peak area counts
Zero	5586
LLOQ	28130
% Contamination	19.9

4.2.3.4 Recovery

Recovery was determined by comparing the responses measured for pre-extraction spiked QC samples (test samples) – with responses measured for post-extraction spiked samples (theoretical, represents 100% recovery) of 4β-OHC-D7 in six-fold, at high, medium and low concentrations, and 4β-OHC-D4 at one working concentration. The ratio of the 4β-

OHC-D7 and 4β-OHC-D4 peak area found after extraction, to the theoretical peak area, was expressed as the percentage recovery. The acceptance mean recovery of a quantitative assay method should be consistent with a % CV of not more than 15 – for any particular concentration of the analyte, at which it is determined. Recovery reproducibility between concentration levels should also not be more than 15%. Tables 4.22 and 4.23 (below) are the recovery results for 4β-OHC-D7 and 4β-OHC-D4, respectively – showing that the mean recovery of 4β-OHC-D7 and 4β-OHC-D4 from the blank human plasma was consistent and precise.

Table 4.22 Absolute recovery for 4β-OHC-D7 using three response factors

Sample	Analyte Concentration (ng/ml)	Mean of peak areas		Absolute Recovery (%)	% CV
		After Extraction	Theoretical Values		
High	400	13108333	17275875	75.9	5.0
Medium	20	791450	1127126	70.2	6.5
Low	3	196433	275137	71.4	10
			Mean:	72.5	7.2

Table 4.23 Absolute recovery for ISTD (4β-OHC-D4) using one response factor

ISTD Concentration ng/ml	Mean of peak areas		Absolute Recovery (%)	% CV
	After Extraction	Theoretical Values		
200	9509200	12899320	73.7	4.9

4.2.3.5 Haemolysis effect

The presence of haemolysed blood in samples could affect the ionisation of the analyte and ISTD. Determination of the haemolysis effect was tested at 1% haemolysed blood in plasma, which was prepared by adding 1 ml of whole human blood in 99 ml of human blank plasma and vortex mixed. A high percentage difference and a % CV of more than 15 of the peak area ratios observed in haemolysed samples and normal samples, indicates that haemolysis has an effect on the assay of the analyte and that the ISTD does not sufficiently compensate for the analyte. The results reported in Table 4.24 (below) show that the

% difference and % CV are within the acceptable range and that 1% haemolysed samples did not adversely influence the precision of the assay.

Table 4.24 Effect of 1% Haemolysis on 4β-OHC-D7

	High Concentration (400 ng/ml)		Low Concentration (3 ng/ml)	
	Observed normal Peak area ratio	**Observed Haemolysed peak area ratio**	**Observed normal peak area ratio**	**Observed Haemolysed peak area ratio**
Sample 1	1.15	1.30	0.018	0.020
Sample 2	1.23	1.27	0.018	0.019
Sample 3	1.17	1.32	0.019	0.020
Sample 4	1.15	1.34	0.019	0.019
Sample 5	1.20	1.36	0.017	0.019
Sample 6	1.11	1.37	0.018	0.019
Average	1.17	1.33	0.018	0.019
ST DEV	0.04	0.03	0.0005	0.0004
% CV	3.4	2.8	2.8	2.5
% Difference		13.7		5.6

4.2.4 Discussion

The method performed well during intra and inter batch validations and passed the acceptance criteria for peak areas with the use of an ISTD. The calibration curve fitted quadratic (weighted by $1/x^2$) regression over the range of 2–500 ng/ml. The S/N ratio at LLOQ (2 ng/ml) was above the minimum international acceptance criteria (European Medicines Agency, 2012; Guidance for industry, 2001).

No significant matrix effects influencing 4β-OHC-D7 and 4β-OHC-D4 were found. During stability assessments, 4β-OHC-D7 and 4β-OHC-D4 were found to be stable at the three freeze-thaw cycles, stable on-instrument for 34 hours, and stable on-bench for 15 hours. Notably, during three freeze-thaw stability assessments, the % CV of 4β-OHC-D7 and 4β-OHC-D4 at high and low concentrations was high, not because of instability – but rather due to the drop of instrument response. The ISTD compensated well and the % CV

of peak-area ratios were within the acceptable range (%CV ≤ 15). The long-term stability of 4β-OHC was not assessed in this assay, because the time period for validation was shorter than the storage period of the clinical samples which the method was applied to. However, long-term stability of 4β-OHC was assessed in previous studies and it was found to be stable after 203 days at -20^{o}C and 120 days at -80^{o}C (Xu *et al.,* 2013; Goodenough *et al.,* 2011). This study's stability results were similar to previous studies which assessed endogenous 4β-OHC directly – instead of SIL analogues for three freeze-thaw, bench-top and on-instrument stability assessment. The mean recovery for 4β-OHC-D7 and 4β-OHC-D4 was 72.5% and 73.7% – which was encouraging considering the multiple steps during the sample extraction process. No significant carry-over was observed in the blank and zero samples, and 1% hemolysis of blood did not affect the ionisation of 4β-OHC-D7 and 4β-OHC-D4 in the human plasma.

It can be concluded, therefore, that the method performed well during the course of the entire three validation batches and fundamental parameters assessed, and with accuracy and precision within the acceptable ranges. This made the developed and validated method suitable for clinical sample analysis – which will be described in the next chapter (Chapter 5).

CHAPTER 5: APPLICATION OF THE DEVELOPED AND VALIDATED METHOD ON CLINICAL SAMPLES AND RESULTS

After the developed and validated method was made suitable for clinical analysis, the method was applied on clinical samples – where plasma 4β-OHC was used as a biomarker to investigate the levels of CYP3A induction in African HIV-infected children with and without treatment containing NNRIs. A quadratic equation (shown in Appendix B Equation 1) was used to calculation plasma 4β-OHC concentrations in the clinical samples. Analysis was done to compare difference in plasma 4β-OHC concentrations between the three different treatment groups and in terms of assessing the effect of time on treatment, age, weight and sex.

5.1 Samples and characteristics of the study population

The plasma samples used in this study were from a previous study (CHAPAS -3 Trial (ISRCTN69078957; UCT HREC/REF 143/2010)), which was aimed at comparing the toxicity and pharmacokinetics of three fixed-dose combination-based antiretroviral regimes for treatment of HIV-infected children in Africa. The CHAPAS -3 Trial enrolled 470 male and female children who were confirmed to have HIV infection. Recruitment of the children for the study occurred over a period of 18 months from one Zambian and three Ugandan paediatric clinical centres, and they were followed for a minimum period of 96 weeks. Children were included in the study according to the eligibility criteria below:

- Children less than 5 years old, who were previously untreated with ART except for exposure to perinatal ART for the prevention of mother-to-child HIV transmission and who met WHO 2010 criteria for starting ART.
- Children 5 years old and above who had a viral load (VL) <50 copies/ml and who were on ART regime containing stavudine for ≥ 2 years.

All children were divided into 2 strata: stratum 1 (ART naïve children); and stratum 2 (ART experienced children). Randomisation was also stratified by clinical centre, age and by NNRTI (NVP or EFV).

Children were randomised 1:1:1 to receive stavudine, zidovudine or abacavir – together with lamivudine and either NVP or EFV. In accordance with paediatric treatment recommendation guidelines, all children <3 years in the study received NVP. Serial blood

samples were collected from the children over the study period.

5.2 Sample selection for this study

In this study, plasma samples were analysed from 66 children. The samples were from three groups: ART naïve children starting NVP (Naïve group); ART experienced children staying on NVP (Nevirapine group); and ART experienced children switching from NVP to EFV (Efavirenz group). The sample selection process was not based on any known factors that could affect the results. The selection of samples was based on availability of samples from the CHAPAS-3 study sites, and for naïve children the samples available were from the younger children. The characteristics of the children and the plasma samples observed are summarised in Table 5.1 (below).

Table 5.1 Characteristics of the children and plasma samples collected

Characteristics	**Naïve group n=27**	**Nevirapine group n=29**	**Efavirenz group n=10**
Sex, n (%)			
Male	12 (44.4%)	14 (48.2%)	7 (70%)
Female	15 (55.6%)	15 (51.7%)	3 (30%)
Baseline age (years), median (iqr)	1.33 (0.82-1.61)	5.5 (5.3-5.79)	7.3 (6.93-8.65)
Baseline weight (kg), median (iqr)	8.6 (7.5-9.8)	17.8 (16.5-19.1)	20.1 (19.3-22.7)
Number of samples at baseline	27	29	10
Number of samples at week 36	12	6	1
Number of samples at week 60	24	16	4
Number of samples at week 84	26	29	10
Number of samples at week 108	15	23	9
Number of samples at week 132	2	10	5
Total number of samples observed	106	113	39

n = number of children, iqr = interquartile range.

5.3 Sample size calculation

The primary objective of this study was to evaluate changes (within individuals) and differences (between patient groups) in 4β-OHC plasma concentrations. A coefficient of variation of 34% in baseline 4β-OHC concentrations, was reported in Swedes and

Tanzanians (Diczfalusy *et al.,* 2008a). Based on this assumption and allowing for an increase in the sample size of 15% to compensate for potentially skewed data, a sample size of at least 20 in each group was necessary to detect a difference in 4β-OHC concentrations between groups with power = 0.9 (or 15 in each group with power = 0.8). The ultimate sample size in this study was 27 in the naïve group, 29 in the NVP group, and 10 in the EFV group. The EFV group had a limited number of samples of 10, as these were the only suitable baseline samples available and therefore the desired sample size was not reached for this group. Considering this, a conservative approach was applied to the naïve and NVP groups – with a larger sample size where these were available.

5.4 Patient blood sample collection

Each child had 4 serial time points selected at different weeks, when plasma samples were collected: baseline, week 36, week 60, week 84, week 108 and week 132 (Table 5.1). For each participant, one sample was taken at baseline and two samples (taken on the same day, 2 or more hours apart) at each of the subsequent selected weeks. A total of 6 samples were lost during laboratory processes.

5.5 Ethics and institutional approval

This was a sub study of the CHAPAS -3 Trial. Approval to use the plasma samples was obtained from the Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (Reference number HREc/REF: 076/2015).

5.6 Statistical analysis

For the baseline plasma 4β-OHC concentration, a single plasma sample was analysed. For subsequent 4β-OHC concentrations of two samples (taken on the same day, 2 or more hours apart), an average of two samples was used – since no substantial difference or consistent trend between the 2 samples was found. Data analysis was done using Stata 12 software (StataCorp, College station, Texas ,USA). The Shapiro-Wilks normality test was used to test the data for normality. Summary statistics were used to provide a general description of the study population by week, age, weight and sex. Descriptive statistics were expressed as means ± SD and medians (IQR) for the continuous data. The plasma 4β-

OHC concentrations were reported as medians because they were non-parametric. The Wilcoxon signed ranked test was used to test the null hypothesis for determining difference in 4β-OHC concentrations between each group from the others (naïve and NVP groups; naïve and EFV groups; NVP and EFV groups). A multi-level mixed-effect model was used to determine the effect of time on treatment, baseline age, weight and sex, on 4β-OHC concentrations in each group. A p-value of less than 0.05 was considered to be statistically significant.

5.7 Determination and comparison of 4β-OHC baseline concentrations in plasma of naïve, NVP and EFV groups

Baseline 4β-OHC concentrations among the groups were compared. Notably, it was found that there was a significant difference between the naïve and NVP group (p = 0.008), and the naïve and EFV group (p = 0.005). There was no significant difference observed between the NVP group and EFV group (p= 0.64). Figure 5.1 (below) presents 4β-OHC concentrations among the three treatment groups.

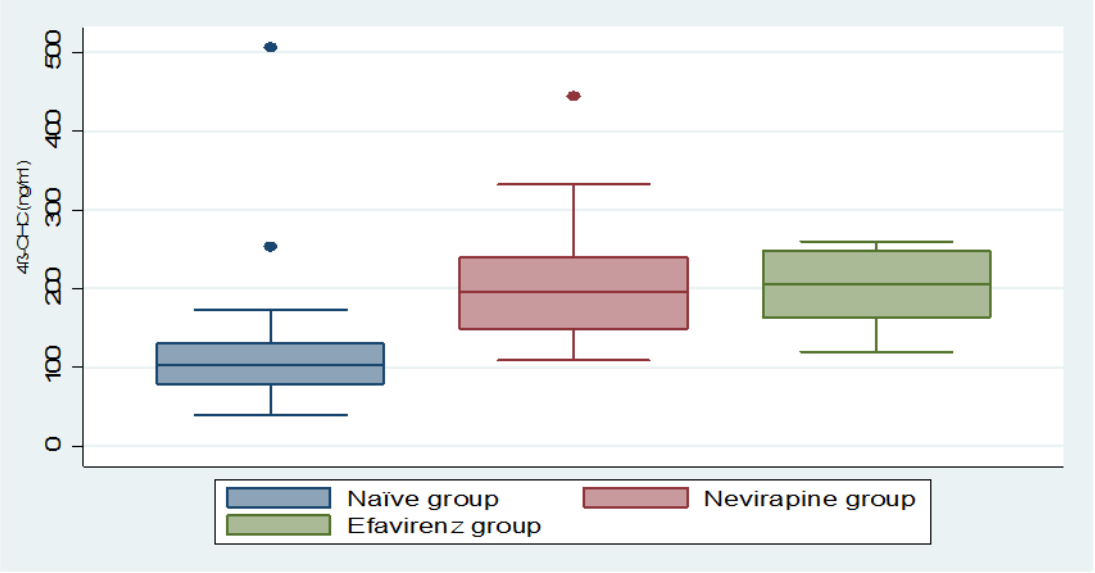


Figure 5.1 Baseline 4β-OHC concentrations among the three treatment groups

(horizontal bars in boxes represent median concentration, whiskers represent minimum and maximum concentrations, boxes represent 25th and 75th percentiles, and "•" represent outliers)

5.8 Determination and comparison of plasma 4β-OHC concentrations between NVP and EFV groups at different weeks on treatment

Plasma 4β-OHC concentrations between the NVP and EFV groups were compared at weeks 60, 84, 108 and 132. Table 5.2 (below) shows the results of a comparison in median 4β-OHC concentrations between the NVP and EFV groups at different weeks on treatment, and the associated p-values. No comparison was done between the NVP and EFV groups at week 36, because the EFV group had only one sample available at that week. A statistically significant difference in 4β-OHC concentrations was only detected at week 84 – where the median 4β-OHC concentration was higher in the EFV group than the NVP group.

Table 5.2 Results of a comparison of median 4β-OHC concentrations between NVP and EFV at different weeks on treatment

Week	Median 4β-OHC in ng/ml (n)				P-value
	NVP group		EFV group		
60	147	(16)	220	(4)	0.11
84	147	(29)	180	(10)	0.03
108	169	(23)	222	(9)	0.23
132	162	(10)	209	(5)	0.39

n= number of samples; NVP = Nevirapine; EFV = Efavirenz

5.9 Effect of time, age, sex, and weight

5.9.1 Naïve group

In this treatment group, a significant increase in 4β-OHC concentrations between baseline and each of the non-baseline weeks on treatment – except at week 132 was observed. No significant difference was observed in 4β-OHC concentrations between non-baseline weeks – with each of the non-baseline weeks as a reference. There was no significant correlation found between 4β-OHC concentrations and age and weight at baseline. In addition, no significant difference was found between 4β-OHC concentrations and sex in this group. Table 5.3 shows the results of a multi-level mixed-effect model determining the effect of time on treatment, baseline age, weight and sex on 4β-OHC concentrations in the naïve group.

Table 5.3 Results of a multi-level mixed-effect model determining the effect of time on treatment, baseline age, weight and sex on 4β-OHC concentrations in the naïve group (n=27)

	Coefficient	Std. Err.	Z	P>z	95% confidence Interval	
Baseline (n=27)	Reference					
Week 36 (n=12)	109.64	29.38	3.73	0.000	52.06	167.23
Week 60 (n=24)	89.45	22.83	3.92	0.000	44.71	134.19
Week 84 (n=26)	85.05	22.24	3.82	0.000	41.46	128.65
Week 108 (n=15)	103.55	26.95	3.84	0.000	50.73	156.38
Week 132 (n=2)	78.94	65.47	1.21	0.23	-49.38	207.25
Sex (Male)	Reference					
Female	17.74	40.02	0.44	0.66	-60.69	96.19
Baseline weight (kg)	2.56	19.96	0.13	0.89	-36.56	41.68
Baseline age (years)	-9.39	66.24	-0.14	0.89	-139.22	120.43

n = number of samples observed

From baseline to week 132 no significant difference was found, though the 4β-OHC level increased. This could be attributed to the smaller sample size at that time point. Figures 5.2 and 5.3 (below) show plots of 4β-OHC concentrations in the naïve group over the study period.

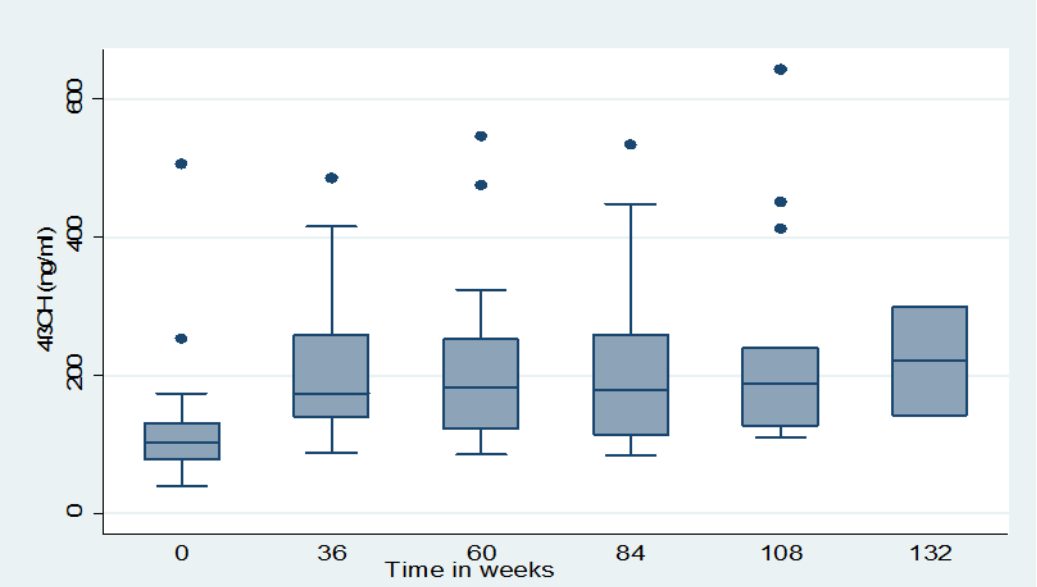


Figure 5.2 4β-OHC concentrations in the naïve group over the study period

(horizontal bars in boxes represent median concentration, whiskers represent minimum and maximum concentrations, boxes represent 25th and 75th percentiles, and "•" represent outliers).

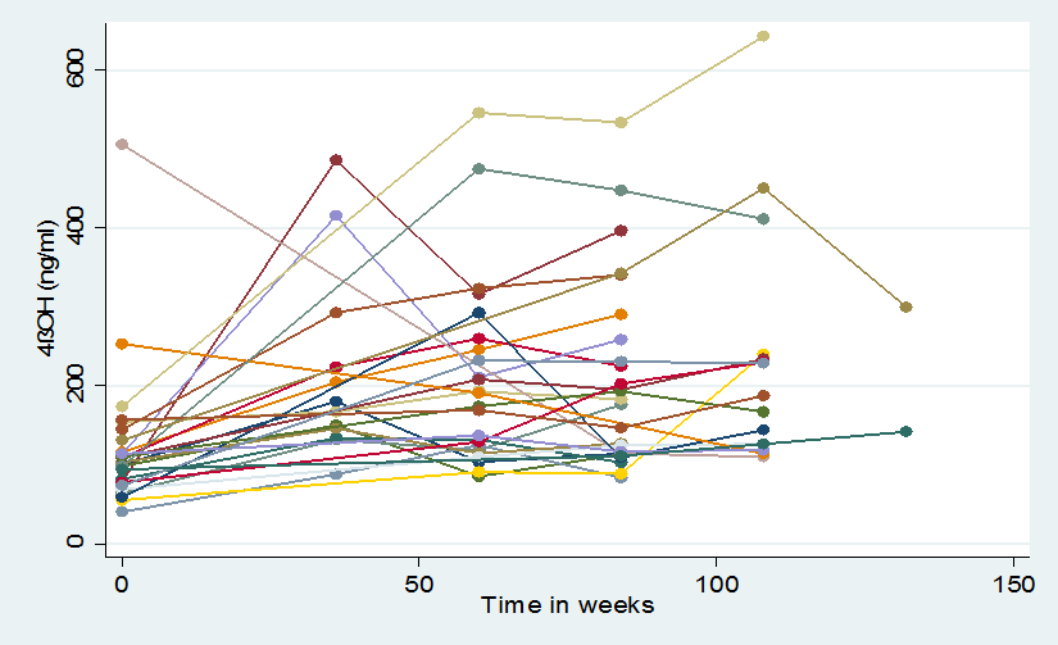


Figure 5.3 4β-OHC concentration trends in individual patients in the naïve group over the study period

Figures 5.4 and 5.5 show lack of correlation between 4β-OHC concentrations and age and weight, respectively, and Figure 5.6 (below) shows the 4β-OHC concentrations in males and females in the naïve group, respectively.

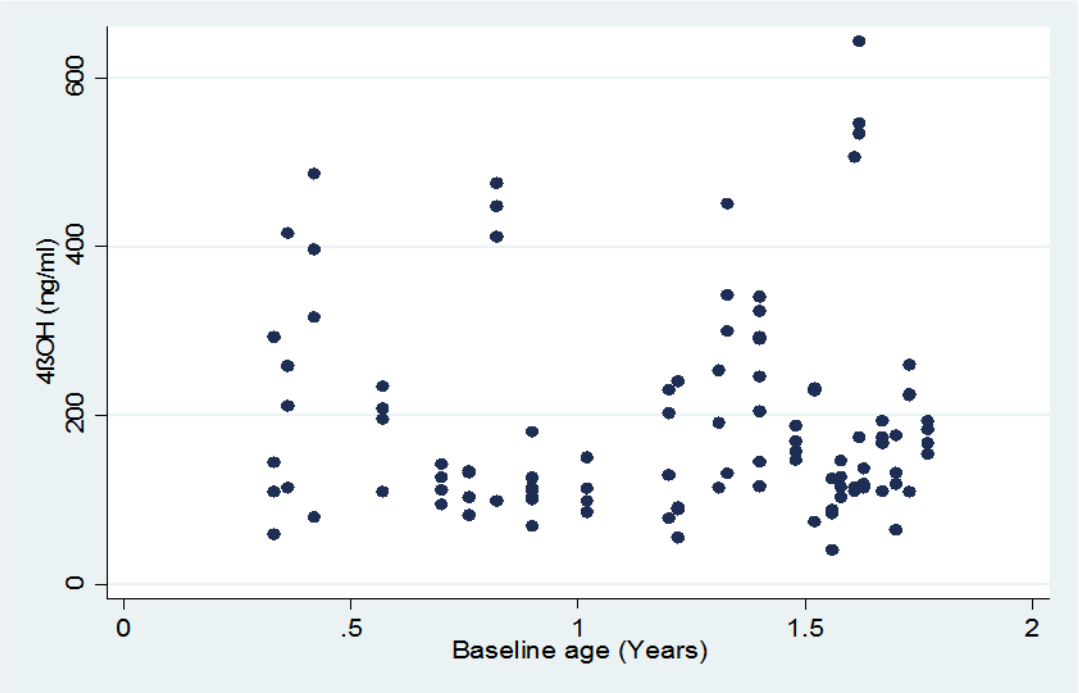


Figure 5.4 Scatter plot illustrating lack of correlation between 4β-OHC concentrations and age at baseline in the naïve group

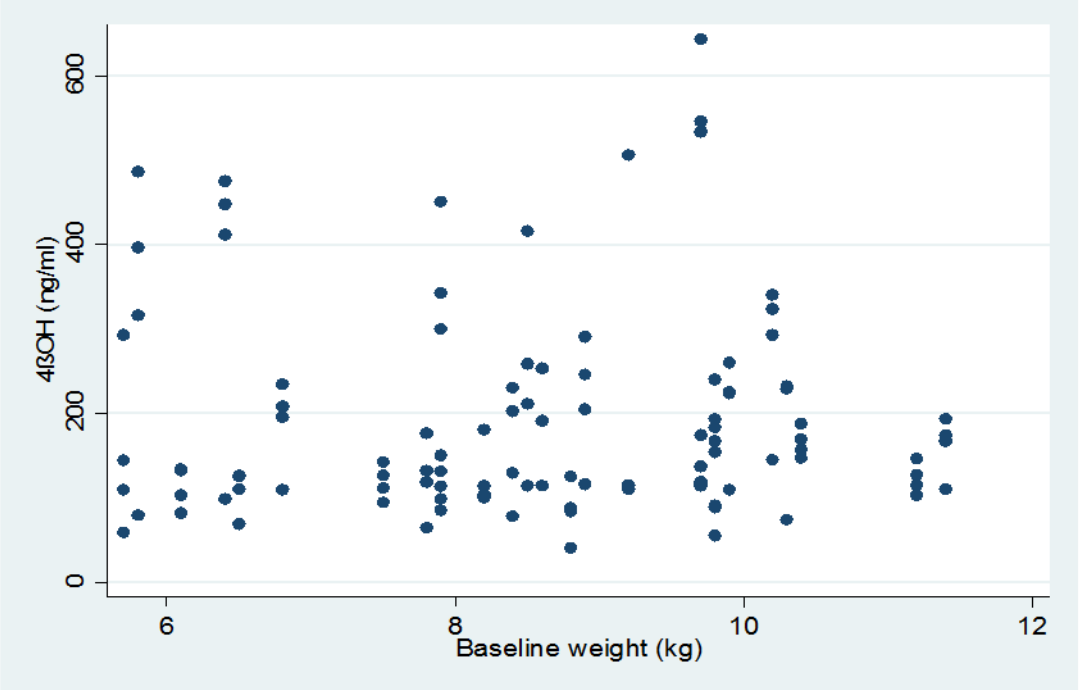


Figure 5.5 Scatter plot illustrating the lack of correlation between 4β-OHC concentrations and weight at baseline in the naïve group

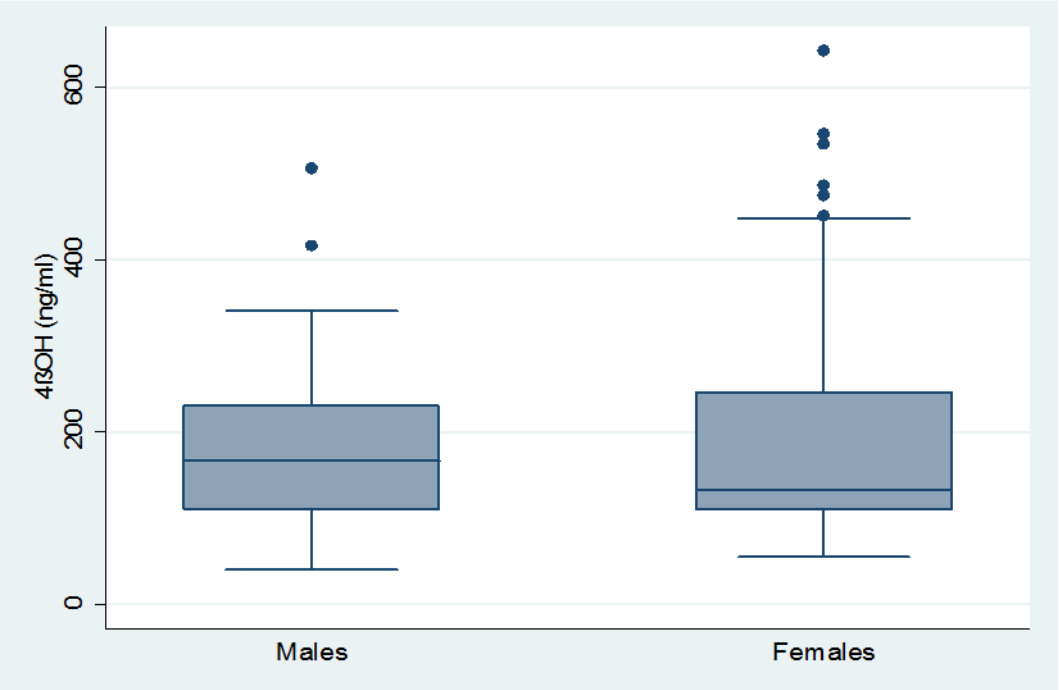


Figure 5.6 4β-OHC concentrations between males and females in the naïve group

(horizontal bars in boxes represent median concentration, whiskers represent minimum and maximum concentrations, boxes represent 25th and 75th percentiles, and "•" represent outliers)

5.9.2 Nevirapine group

We found that children continuing on NVP had a significant decrease in 4β-OHC concentrations between baseline and each of the non-baseline weeks. However, no significant difference was observed in 4β-OHC concentrations between non-baseline weeks – with each of the non-baseline weeks as a reference. Like the naïve group, there were no significant correlations between 4β-OHC concentrations and age or weight at baseline in the NVP group. There was also no significant difference observed in 4β-OHC concentrations between males and females in the group. Table 5.4 shows the results of a multi-level mixed-effect model determining the effect of time on treatment, baseline age, weight and sex on 4β-OHC concentrations in the NVP group.

Table 5.4 Results of a multi-level mixed-effect linear regression determining the effect of time on treatment, baseline age, weight and sex on 4β-OHC concentrations in the NVP group (n=29)

	Coefficient	Std. Err.	z	P>z	95% Confidence Interval	
Baseline (n=29)	Reference					
Week 36 (n=6)	-38.64	13.19	-2.93	0.003	-64.49	-12.79
Week 60 (n=16)	-45.89	8.56	-5.36	0.000	-62.67	-29.11
Week 84 (n=29)	-47.26	6.92	-6.83	0.000	-60.82	-33.71
Week 108 (n=23)	-29.29	7.51	-3.90	0.000	-44.02	-14.57
Week 132 (n=10)	-40.95	10.40	-3.94	0.000	-61.34	-20.56
Sex (Male)	Reference					
Female	-15.35	20.07	-0.76	0.44	-54.69	23.99
Baseline weight (kg)	-7.32	5.14	-1.42	0.15	-17.39	2.76
Baseline age (years)	-13.81	23.96	-0.58	0.56	-60.78	33.16

n = number of samples observed

Figures 5.7 and 5.8 (below) show 4β-OHC concentrations over the study period in the NVP group.

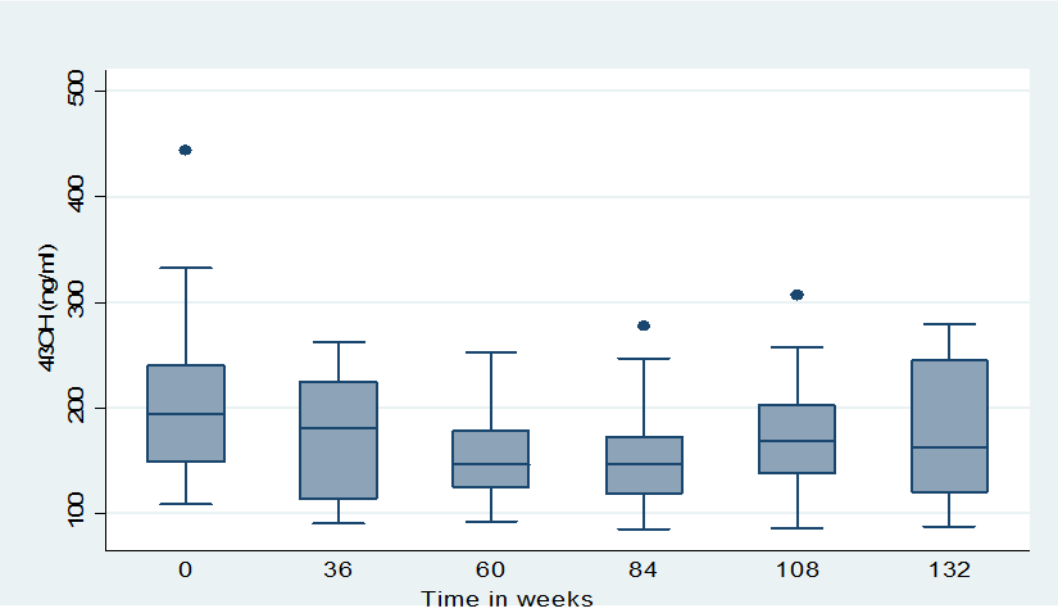


Figure 5.7 4β-OHC concentrations over the study period in the NVP group

(horizontal bars in boxes represent median concentration, whiskers represent minimum and maximum concentrations, boxes represent 25th and 75th percentiles, and "•" represent outliers)

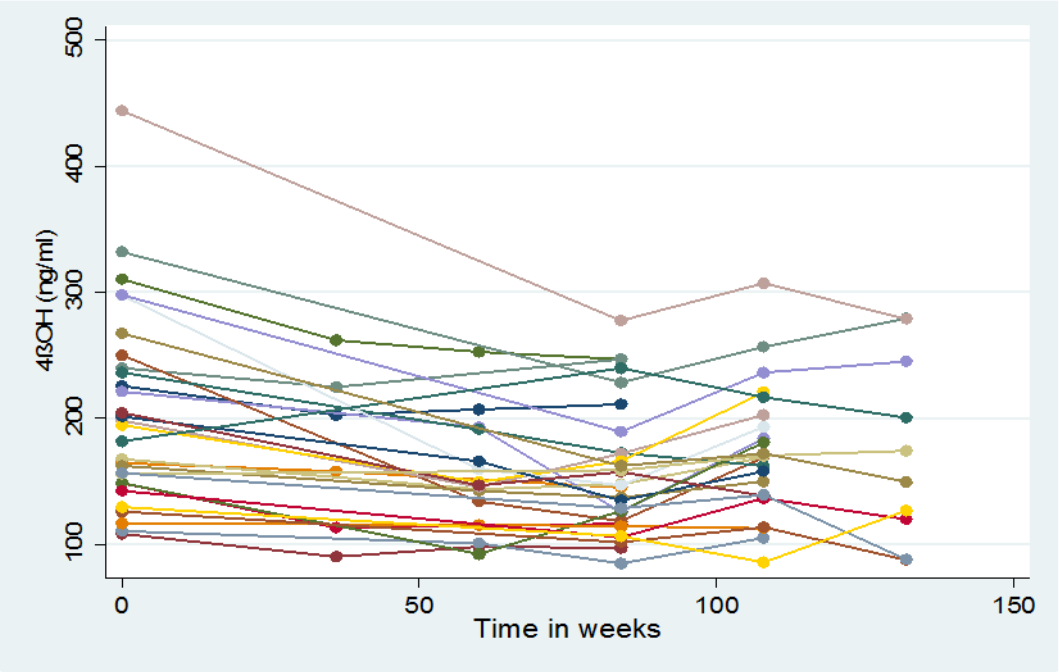


Figure 5.8 4β-OHC concentration trends in individual patients in NVP group over the study period

Figures 5.9 and 5.10 (below) show lack of correlation between 4β-OHC concentrations and age and weight respectively. Figure 5.11 (below) shows 4β-OHC concentrations between males and females in the NVP group.

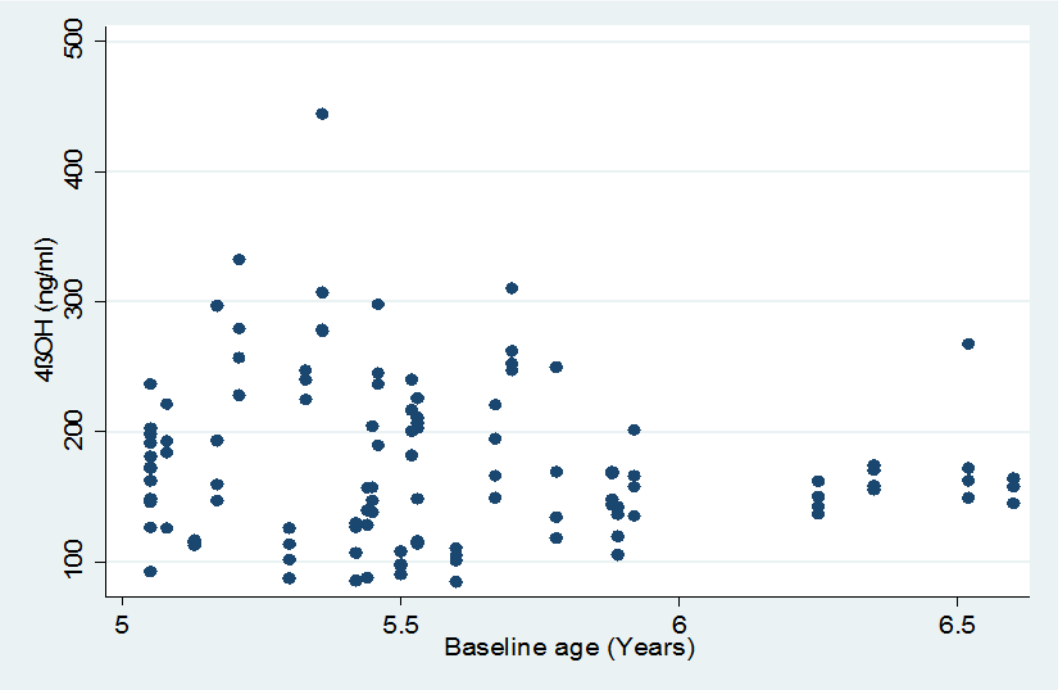


Figure 5.9 Scatter plot illustrating the lack of correlation between 4β-OHC concentrations and age at baseline in the NVP group

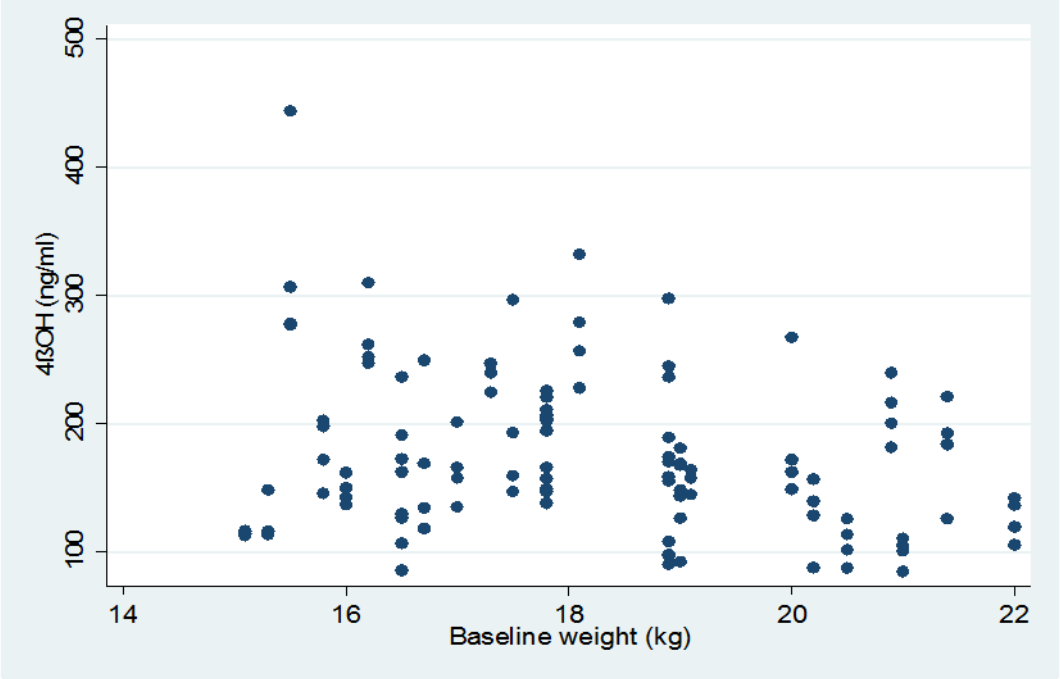


Figure 5.10 Scatter plot illustrating the lack of correlation between 4β-OHC concentrations and weight at baseline in the NVP group

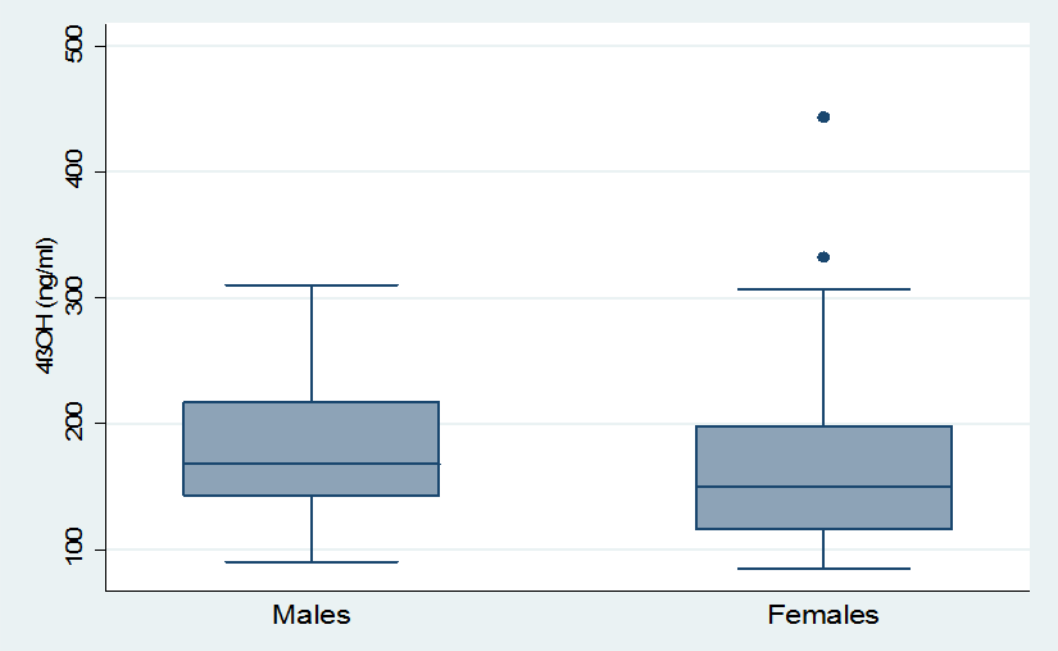


Figure 5.11 4β-OHC concentrations between males and females in the NVP group

(horizontal bars in boxes represent median concentration, whiskers represent minimum and maximum concentrations, boxes represent 25th and 75th percentiles, and "•" represent outliers)

5.9.3 Efavirenz group

In this group, no significant difference was found between baseline 4β-OHC concentrations and each of the non-baseline weeks or 4β-OHC concentrations between non-baseline weeks. There was no significant correlation observed between 4β-OHC concentrations and age and weight at baseline. Furthermore, there was no significant difference in 4β-OHC concentrations between males and females in this group. Table 5.5 (below) shows results of a multi-level mixed-effect model determining the effect of time on treatment, baseline age, weight and sex on 4β-OHC concentrations in the EFV group.

Table 5.5 Results of a multi-level mixed-effect model determining the effect of time on treatment, baseline age, weight and sex on 4β-OHC concentrations in the EFV group (n=10)

	Coefficient	Std. Err.	z	P>z	95% Confidence Interval	
Baseline (n=10)	Reference					
Week 36 (n=1)	-85.67	89.68	-0.96	0.33	-261.43	90.10
Week 60 (n=4)	43.58	47.49	0.92	0.36	-49.51	136.67
Week 84 (n=10)	20.79	34.18	0.61	0.54	-46.19	87.78
Week 108 (n=9)	48.35	35.39	1.37	0.17	-21.03	117.72
Week 132 (n=5)	23.89	43.64	0.55	0.58	-61.64	109.42
Sex (Male)	Reference					
Female	-60.61	55.61	-1.09	0.28	-169.61	48.38
Baseline weight (kg)	-30.52	15.59	-1.96	0.05	-61.07	.03
Baseline age (years)	62.66	31.39	2.00	0.05	1.12	124.21

n= number of samples observed

Figures 5.12 and 5.13 (below) show 4β-OHC concentrations over the study period in the EFV group.

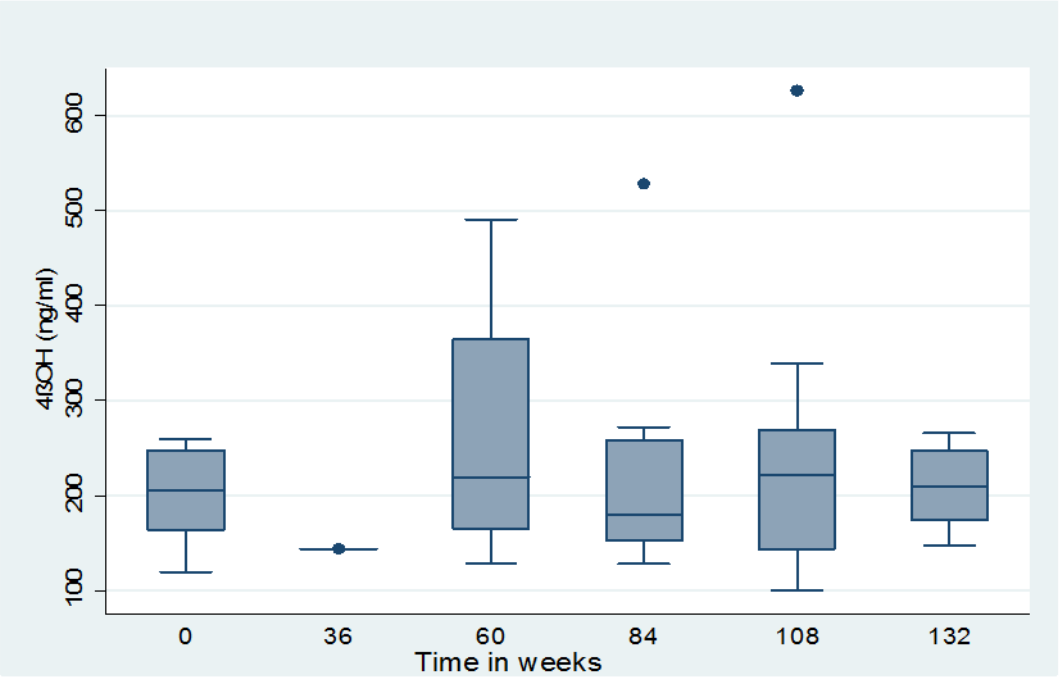


Figure 5.12 4β-OHC concentrations over the study period in the EFV group

(horizontal bars in boxes represent median concentration, whiskers represent minimum and maximum concentrations, boxes represent 25th and 75th percentiles, and "•" represent outliers)

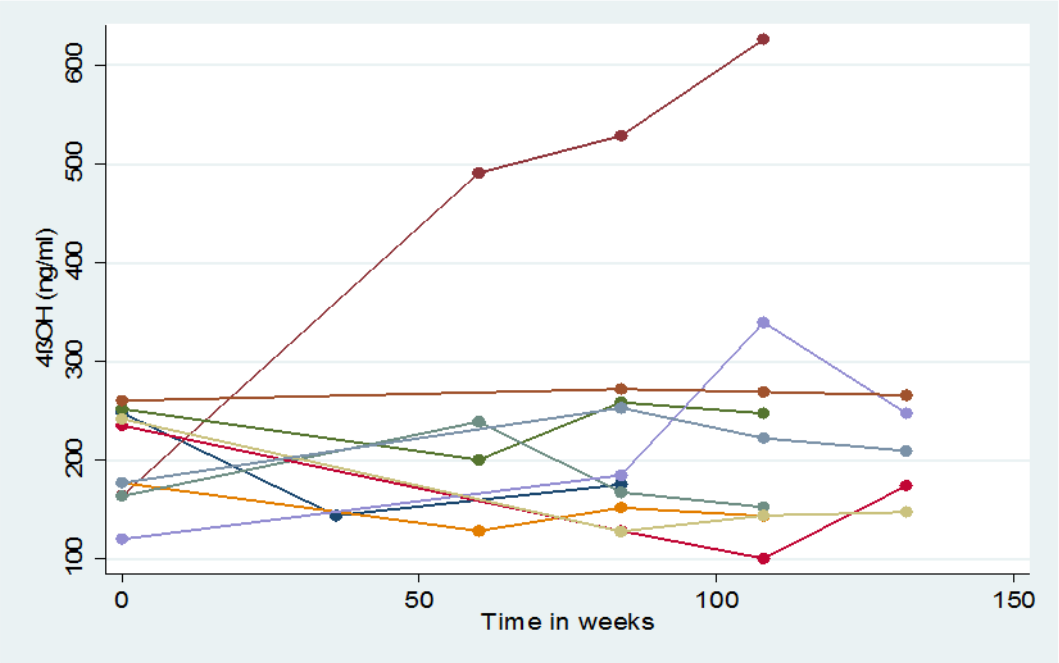


Figure 5.13 4β-OHC concentrations trends in individual patients in the EFV group over the study period.

Figures 5.14 and 5.15 (below) show lack of correlation between 4β-OHC concentrations and age and weight respectively. Figure 5.16 (below) shows 4β-OHC concentrations between males and females in the EFV group.

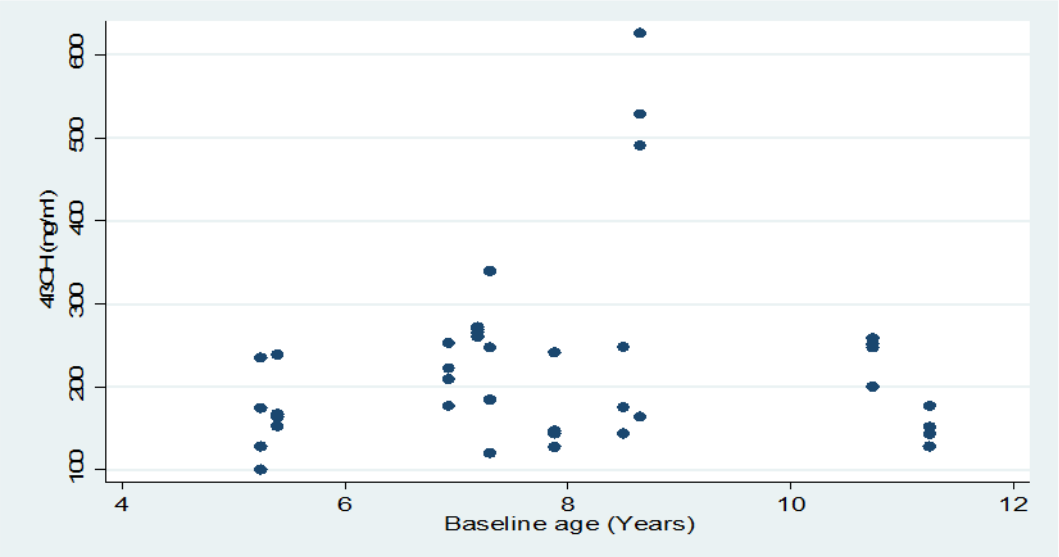


Figure 5.14 Scatter plot illustrating the lack of correlation between 4β-OHC concentrations and age at baseline in the EFV group

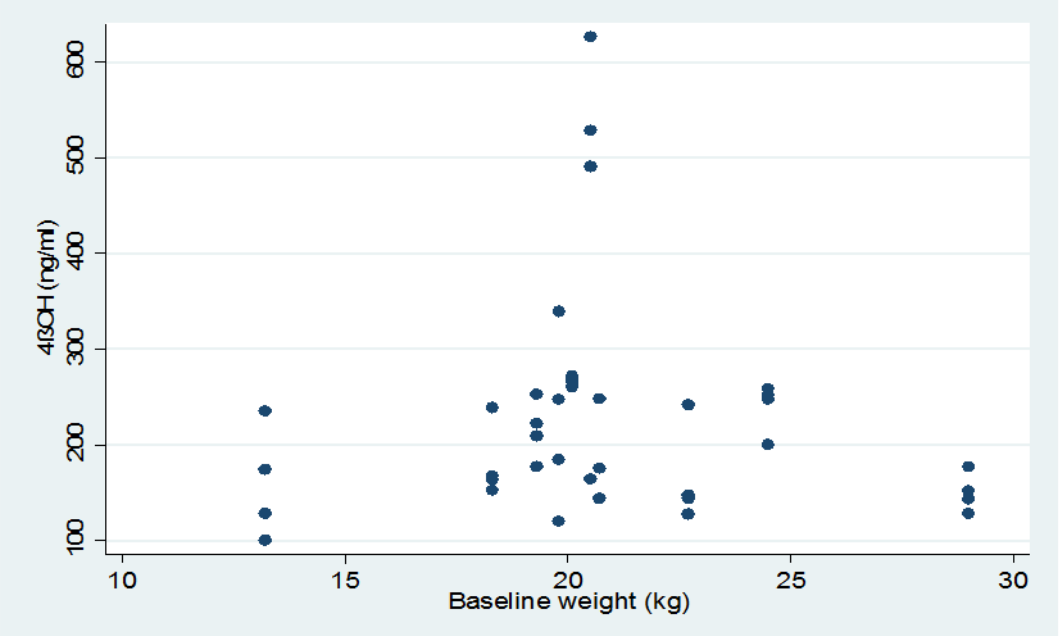


Figure 5.15 Scatter plot illustrating lack of correlation between 4β-OHC concentrations and weight at baseline in the EFV group

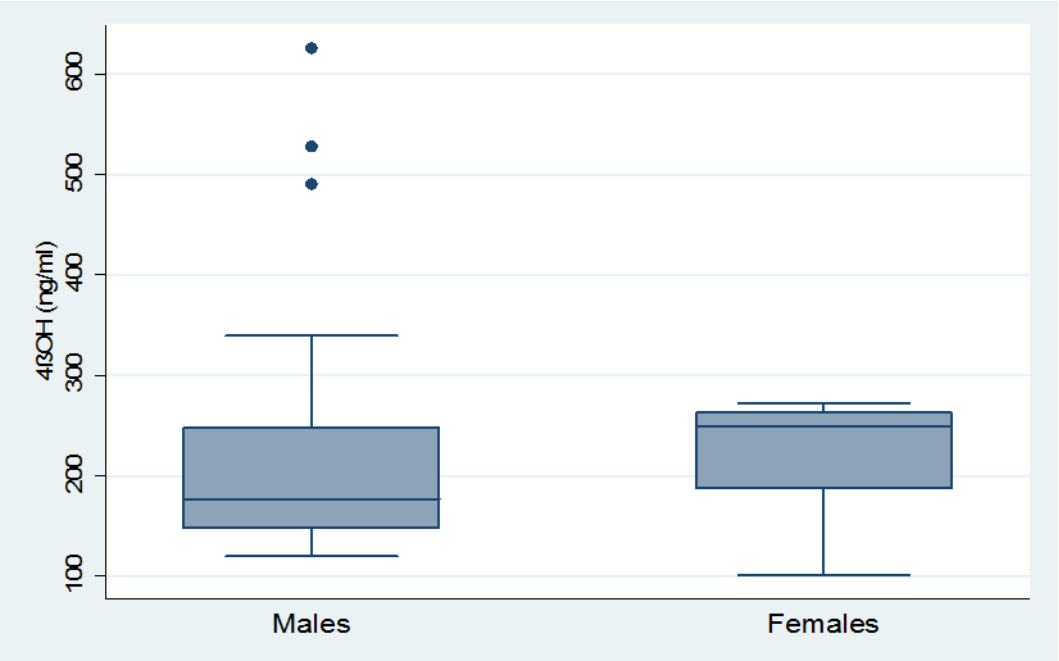


Figure 5.16 4β-OHC concentrations between males and females in the EFV group

(horizontal bars in boxes represent median concentration, whiskers represent minimum and maximum concentrations, boxes represent 25th and 75th percentiles, and "•" represent outliers)

5.10 Results summary

In this study, it was found that plasma 4β-OHC concentrations at baseline were significantly lower in children belonging to the naïve group compared to the NVP and EFV groups. Comparing the NVP and EFV groups, a statistically significant difference in 4β-OHC concentrations was only detected at week 84 – where the median 4β-OHC concentration was higher in the EFV group than the NVP group by 22.45%. Regarding the effect of time on treatment, in the naïve group a significant increase in 4β-OHC concentration from baseline to each of the non-baseline weeks after commencement of NVP treatment, was noted. Nevertheless, there was no significant difference observed between the non-baseline weeks following commencement of NVP in this group. In the NVP group, baseline 4β-OHC concentration was significantly higher compared to each of the non-baseline weeks. There was no significant difference in 4β-OHC concentrations between non-baseline weeks in the NVP group. In the EFV group, time did not show any significant change in 4β-OHC concentrations. Furthermore, in all the three treatment

groups, no significant correlations were found between 4β-OHC concentrations and age or weight at baseline. There was also no significant difference in 4β-OHC concentrations between males and females.

5.11 Discussion

5.11.1 The use of 4β-OHC as a biomarker of CYP3A activity in clinical practice and research

Cytochrome (CYP) 3A is quantitatively the most important drug-metabolising enzyme in humans. Members of the cytochrome CYP3A (CYP3A4/5/7) enzymes, specifically CYP3A4 and to a lesser extent CYP3A5 and CYP3A7, are involved in the metabolism of up to 50% of the commonly used drugs – such as antiretroviral, anticonvulsant, antibacterial and antifungal drugs (Li, Kaminski & Rasmussen, 1995). These drugs may cause induction or inhibition of CYP3A enzyme activity (Josephson *et al.,* 2008; Bodin *et al.,* 2001). This may be a challenge in the context of ART, because induction or inhibition of the CYP3A enzyme may lead to loss of therapeutic effects or increased side effects of the drug itself or other co-administered CYP3A drug substrates used to treat coincidental or complicating diseases (Kappelhoff *et al.,* 2005; Moltke *et al.,* 2001; Evans & Relling, 1999). This is thus one of the clinical reasons for monitoring CYP3A activity.

Previous studies have reported the use of probe drugs such as midazolam or erythromycin, and biomarkers such as urinary 6β-hydroxycortisol to cortisol ratio for assessing CYP3A activity (Galteau & Shamsa, 2003; Gorski *et al.,* 2003). Unlike these biomarkers, 4β-OHC is safer because no administration of probe drugs is involved – which may put the patients at risk of drug-related side effects. The other advantage of 4β-OHC compared to 6β-hydroxycortisol, is that it has a long half-life of approximately 17 days, that makes its concentrations relatively stable from day-to-day within an individual (Diczfalusy *et al.,* 2008b). The use of 4β-OHC as a biomarker of CYP3A activity may not be best indicated for the detection of rapid changes in CYP3A activity – such as when CYP3A inhibitor is introduced. With such rapid changes, the use of a probe drug may be more suitable. The use of cholesterol to 4β-OHC ratio could also be used as a biomarker in conditions where the cholesterol concentration is changing (Diczfalusy *et al.,* 2011). In this study, a cholesterol-validated method was not available and therefore only 4β-OHC concentrations were measured in the clinical samples.

Other than prediction of drug-drug interactions, 4β-OHC as a biomarker could be used in predicting other metabolic processes affected by regulators of CYP 3A activity – such as PXR and CAR (Ihunnah, Jiang & Xie, 2011; Chang, 2009; Moreau *et al.,* 2008). Some of the affected metabolic processes include lipid and glucose metabolism. These effects may result from treatment with drugs that induce or inhibit CYP3A enzyme activity – such as ART. NVP and EFV, for example, are classified as weak PXR activators, but demonstrate their CYP3A-inductive effects predominantly through CAR activation (Faucette *et al.,* 2007). It thus follows that people on long-term ART containing NVP or EFV, are at risk of metabolic dysfunction and associated non-communicable diseases.

5.11.2 4β-OHC concentration in the different study groups

In this study, plasma 4β-OHC concentrations at baseline were significantly lower in children belonging to the naïve group – compared to the NVP and EFV groups. This was in agreement with the hypothesis of this study, that plasma 4β-OHC concentrations of children naïve to ART would be lower than ART-experienced groups. Previous studies in adults have shown that ART therapy with CYP3A inducers such as EFV have increased plasma 4β-OHC concentrations (Josephson *et al.,* 2008). One *in vitro* study assessed the capacity of NNRTIs to inhibit CYP450 enzymes under acute exposure using human liver microsomes (Moltke *et al.,* 2001). Moltke *et al*. reported that NVP had a weak inhibition of CYP3A activity under acute conditions – which was not significant and probably not clinically important. The authors of this *in vitro* study also acknowledged that NVP and EFV are significant CYP3A inducers, and that the weak inhibitory effects displayed in their study were only immediate. It follows therefore, that the children on ART in the present study were at risk of effects of CYP3A induction – as indicated by the increased plasma 4β-OHC concentrations in the NVP and EFV groups.

Comparing the NVP and EFV groups, a statistically significant difference in 4β-OHC concentrations was only detected at week 84, where the median 4β-OHC concentration was higher in the EFV group than the NVP group. However, this difference was not detected at weeks 60, 108 and 132. Maganda *et al.* (2015) report that EFV seems to be a more potent inducer of CYP3A activity than NVP. The study was, however, done in an adult population. Nevertheless, if this effect is also expressed in children, then we would have expected higher concentrations of 4β-OHC in the EFV groups in the present study in

all the weeks compared to the NVP group. Perhaps, we were unable to detect statistically higher levels in the EFV group than the NVP group due to a small sample size – especially in the EFV group. The statistically significant higher 4β-OHC concentration in the EFV group, detected at week 84, may support the effect of sample size – because it is at this week that the sample size was highest in both the EFV and NVP groups.

5.11.3 Effect of time on treatment

The naïve group in this study had a statistically significant higher plasma 4β-OHC concentration at the non-baseline weeks (36^{th}, 60^{th}, 84^{th} and 108^{th}) after commencement of NVP treatment, compared to baseline. However, there was no steady, significant increase with time in 4β-OHC concentrations observed over the period following commencement of NVP in this group. Like these findings, one previous study which treated paediatric patients aged 0-18 years with carbamazepine – an inducer of the CYP3A enzyme – reported that CYP3A induction was complete after two weeks on treatment (Wide *et al.,* 2008). Wide *et al.* (2008) also reported that CYP3A induction was associated with a significant increase of plasma 4β-OHC concentrations in the first two weeks, after which there was a slow increase until 8 weeks when the plasma 4β-OHC concentration became relatively stable. In the current study, since 4β-OHC measurement after baseline was only done at 36 weeks (28 weeks after the first 8 weeks), one would expect no further increase in the levels if the same trend occurs in the use of ART as in the study by Wide *et al.* (2008). Other authors have suggested that plasma 4β-OHC is transported slowly over biological membranes (Meaney *et al.,* 2002), and that steady levels at certain concentrations may possibly be due to slow equilibrations between different compartments. It is therefore plausible that significant CYP3A enzyme activity induction occurs within a limited period of time after initiation of treatment – and then weakens thereafter. The length of this period with regard to the use of ART in children – warrants further research.

In the NVP group, baseline plasma 4β-OHC concentration was significantly higher compared to each of the non-baseline weeks. Still, there was no difference in 4β-OHC concentration between the non-baseline weeks. It should be noted, however, that at baseline, these children had already been on NVP for at least 2 years. Interestingly, even though the plasma 4β-OHC concentrations of the non-baseline weeks in the NVP group

were lower than at baseline, these concentrations were certainly higher than the naïve group. Possibly this finding may be due to better viral suppression whilst on treatment. It has been suggested that inflammatory mediators influence nuclear receptors which affect CYP3A enzyme activity (Jetter *et al.,* 2010; Morgan, 2009; Morgan *et al.,* 2008). Also, the study environment which offered a higher level of care may have led to more normal immunological environment. However, this hypothesis requires further studies to be explained.

In the EFV group, time did not show any significant change in plasma 4β-OHC concentrations between baseline and non-baseline weeks or non-baseline to non-baseline weeks. This might be explained by the fact that these children had already been on NVP for 2 years – another CYP3A enzyme inducer – and hence there was no significant change of plasma 4β-OHC concentration after switching to EFV. A study in adult patients that used the 4β-OHC/cholesterol ratio as a biomarker for CYP3A activity in ART-naive HIV individuals, reported that EFV increased the median plasma 4β-OHC/cholesterol ratio from baseline to week 4, 16, 48 – by 257%, 291% and 165% respectively (Habtewold *et al.,* 2013). Unlike in the current study, the Habtewold *et al.* study was done in adults and the patients were ART-naïve before commencing EFV. In the current study, the children were on NVP before they were switched to EFV. Nevertheless, Habtewold *et al.* (2013) showed that the rate of increase in the 4β-OHC/cholesterol ratio decreased between week 16 and 48 – possibly a comparable trend of insignificant increase in 4β-OHC concentration observed in the present study. Another explanation for the insignificant change could be lack of power – considering that this group had the smallest sample size.

5.11.4 Effect of age, weight and sex on 4β-OHC

In all the three treatment groups, there were no significant correlations between plasma 4β-OHC concentrations and age at baseline. It is well documented that CYP3A activity matures fairly rapidly after birth (Cresteil, 1998; Lacroix *et al.,* 1997). One study reported that neonates had median 4β-OHC/cholesterol ratios comparable to those of adults at birth (Nylén *et al.,* 2011).

No significant correlation was observed in all three groups between plasma 4β-OHC concentrations and weight at baseline. The effect of weight on plasma 4β-OHC

concentrations in HIV-infected children with or without treatment has apparently not yet been reported. Additionally, in all three groups there was no significant difference in plasma 4β-OHC concentrations between males and females. Currently there are no available data on plasma 4β-OHC concentrations and sex in paediatric patients to compare these findings to. Previous adult studies reported that females have higher 4β-OHC concentrations than males (Diczfalusy *et al.,* 2011). One study that used human liver samples stated that females have a 2-fold higher expression of hepatic CYP3A enzyme than males (Wolbold *et al.,* 2003).

This study has an important novel contribution to the existing literature on the levels of plasma 4β-OHC as a biomarker of CYP3A activity in children on ART in an African setting. The results of this study may be used to inform the design of future studies to evaluate the effects of CYP3A induction on drug-drug interactions, and ART side effects.

5.12 Limitations

The results of this study should be interpreted in the context of its design as a retrospective study. The selection of samples analysed in this study was in part based on availability – and hence the results may be affected by selection bias. There was no actual control group to compare the study groups to, but analysis of the pre-dose samples enabled each subject to serve as its own control, so providing greater accuracy in determining the magnitude of change in 4β-OHC concentrations as a result of treatment.

Our sample sizes for the naïve and NVP groups were adequate to answer the research question in the study. However, the sample size for the EFV group was 50% of the required estimate due to lack of availability of suitable baseline samples, and this was sufficient only to detect major effects. In this regard, the small sample size may have lessened the power to detect significance in some of the results.

In this study, only plasma 4β-OHC levels were determined – not cholesterol levels. ART-containing NNRTIs have been reported to yield changes in plasma cholesterol (Franssen *et al.,* 2009). It has been suggested that calculating the 4β-OHC/cholesterol ratio may be more appropriate to determine CYP3A enzyme activity under conditions where plasma cholesterol concentrations are significantly changing (Diczfalusy *et al.,* 2011). Cholesterol

levels could not be measured in this study due to the unavailability of a validated assay in the laboratory. Thus, interpretation of the results in this study was done in the light of the assumption that the cholesterol levels were not significantly changing. Diczfalusy *et al.* (2008a) suggested that variations in plasma cholesterol within normal limits are unlikely to significantly influence 4β-OHC concentrations. Finally, factors such as environmental effects, diet and pharmacogenetic variability, such as CYP3A5 genotype, were not assessed, as this was beyond the scope of the research project. Nevertheless, these have been reported to also affect CYP3A enzyme activity (Habtewold *et al.,* 2013; Diczfalusy *et al.,* 2008a).

CHAPTER 6: CONCLUSION

In this study, LC/MS/MS derivatised and underivatised methods for determination of plasma 4β-OHC were developed and compared. A highly sensitive, selective, and reproducible derivatisation method was successfully developed and validated according to FDA guidance for industry (2001) and EMA guidelines on bioanalytical method development (2012). Chromatographic separation was achieved on an Agilent SB phenyl column (100 x 2.1 mm x 1.8 µm), with an isocratic mobile phase consisting of 0.1% formic acid in methanol: acetonitrile (50:50; v/v) – at a flow rate of 200 µl/min.

The derivatisation method performed well with regard to accuracy, precision, linearity, sensitivity, selectivity, recovery, and stability. This method was accurate and precise, with the results within the acceptable ranges (accuracy within ± 15% of the nominal values and precision, %CV ≤ 15). The calibration curve of the developed method fitted a quadratic (weighted by $1/x^2$) regression over the range 2-500 ng/ml. Selectivity was confirmed by analysing a blank matrix extract and no interferences from endogenous matrix components were observed at or near the retention time – of either 4β-OHC, 4β-OHC-D7 and 4β-OHC-D4. The assay's recovery after LLE with derivatisation reaction, was >70%. This high extraction recovery confirmed that derivatisation of analytes with picolinic acid was successful and also that the assay did not suffer from interferences by the endogenous matrix components. The assay demonstrated stability of 4β-OHC-D7 and 4β-OHC-D4 in stock solution (ethanol) at 4^oC and room temperature, for 14 days, on-bench for 15 hours, on-instrument for 34 hours, and after being subjected to three freeze-thaw cycles. The approach of surrogate analyte in preparation of calibration STDs and QCs alleviated the challenge posed by the endogenous compounds available in blank plasma.

The method was successfully applied on clinical samples, where 4β-OHC was used as a biomarker to investigate the levels of CYP3A induction in African HIV-infected children with and without treatment containing NNRTIs. It was found that plasma 4β-OHC concentrations at baseline were significantly lower in children belonging to the naïve group compared to the NVP and EFV groups. When the NVP and EFV groups were compared at non-baseline treatment weeks, the median 4β-OHC concentrations were significantly higher in the EFV group compared to the NVP group. Regarding the effect of time on treatment, a significant increase in 4β-OHC concentrations was observed from baseline to each of the non-baseline weeks in the naïve group. Conversely, in the NVP

group, there was a significant decrease in 4β-OHC concentrations from baseline to each of the non-baseline weeks. Time did not show any significant effect on 4β-OHC concentrations in the EFV group. Furthermore, at baseline – age, sex and weight did not affect 4β-OHC concentrations in all the three groups.

The results of this study suggest that children on ART are at risk of effects of CYP3A induction, as indicated by the increase of 4β-OHC concentrations in the NVP and EFV groups. Additionally, prolonged use of the ART may activate some nuclear receptors that regulate CYP3A enzyme activity – thereby negatively affecting the regulation of lipid and glucose metabolism, for example. This developed method may therefore be used to predict such events.

This study has provided a method that could be utilised to determine 4β-OHC concentrations using relatively small plasma volumes – typical of samples taken from children. The method may be useful in predicting drug-drug interactions in the context of multiple therapy, and may also be used in predicting other metabolic processes affected by regulators of CYP3A activity. The novel results of this study contribute to the body of knowledge and may be used to inform the design of future studies to evaluate the effects of CYP3A induction on drug-drug interactions, and also ART side effects. Given the limitations in the design and sample size of the clinical part of the present study, further research – preferably prospective studies with larger sample sizes – are warranted to confirm and build on the evidence demonstrated in this study.

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APPENDIX A: REAGENTS, CHEMICALS, SOLUTIONS, CONSUMABLES AND EQUIPMENT USED

Table A1: Reagents and Chemicals

Reagent	**Catalogue No.**	**Grade**	**Supplier**	**Lot no.**
Formic Acid	1.00264.2500	HPLC	Merck	BCBM788IV
Acetonitrile	015-4	HPLC	Merck	DK 621
Methanol	LC230-2.5	Gradient grade for LC	Merck	DK 558
Ethanol	1.09634.2500	For Analysis	Merck	K43192934 232
Water	LC 365-2.5	HPLC	Honeywell Burdick	DK 706
Hexane	N/A	For analysis	SIGMA	S2BE162CV
Picolinic Acid	101441569	For analysis	SIGMA	BCBK3582V
2-methyl-6-nitrobenzoic anhydride	101300218	For analysis	SIGMA	BCBK8805V
4-dimethyl aminopyridine	101284547	For analysis	SIGMA	MKBL4922V
Triethylamine	101447733	For analysis	SIGMA	STBD8779V
Pyridine	1001782923	For analysis	SIGMA	SHBF1536V
Butylated hydroxytoluene	PHR1117-1G	For analysis	SIGMA	P500117
Potassium Hydroxide	1310-58-3	For analysis	SIGMA	SZBB2210V
Molecular sieve (UOP type 3A) beads	2D-89555	Drying agent	SIGMA	BCBG6930V

Table A2. Necessary Consumables

Description	**Catalogue Number**	**Supplier**
5 ml polypropylene tubes	1505	Lasec
96 well plates	PAXGP-96-450R-C-S	Lasec
Silicone Sealing mats	AM-2ML-RD	AXYMAT
Pipette tips (yellow)	73290	Lasec SA
Analytical Column: SB phenyl 1.8 µm 2.1x100 mm	_	Agilent

Table A3. Equipment

Name	**Model**	**Manufacturer**
Vortex	G560E	Scientific Industries
Centrifuge	5415D	Magnus
Timer	HFLSPS 300A	Lasec
Balance	CPA2P	Sanas calibration Laboratory
Pipette: 2-20 µl	M20 (13514590)	Biohit
Pipette: 20-200 µl	M200 (12563002)	Biohit
Pipette: 200-1000 µl	M1000 (13541980)	Biohit

A2. Preparation of derivatisation solution

Derivatisation solution contained a mixture of; 240 mg of 2-methyl-6 nitrobenzoic anhydride, 74 mg of 4-dimethylaminopyridine, 200 mg of picolinic acid in dry 7.5 ml pyridine and 1 ml triethylamine. Molecular sieve (schematic diagram shown in Figure A1) was added to the solvents first- to absorb the moisture. Fresh derivatisation solution was

prepared just before sample preparation.

Figure A1. Schematic diagram of the molecular sieve (*Source: www.silicagel-co.com)*

A3. Preparation of saponification solution

Dissolve 5.6 g of KOH in 100 ml ethanol

A4. Preparation of antioxidant

Dissolve 5 mg of BHT in 5 ml ethanol (stored over -20^{o}C)

APPENDIX B : UNDERIVATISED AND DERIVATISED METHODS ASSESSMENTS

B1. Underivatisation method

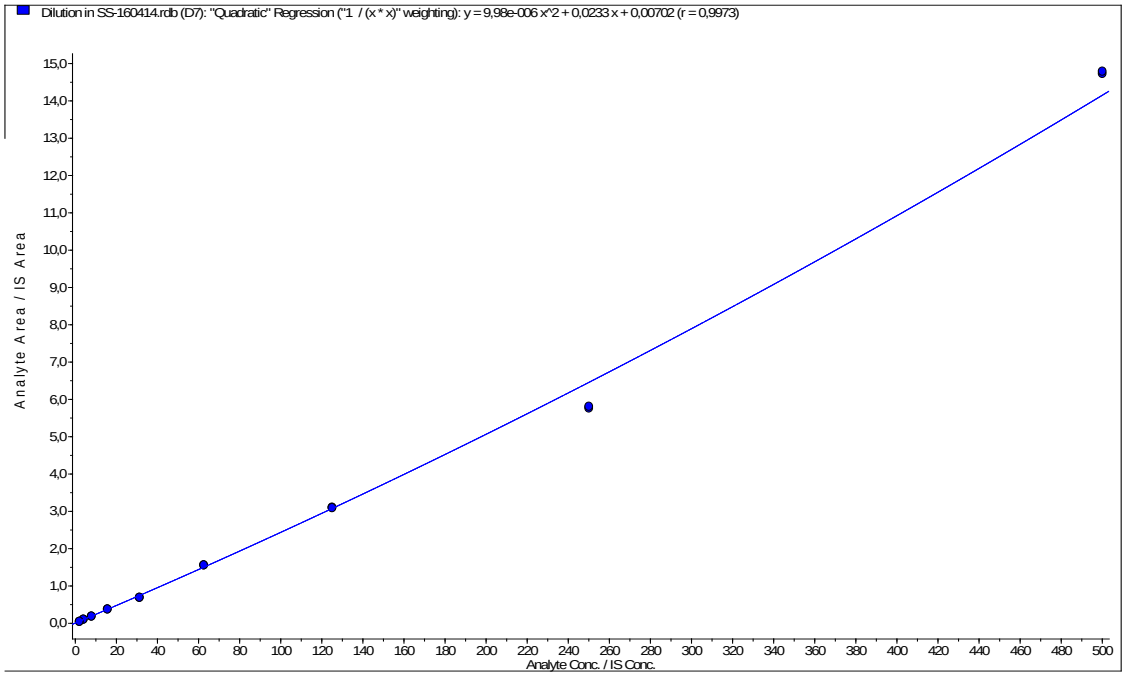


Figure B1. Dilution sequence of 4β-OHC-D7 in injection solution

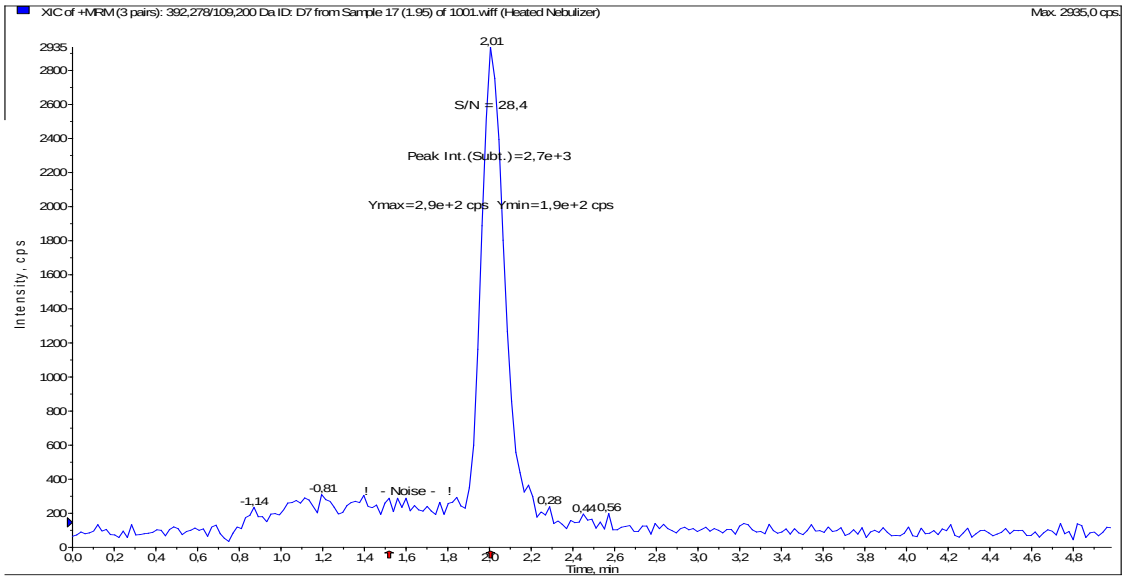


Figure B2. S/N ratio of 4β-OHC-D7 at LLOQ 2 ng/ml in injection solution

Table B1. Instrument response repeatability in injection solution n=52

	4β-OHC-D7 /4β-OHC-D4 Peak area ratio
Average	2.32
ST DEV	0.163
% CV	7.05

B2. Derivatisation method

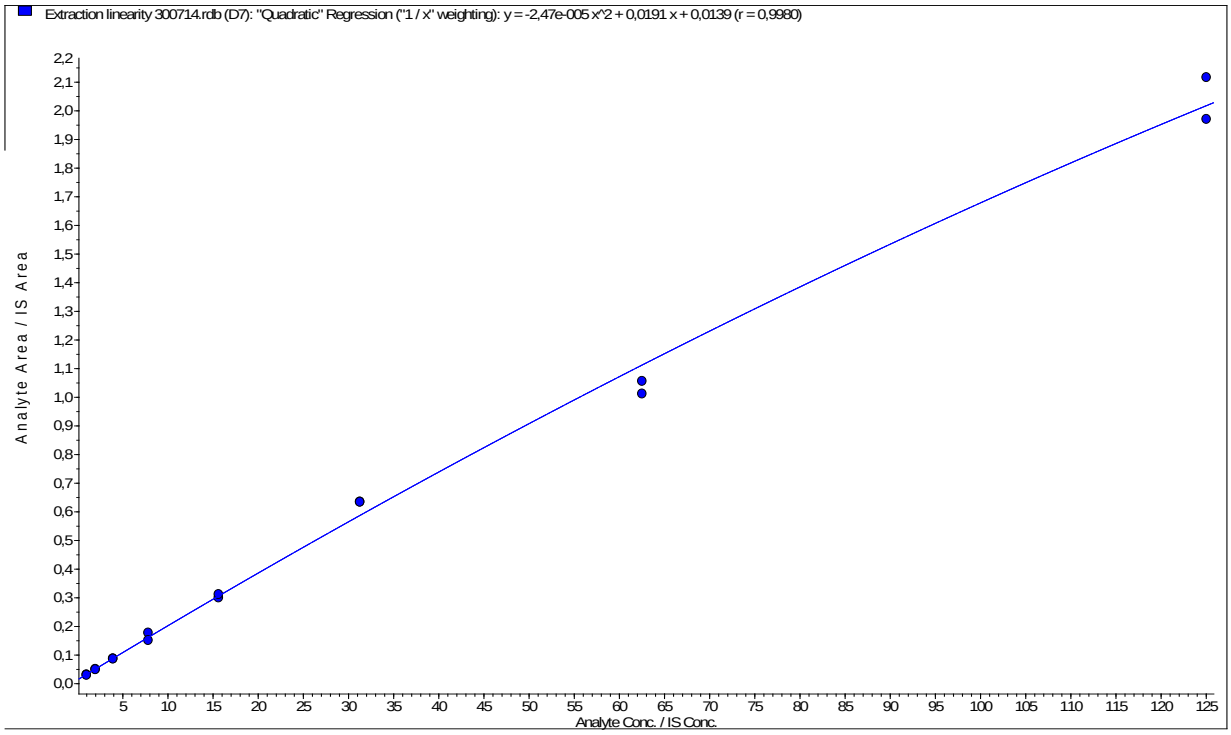


Figure B3. Dilution sequence of 4β-OHC-D7 in injection solution

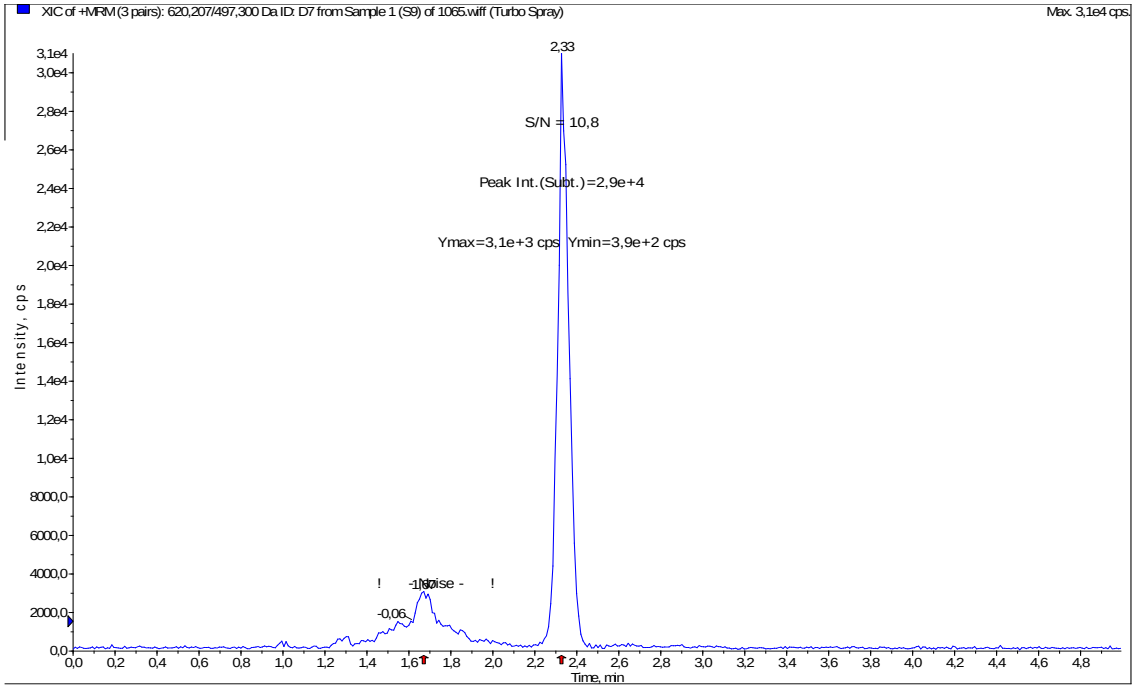


Figure B4. S/N ratio of derivatised 4β-OHC-D7 at LLOQ (2 ng/ml) in injection solution

Table B3. Instrument response repeatability in injection solution n=117

	4β-OHC-D7 /4β-OHC-D4 Peak area ratio
Average	1.52
ST DEV	0.03
% CV	2.26

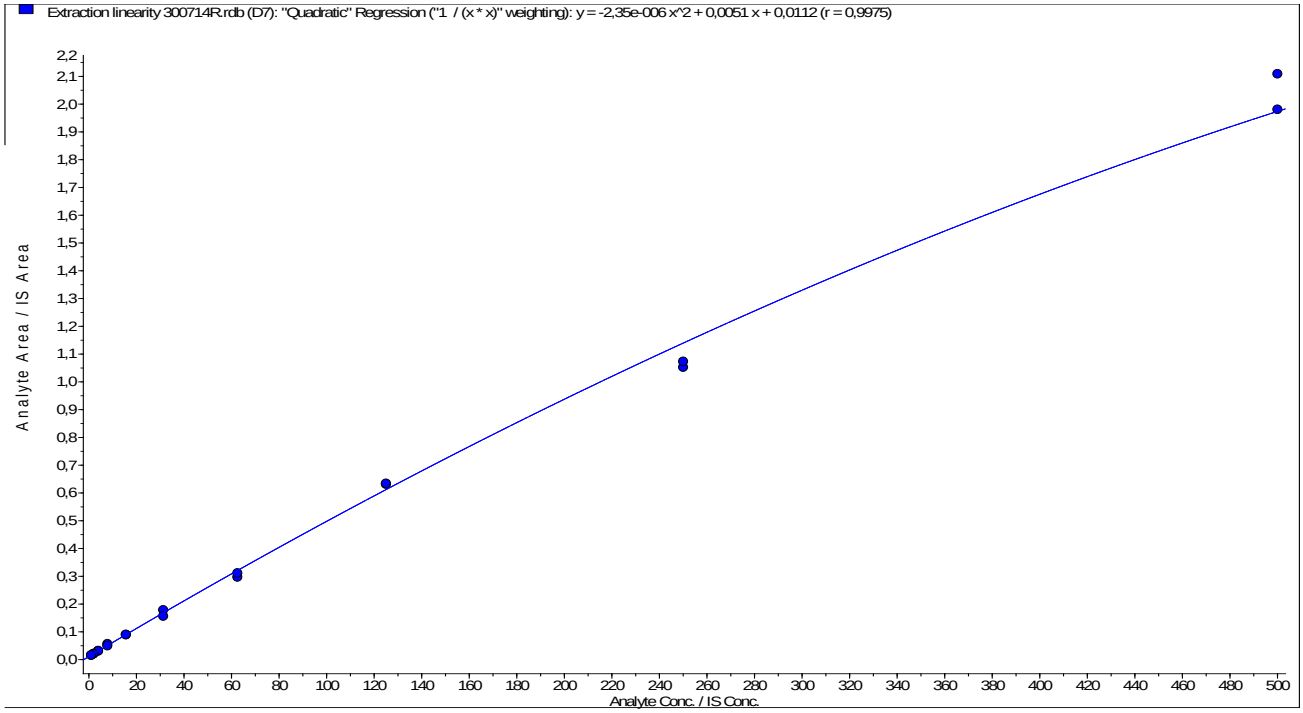


Figure B5. Extraction linearity of derivatised 4β-OHC-D7 in blank human plasma

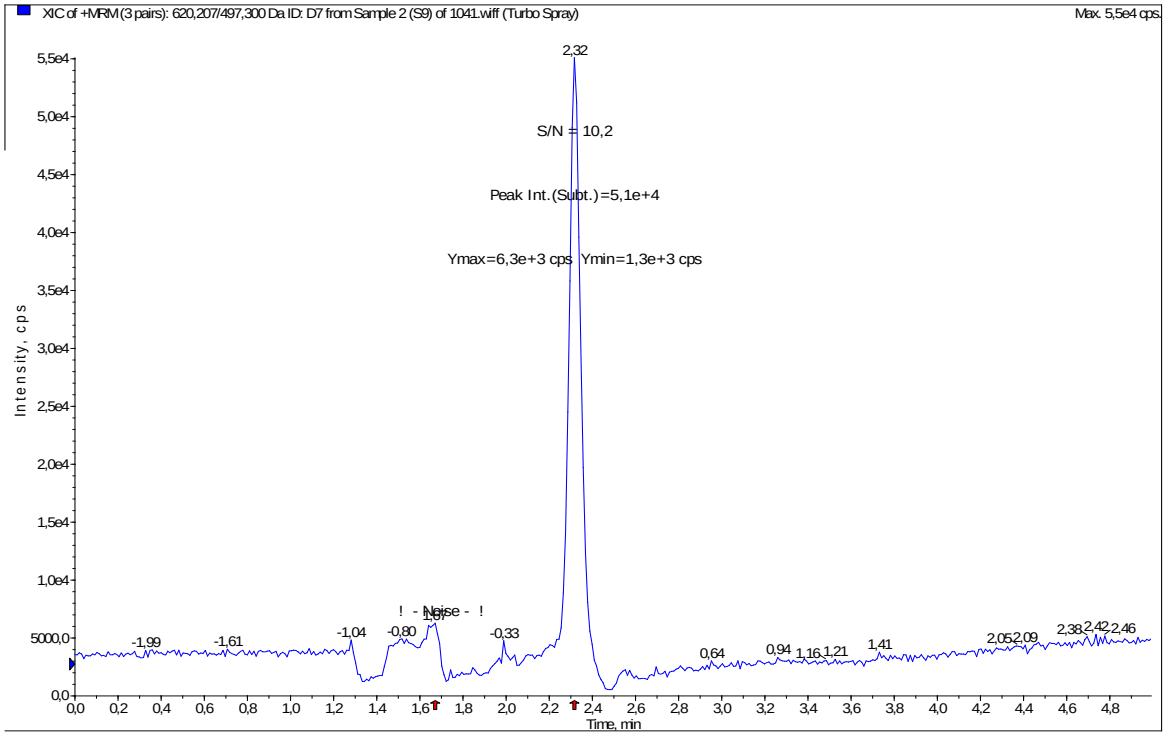


Figure B6. Signal to noise ratio of derivatised 4β-OHC-D7 at LLOQ 2 ng/ml in blank human plasma

Table B4. Extraction repeatability of 4β-OHC-D7 in blank human plasma n= 40

	4β-OHC-D7 /4β-OHC-D4 Peak area ratio
Average	1.28
ST DEV	0.18
% CV	14.0

Table B5. Extraction repeatability of endogenous 4β-OHC in blank human plasma n= 40

	4β-OHC-D7 /4β-OHC-D4 Peak area ratio
Average	0.47
ST DEV	0.05
% CV	10.7

B3. Quadratic equation used for the calculation of unknown 4β-OHC in clinical samples:

$Y = a.x^2 + b.x + c.$

Where the relationship describes the reading on the mass spectrometer as a quadratic function of concentration in the study sample.

Given the mass spectrometer reading as y (ratio of; study sample 4β-OHC peak area/ISTD 4β-OHC-D4 peak area), the concentration was obtained by calculating the corresponding x solutions of the quadratic equation. a, b and c were the standard curve coefficients.

X = (- b +- sqrt(b^2 - 4.a.c)) / (2.a)

APPENDIX C: MNR-ESD OUTLIER TEST TABLES

C1. MNR-ESD outlier test for Freeze Thaw stability

Table C1. High concentration 4β-OHC- D7 (400 ng/ml)

Replicates	Flag	Data	Absolute Difference From Mean	
1		7751000	2417666,667	
2		6785000	1451666,667	
3		5443000	109666.667	
4		5003000	330333.333	
5		4204000	1129333.333	
6	MDV	2814000	2519333.333	Outlier
Mean		5333333		
N		6	0.84396	
MNR		0.635902		

Table C2. ISTD 4β-OHC- D4 (200 ng/ml) (spiked in high 4β-OHC-D7 samples)

Replicates	Flag	Data	Absolute Difference From Mean	
1		7631000	2434833.333	
2		6843000	1646833.333	
3		5057000	139166.667	
4		4867000	329166.667	
5		4099000	1097166.667	
6	MDV	2680000	2516166.667	Outlier
Mean		5196167		
N		6	0.84396	
MNR		0.623167		

Table C3. Low concentration 4β-OHC-D7 (3 ng/ml)

Replicates	Flag	Data	Absolute Difference From Mean	
1		137600	22595	
2		149800	34795	
3		88230	26775	
4		135100	20095	
5		106200	8805	
6	MDV	73100	41905	Outlier
Mean		115005		
N		6	0.84396	
MNR		0,612847		

Table C4. ISTD 4β-OHC- D4 (200 ng/ml) (spiked in low 4β-OHC-D7 samples)

Replicates	Flag	Data	Absolute Difference From Mean	
1		7321000	1685333.333	
2		7451000	1815333.333	
3		4554000	1081666.667	
4		6778000	1142333.333	
5		5351000	284666.667	
6	MDV	2359000	3276666.667	Outlier
Mean		5635667		
N		6	0.84396	
MNR		0.743386		

C2. MNR-ESD outlier test for bench-top stability

Table C5. High concentration 4β-OHC-D7 (400 ng/ml)

Replicates	Flag	Data	Absolute Difference From Mean	
1		6989000	906333.333	
2		6423000	340333.333	
3		7489000	1406333.333	
4		5985000	97666.667	
5		6025000	57666.667	
6	MDV	3585000	2497666.667	Outlier
Mean		6082667		

N		6	0.84396	
MNR		0.824971		

Table C6. ISTD 4β-OHC- D4 (200 ng/ml) (spiked in high 4β-OHC-D7 samples)

Replicates	Flag	Data	Absolute Difference From Mean	
1		6886000	936500	
2		6415000	465500	
3		7292000	1342500	
4		5759000	190500	
5		5851000	98500	
6	MDV	3494000	2455500	Outlier
Mean		5949500		
N		6	0.84396	
MNR		0.819798		

Table C7. Low concentration 4β-OHC-D7 (3 ng/ml)

Replicates	Flag	Data	Absolute Difference From Mean	
1		149100	26358.333	
2		117800	4941.667	
3		134100	11358.333	
4		125100	2358.333	
5		125200	2458.333	
6	MDV	85150	37591.667	Outlier

Mean		122741		
N		6	0.84396	
MNR		0.788493		

Table C8. ISTD 4β-OHC- D4 (200 ng/ml) (spiked in low 4β-OHC-D7 samples)

Replicates	Flag	Data	Absolute Difference From Mean	
1		8019000	1863833.333	
2		6185000	29833.33333	
3		7329000	1173833.333	
4		6179000	23833.33333	
5		5842000	313166.6667	
6	MDV	3377000	2778166.667	Outlier
Mean		6155167		
N		6	0.84396	
MNR		0.780510		

C3.MNR-ESD outlier test for ISTD recovery

Table C9. ISTD recovery at 200 ng/ml

Replicates	Flag	Data	Absolute Difference From Mean	
1	MDV	12310000	2334000	Outlier
2		8560000	1416000	
3		9520000	456000	
4	MDV	12010000	2034000	Outlier
5		8775000	1201000	
6		8681000	1295000	
Mean		9976000		
N		6	0.84396	
MNR		0.604307		

APPENDIX D; 4B-OHC, 4B-OHC-7 AND 4B-OHC-D4 CERTIFICATE OF ANALYSIS

CERTIFICATE OF ANALYSIS

Toronto Research Chemicals Inc.
2 Brisbane Road, North York, ON, M3J 2J8 Canada Tel: (416) 665-9696 Fax: (416) 665-44
E-mail: orders@trc-canada.com Website: www.trc-canada.com

1. Identification

CAS Number:
17320-10-4

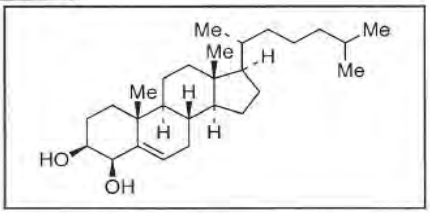
Catalogue Number:
H917980

#354 and #355

Product:
4β-Hydroxy Cholesterol

Synonyms:
(3β,4β)-Cholest-5-ene-3,4-diol; Cholest-5-ene-3β.4β-diol; 3β,4β-Dihydroxycholest-5-ene; cis-3,4-Dihydroxy-5-cholestene; cis-5-Cholestene-3,4-diol;

Structure:



Molecular Formula:
$C_{27}H_{46}O_2$

Molecular Weight:
402.65

Source of Product:
Synthetic

2. Analytical Information

Lot Number:
4-MNZ-147-1

Melting Point:
172-174°C

Boiling Point:
N/A

Atmosphere:
Air

Appearance of Product:
White Solid

Solubility
Chloroform, Ethyl Acetate

Method for Determining Identity:
^{1}H NMR ($CDCl_3$) Spectroscopic and Mass Spectrometric Analysis

Stability
Not determined

Purity:
98%

Long Term Storage Condition:
Refrigerator

Additional Information:
TLC Conditions: SiO_2; Ethyl Acetate: Hexane = 1: 1; Visualized with UV and AMCS; Single spot; Rf=0.6.
^{1}H NMR and mass spectra conform to structure.
Specific rotation: -61.5°(c=0.54, Chloroform)

#received! 31/05/2013

Philip Chan, Head of Quality Assurance

QC Test Date
July 6, 2011

Retest Date
July 6, 2014

CERTIFICATE OF ANALYSIS

2 Brisbane Road, North York, ON. M3J 2J8 Canada Tel: (416) 665-9696 Fax: (416) 665-4439
E-mail: orders@trc-canada.com Website: www.trc-canada.com

1. Identification

CAS Number:
1246302-80-6

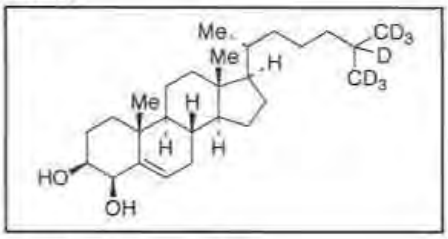
Catalogue Number:
H917982

#IS198 9-2FS199

Product:
4β-Hydroxy Cholesterol-d7

Synonyms:
(3β,4β)-Cholest-5-ene-3,4-diol-d7; Cholest-5-ene-3β,4β-diol-d7; 3β,4β-Dihydroxycholest-5-ene-d7; cis-3,4-Dihydroxy-5-cholestene-d7; cis-5-Cholestene-3,4-diol-d7;

Structure:



Molecular Formula:
$C_{27}H_{39}D_7O_2$

Molecular Weight:
409.70

Source of Product:
Synthetic

2. Analytical Information

Lot Number:
1-GAB-60-2

Melting Point:
171 - 173°C

Boiling Point:
N/A

Atmosphere:
Air

Appearance of Product:
Off-White to Light Grey Solid

Solubility
Chloroform, Methanol (Sparingly)

Method for Determining Identity:
^{1}H NMR ($CDCl_3$) and MS

Stability
Not Determined

Purity:
Chemical Purity: 98%
Isotopic Purity: 99.5%

Long Term Storage Condition:
Refrigerator

Additional Information:
TLC Conditions: SiO_2; Ethyl Acetate : Hexane = 1 : 1; Visualized with AMCS and $KMnO_4$; Single Spot, Rf = 0.45.
^{1}H NMR and MS conform to structure.
Elemental Analysis: (Found) %C: 79.15, %H: 11.35; (Calculated) %C: 79.15, %H: 11.32
Normalized Intensity: d_0 = 0.14%, d_1 = 0.00%, d_2 = 0.03%, d_3 = 0.03%, d_4 = 0.08%, d_5 = 0.09%, d_6 = 1.68%, d_7 = 97.96%

Philip Chan, Head of Quality Assurance

QC Test Date
May 24, 2013

RECEIVED
05 AUG 2016

Retest Date
May 22, 2016

CERTIFICATE OF ANALYSIS

2 Brisbane Road, North York, ON. M3J 2J8 Canada Tel: (416) 665-9696 Fax: (416) 665-4439
E-mail: orders@trc-canada.com Website: www.trc-canada.com

1. Identification

CAS Number:

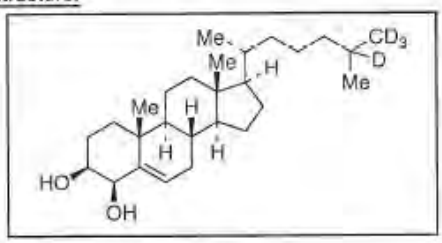
Catalogue Number:
H917983

#IS114 & #IS115

Product:
4β-Hydroxy Cholesterol-d4 (Major)

Synonyms:
(3β,4β)-Cholest-5-ene-3,4-diol-d4; Cholest-5-ene-3β,4β-diol-d4; 3β,4β-Dihydroxycholest-5-ene-d4; cis-3,4-Dihydroxy-5-cholestene-d4; cis-5-Cholestene-3,4-diol-d4;

Structure:



Molecular Formula:
$C_{27}H_{42}D_4O_2$

Molecular Weight:
406.68

Source of Product:
Synthetic

2. Analytical Information

Lot Number:
8-QFY-109-1

Melting Point:
172-174°C

Boiling Point:
N/A

Atmosphere:
Air

Appearance of Product:
Light Pink Solid

Solubility
Chloroform, Ethyl Acetate

Method for Determining Identity:
^{1}H NMR ($CDCl_3$) Spectroscopic and Mass Spectrometric Analysis

Stability
Not determined

Purity:
98%

Long Term Storage Condition:
Refrigerator

Additional Information:
TLC Conditions: SiO_2; Ethyl Acetate : Hexane = 1 : 1; Visualized with $KMnO_4$; Single spot, Rf=0.5.
^{1}H NMR and Mass spectra conform to structure.
Normalized Intensity: d_0 = 0.06%, d_1 = 0.14%, d_2 = 2.15%, d_3 = 16.02%, d_4 = 81.63%

Received 31/05/2013

Philip Chan, Head of Quality Assurance

QC Test Date
April 27, 2012

Retest Date
April 27, 2015