An assessment of the effects of selected antiretroviral drugs on steroid hormone production using an *in vitro* steroidogenesis assay

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ABSTRACT

Various contaminants already exist in the environment, with knowledge of more emerging each day. Some of these emerging contaminants are pseudo-persistent. Examples of these are pharmaceuticals and personal care products (PPCPs). PPCPs are designed to have a biological effect at very low doses, and therefore they are capable of causing a lot of harm in the environment. Due to the high prevalence of human immunodeficiency virus in South Africa, there are copious amounts of antiretroviral (ARV) treatments being administered. The ARVs that are unused or not fully metabolised make their way into the receiving environment, inter alia via wastewater treatment plants. ARVs along with many other PPCPs have already been detected in various water sources globally. However, the effects that these environmental ARVs may have on various non-target organisms, is not well researched. Various PPCPs are known to be endocrine disruptive *in vitro.* Therefore, this study aimed to determine the effect of known environmentally relevant concentrations of ARV active ingredients on steroidogenesis *in vitro*.

The H295R human adrenocortical carcinoma cells were exposed to six concentrations (between 0.0008 and 80 ng/L) of six ARVs for 48 hours. The change in hormone concentrations in the nutrient growth medium was compared to that of solvent exposed control cells and expressed as a fold-change. Data was corrected for the evaporation of nutrient media during exposure, as well as the viability of the cells. The six hormones quantified were testosterone, 17β-oestradiol, aldosterone, cortisol, androstenedione, and 17α-hydroxyprogesterone. The method to quantify six steroid hormones was developed and validated for the purpose of this study using an ultra-high-pressure liquid chromatograph, quadrupole time-of-flight mass spectrometer.

Results were variable and not always dose-dependent: a single ARV would decrease hormone levels at high concentrations, just to increase again at a lower concentration, followed by an increase again at an even lower concentration. In general, lopinavir, lamivudine, stavudine and efavirenz exposures decreased 17β-oestradiol levels, while ritonavir increased 17β-oestradiol levels. Testosterone levels decreased with exposures to ritonavir, lamivudine, stavudine and efavirenz. Furthermore, ritonavir, efavirenz and stavudine exposures resulted in decreased cortisol levels, while stavudine, didanosine and efavirenz decreased androstenedione. 17α-hydroxyprogesterone decreased with efavirenz and stavudine exposures, but increased with exposures to ritonavir, lopinavir, didanosine and lamivudine. Therefore, the results from this study show that all the ARVs tested influenced the steroidogenesis process in the H295R cells to some extent, possibly causing endocrine

disrupting effects in vertebrate organisms exposed to concentrations occurring in the natural environment.

Keywords: ARV; H295R cells; PPCP; endocrine disruption; EDC; UHPLC-QTOF; adrenal gland.

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17-OH progesterone	—	17α-hydroxyprogesterone
17-OH pregnenolone	—	17α-hydroxypregnenolone
Oestradiol	—	17β-oestradiol
AIDS	—	Acquired immunodeficiency syndrome
AJS	—	Agilent Jet Stream
ART	—	Antiretroviral therapy
ARV	—	Antiretroviral
C18	—	Agilent ZORBAX C18 column
CV	—	Coefficient of variation
DDT	—	Dichlorodiphenyltrichloroethane
DHEA	—	Dehyroepiandrosterone
DHEAS	—	Dehyroepiandrosterone sulfate
DMEM	—	Dulbecco’s modified eagle’s medium
DMSO	—	Dimethyl sulphoxide
DNA	—	Deoxyribonucleic acid
DPBS	—	Dulbecco’s phosphate buffered saline

EDC	—	Endocrine disrupting chemical
ELISA	—	Enzyme-linked immunosorbent assay
ESI	—	Electrospray ioniser
FC	—	Fold change
FDA	—	United States Food and Drug Administration
FIA	—	Flow injector analysis
GC	—	Gas chromatography
HAART	—	Highly active antiretroviral therapy
HIV	—	Human immunodeficiency virus
HPA	—	Hypothalamus-pituitary-adrenal axis
HSD	—	Hydroxysteroid dehydrogenases
LC	—	Liquid chromatography
LLE	—	Liquid-liquid extraction
LOD	—	Limit of detection
LOQ	—	Limit of quantification
MS	—	Mass spectrometer

MS/MS	—	Tandem mass spectrometry
MTT	—	2-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NNRTI	—	Non-nucleoside reverse transcriptase inhibitor
NRTI	—	Nucleoside/nucleotide reverse transcriptase inhibitor
OECD	—	Organisation for Economic Cooperation and Development
PLWHIV	—	Person living with human immunodeficiency virus
PI	—	Protease inhibitor
PPCP	—	Pharmaceuticals and personal care product
QC	—	Quality control
QTOF	—	Quadrupole time-of-flight mass spectrometer
QQQ	—	Triple quadrupole mass spectrometer
RNA	—	Ribonucleic acid
RSD	—	Relative standard deviation
SA	—	South Africa
SC	—	Solvent exposed H295R cells

SD	—	Standard deviation
SPE	—	Solid phase extraction
TOF	—	Time-of-flight mass spectrometer
UHPLC	—	Ultra-high pressure liquid chromatograph
USA	—	United States of America
USEPA	—	United States Environmental Protection Agency
WHO	—	World Health Organization
WWTP	—	Wastewater treatment plant

CHAPTER 1: INTRODUCTION

1.1 Background and motivation

Compared to the rest of the world, South Africa (SA) has the greatest number of people living with the Human Immunodeficiency Virus (HIV) (17.52 million in 2018) (STATSSA, 2018, UNAIDS, 2019). Furthermore, SA has the largest number of antiretroviral drugs (ARVs) being used per capita in the world (66% of the people with HIV in SA are on ARVs) (STATSSA, 2018, UNAIDS, 2019). The unused ARVs, as well as the metabolised ARVs, are entering the natural environment, inter alia via wastewater treatment plants (WWTPs) and have already been detected in various SA rivers (Abafe et al., 2018, Schoeman et al., 2017, Wood et al., 2015). It is known that ARVs are capable of causing endocrine disrupting effects in HIV patients receiving therapeutic doses. It is for this reason that the possible endocrine disrupting capabilities of ARVs at lower, environmentally relevant, exposures on non-target organisms and humans in the aquatic environment were investigated further in this study.

Endocrine disrupting chemicals (EDCs) are exogenous substances that are capable of modulating the endocrine (hormone) systems of various animals, including humans. EDCs have various modulating mechanisms of action, such as by direct protein degradation, co-activator recruitment, deoxyribonucleic acid (DNA) interference, and dysregulation of hormone metabolism (which includes how the enzymes assist in the metabolism) (Swedenborg et al., 2009). There is a lack of literature on the endocrine disrupting capabilities of ARVs, and in particular on their influence on the steroidogenesis pathway in the adrenal glands. The Organisation of Economic Cooperation and Development (OECD) has an established *in vitro* screening method for detecting the effects of compounds on the steroidogenic process, and quantifying the up or down regulation of various hormones in the nutrient media of H295R cells (OECD, 2011). More details on endocrine disrupting chemicals is to follow in the literature review (see section 2.3).

The H295R human adrenocortical carcinoma cells had been used to study the *in vitro* effects of various drugs and chemicals on the adrenal glands. Examples of such chemicals that had been studied include polybrominated diphenyl ethers (He et al., 2008), 2,4-dichlorophenol (Ma et al., 2012), forskolin, atrazine, letrozole, prochloraz, ketoconazole, aminoglutethimide and prometon (Higley et al., 2010), polychlorinated biphenyls and methyl sulfone polychlorinated biphenyls (Xu et al., 2006). However, very few studies have been done exposing the H295R cell line to ARVs, to determine their endocrine disrupting effects (Malikova et al., 2019).

Enzyme-linked immunosorbent assays (ELISAs) are the most common tool used to determine the concentrations of steroid hormones produced from these H295R cells in the above studies. However, each hormone requires a different immunoassay, which is time consuming and costly. Furthermore, these tests may have various cross-reactive substances, depending on how exclusive an analyte is to an antibody (Murtagh et al., 2013). Recently, some studies developed better methods for quantitative analysis of multiple hormones in one run, using liquid chromatography (LC) combined with a mass spectrometer (MS), on extracts from various biological mediums. LC-MS methods are more accurate than immunoassays with the added benefit of detecting and quantifying multiple analytes simultaneously (Gaikwad, 2013, Murtagh et al., 2013, Peters et al., 2010) (the benefits of LC-MS to immunoassays are described in more detail in the literature review (see section 2.8)). Although there are LC-MS methods available, these are not often performed using the H295R cell medium, but rather using other biological matrices such as serum, urine, and faeces (Murtagh et al., 2013). Furthermore, these analyses are performed using a triple quadrupole MS (QQQ), whereas our laboratory is equipped with a quadrupole-time-of-flight MS (QTOF) for this study. In addition, studies involving the H295R assay often only focus on two key steroid hormones: oestradiol and testosterone. In this study, four more steroid hormones were included.

The current study is novel in that it attempts to develop and validate a method to quantify steroid hormones on an ultra-high pressure liquid chromatograph (UHPLC)-QTOF instrument. Secondly, the steroid hormones produced by H295R cells were quantified after exposure to various ARVs active ingredients. Based on the literature research available (see section 2.5.2), our hypothesis was realised (Cardoso et al., 2007, Kibirige and Ssekitoleko, 2013, Sinha et al., 2011). The hypothesis was that ARVs at environmentally relevant levels will affect steroid hormone production *in vitro*.

1.2 Research aim and objectives

The aim of this study was to determine the effect of known concentrations of the active ingredients of six ARVs (see chemical structures in addendum A) on steroidogenesis. In order to achieve this, the following objectives were set:

- To develop and validate a method to detect steroid hormones using the UHPLC-QTOF.
- To expose H295R human adrenal carcinoma cells to known concentrations of six ARV active ingredients.
- To use the validated method to quantify six steroid hormones extracted from the nutrient media of the H295R cells after exposure.

- To compare the concentrations of six steroid hormones between exposed and control cells.

CHAPTER 2: LITERATURE REVIEW

2.1 Environmental pollutants

Our ecosystem is detrimentally affected by environmental pollution, with approximately 16% of human deaths per annum globally attributed to pollutant exposures from, inter alia, industrial discharges, exhaust fumes, and toxic chemicals (Landrigan et al., 2018). These contaminants pose a risk to both the environment, as well as humans (Nweke and Sanders, 2009, Swanepoel et al., 2015).

Chemical pollutants that have been extensively studied include the inorganic heavy metals, such as mercury, lead, chromium and cadmium; the persistent organic pollutants, such as dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls, hexabromobiphenyl and lindane; and the radionuclides, such as uranium (Landrigan et al., 2018, Nweke and Sanders, 2009, WHO, 2019b). These pollutants are persistent in the environment and are of concern globally due to their capability to resist degradation for many decades, their long-range transport potential, and their significant negative effects on the ecosystem health (Nadal et al., 2015).

Pollutants that are suspected or known to cause human or ecological effects, but for which there is limited understanding of their deposition, occurrence and fate, and are not commonly monitored in the environment, are commonly referred to as emerging contaminants (Rosenfeld and Feng, 2011). The list of emerging contaminants is continuously changing as new compounds are produced and science works to understand better the various contaminants (Sauve and Desrosiers, 2014). Currently, the list of emerging contaminants includes industrial chemicals, surfactants, pharmaceuticals and personal care products (PPCPs) (Rosenfeld and Feng, 2011). PPCPs are classified as pseudo-persistent, because they are constantly being released into the environment and therefore constantly present. Once PPCPs bio-accumulate, their concentrations may rise to toxic levels, and they are then able to influence various ecological processes and functions (Caliman and Gavrilescu, 2009, Richmond et al., 2017).

2.2 Pharmaceuticals and personal care products

PPCPs are “any product used by individuals for personal health or cosmetic reasons or used by agribusiness to enhance the growth or health of livestock”, and consists of various chemicals (Cizmas et al., 2015). Personal care products or cosmetics include products such as lipsticks, shampoos, toothpaste, skin moisturizers, deodorants, perfumes, nail polishes,

and makeup, which are used to improve the quality of daily life. Pharmaceuticals are more commonly known as medicines and drugs. Pharmaceuticals of concern in the global environment include steroidal drugs, such as hormones; and non-steroidal drugs, such as antiretrovirals (ARVs), antibiotics, analgesics and antiepileptics (Schoeman et al., 2017). Pharmaceuticals are designed to, at very low concentrations, improve human and animal health, by preventing and treating diseases. However, after their consumption, these compounds undergo metabolic degradation, but are not always completely degraded in the body, and therefore some of these compounds are excreted from the body unchanged (Madikizela et al., 2017). The significant increase in pharmaceutical production and use worldwide have brought about a noticeable number of these compounds being released into ecosystems (Mezzelani et al., 2018). PPCPs are continuously being discharged into the environment, and due to their bioactivity, are able to interfere with the health of organisms, including their life cycles (Mezzelani et al., 2018, Sun et al., 2015). Furthermore, there are many substandard and falsified PPCPs available globally, which cause various unknown effects on an organism (WHO, 2018).

Along with the significant consumption of pharmaceuticals, the improper disposal of the unused medications, contributes to the increase in the concentrations of these compounds in our ecosystem. The major sources of pharmaceuticals in the environment are from industrial and hospital discharges, WWTPs, agriculture (including bio solid sewage sludge usage), and soil runoff (Al-Rajab et al., 2010, Madikizela et al., 2017, Mezzelani et al., 2018, Sun et al., 2015). A major concern is that environmental legislations lack the obligation to perform routine monitoring of PPCPs in the environment and can therefore not identify the sources or prosecute the companies responsible. The current PCPP concentrations in the environment are expected to increase for the foreseeable future (Padhye et al., 2014).

The majority of the PPCPs are only partially removed in conventional WWTPs, and are therefore present in distribution waters (Mezzelani et al., 2018, Sun et al., 2015). Most WWTPs use a biological treatment process, which removes solid waste, dissolved organic matter, and nutrients. Depending on the varied physiochemical properties, environmental conditions and operational parameters, some PPCPs may also be removed during this process (Wang and Wang, 2016). However, the most effective treatment for the majority of PPCPs is ozonation, which is very costly, and therefore only suitable for developed countries (Wang and Wang, 2016). Furthermore, some African communities do not even have proper sanitation, with no WWTPs. In these communities, human waste is excreted directly onto the ground and into the surface water, where it is then washed into the rivers during the rainy

seasons, causing various health dangers to humans and aquatic species alike (Madikizela et al., 2017, Wood et al., 2015).

PPCPs have been detected in various types of environmental compartments globally, for example (but not limited to):

- sediment of the upper Danube river (Germany) (Grund et al., 2011);
- surface water (SA) (Wood et al., 2015, Wood et al., 2016, Wood et al., 2017);
- the Nairobi river basin (Kenya) (Ngumba et al., 2016);
- WWTPs influents and effluents in Germany (Funke et al., 2016), SA (Abafe et al., 2018, Mosekiemang et al., 2019, Schoeman et al., 2017), and Sweden (Björklund and Svahn, 2018);
- various German rivers and streams (Funke et al., 2016);
- drinking water in China (Sun et al., 2015), Germany (Funke et al., 2016) and the United States of America (USA) (Ferrer and Thurman, 2012);
- and agricultural soil in Canada (Al-Rajab et al., 2010).

The concentrations of PPCPs in the environment are affected by their physicochemical characteristics as well as environmental factors (Mezzelani et al., 2018). The chemical characteristics that influence PPCP concentrations include whether a chemical is hydrophilic (will partition into water) or hydrophobic (will partition into sediment and suspended organic matter) (Madikizela et al., 2017, Mezzelani et al., 2018). The hydrophobic PPCPs that partition to sediment are more persistent than the hydrophilic ones, as they stay in the sediment and then re-suspend or diffuse at a later stage into the surrounding water (Zhang et al., 2003). Other physiochemical properties of PPCPs affecting their concentrations in the environment include their acidity, volatility and sorption properties. Environmental factors that influence the concentration of PPCPs in the water include decreased water quality (due to increased loading from WWTPs and agricultural run-off), prolonged droughts, climate change effects (such as rising temperatures worldwide) and an increase in the human population (Padhye et al., 2014). Additionally, the concentration of chemicals in water will be affected at different periods by precipitation and rainfall; evaporation of water at higher temperatures (as is the case in warmer countries and during summer); seasonal changes (N,N-diethyl-m-toluamide (DEET) is used more commonly as an insect repellent during summer); and increased biodegradation and photolysis with increased sunshine (Padhye et al., 2014, Sun et al., 2015).

PPCPs with negative effects (such as alteration of immunological parameters, lipid peroxidation, DNA fragmentation, oxidative stress, and transcriptional gene changes) on the environment (especially on the marine species), include: non-steroidal anti-inflammatory

drugs, antibiotics, steroid hormones, psychiatric drugs, hypocholesterolaemia drugs, anti-inflammatory, antidiabetic and cardiovascular drugs (Mezzelani et al., 2018). However, the long-term exposure effects (especially at certain sensitive developmental stages) of PPCPs on human health and non-target organisms require further investigation, as the ecotoxicological effects of many PPCPs have not yet been established (Mezzelani et al., 2018, Padhye et al., 2014, Wood et al., 2015). Furthermore, low levels of individual PPCPs could have adverse effects due to their bioaccumulation properties. Some PPCPs that may not cause any adverse effects individually at trace levels, could create adverse effects when present as mixtures with other PPCPs with similar toxicological mechanisms of action (Ebele et al., 2017, Padhye et al., 2014). An example of the effect of PPCPs on the aquatic ecosystem were shown where low level of exposure to selective serotonin re-uptake inhibitors caused some fish to become more aggressive and alter their mating behaviour, as well as altered the social behaviour and development of amphibians (Sehonova et al., 2018).

Furthermore, the presence of antibiotics in the environment threatens the prevention and treatment of various infectious diseases due to antibiotic resistance developing, as well as killing off the “good” natural bacteria (microbiomes) present in the environment (Ebele et al., 2017). A microbiome is a collection of bacteria, eukaryotes and viruses that are found in the body, and its functions include vitamin production, supply of nutrients, and immunity against other pathogens. When this microbiome is not functioning correctly, as is the case when antibiotics are used, disorders of the immune system, metabolism and even development can occur (Langdon et al., 2016).

Finally, a major concern of PPCPs in the environment is their ability to interfere with the endocrine system of various organisms (Madikizela et al., 2017, Padhye et al., 2014). Endocrine disruption causes various effects including disruption of homeostasis, abnormal growth and development patterns, altered reproductive functions, neurological alterations, immune function changes, and even increased risk of breast cancer and other cancerous tumours (WHO, 2019a).

2.3 Endocrine disrupting chemicals

The World Health Organisation (WHO) defines an EDC as “an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations” (WHO, 2002). EDCs are capable of interfering with hormone homeostasis by disrupting one or more of the various hormonally mediated processes and interfering with their mechanisms of action. This is done by direct protein degradation, co-activator recruitment, DNA interference, or dysregulation of

hormone metabolism (which includes how enzymes assist in the metabolism) (Nielsen et al., 2012, Swedenborg et al., 2009). The term endocrine disruption, in the context of this study, refers to a chemical's ability to disrupt any endocrine process, by any of the above-mentioned mechanisms of action. Pathologies linked to steroid dysregulation from EDC exposure include some cancer types, diabetes, obesity, metabolic disorders, reproductive dysfunctions, and neural development defects (Boccard et al., 2019). Most EDCs are fat-soluble, and for this reason are able to bio-accumulate in adipose tissue, creating higher concentrations, which would have increased effects the longer the exposure to a particular EDC lasts (Diamanti-Kandarakis et al., 2009). Therefore, humans should take care to limit their exposures to known EDCs as much as possible, consequently decreasing the EDCs released/re-released into the environment, thereby decreasing the EDC effects on the ecosystem.

EDCs include natural and synthetic chemicals, and are widely present in the environment, where they have the potential to be toxic to humans (Boccard et al., 2019). Various chemical classes contain compounds that may interfere with the endocrine system, making them a challenging group of chemicals to study. The different groups can be divided as follows: hormones in their natural and metabolised states (e.g. 17β-oestradiol, testosterone, cortisol); synthetic forms of hormones (e.g. contraceptive pill steroids, diethylstilboestrol); PCPPs (e.g. sunscreen, soaps, cosmetics); additives in food (e.g. preservatives and colourings); pesticides and insecticides (including their metabolites) (e.g. lindane, endosulfan, DDT); myco- and phytoestrogens (e.g. isoflavones, lignans); chemicals used in industrial and household settings, including their combustion by-products (dioxins, polycyclic aromatic hydrocarbons, polychlorinated/brominated biphenyls); heavy metals (lead, mercury, arsenic, cadmium); and flame retardants, paints and plasticisers (bisphenol A, phthalates) (Burkhardt-Holm, 2010).

Although there are numerous classes of EDCs (as mentioned above), the one of interest for this study was the PPCPs, with specific attention to drugs used in antiretroviral therapy (ART). Previous studies had shown that they cause endocrine disruption (Anuurad et al., 2009, Kibirige and Ssekitoleko, 2013, Malikova et al., 2019, Sinha et al., 2011, Strajhar et al., 2017), and they prevail in the environment (Archer et al., 2017, Ncube et al., 2018), but not that their presence in the environment may elicit endocrine disruption.

2.4 HIV globally & in South Africa

The medications used to treat retroviral infections, such as human immunodeficiency virus (HIV), is known as ARVs. HIV is a virus that affects the body's ability to fight an infection, by attacking the body's immune cells. The body of a person living with HIV (PLWHIV) then becomes unable to fight off other infections and diseases, and if left untreated, HIV can

progress to acquired immunodeficiency syndrome (AIDS), in which the immune system is damaged to a point of complete failure.

A retrovirus is a ribonucleic acid (RNA) based virus that contains reverse-transcriptase, which permits it to integrate into the DNA of the host cell (Levy, 1986). HIV requires various components from the host to reproduce itself and therefore it injects its genetic material into the CD4 cells (immune cells in the body). The growth, maturation and replication of the virus are dependent on the HIVs' protease enzyme. Furthermore, the reverse transcriptase enzyme is required by HIV in order to transcribe its RNA into DNA before it can incorporate itself into the host DNA. ARVs are designed to disrupt this reproductive cycle of the retrovirus. Although the virus cannot be cured or killed by ARVs, they help to slow down and in some cases even stop the multiplication of the HIV virus, and with their consistent use, one can live a long and healthy life (Ncube et al., 2018).

AIDS is a worldwide epidemic, which although it reached its peak in the western world in 1985, it only reached its peak in SA in 2006 (HIV.GOV, 2019b). HIV is transmitted to an uninfected human through contact with any bodily fluid of an infected person (HIV.GOV, 2019a).

AIDS was first encountered in 1981, but received its official naming in 1982, while the retrovirus (now known as HIV) causing AIDS was discovered in 1983. The first commercial blood test to detect HIV was released in 1985. It was in 1987, with the approval of the first ARV, zidovudine, by the United States Food and Drug Administration (FDA), that there was any hope for the eradication of this highly transmittable, deadly virus (HIV.GOV, 2019b). Nevertheless, despite all the research and funding received to stop HIV/AIDS, there were still 37.9 million PLWHIV globally in 2018, with more becoming infected daily (UNAIDS, 2019). There were approximately 5 000 new HIV infections per day worldwide in 2018 (UNAIDS, 2019). Of those infected with HIV, 23.3 million people are on ART, while 770 000 people died from AIDS related deaths in 2018 alone (UNAIDS, 2019). Although these statistics have improved from previous years, these numbers are alarming, especially when considering that the majority of these cases are in eastern and southern Africa (19.6 million) and that SA is the highest HIV prevalent country (17.52 million) in the world (STATSSA, 2018, UNAIDS, 2018). The population of SA in 2018 was approximately 57.7 million, meaning that 13.1 % of South Africans are infected with HIV (STATSSA, 2018), while 66% of PLWHIV are receiving ART (UNAIDS, 2018). This concludes the fact that SA has the largest amount of ARVs being used per capita in the world (Abafe et al., 2018, Wood et al., 2015).

The ARVs currently (2019) recommended globally by WHO for first-line regimen for adults and adolescents living with HIV include tenofovir, with lamivudine or emtricitabine, and dolutegravir

or efavirenz. The second-line regimen includes zidovudine, with lamivudine, and atazanavir/ritonavir or lopinavir/ritonavir or dolutegravir (WHO, 2019c). Similarly, in SA, the National Department of Health (DOH) stipulates the use of tenofovir, with lamivudine, and dolutegravir; or tenofovir, with emtricitabine and efavirenz, for the first-line regimen, and zidovudine or tenofovir, with lamivudine or emtricitabine, and lopinavir/ritonavir or atazanavir/ritonavir for the second-line regimen (SA-DOH, 2019). Therefore, these ARVs are most likely used by PLWHIV and are later excreted into the environment.

The increased use of ARVs globally, but mainly in SA, to curb the AIDS pandemic, is causing more ARVs to end up in the environment. The high concentrations of ARVs being found in the environment are causing chronic exposure of non-target organisms to ARVs, which have the potential to cause harm, with endocrine disruption being a probable effect (Kibirige and Ssekitoleko, 2013). Therefore, due to the high levels of ARVs being consumed and found it the environment, the effects of commonly used ARVs on the endocrine glands of HIV-negative people and other organisms, requires further research.

2.5 ARVs

2.5.1 Classes of ARVs

There are six main classes of ARVs currently available worldwide to help prevent the spread of HIV. The nucleoside/nucleotide reverse transcriptase inhibitor (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), inhibits the reverse transcriptase enzyme, thereby preventing reverse transcription of the viral RNA into the host DNA, preventing the virus from integrating into the host cells. The protease inhibitors (PIs) prevent the HIV RNA strands from being produced, by inhibiting the HIV DNA from fragmenting into the necessary components, and are used to inhibit multiple viral targets. To prevent the HIV virus from attaching to the cell body, entry or fusion inhibitors are used. Integrase inhibitors prevent the HIV integrase enzyme from inserting the viral DNA into the host cells' DNA, and is the ARV drug class of choice in patients with ARV resistance. The last class (cytochrome P450-3A inhibitors) is not an ARV, but rather a pharmacokinetic enhancer of ARVs, inhibiting cytochrome P450-3A isoforms from metabolising the ARVs (Ncube et al., 2018).

Examples of ARVs in the various groups are:

- NRTIs: abacavir, emtricitabine, lamivudine, zidovudine, stavudine, didanosine and tenofovir;
- NNRTIs: efavirenz, etravirine, and nevirapine;

- PIs: atazanavir, darunavir ritonavir, and lopinavir;
- Integrase inhibitors: dolutegravir, and raltegravir;
- Entry & fusion inhibitors: enfuvirtide, and maraviroc;
- P450-3A inhibitors: cobicistat (Ncube et al., 2018).

2.5.2 Side-effects of ARVs

Multiple studies have been done on the efficacy of ARVs and their associated side effects in PLWHIV (Abers et al., 2014, Boesecke and Cooper, 2008, Hawkins, 2010, Ncube et al., 2018). HIV itself, as well as its treatment with ARVs, cause various symptoms. One of the known symptoms of HIV is its ability to cause abnormalities of the various endocrine pathways (Cardoso et al., 2007, Kibirige and Ssekitoleko, 2013, Sinha et al., 2011). The most common endocrine abnormality in PLWHIV is adrenal insufficiency, with others including hypogonadism, thyroid dysfunction, lipodystrophy, and insulin resistance (Kibirige and Ssekitoleko, 2013). Some of the negative health effects of the ARVs include diarrhoea, vomiting, nausea, neurotoxicity, rash, lipodystrophy, insulin resistance, renal and respiratory system problems, mitochondrial toxicity, hepatotoxicity, and bone demineralization (Ncube et al., 2018). In addition, with the introduction of highly active antiretroviral therapy (HAART), a rise in incidence of endocrinopathies have become evident (Kibirige and Ssekitoleko, 2013, Sinha et al., 2011), which is of concern, as these cause an increased risk in morbidity and mortality if untreated (Anuurad et al., 2009, Kibirige and Ssekitoleko, 2013). HAART is a regimen that uses a combination of multiple classes of ARVs, that target the virus at various points in its reproductive cycle (Brechtl et al., 2001). In a study to evaluate the endocrine disrupting effects of ARVs, there had been two cases of Cushing's syndrome with secondary adrenal suppression in children, and 12 cases in adults, with the concomitant use of ritonavir and fluticasone (Johnson et al., 2006). Cushing's syndrome occurs when there is too much cortisol in the body, whether from an overproduction by the adrenal glands, or from external sources (such as steroid drug use). Another study reported transient adrenal dysfunction in neonates due to lopinavir-ritonavir treatment (Simon et al., 2011). Although the endocrine effects of various ARVs at clinical levels on their target population (PLWHIV) had been studied, their endocrine disrupting capabilities in the untargeted population, and the environment at lower levels, have not been extensively studied, with only a few studies done to assess their effects (Ncube et al., 2018).

The effects of ARVs on the untargeted population and the biota in the environment urgently require further investigation, as they have been found to be present in the environment, as discussed below.

2.5.3 ARVs in the environment

ARVs are regarded as emerging contaminants, with the potential to affect the environment negatively. Studies show that ARVs are only somewhat metabolised in the body and both their original and metabolised forms are excreted in the urine and faeces (Ncube et al., 2018). The large number of ARVs released into the environment through human excreta and urine are of major concern, due to their potential to impact the ecosystem and the development of viral resistance (Aves et al., 2018, Talman et al., 2013). However, limited information is available on the fate of all the ARVs in our ecosystem (Ncube et al., 2018).

Current technologies to treat wastewater, are ineffective at removing many complex chemicals, including ARVs (Swanepoel et al., 2015). As mentioned earlier, at the moment ozonation is the most effective technique for the removal of many PPCPs and EDCs, but it is a specialised and costly procedure, meaning that only developed countries can effectively implement it on a large scale (Padhye et al., 2014, Schoeman et al., 2017). A number of studies done in Africa detected ARVs in various water sources, including wastewater and rivers. Wood et al. (2015) tested for ARVs in various SA water systems including the Roodeplaat Dam system where they detected lamivudine (94.5–242 ng/L), stavudine (102–778 ng/L), zidovudine (156–973 ng/L), tenofovir (243 ng/L) and nevirapine (177–1480 ng/L). Furthermore, in the Orange River system they found zalcitabine (71.3 ng/L), tenofovir (145-189 ng/L), and didanosine (54 ng/L) (Wood et al., 2015). Whereas, in the Hartebeespoort Dam system, they detected zalcitabine (8.4–28.2 ng/L), didanosine (54.1 ng/L), zidovudine (72.7–452 ng/L), nevirapine (130–143 ng/L), and lopinavir (130–305 ng/L) (Wood et al., 2015). In another study, efavirenz and nevirapine were found in wastewater influent and effluent in Gauteng (SA), proving that the wastewater treatment available currently is not sufficient to remove these ARVs (Schoeman et al., 2017). Abafe et al. (2018) also reported ARVs in WWTP influents and effluents in KwaZulu-Natal (SA), including, but not limited to, ritonavir (460–320 ng/L), lopinavir (1 200–3 800 ng/L), lamivudine (60–2 200 ng/L) and efavirenz (20 000–34 000 ng/L). The ARVs detected in river water and WWTPs in Nairobi, Kenya, included lamivudine (3 985–5 428 ng/L), zidovudine (513–7 684 g/L) and nevirapine (1 357–4 859 ng/L) (Ngumba et al., 2016). In Germany, the ARVs lamivudine, acyclovir and abacavir were removable from the water by sewage treatment, while nevirapine, zidovudine and oseltamivir were not (Prasse et al., 2010). Nevirapine is a NNRTI ARV, that is widely used, highly persistent in the environment, resistant to degradation at the relevant chlorine levels used in SA WWTPs, and non-biodegradable, and therefore it is commonly detected in the environment (Schoeman et al., 2017, Wood et al., 2015).

Additionally, due to the popularity of using biosolids in commercial agriculture, there is a concern for the increase in soil bound PPCPs (including ARVs). Biosolids are used to improve the organic matter of soil, thereby increasing the source of nutrients available to the crops. This solid organic matter absorbs the PPCPs (efavirenz and nevirapine) that are stable through the sewage treatment process, and then when applied to the soil, the ARVs are able to be transported into nearby surface and ground water (USEPA, 2009). Moreover, a study in Canada, found that the ARV tenofovir was persistent for several weeks in agricultural soil (Al-Rajab et al., 2010).

From the preceding section, it is clear that non-target humans and the aquatic biota are possibly being chronically exposed to ARVs due to its presence in river water, as well as the soil in which crops are grown. Additionally, the concentrations of the various ARVs in the environment will continue to increase as the use of ARVs increases, in an attempt to eradicate HIV, especially in SA, where there are a large number of people living with HIV.

2.6 Steroidogenesis pathway

2.6.1 Adrenal (suprarenal) glands

Adrenal glands are responsible for the production of various hormones that vertebrates, including humans are unable to live without, and therefore it is very important that they function correctly (Silverthorn et al., 2007).

Each adrenal gland consists of two sections: the outer adrenal cortex and the inner adrenal medulla. The adrenal cortex secretes the essential steroid hormones such as the corticosteroids and the sex hormones. The adrenal medulla secretes nonessential hormones, which activates the body for the fight-or-flight response. These include epinephrine, norepinephrine and dopamine (Silverthorn et al., 2007). The release of hormones is regulated by the hypothalamic-pituitary-adrenal (HPA) axis, which is able to up or down regulate the release of the steroid hormones to maintain homeostasis. This happens according to the body's needs internally or is triggered/activated through external factors, such as certain pharmaceuticals. When the HPA negative feedback loop is disrupted, the outcome involves various steroid hormones being hyper- or hypo- secreted, causing various negative effects in the body (Silverthorn et al., 2007).

The corticosteroids in the adrenal cortex are divided into two groups: glucocorticoids and mineralocorticoids. The mineralocorticoids, with aldosterone being the primary one, are responsible for electrolyte and fluid balance, which in turn assists with controlling blood

pressure. The glucocorticoids assist in the immune, metabolic, developmental, arousal and body fluid homeostasis systems. Cortisol, the primary glucocorticoid, is essential for life, as it regulates important functions, such as homeostatic, immunologic, cardiovascular and metabolic functions. The sex steroids are only released in small amounts by the adrenal cortex, with much larger amounts being released by the ovaries and testes (Silverthorn et al., 2007).

The adrenal cortex as a whole, is capable of producing more than 30 steroids, of which the most commonly known are: aldosterone, pregnenolone, 17α-hydroxypregnenolone (17-OH pregnenolone), progesterone, 17α-hydroxyprogesterone (17-OH progesterone), 11-deoxycorticosterone, corticosterone, cortisone, 11-deoxycortisol, deoxycorticosterone, cortisol, androstenediol, androstenedione, dehyroepiandrosterone (DHEA), dehyroepiandrosterone sulfate (DHEAS), oestrone sulfate, oestriol, 17β-oestradiol (oestradiol), testosterone, and dihydrotestosterone (Ahmed et al., 2019, Holst et al., 2004, Nakano et al., 2016). However, only a few of these steroid hormones are produced exclusively in the adrenal glands, while the rest are also produced by the gonads or placenta. In order for the steroid pathway to produce these hormones, various enzymes, which can be categorised into different groups, are required. The majority of the enzymes required for steroidogenesis comes from one of mainly two classes of enzymes: cytochrome P450 hydroxylases or hydroxysteroid dehydrogenases (HSD). Other enzymes that also occur include 5α-reductase and sulfotransferase (Sult2A1) (Nakano et al., 2016). Some examples of cytochrome P450 hydroxylases (and their responsible genes) include 11β-hydroxylase (CYP11B1), 17α-hydroxylase (CYP17), 18-hydroxylase (CYP11B2), and 21-hydroxylase (CYP21). Some examples of the HSDs include 3α-HSD, 3β-HSD, 11β-HSD, 17β-HSD, 20α-HSD, 20β-HSD.

The main target for EDCs is the adrenal cortex, where the EDCs are capable of directly affecting the enzymes involved in the steroidogenesis pathway (Figure 2-1) (Ahmed et al., 2018). Furthermore, due to the complexity and various elements involved in steroidogenesis, simultaneous determination of various hormones is required in order to understand the dysregulation of the pathway by means of exogenous compounds or hormone pathologies (Nakano et al., 2016, Nielsen et al., 2012).

The steroid hormones selected in this study, were based on their importance in the steroidogenesis pathway. These steroid hormones are frequently screened in a clinical laboratory setting for diagnosis of adrenal disorders, as well as their relation to the enzymes they represent in the steroidogenesis pathway. If there were an inhibition or stimulation of any specific enzyme by a particular EDC, the steroid preceding or following that enzyme in the sequence of events in steroidogenesis (Figure 2-1) would decrease or increase accordingly.

When an enzyme is stimulated, more substrate (hormone) would undergo the chemical reaction triggered by the enzyme, and therefore more product hormones form, as the substrate hormone levels decrease. If an enzyme is inhibited, then it is unable to trigger a chemical reaction with the substrate and therefore less product hormones form, while the levels of substrate hormones increases. The enzymes' stimulation or inhibition may be characterised by the levels of substrate and/or product hormones present. The following product hormones' levels, would represent the respective preceding enzymes' activity (inhibition or stimulation) in the steroidogenesis pathway: aldosterone for aldosterone synthase; cortisol for 11β-hydroxylase and 21-hydroxylase; 17-OH progesterone for 17α-hydroxylase and 3β-HSD, androstenedione for 17α-hydroxylase and 3β-HSD; testosterone for 17β-HSD; and oestradiol for aromatase activity (Figure 2-1) (Sanderson, 2006). However, some substrate hormones' levels could also be an indicator of the following enzymes' activity that it binds to, such as 17-OH progesterone, which increases when there is an inhibition of the 21-hydroxylase enzyme. The majority of these hormones (whether substrate or product) are therefore used to identify specific disorders and diseases of the adrenal gland in various pathology laboratories (ie. 17-OH progesterone testing is performed to evaluate 21-hydroxylase deficiencies during newborn screening). Therefore, if these hormone levels are being affected by EDCs, there is a possibility of a false diagnosis of a disease, and consequently the wrong treatment could be recommended.

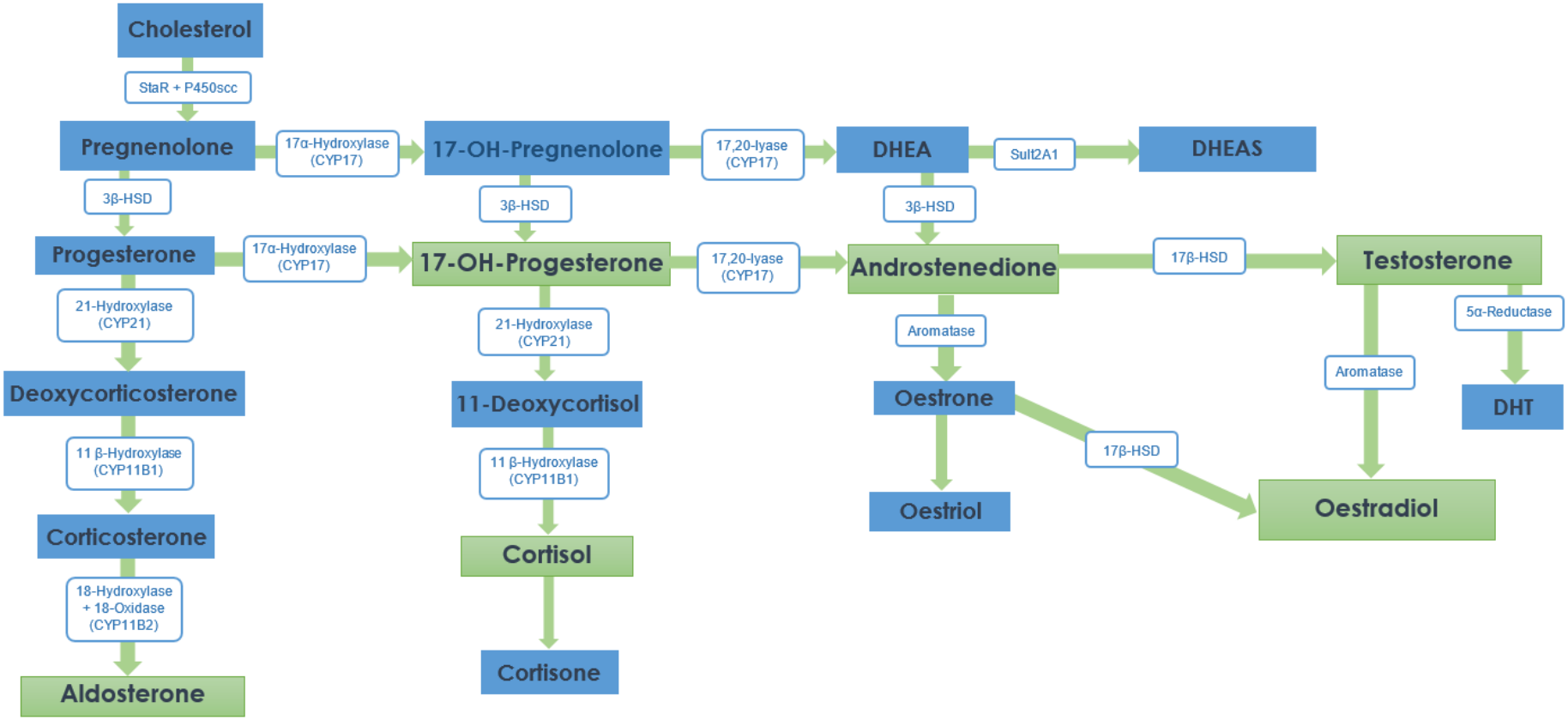

Figure 2-1: Schematic diagram of the steroidogenesis pathway, including the key steroid hormones and the respective steroidogenesis enzymes. The green blocked hormones are the hormones of the pathway that were analysed in this study, whereas the blue blocked hormones are the other hormones not analysed. The white blocks are the enzymes involved in each relevant hormone conversion in the pathway. (Adapted from Xu et al. (2006))

2.6.2 Adrenal gland disorders and diseases

Dysregulation of steroidogenesis causes various disorders, such as impaired memory, cognitive defects, hypertension, infertility, cancer, reduced immunity, metabolic disorders, and cardiovascular complications (Ahmed et al., 2019, Mangelis et al., 2016). An adrenal gland dysregulation could result in either overproduction or underproduction of any of the steroid hormones. There are numerous causes of these disorders, which include genetic mutations, infections, tumours, problems within the HPA axis, or from certain medications. (Silverthorn et al., 2007). The most common adrenal disorders include Addison's disease (insufficient aldosterone and cortisol production), Cushing's syndrome (overproduction of cortisol), adrenal cancer, and congenital adrenal hyperplasia (a genetic disorder affecting various hormone production) (Silverthorn et al., 2007). These disorders in humans are detected by determining the levels of 17-OH progesterone, cortisol, testosterone, DHEA, and oestradiol in blood, urine, and saliva.

EDCs are not only affecting hormone production, but could also cause false diagnosis of other endocrine disorders, thereby causing further damage to humans that are receiving the incorrect treatments. Therefore, it is important to screen emerging contaminants (for example PCPPs) for possible endocrine disruptive effects. Due to the need to identify potential EDCs, the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), Organisation for Economic Cooperation and Development (OECD), and United States Environmental Protection Agency (US EPA) approved a standard assay using H295R human adrenocortical carcinoma cells to measure the effects of EDCs *in vitro,* from which the results can be used in regulations.

2.7 H295R *in vitro* assay

The OECD developed test guidelines to use H295R cell assays to investigate various chemicals' effects on the human steroidogenesis pathway, as an OECD level 2 screening assay and US EPA tier 1 assay (OECD, 2011, USEPA, 2011). The H295R cell line is an excised human adrenocortical carcinoma (parent NCI-H295 cell line) (OECD, 2011). H295R cells are a good model to study the toxicological effects of EDCs on the adrenal cortex, as they are less sensitive to cytotoxicity, express all the key enzymes necessary for steroidogenesis (capable of producing all the various steroids derived from cholesterol, except dihydrotestosterone), are zonally undifferentiated (can produce steroid hormones from all the

adrenal cortex zones), are able to express up- and downregulation of the steroidogenic enzymes, and gene expression does not result in alteration of catalytic activity (OECD, 2011).

Although the main objective of the H295R assay is to identify xenobiotics that affect oestradiol and testosterone production from cholesterol, they can be used to identify the effects on specific enzymes and intermediate hormones as well (OECD, 2011). They are therefore unique in that they allow for *in vitro* testing for effects on both corticosteroid and sex steroid hormone synthesis. However, the changes in the steroid production could result from a multiple of different interactions between the test chemical and the H295R steroidogenic functions, such as the cells' ability to express and synthesise enzymes, which are responsible for the production, transformation and elimination of the steroid hormones. The changes in the steroidogenic pathway can be through three mechanisms of action i) direct competitive binding to an enzyme; ii) influencing of cofactors (such as nicotinamide adenine dinucleotide hydrogen and cyclic adenosine monophosphate); and iii) changes in the gene expression of the steroidogenesis enzymes (OECD, 2011).

Various chemicals have been studied by the H295R assay including 2,4-dichlorophenol (Ma et al., 2012); forskolin, atrazine, letrozole, prochloraz, ketoconazole, aminoglutethimide and prometon (Higley et al., 2010); polybrominated diphenyl ethers (He et al., 2008); and pentachlorophenol, and 2,4,6-trichlorophenol (Ma et al., 2011). Furthermore, environmental extracts have also been tested with the H295R assay, to determine the effects of unknown contaminant mixtures present in environmental matrices on the steroidogenesis pathway of these cells. Some examples include exposure of H295R cells to sediment extracts from: the Upper Danube River in Germany (Grund et al., 2011); the Awba Dam in Nigeria (tropical freshwater) (Natoli et al., 2019); the coastline near the Hebei Spirit oil spill (HSOS) site in Taean, Korea (Liu et al., 2018); and water extracts from coastal areas and the influents and effluents of WWTPs in Hong Kong, China (Gracia et al., 2008). All of these above mentioned studies measured the concentrations of oestradiol and testosterone produced by the exposed H295R cells, using ELISA.

Several studies evaluated different xenobiotic chemicals using modified methods of the OECD H295R cell assay, in order to test for other steroid hormone concentrations produced by these cells. Some of these authors used some form of instrumental analysis (see section 2.8) to quantify a variety of hormones. Some examples include:

- androstenedione, pregnenolone, DHEA, testosterone, oestradiol, oestrone, and progesterone when exposed to either prochloraz, ketoconazole or genistein (Nielsen et al., 2012);
- untargeted scanning of 130 putative steroid metabolites, when exposed to either acetyl tributylcitrate, octyl methoxycinnamate, torcetrapib, forskolin, linuron, or octocrylene (Boccard et al., 2019);
- pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 17-OH progesterone, androstenedione, 11-deoxycortisol, DHEA and cortisol when exposed to either angiotensin II, forskolin or abiraterone (Mangelis et al., 2016);
- cholesterol, pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 17-OH progesterone, 17-OH pregnenolone, oestrone, testosterone, oestradiol, dihydrotestosterone, androstenediol, androstenedione, 11-deoxycortisol, DHEA and cortisol, when exposed to either forskolin or prochloraz (Nakano et al., 2016);
- untargeted scanning of 14 steroids, and then quantification of progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 17-OH progesterone, testosterone, androstenedione, 11-deoxycortisol, DHEA, DHEAS and cortisol, when exposed to either of 31 various chemicals including etomidate, chlorophene, mitotane, sotalol, digitoxin, clofazimine, and zidovudine (Strajhar et al., 2017);
- pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 17-OH progesterone, 17-OH pregnenolone, testosterone, androstenediol, androstenedione, 11-deoxycortisol, DHEA, cortisone and cortisol, when exposed to either atorvastatin (Munkboel et al., 2018a) or promethazine, cetirizine or fexofenadine (Munkboel et al., 2018b);
- testosterone, progesterone and oestradiol when exposed to either prochloraz, ketoconazole, fadrozole, aminogluthetimide, forskolin or vinclozolin (Hecker et al., 2006);
- DHEA, oestradiol, androstenedione, testosterone, pregnenolone, progesterone, 17-OH progesterone, deoxycorticosterone, and aldosterone, when exposed to either of 11 types of polyphenols (Hasegawa et al., 2013);
- testosterone, progesterone and oestradiol when exposed to either acetaminophen, clofibrate, dexamethasone, doxycycline, DEET, erythromycin, ibuprofen, trimethoprim, tylosin, amoxicillin, cephalexin, cyproterone, ethynylestradiol, fluoxetine, oxytetracycline, salbutamol, trenbolone, or α-zearalanol (Gracia et al., 2008).

The two reference compounds recommended to be used in the H295R assay are prochloraz and forskolin, as their effects on the pathway in this assay are known. Forskolin is an activator of adenylate cyclase, and therefore would stimulate the cells to produce more of all the steroids in the steroidogenesis pathway (Sanderson, 2006). Prochloraz on the other hand inhibits various enzymes in H295R cells, including aromatase , 17α-hydroxylase, and 21-hydroxylase, thereby decreasing various steroids *in vitro* (Sanderson, 2006).

Although there have been many studies about the effects of xenobiotics using the H295R assays, there have been limited studies on PPCPs such as ARVs using this model. Only two studies were found that used H295Rs, in which these cells were exposed to ARVs. The study by Strajhar et al. (2017) screened the effects of zidovudine, but found that at 10 µM, it had little effect on the steroidogenesis pathway. All the steroid hormones had a fold change (FC) of > 1 (1.06-1.29), except aldosterone, which was 0.92 (Strajhar et al., 2017). The study by Malikova et al. (2019) screened the effects for efavirenz, tenofovir, emtricitabine, and zidovudine on the H295R cells. Tenofovir, emtricitabine, and zidovudine were found to have no effect on the steroidogenesis pathway (Malikova et al., 2019). Efavirenz, however, was found to have a significant dose dependent effect on CYP21A2 activity and cell viability at various concentrations (5, 10, 50 µM) after 3 hours of exposure, but only at 50 µM after 24 hours (Malikova et al., 2019). This study, however, never reported the concentrations of 17-OH progesterone, but rather the percentage conversion of 17-OH progesterone to 11-deoxycortisol, which is the function of 21-hydroxylase. Furthermore, the OECD guidelines were not followed with the H295R assay, and therefore the results are not sufficient for an accurate conclusion on the endocrine disrupting effects of efavirenz (Malikova et al., 2019).

In order to measure the steroid hormone concentrations in the H295R cell medium, various methodologies such as ELISA, gas chromatography (GC)-MS or LC-MS can be used (OECD, 2011).

2.8 Steroid hormone analysis

Steroid hormones are commonly quantified by various immunological assays, such as fluoroimmunoassays, radioimmunoassays, and ELISAs. However, for these types of assays each hormone requires a different immunoassay, which is time consuming and not cost effective. Furthermore, these tests could have various cross-reactivities, depending on how exclusive an analyte is to an antibody, with poor accuracy at low concentrations, as the

different steroids have very similar structures (Ahmed et al., 2018, Murtagh et al., 2013, Nakano et al., 2016).

Various analytical methods have been developed to quantify multiple hormones simultaneously in different biological mediums using LC or GC combined with a MS (QQQ or QTOF). These analytical methods have been shown to be more sensitive and accurate than immunoassays, as multiple analytes can be detected at low concentrations, in one run (Ahmed et al., 2018, Gaikwad, 2013, Murtagh et al., 2013, Peters et al., 2010). Furthermore, LC-MS methods are more robust and suitable in high throughput environments, and are consequently becoming the instrument of choice for steroid hormone analysis (Ahmed et al., 2019). Most LC-MS methods available analyse biological matrices such as serum, urine, and faeces (Murtagh et al., 2013), but a few studies have also been published where the tissue culture medium of H295R cells is extracted and analysed for various steroid hormones on an LC-MS (Karmaus et al., 2016, Weisser et al., 2016) or GC-MS (Nakano et al., 2016, Nielsen et al., 2012). However, for untargeted analytical assessment of steroid analysis, UHPLC coupled to high resolution MS (Orbitrap or time-of-flight (TOF)) is showing much promise (Boccard et al., 2019).

The present study aimed to develop and validate an analytical method to quantify steroid hormones in H295R medium using a UHPLC-QTOF, after the cells were exposed to various concentrations of ARVs with potential endocrine disrupting properties.

CHAPTER 3: ANALYTICAL METHOD DEVELOPMENT & OPTIMISATION

3.1 Background

A sensitive and specific method was required to quantitate steroid hormones using a single extraction technique of the cell medium, and running it on an UHPLC-QTOF.

The hormones to be analysed and quantified were oestradiol, testosterone, 17-OH progesterone, cortisol, aldosterone and androstenedione, which were chosen due to their reference standards' availability for this project and their importance in the steroidogenesis pathway (see section 2.6.2).

3.2 Instrumentation

The samples were analysed on a UHPLC-QTOF, consisting of the following parts: an Agilent 1290 Infinity binary pump (G4220A); 1290 Infinity autosampler (G4226A); and 1290 Infinity thermostatted column compartment (G1316C), coupled to an Agilent 6540 accurate mass QTOF (G6540A) (Agilent Technologies, Santa Clara, CA, USA). A dual Agilent Jet Stream (AJS) technology electrospray ioniser (ESI) was used in positive and negative ionisation mode for the desolvation and ionisation of the samples.

The software used included MassHunter data acquisition (version B.05.00), MassHunter qualitative analysis (version B.05.00) and quantitative analysis for QTOF (version B.05.01). Tuning mixes (Agilent Technologies; Chemetrix) were used to do a mass axis calibration of the QTOF before each run, for positive and negative ionisation (G1969-85000, Agilent). A reference solution (Agilent Technologies; Chemetrix) was constantly infused throughout the runs as an accurate mass reference. For positive ionisation the reference masses used were 121.050873 m/z and 322.048121 m/z, while for negative ionisation, the reference masses used were 119.03632 m/z and 301.998139 m/z.

3.3 Chemicals

Aldosterone (CAS# 52-39-1), cortisol (CAS# 50-23-7), oestradiol (CAS# 50-28-2), 17-OH progesterone (CAS# 68-96-2), and testosterone (CAS# 58-22-0) were purchased from Sigma-Aldrich, South Africa. Androstenedione (CAS# 63-05-8) was obtained from Steraloids, Inc. The internal standards, namely, 17-OH progesterone-d8 (CAS# 850023-80-2), oestradiol-d3 (CAS# 79037-37-9), and cortisol-d4 (CAS# 73565-87-4) were all obtained from Sigma-Aldrich,

South Africa. The diethyl ether (CAS# 60-29-7), as well as all the additives for the mobile phases, (ammonium acetate (CAS# 631-61-8), ammonium fluoride (CAS# 12125-01-8), and ammonium formate (CAS# 540-69-2)) were also purchased from Sigma-Aldrich, South Africa. Honeywell Burdick & Jackson, spectrometry grade acetonitrile (CAS# 75-05-08), spectrometry grade methanol (CAS# 67-56-1) and LC-MS grade formic acid (CAS# 64-18-6), were purchased from Anatech, South Africa.

3.4 Solutions preparation

The powdered steroid standards were dissolved in methanol. These steroid solutions (varying concentrations) were used to prepare a final working standard solution of 200 µg/L in nutrient growth media for the H295R cells. The calibration curve standards for each steroid was prepared using growth media to create matrix-matched calibration curves. The calibration curve standards were prepared by spiking the blank growth media to final concentrations of 1 µg/L, 5 µg/L, 10 µg/L, 20 µg/L, 50 µg/L, 100 µg/L and 200 µg/L. These concentrations were decided on after a literature search provided insight on the expected concentrations from the cell media (Nakano et al., 2016, Nielsen et al., 2012). Furthermore, cell media that was harvested from the cells before exposure, extracted and run on the UHPLC-QTOF, was also used to assist in determining the expected concentrations of the steroid hormones that are produced by the cells naturally without any exposures. This was in order to ensure that the linear range (0–200 µg/L) covers the expected concentrations. The mean concentrations and standard deviation (SD) of the target hormones of these unexposed cells are summarised in Table 3-1.

Table 3-1:The mean concentrations (µg/L) and standard deviations (SDs) of each of the steroids quantified in the unexposed cells' media, which was used to estimate the expected concentrations of the steroids produced by the cells.

Steroid hormone	Mean ± SD
Aldosterone	**0.033** ± 0.026
Cortisol	**23.085** ± 4.104
Testosterone	**13.760** ± 1.971
Androstenedione	**139.052** ± 55.466
17-OH progesterone	**6.165** ± 0.307
Oestradiol	**0.424** ± 0.154

In addition to the calibration curve standards, matrix-matched quality controls (QCs) were prepared at concentrations at the lower (6 µg/L) and upper (80 µg/L) ends of the calibration curve. These QCs were prepared from different actual solutions (to determine the variability), but were prepared the same way as the calibration curve standard solutions. These QCs were prepared and run at various time intervals, in order to monitor any deviations of the analytical error, within a run. The internal standard mixture was prepared by combining individual stock solutions (varying concentrations) into a mixed internal standard working solution (1 000 µg/L) using the following deuterated analytes: 17-OH progesterone-d8, oestradiol-d3, and cortisol-d4. All calibration standards and QCs underwent the same sample extraction process as the cell media samples (as explained in section 4.6.1).

3.5 Sample extraction and clean-up method optimisation

The preparation of the samples for analysis on the UHPLC-QTOF in this study, required extraction and clean-up. The feasibility of different extraction methods was investigated. The methods considered were based on a literature search, costs involved, and job related experience as a medical scientist. The extraction of hormones from various matrices is often done using solid phase extraction (SPE) methods, such as the Bond Elut C18 SPE cartridges (Nielsen et al., 2012, Weisser et al., 2016), or the OASIS WCX-96 well SPE plates (Mangelis et al., 2016). However, these were considered too costly for this study, as it required additional equipment and expensive consumables. Another method from literature used acetonitrile and it involved centrifugation to remove protein pellets (protein precipitation) (Kay et al., 2008), but this method was discarded in the early stages because it was inconsistent in making pellets from the cell media. Multiple methods suggested to do a liquid-liquid extraction (LLE) using different organic solvents, such as dichloromethane, ethyl acetate, hexane, or diethyl ether, or a combination of a few them (Ahmed et al., 2018, Hecker and Giesy, 2008, Higley et al., 2010, Schloms et al., 2012).

The extraction method used for this study involved LLE, whereby an organic solvent (non-polar) is added to a water based (polar) liquid, and the analyte of interest can then distribute itself in a certain ratio between the two immiscible solvents (Siek, 1978). Diethyl ether, one of the most versatile organic solvents, has a hydrogen bond acceptor molecule, which allows it to extract electron donor solutes more easily (Siek, 1978). Diethyl ether was chosen for the LLE in this study, because it was used by other researchers using the H295R cells (Hecker and Giesy, 2008, Higley et al., 2010, Topor et al., 2011), as well as by application notes published on the internet by commercial laboratories, such Oxford Biomedical Research (Oxford-Biomedical-Research, 2015). Added benefits of using it were that diethyl ether is

affordable, readily available in the laboratory, and it is versatile for various other applications in future.

The media harvested from the cells were stored at -80°C until extraction and analysis could be done, and only then, the media were thawed and subjected to extraction. Once 500 µL of the solutions (calibration standards and quality controls) and samples (harvested cell media), together with 100 µL mixed internal standard working solution were added to the tubes, 2 mL diethyl ether was added. The tubes were mixed for a minimum of five minutes to ensure proper mixture of the two immiscible solvents, and thereby ensuring that the maximum amount of analyte of interest could be extracted to the organic layer. To separate the two layers after mixing, the tubes were centrifuged at 2 000 g for five minutes. Freezing at -80°C was attempted, but by the time the samples were transported to the fume hood for the transfer of the organic layer, the aqueous layer had already started to thaw. Therefore, this idea was discarded early on, and instead, centrifugation and transfer of the organic layer using a glass Pasteur pipette was used. At first, transferring of the top organic layer was done as a whole (the full 2 mL at once), but it was discovered that the reconstitute volume did not reach the higher parts of the tube, and therefore was unable to reconstitute everything adequately. Therefore, at first, only 1 mL of the top organic layer was transferred to a corresponding clean 1.5 mL amber vial, and dried completely under a steady flow of nitrogen gas. This was followed by the remainder of the top organic layer being transferred into the same vial, and once again dried. In this way, targeted hormones were restricted to the lower surface area of the amber vial, and not spread over the entire inside of the vial. This allowed for a higher extraction efficiency.

Once completely dry, the samples were reconstituted with 100 µL of 1:1 methanol:water (similar constituent as the mobile phase, in order for the sample to be carried better through the LC column), and vortexed well to reconstitute as much of the analyte of interest as possible. Different volumes of the reconstitute were experimented with, to ensure that the analytes were not diluted down too much, but that the volume was sufficient to reach the dried analyte in the vial, and that it could be injected multiple times if necessary. The reconstitute was finally transferred to low volume inserts (Cat#702813, Macherey-Nagel, Germany), to ensure that the needle would be able to reach the sample, and placed into the 1.5 mL amber vials, to inject into the UHPLC-QTOF for analysis.

3.6 QTOF development and optimisation

The QTOF parameters were optimised using a flow injector analysis (FIA) program. This consisted of various injections of each analyte, while changing one parameter at a time, but

keeping all the other default values consistent throughout the optimisation. The first few source parameters were analysed and the optimal values were then adjusted accordingly, before completing the rest of the FIA program. The various parameters that were optimised included drying gas temperature (°C) and flow (L/min), nebuliser pressure (psi), sheath gas temperature (°C) and flow (L/min), VCap (V) and nozzle voltages (V), fragmentor (V), skimmer (V) and OCT RF Vpp (V).

After the source optimisation, the optimised parameters were used to set up the QTOF method to obtain the highest possible abundance for each analyte (QTOF optimisation data available in Table 3-2.

Table 3-2: Optimisation data for the positive ionisation optimisation, showing the various parameters and how the different steroids' abundance was affected with each changed parameter.

Parameter on QTOF		17-OH progesterone	Androstenedione	Cortisol	Testosterone
Drying gas flow (L/min)	6	569781	2104122	583287	17194
	8	597330	1899618	596535	14561
	10	602145	1755788	556282	15657
	12	585707	1698998	549777	14171
Nozzle voltage (V)	**0**	597387	1668199	527490	14260
	100	588260	1616530	519696	15432
	500	611440	1578675	540534	14197
	1000	519900	1305434	438653	11278
Fragmentor (V)	**130**	789031	2140120	665304	21328
	175	764708	2017238	623757	19054
	200	573838	1468884	448559	3045
	250	46867	95017	31087	258
Skimmer (V)	45	430915	1037590	317919	10628
	55	523830	1301480	374139	11668
	65	577100	1419456	419426	5686
	75	572454	1440687	422770	14562
Vcap (V)	**2500**	695453	1848287	594764	21183
	2750	643746	1614449	494493	11721
	3000	575545	1387960	407528	13979
	4000	419586	907907	247014	7920
Nebuliser pressure (psi)	15	938903	2869323	999680	27388
	30	1117930	3598858	1350794	36437
	45	1049970	4133799	1979611	45794
	60	900348	3675239	1943250	42829
Drying gas temperature (°C)	250	834569	2761190	967159	24229
	275	1003000	3468678	1331945	33638
	300	978662	4044684	1904244	42607
	350	852123	3565879	1884371	39296
Sheath gas temp and flow (°C / mL/min)	250 / 5.5	635044	1843793	1232675	31109
	300 / 6	582412	2155392	1114279	28426
	350 / 7	645991	2639009	1266874	32305
	400 / 10	811932	3270237	1511840	35165
	400/ 11	961317	3358944	1717206	41208

**The parameters in bold were selected for the optimised QTOF method, as it best suited the majority of the steroids, while the grey scaled boxes are the highest abundance for each steroid, based on the changed parameters.*

3.7 UHPLC development and optimisation

Different LC parameters were tested based on information from literature (Gaikwad, 2013, Long, 2012, Strajhar et al., 2017, Weisser et al., 2016, Yang, 2011, Zhang et al., 2011a). Trials were attempted on the following available columns: an Agilent ZORBAX C18 column (C18), a Phenomenex Synergi RP column and a Phenomenex PFP column (results not shown, because no peaks were found). Literature showed that a phenyl hexyl column had the best separation of the steroid hormones to be analysed (Long, 2012), which through my experimenting using an Agilent InfinityLab Poroshell 120 Phenyl-Hexyl, 2.1 x 100 mm 1.9 - µm column (phenyl hexyl), was found to be true.

The chromatogram in Figure 3-1 shows four peaks (cortisol, androstenedione, testosterone and 17-OH progesterone) produced by the C18 column, but due to their relative peak sizes, the testosterone peak is barely visible (orange peak in Figure 3-1). The peak shapes are not of good quality and overlap each other. The fact that all the peaks came off of the column in under 2 minutes, demonstrates that there was very little or no retention of these analytes on this specific column.

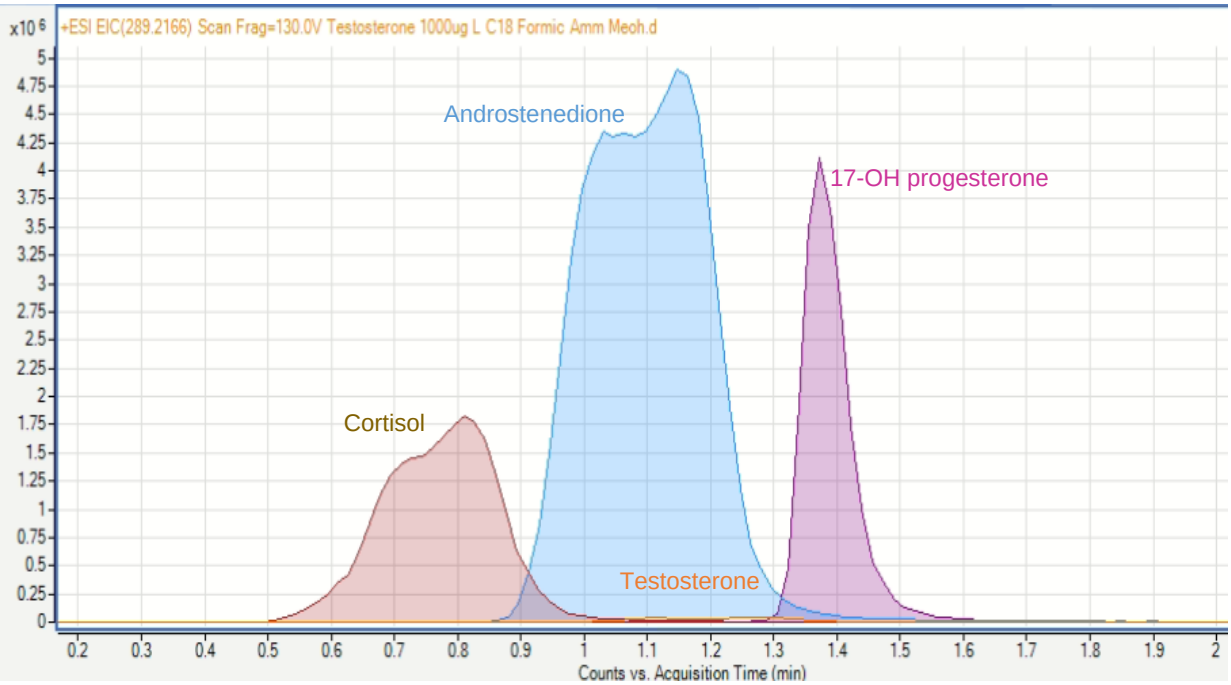

Figure 3-1: Extracted ion chromatogram showing the four different steroids' peak shapes and separation on a C18 column, with methanol:water mobile phase (0.1% formic acid and 2 M ammonium acetate additives).

Using a phenyl hexyl column under exactly the same UHPLC-QTOF conditions produced much narrower peak shapes and much improved separation between the peaks (Figure 3-2), when compared to the C18 column (Figure 3-1). Furthermore, it took over 4 minutes for all four peaks to elute on the phenyl hexyl column, meaning that they were retained on the column more efficiently than with the C18 column. Unfortunately, the testosterone peak could still not be discerned.

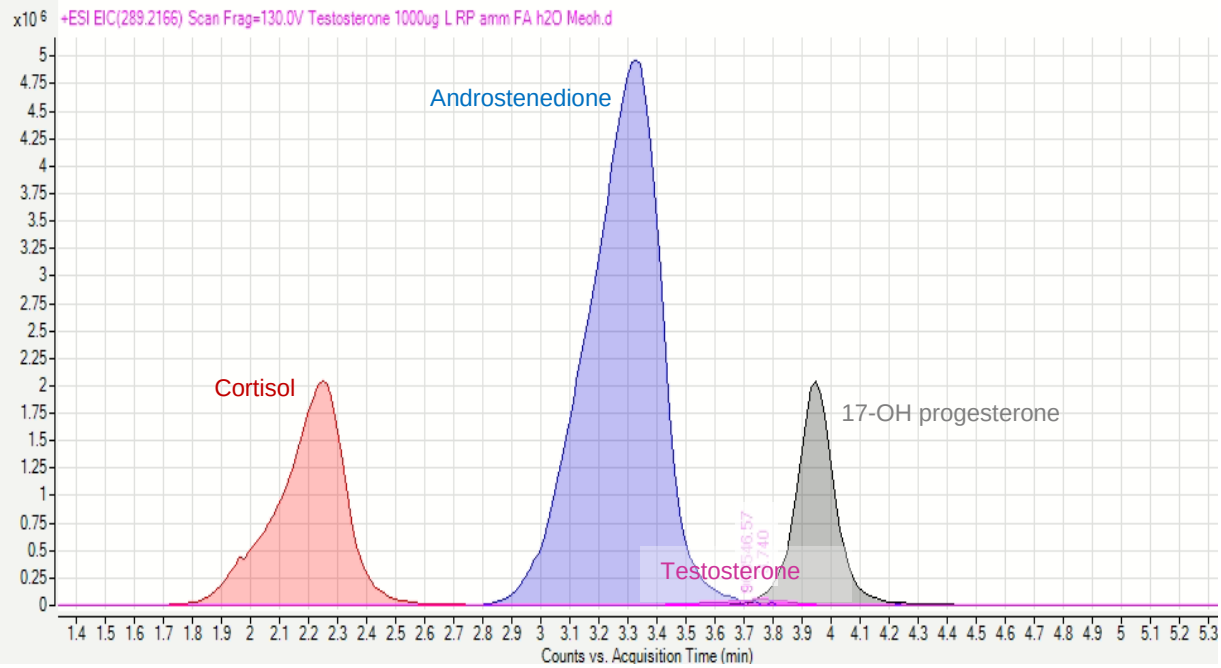

Figure 3-2: Extracted ion chromatogram showing the four different steroids' peak shapes and separation on a phenyl hexyl column with methanol:water mobile phase (0.1% formic acid and 2 M ammonium acetate additives).

Further proof of the better chromatography provided by the phenyl hexyl column was observed when the chromatograms for the same steroid hormone were overlaid. The testosterone peak from the C18 column (orange) was much broader and had a lower peak height than the one from the phenyl hexyl column (pink) (Figure 3-3). Furthermore, the shift in the retention time from 1.2 to 3.8 minutes, demonstrated that the testosterone had been retained on the column for longer, meaning that the column was more suitable for that specific analyte. The same was true for the androstenedione peaks in Figure 3-4, where the C18 column peak is the light blue peak, while the improved peak on the phenyl hexyl column is shown in dark blue.

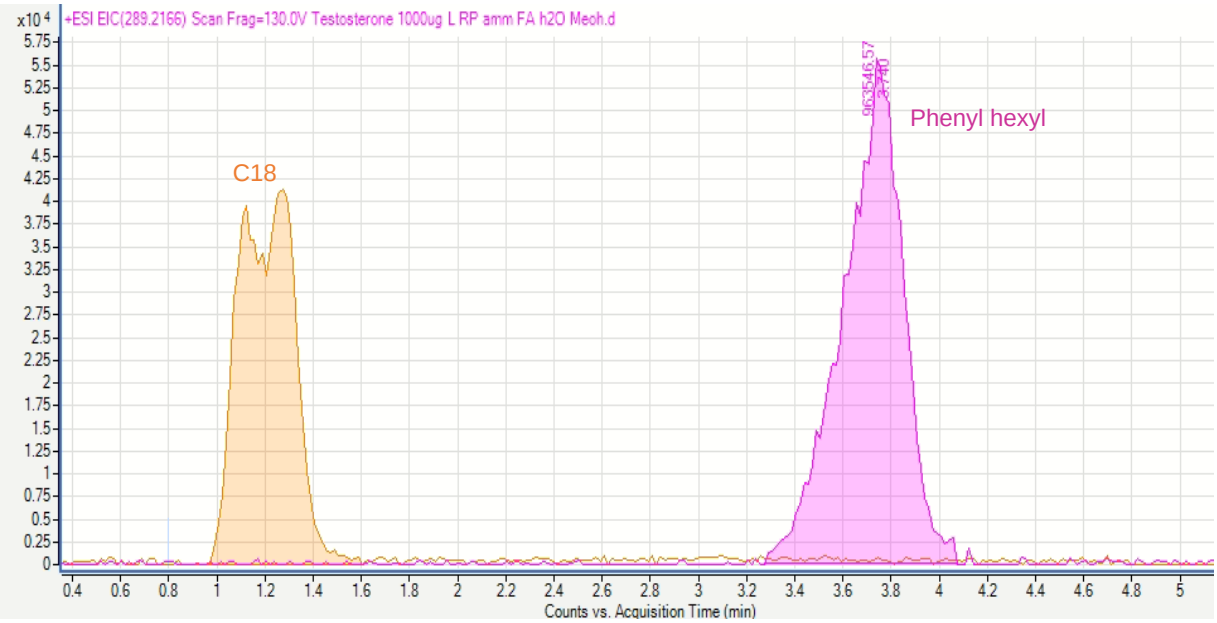

Figure 3-3: Overlaid extracted ion chromatograms, demonstrating the comparison of the testosterone peak through the C18 column (orange) versus the phenyl hexyl column (pink), with methanol:water mobile phase (0.1% formic acid and 2M ammonium acetate additives).

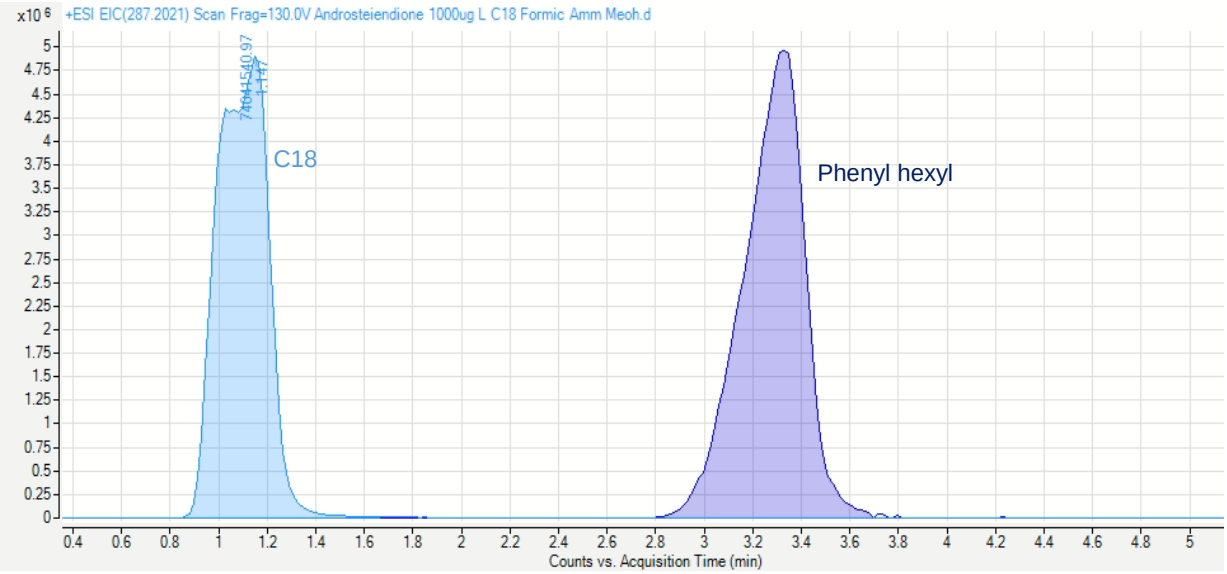

Figure 3-4: Overlaid extracted ion chromatograms, demonstrating the comparison of the androstenedione peak, through the C18 column (light blue) versus the phenyl hexyl column (dark blue), with methanol:water mobile phase (0.1% formic acid and 2M ammonium acetate additives).

The following authors, Yang (2011), Long (2012), Gaikwad (2013), Weisser et al. (2016), Zhang et al. (2011a), and Strajhar et al. (2017) all suggested either acetonitrile or methanol, and water for the mobile phases. Both organic phases were attempted, altering their percentages with water. Methanol was a better organic phase than acetonitrile on the phenyl hexyl column, as it produced the greater abundances for the various steroid peaks (Table 3-3), as well as the best peak shapes overall for the majority of the steroids. Furthermore, the lower percentage of methanol that was used produced an increase in the abundance of the various steroid peaks (Table 3-3).

Table 3-3: A comparison of the peak abundances using methanol vs acetonitrile as the organic mobile phase, as well a comparison of the peak abundances using different percentages of methanol in the mobile phases.

	Abundance			
Steroid hormone	**Acetonitrile**	**Methanol**	**Methanol:Water 70:30**	**Methanol:Water 50:50**
17-OH progesterone	146 318	569 781	897 244	1 117 930
Androstenedione	110 642	2 104 122	2 577 786	4 133 799
Cortisol	49 265	583 287	1 307 271	1 979 611
Testosterone	977	17194	32 119	45 794

Ammonium acetate, ammonium fluoride and ammonium formate were all evaluated as additives to the mobile phases because they are commonly used in LC applications. Additives assist with the ionisation process of compounds. Using positive ionisation, the ammonium acetate performed the best overall with regards to peak shape and intensity for all the analytes, and therefore was the additive of choice (Figure 3-5, Table 3-4). However, negative ionisation worked better with the ammonium fluoride, and therefore was used for the oestradiol analysis (data not shown).

Table 3-4: A comparison of the various steroid hormone peak abundances, either with or without the ammonium acetate additive in the mobile phase, and comparison of the peak abundances with and without the addition of formic acid to the mobile phase.

	Abundance			
Steroid hormone	**Without ammonium acetate**	**2 M ammonium acetate**	**Without formic acid**	**0.1% formic acid**
17-OH progesterone	14 762 754	20 920 588	146 318	407 437
Androstenedione	49 760 189	74 035 810	110 642	748 936
Cortisol	14 136 259	24 196 980	49 265	458 067
Testosterone	619 611	699 361	977	11 383

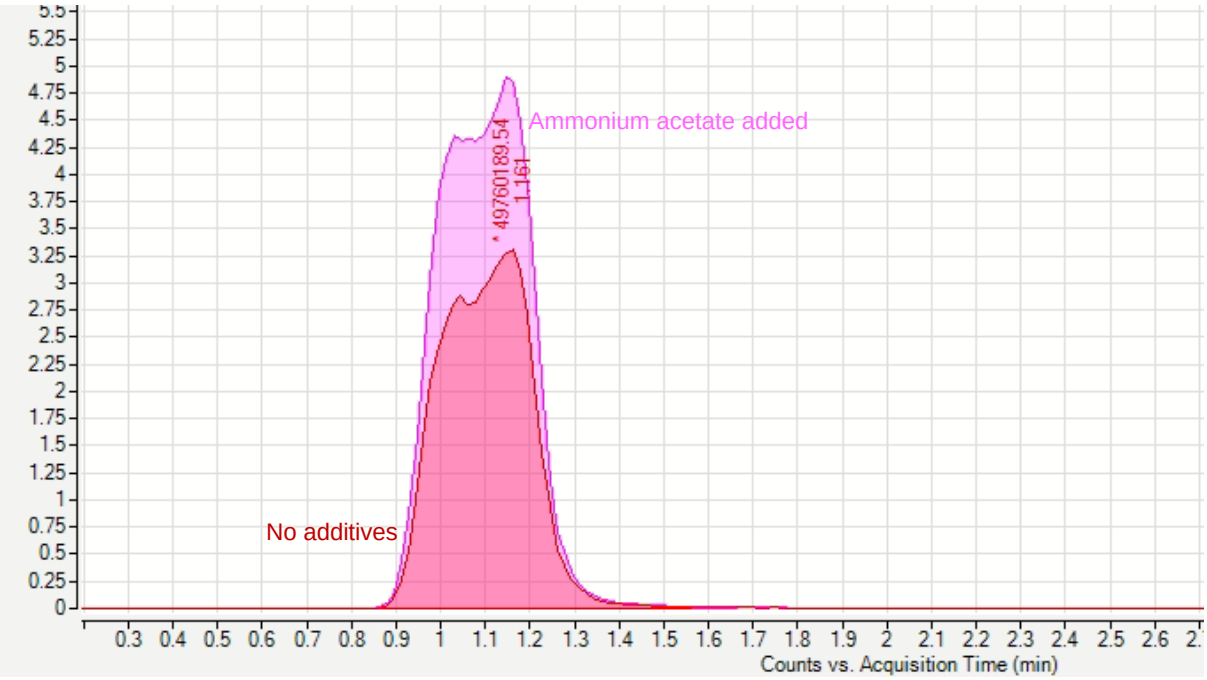

Figure 3-5: An overlaid extracted ion chromatogram of the androstenedione peak, to demonstrate the comparison of methanol:water mobile phases, with only 0.1% formic acid added (red), versus with ammonium acetate additive also being added (pink).

Mobile phases with additives were prepared with and without formic acid. The formic acid was added to the mobile phases for the positive ionisation run because it improved the abundances further (Table 3-4). Formic acid was not expected to have any significant effect on the negative ionisation run, which was found to be true on testing, and it was therefore not included in those mobile phases (data not shown).

To ensure that all the peaks had as close to baseline separation as possible, a solvent gradient was introduced. This was altered until eventually the best baseline separation was successfully achieved for all the steroids. The chromatogram in Figure 3-6 shows how the peaks were separated out using a mobile phase gradient, versus Figure 3-7 with a lot of peak overlay, where an isocratic mobile phase percentage was used.

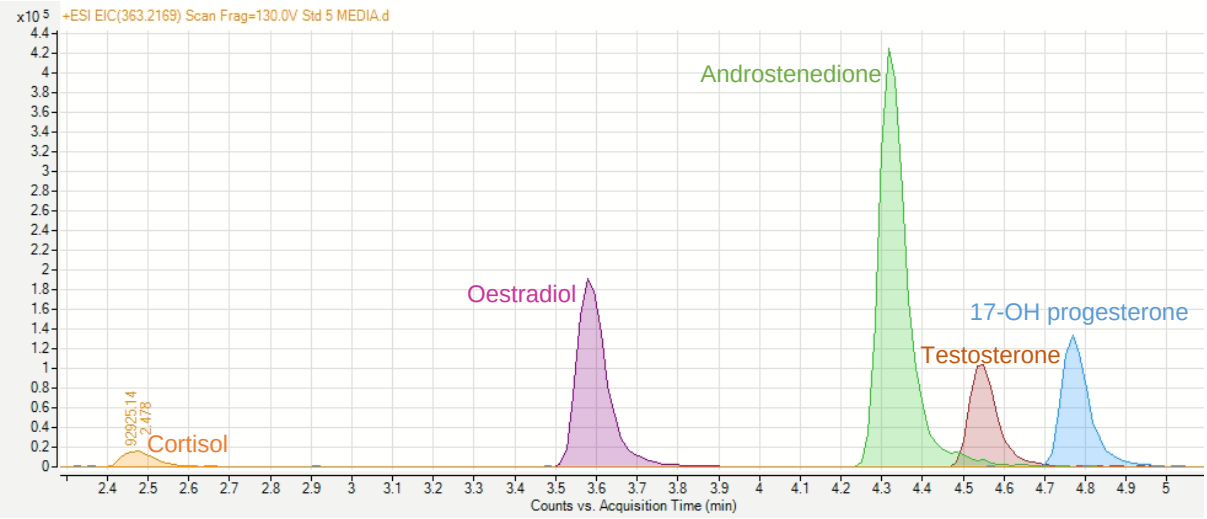

Figure 3-6: An extracted ion chromatogram showing peaks for cortisol (orange), oestradiol (purple), androstenedione (green), testosterone (brown) and 17-OH progesterone (blue), with a gradient flow introduced.

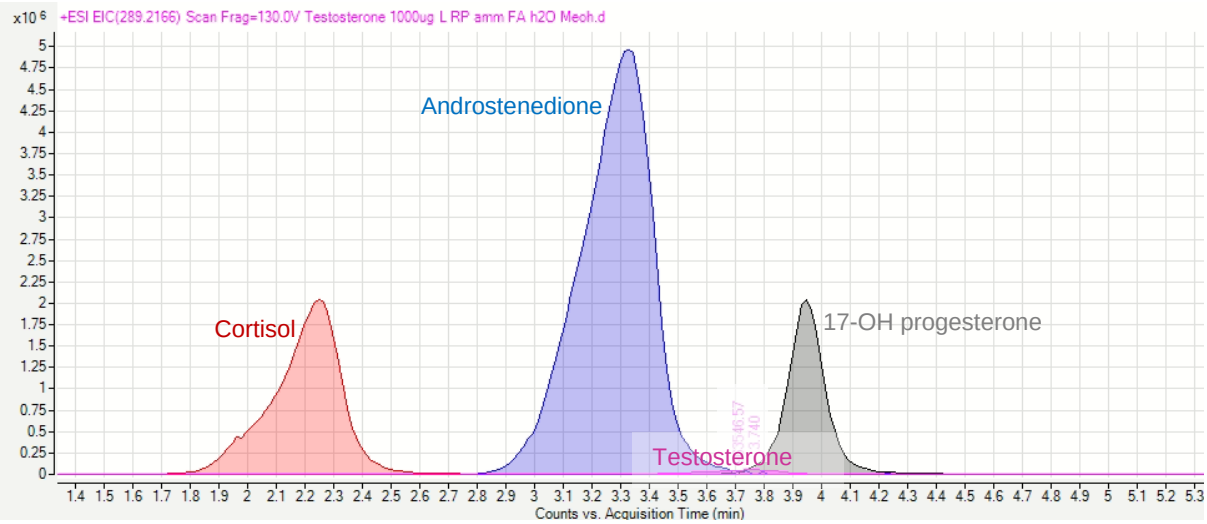

Figure 3-7: An extracted ion chromatogram showing peaks for cortisol (red), testosterone (pink), androstenedione (blue), and 17-OH progesterone (black) during isocratic flow.

As a precautionary measure to extend the lifetime of the detector and limit analyte carry over, the diverter valve was switched so that the UHPLC flow went to waste after the analytes of interest eluted (at 6.5 mins).

Aldosterone was added into the method after most of the method development was completed, and therefore the various aspects considered for the method, were not compared for aldosterone. Aldosterone had an acceptable peak shape and was included in the method validation.

3.8 Method validation

The method was validated by preparing and running various blank media samples, spiked matrix matched calibration curve standards, as well as matrix matched quality control samples, over five different days. This information was used to determine linearity, sensitivity, stability, precision (reproducibility) and accuracy.

3.8.1 Linearity

The reportable range was assessed by analysing the matrix matched calibration curve standards in triplicate (Westgard, 2008). Linearity is determined using the R^2 value of relative response versus relative concentration. Good linearity is indicated when the R^2 value is as close to 1 as possible (0.9 minimum) (Miller and Miller, 2010). All the hormones analysed, were found to have linear calibration curves within the ranges tested ($R^2 \geq 0.999$) (Figure 3-8: A-F).

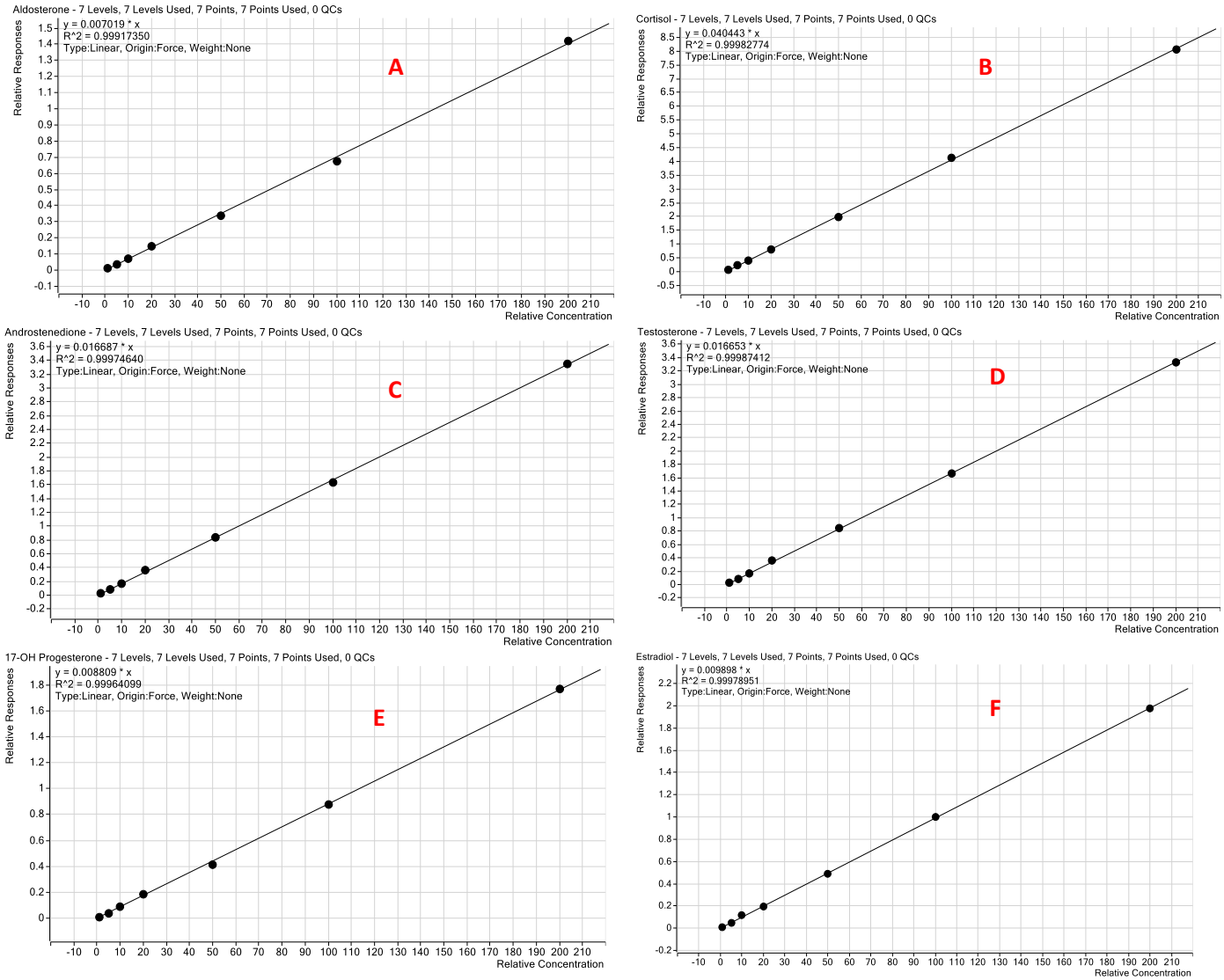

Figure 3-8: The various calibration curves demonstrating the linearity of all the steroids.

A = Aldosterone (R^2=0.999); B = Cortisol (R^2=0.999); C = Androstenedione (R^2=0.999);
D = Testosterone (R^2=0.999); E = 17-OH Progesterone (R^2=0.999); and
F = Oestradiol (R^2=0.999).

3.8.2 Sensitivity

Blank media extracts were run fifteen times in total, over the five validation days. The calculations used to determine the limit of detection (LOD) and the limit of quantification (LOQ) were as follows (Shrivastava and Gupta, 2011):

LOD = Mean of blanks + (3 x standard deviation of the blanks)

LOQ = Mean of blanks + (10 x standard deviation of the blanks)

The LODs and LOQs for each steroid hormone are displayed in Table 3-5.

Table 3-5: Limit of detection (LOD) and the limit of quantification (LOQ) of each of the steroid hormones.

Steroid hormone	**LOD** (µg/L)	**LOQ** (µg/L)
Oestradiol	0.10	0.26
Aldosterone	0.11	0.30
Cortisol	0.09	0.24
Testosterone	0.07	0.20
Androstenedione	0.04	0.12
17-OH progesterone	0.09	0.24

3.8.3 Stability

Calibration curve standards and QCs were prepared and run, and then rerun 3 months later to determine whether the analytes are chemically stable over time. The samples were stored at -20°C between analyses. Although the abundances of the peaks differed (increased mainly due to evaporation), the concentrations of the results were not significantly different between the two runs, due to the internal standards that were of similar chemical structures, which compensated for any evaporation or analyte degradation (data not shown).

3.8.4 Precision and accuracy

By analysing two different concentration QC samples four times on five different days, the precision and accuracy were determined. The accuracy was determined by comparing the achieved QC concentration to the target concentration, and then represented as a percentage (Table 3-6). The precision (reproducibility) was calculated using the % RSD (relative standard deviation) (percentage of the mean of the standard deviation of the QCs divided by the mean of the QCs) (Table 3-6).

Table 3-6: Precision and accuracy of the QC samples for each of the steroid hormones.

Steroid hormone	Precision (% RSD)		Accuracy (%)	
	Low (6 µg/L)	High (80 µg/L)	Low (6 µg/L)	High (80 µg/L)
Oestradiol	6.47	2.59	99.66	101.18
Aldosterone	6.76	6.48	101.62	101.30
Cortisol	6.20	3.51	102.80	98.98
Testosterone	3.99	4.27	102.18	101.41
Androstenedione	7.08	3.91	100.73	103.49
17-OH progesterone	7.19	1.68	98.76	100.28

The use of a MS introduces ion suppression or enhancement of compounds in certain matrices. Matrix effects should therefore be accounted for with a matrix matched calibration curve. Calibration curve standards were prepared in deionised water and blank nutrient media, and compared to one another to evaluate the matrix effects (Van Eeckhaut et al., 2009). Figure 3-9 demonstrates that there were no negative matrix effects (no ion suppression) on the calibration curve standards. However, it was decided to use matrix-matched standards anyway, for thoroughness.

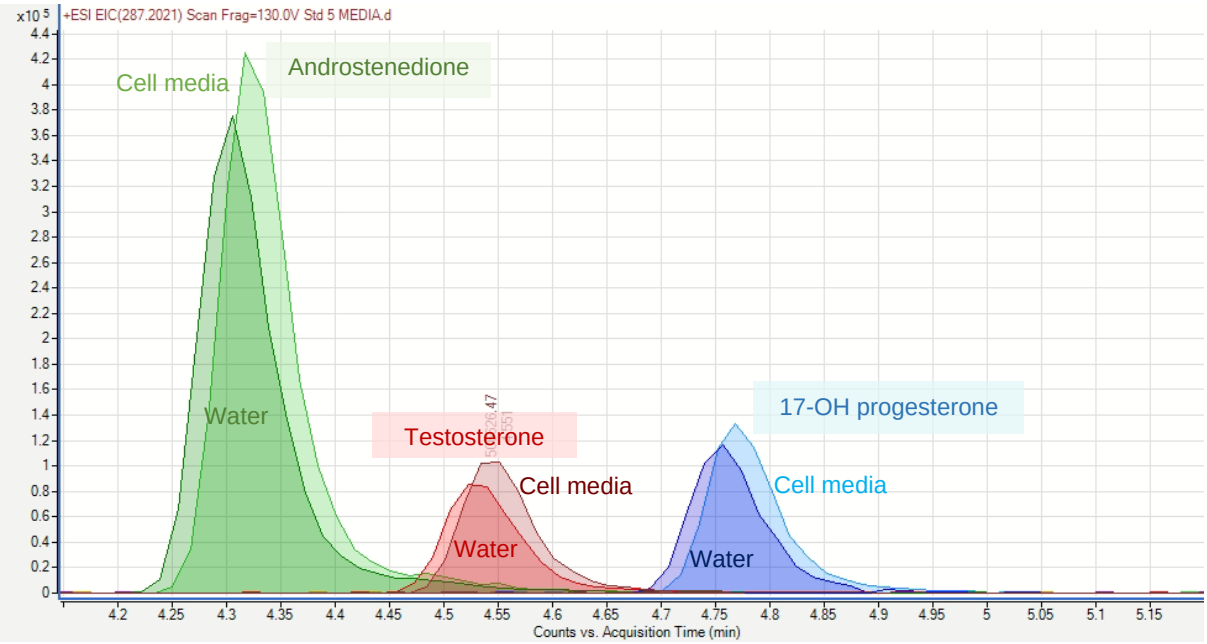

Figure 3-9: An extracted ion chromatogram showing peaks for androstenedione (green), testosterone (red) and 17-OH progesterone (blue) from deionised water (darker peaks) and cell media (lighter peaks).

3.9 Data analysis

Data was analysed using Agilent software available. The software used was MassHunter qualitative analysis (version B.05.00) and quantitative analysis for QTOF (version B.05.01). The qualitative analysis software was used in the development stages of the project, to obtain information on accurate masses and retention times of the different steroid hormones; and to check for sufficient peak separation from the other peaks. Once the method was developed and optimised, the quantitative analysis software was used for the method validation and quantitation of the concentrations of hormones in the cell media extracts. The concentrations were calculated based on a matrix matched calibration standard curve that was run at known concentrations (x), to obtain their corresponding peak area (abundance) responses (y). This calibration standard curve uses a linear regression with the formula of y=mx+c (where *y* is the relative response, *m* is the slope, *x* is the concentration and *c* is the y-intercept). The samples of unknown concentration are then run, and based on the calibration curve formula, their relative responses (y) are used to calculate their relative concentrations (x).

Furthermore, the internal standard is used by the software in the calculation of the concentration of each steroid hormone, based on the steroids' concentration as a ratio to the appropriate internal standard concentration. Cortisol concentrations were calculated using the cortisol-d4 internal standard. While aldosterone, androstenedione, testosterone and 17-

OH progesterone used the 17-OH progesterone-d8 internal standard for their ratio calculations. Oestradiol concentrations used the oestradiol-d3 internal standard.

The method was found to be robust and fit for the purpose of this study.

CHAPTER 4: MATERIALS & METHODS RELATED TO STEROIDOGENESIS ASSAY

4.1 Background

Human adrenocortical carcinoma cells, known as H295Rs, were used to determine the effects of the active ingredients of six ARV chemicals on the steroidogenesis pathway. Although oestradiol and testosterone are the most commonly measured hormones to determine the effects of a compound on steroidogenesis (OECD, 2011), this research included the quantification of various steroid hormones. This was to assist in getting a more complete representation of the possible effects of the compounds on the steroidogenic pathway. The nutrient media of the cells was harvested, extracted and then quantified for oestradiol, testosterone, androstenedione, cortisol, aldosterone, and 17-OH progesterone, using analytical instrumentation. The quantified results of the exposed cells were then compared to those of the solvent exposed control (SC) cells, to determine if any statistically significant differences occurred.

4.2 Chemicals

The Corning Costar 24-well plates, Nu-serum (Catalogue# 355500) and ITS+ (insulin-transferrin-selenium) Premix (Catalogue# 354351) were purchased from BD Biosciences. The ARV active ingredients were all LGC chemicals and included efavirenz (CAS# 154598-52-4), didanosine (CAS# 69655-05-6), lamivudine (CAS# 134678-17-4), stavudine (CAS# 3056-17-5), ritonavir (CAS# 155213-67-5), and lopinavir (CAS# 192725-17-0). The Burdick and Jackson spectrophotometry grade methanol (CAS# 67-56-1) was purchased from Anatech. Forskolin (CAS# 66575-29-9), prochloraz (CAS# 67747-09-5), 2- (4,5- dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) (Catalogue# M5655), Dulbecco's phosphate buffered saline (DPBS) (Catalogue# D5252), dimethyl sulphoxide (DMSO) (Catalogue# 224), and Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 mixture (Catalogue# D2906), were obtained from Sigma-Aldrich, South Africa. Trypsin (Catalogue# T360-500) was obtained from Celtic Molecular Diagnostics, South Africa.

4.3 Maintenance of cells

The H295R cell line (ATCC® CRL2128™) was obtained from the American Type Culture Collection (ATCC). The H295R cells were grown in 50% DMEM and 50% Ham's F-12 (stock medium) (pH 7.3), containing 2.5% Nu-serum and 1% ITS+ Premix (supplemented medium),

and was kept at 37°C in humidified incubators supplemented with 5% carbon dioxide (OECD, 2011). The cells were passaged once the tissue culture dishes were at around 95-100% confluency. This involved rinsing the cells three times with DPBS, after which they were lysed using trypsin. The action of the enzyme was stopped after a 5-minute incubation period, when the cells were resuspended in supplemented media. Cells that were between passages 3-10, were seeded for the steroidogenesis assay (OECD, 2011).

4.4 Steroidogenesis assay

Cells were seeded into 24-well plates at 300 000 cells/mL, with1 mL working volume added into each well (Hecker and Giesy, 2008). The cells were incubated for 24 hours, before being dosed with 1 µL of the ARV active ingredients (efavirenz, didanosine, lamivudine, stavudine, ritonavir, lopinavir) and controls. All the ARV active ingredients, dissolved in methanol (1 mg/mL), were tested in triplicate on the H295R cells. Six concentrations (0.8, 8, 80, 800, 8 000, and 80 000 ng/mL) of ARVs were chosen based on their therapeutic dose concentrations and from previously reported values of ARVs in the aquatic environment in South Africa (Wood et al., 2015). Each plate also included a solvent control of methanol, as well as a blank control (only cells and their media), in triplicate. Each experiment included a QC plate, which received a known inducer and inhibitor to evaluate the cells' responses. Six blank control wells, six SC wells, triplicate wells of 1 µM and 10 µM forskolin, and triplicate wells of 0.1 µM and 0.3 µM[1] prochloraz were all included in the QC plate. These concentrations were selected to cover the recommended concentrations according to the OECD guideline for the steroidogenesis assay (OECD, 2011). Forskolin is known to be an inducer, while prochloraz is known as an inhibitor of both testosterone and oestradiol (Nielsen et al., 2012).

After a 48 h exposure period, the cell medium of each exposed well was harvested and stored in 1.5 mL amber glass vials at -80°C, until extraction of the steroid hormones could commence (refer to section 4.6.1 for the extraction procedure). During the 48 h exposure period, the media evaporated from some wells and in order to determine the degree of enrichment of the targeted hormones, the exact nutrient volume that was harvested was determined gravimetrically. Care was taken to remove all the liquid media from the wells after the exposure period. The masses recorded were compared to the initial 1 mL of the media that

[1]It was later discovered that the prochloraz dose concentrations were supposed to be 0.1 µM and 1 µM according to the OECD guidelines. However, it was too late to repeat the assays because by the time the error was discovered the entire H295R cell line stock was lost and newly imported ones also failed to grow. This impacted only on the quality control plates, but not on the experimental exposures.

were seeded at the beginning of the assay. This was then taken into account during the calculations of the results.

4.5 Viability assay

A viability assay was performed on the cells in each well of the 24-well plate, to ensure that the dosed compounds had not affected the cells' survival and growth. This was done on the same cells that were exposed, after the nutrient media was removed for hormone concentration quantification. The viability assay is a colorimetric assay that works on the principle that only viable cells are able to metabolise the yellow MTT into blue formazan crystals (Mosmann, 1983). A decrease in hormone levels could be due to decreased cell viability, and not due to a change in steroidogenesis in response to an ARV drug. The MTT assay enables distinguishing between these two reasons for the decrease in hormone levels.

On the final day of the assay, once the nutrient media was removed for analysis, the cells were rinsed with DPBS and treated with 0.5 mg/mL MTT solution prepared with stock nutrient medium. The plates were incubated for 30 minutes at 37°C, after which the MTT was replaced with DMSO to dissolve the blue formazan crystals. The solution from each well of the 24-well plate was transferred to three respective wells in a 96-well plate (because the plate reader cannot fit 24-well plates). The absorbance was quantified at 560 nm using a multimode microplate reader (Berthold TriStar LB 941, Germany). The viability of the cells was expressed in terms of the percentage of the absorbance of the exposed wells to that of the unexposed wells, and the non-parametric Mann-Whitney U test was used to determine if there was any statistically significant ($p < 0.05$) difference between the viability of the exposed and the control (SC) cells. The percentage cell viability was also taken into account for the calculations of the final concentrations of steroids. This was based on the principle that only cells that are viable would produce the hormones of interest, and therefore by correcting for them, one would remove the variability of the cells that are alive on the different plates.

4.6 Quantification of steroid hormones

Extraction and quantification of the steroid hormones were performed using the developed and validated method explained in Chapter 3. It is briefly summarised below for the sake of completeness.

4.6.1 Extraction of steroid hormones

The extraction method involved adding the following into a 5 mL glass tube: 500 µL of harvested and thawed cell media, 100 µL mixed internal standard working solution (see

section 3.4 for preparation of the working solution) and 2 mL diethyl ether. Each tube was capped with a stopper and mixed for a minimum of 5 minutes. The tubes were centrifuged at 2 000 g for 5 minutes. Approximately 1 mL of the top organic layer was transferred to a corresponding clean 1.5 mL amber vial, and dried completely under a steady flow of nitrogen gas. The remainder of the top organic layer, from the 5 mL tube, was then transferred to the same corresponding 1.5 mL amber vial, and once again dried completely. Once completely dry, the samples were reconstituted with 100 µL of 1:1 methanol:water, and vortexed well. The reconstitute was transferred to low volume inserts, placed into the 1.5 mL amber vials, and subjected to the UHPLC-QTOF for analysis.

4.6.2 UHPLC-QTOF method

The UHPLC column used was an Agilent InfinityLab Poroshell 120 Phenyl-Hexyl (2.1 x 100 mm 1.9-µm) (P.N.:695675-912; S.N.USJTE01014).

The mobile phases used for the positive ionisation were: A1 = 2 M ammonium acetate and 1 mL formic acid in 1 L H_2O (deionised water 18.2 MΩ·cm from an in-house ELGA water purification system). B1 = 2 M ammonium acetate and 1 mL formic acid in 1 L spectrophotometry grade methanol. The mobile phases used for the negative ionisation were: A2 = 1 mM ammonium fluoride in 1 L H_2O, and B2 = 1 L spectrophotometry grade methanol.

4.6.2.1 LC parameters

A volume of 5 µL of sample was injected into the UHPLC system, and carried through the system at a flow rate of 0.35 mL/min for 10 minutes. The mobile phase gradient is summarised in Table 4-1. The column was kept at 45°C in the column compartment, throughout the run. The relevant retention times for each steroid hormone is listed in Table 4-2.

Table 4-1: The mobile phase gradient percentages used on the UHPLC.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	50	50
5	30	70
9	10	90
10	50	50

4.6.2.2 QTOF and source parameters

The Dual AJS ESI was set to positive or negative polarity for ionisation. For 6.5 minutes the flow was set to go to the QTOF to be ionised, after which the valve was set so that the flow goes to waste for the remainder of the run time (10 mins). The ion scan range was set to scan for masses of 50–400 m/z, at a scan rate of 1 spectrum per second. The relevant accurate masses for each steroid hormone is listed in Table 4-2.

The source parameters included a gas flow of 8 L/min at 300°C, the nebuliser at 45 psi, and the sheath gas flow was 11 L/min at 400°C. A 2 500 V VCap was used, with the fragmentor set to 130 V, the skimmer to 75 V, the nozzle to 0 V and the OctopleRF peak to 750 V.

Table 4-2: The retention times and accurate masses used for the different steroid hormones in this study.

Steroid hormone	Retention time (mins)	Accurate mass (m/z)
Oestradiol	3.50	271.1692
Oestradiol-d3	3.48	274.1882
Aldosterone	2.43	361.2034
Cortisol	2.56	363.2169
Cortisol-d4	2.54	367.2429
Testosterone	4.47	289.2166
Androstenedione	4.67	287.2021
17-OH progesterone	4.87	331.2296
17-OH progesterone-d8	4.82	339.2781

4.7 Calculations and statistical analysis

The raw data that was collected from the quantitative software on the UHPLC-QTOF required no manual calculations to be done to correct for the internal standard responses, since the software did all those calculations.

The difference in hormone concentrations produced by ARV exposed and SC cells were tested for significance using the Mann-Whitney U test ($p \leq 0.5$). Known inhibitor (prochloraz) and stimulator (forskolin) of testosterone and oestradiol were used to confirm that the H295R assay was performing satisfactorily. According to the OECD specifications 10 µM forskolin

should cause a 1.5-fold increase in testosterone concentration and >7.5-fold increase in oestradiol concentration in H295R cells, while 1 µM prochloraz should cause a <0.5-fold decrease in testosterone and oestradiol concentrations (OECD, 2011).

For cell viability, the mean absorbance of the exposed cells, were compared to the mean absorbance from the SC cells from the same plate. The percentage (%) cell viability was calculated by dividing the mean absorbance values of the exposed cells by the mean absorbance values of the control (SC cells), from the MTT assay. The concentrations for the hormones were corrected for the viability of the cells as displayed by the MTT viability results. This means that the concentrations were adjusted for the viability of the cells producing those hormones: i.e. a concentration of 3 µg/L would increase to 5 µg/L if the viability was only 60% compared to the viability of the solvent control wells. Whereas, if the viability was 110% the corrected concentrations would have been 5.5 µg/L.

Corrections were also made for loss of volume of nutrient medium in the 24-well plates during the exposure period in the incubator. To do so, the mass of the medium collected from the wells after exposure was compared to the mass that was initially added to each well. The percentage loss was incorporated into the concentration of the hormones.

After these adjustments, the concentrations of the various hormones produced by the ARV exposed cells were compared to the respective concentrations of hormones produced by the SC cells. This ratio was presented as a fold change (FC). The FC was calculated to limit any variation that the different cell plates may have. A FC of 1 means there is no difference between their steroid hormone productions, a FC of less than 1 means there is a decrease in the steroid hormone production of the exposed cells, and a FC of greater than 1 means there is an increase in steroid hormone production of the exposed cells, compared to the SC cells. FC trends were monitored, to see if the dose concentrations of the various ARVs effected the steroid hormone production.

Outliers were only removed if the coefficient of variation (CV) was above 25%, however, if the variable calculations corrected for the large CV, the data point was not omitted, since the viability and mass calculations were taken into account to remove any such bias. All calculations were done on the data set before removing outliers, and again after removing outliers, to determine how the outliers were affecting the results, and to determine if they were in fact influencing the results in any way.

CHAPTER 5: RESULTS AND DISCUSSION

5.1 Introduction

The influence of various ARV active ingredients on the steroidogenesis pathway was determined by exposing H295R cells to various concentrations of each ARV, and then measuring the concentration of six steroid hormones produced by these H295R cells. H295R cells are the ideal assay to assess the effects on the steroidogenesis pathway, because they express all the necessary steroidogenic enzymes, enabling them to produce mineralocorticoids, glucocorticoids and adrenal androgens. The quantification of these six steroids (aldosterone, androstenedione, cortisol, 17-OH progesterone, testosterone and oestradiol) was done using a method that was developed and validated on a UHPLC-QTOF. The results for aldosterone were all below the LOQ, and were therefore excluded from any further calculations, statistical analysis and discussion. The SC cells are those cells on each H295R assay plate that were exposed to only the solvent (methanol in this study) in which the ARVs were dissolved, thereby demonstrating the response caused by the solvent, and is used as a control for each assay plate. The variation in concentrations of steroid hormones between studies can be expected when using tissue cultures, because cells proliferate at different rates, and different generations do not respond identically. Fold changes (FC) are therefore used for comparisons as responses are expressed in terms of the SC cells of the same batch of cells. FC is the comparison of a steroid hormone from the exposed cells, to that of the same steroid hormone of the SC cells from the same assay plate.

5.2 Cytotoxicity of reference compounds and ARVs

The cytotoxic effects of the selected ARVs on H295R cells were evaluated by using the MTT assay. The percentage (%) cell viability was calculated using the mean absorbance values of the exposed cells compared to the mean absorbance values of the SC cells multiplied by 100. The reason why this assay was included in the research, was to make sure that if less steroid hormones were produced by the exposed cells compared to the SC cells, than it was because of the effect of the tested ARV on the steroidogenesis process itself and not because of its cytotoxicity. All the cells were viable after exposure to selected ARVs with viability ranging from 79–114% (Figure 5-1).

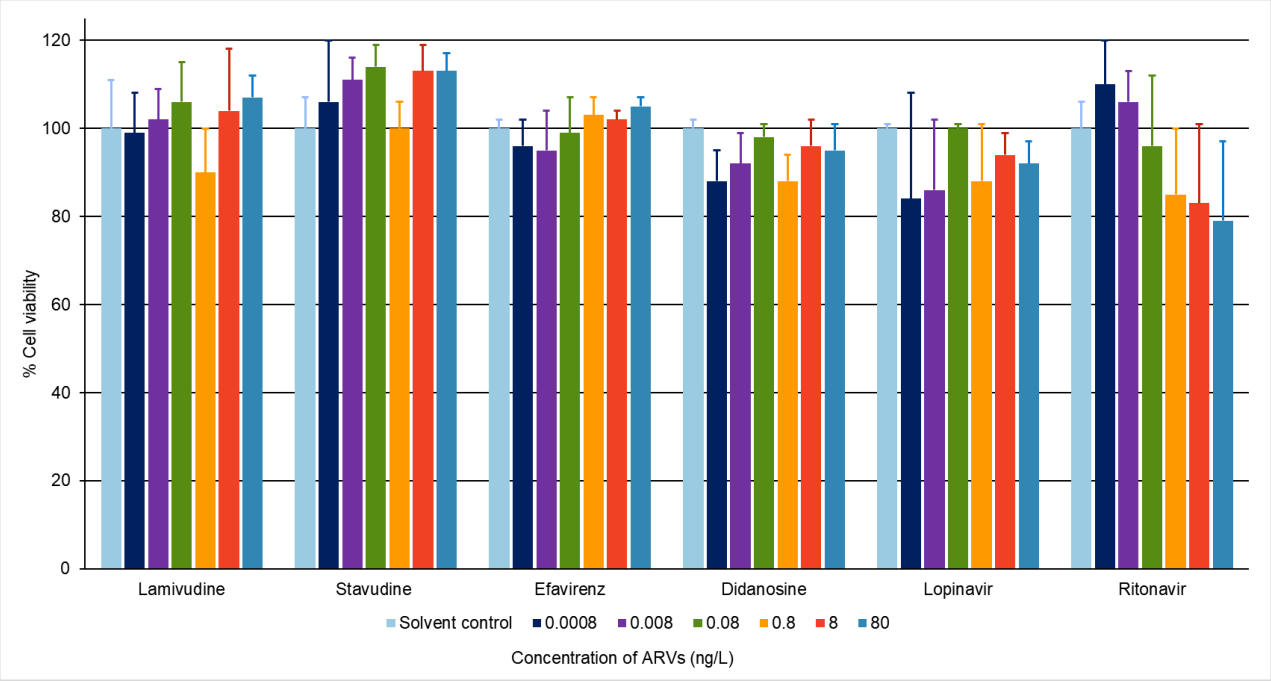

Figure 5-1: The mean percentage (%) of viable cells per dose of each ARV (ng/L). *Standard deviations are indicated by error bars.*

Ritonavir was the only ARV that seemed to have had caused a slight decrease in viability as its concentration increased (Figure 5-1). The OECD guidelines suggest that any inhibition of steroidogenesis in cells which have close to 20% cytotoxicity, should be carefully assessed to confirm that the cytotoxicity is not the source of the inhibition (OECD, 2011). However, the steroid hormone concentrations in this study were corrected for % cell viability (see section 4.7).

Malikova et al. (2019) reported that efavirenz was cytotoxic at concentrations above 1 578 375 ng/L, which is much higher than the maximum concentration of 80 ng/L used in this study.

All the cells exposed to the reference compounds prochloraz and forskolin were viable ranging from 80–108% and 102–120%, respectively (Figure 5-2). Prochloraz is known to be cytotoxic at concentrations above 3 µM (Hecker et al., 2006). This fact was supported by the cell viability reaching just 80% when exposed to 0.3 µM prochloraz in this study. The percentage cell viability for prochloraz was within the 20% cytotoxicity OECD guidelines, and therefore this was not significant enough cytotoxicity to affect our findings (OECD, 2011).

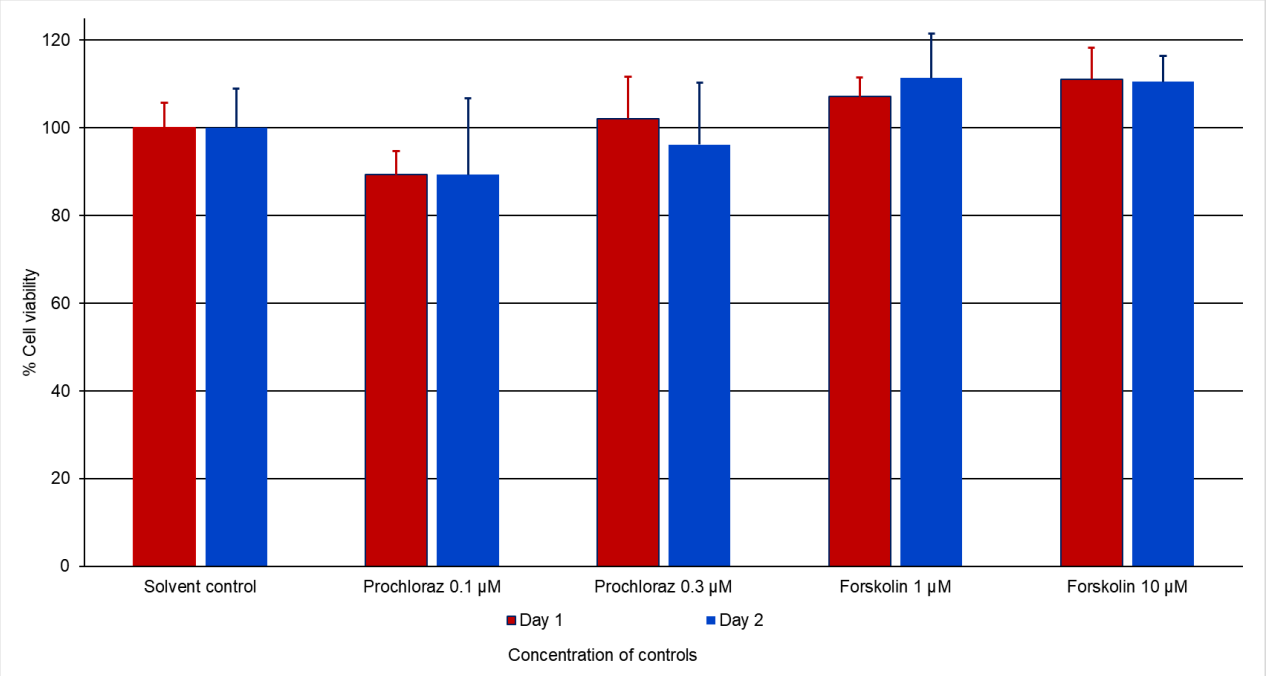

Figure 5-2: The mean % of viable cells per dose of reference compound (prochloraz and forskolin) on the control plates.

Standard deviations are indicated by error bars.

5.3 Hormone production in response to reference compounds (quality control plates)

The control plates from the two days' H295R assays were assessed to evaluate whether the assays were successful and relatable to the requirements as proposed by the OECD guidelines (OECD, 2011). The mean steroid hormone concentrations (and their SD) produced by the H295R cells after exposure to the reference compounds (forskolin and prochloraz) at their different doses, are summarized in Table 5-1. Figure 5-3 and Figure 5-4 show the FCs as compared to the SC cells for the different doses of forskolin and prochloraz. Each H295R assay plate contained SC cells, which was used to calculate the FC.

Table 5-1: Mean and standard deviation of the steroid hormone concentrations (µg/L) of the quality control plates from two repeats.

		Oestradiol	Cortisol	Testosterone	Androstene-dione	17-OH progesterone
Day 1	**Solvent control**	0.31 ± 0.03	8.14 ± 1.49	2.27 ± 0.55	45.08 ± 2.58	22.72 ± 3.00
	Prochloraz 0.1 µM	0.55 ± 0.14	*4.29 ± 0.94	1.57 ± 0.04	79.63 ± 0.27	*65.41 ± 4.02
	Prochloraz 0.3 µM	0.58 ± 0.07	*2.32 ± 0.40	1.91 ± 0.31	26.16 ± 10.75	*62.37 ± 4.61
	Forskolin 1 µM	0.82 ± 0.08	*29.10 ± 1.00	2.92 ± 0.61	178.45 ± 33.66	*49.89 ± 2.66
	Forskolin 10 µM	1.24 ± 0.30	*56.33 ± 6.63	*4.10 ± 0.97	182.72 ± 9.10	*43.46 ± 2.09
Day 2	**Solvent control**	0.54 ± 0.13	9.25 ± 0.86	1.62 ± 0.17	109.75 ± 20.95	74.89 ± 3.44
	Prochloraz 0.1 µM	0.58 ± 0.06	3.95 ± 0.54	1.26 ± 0.30	34.58 ± 5.48	181.82 ± 23.67
	Prochloraz 0.3 µM	0.61 ± 0.12	3.65 ± 0.99	1.08 ± 0.22	56.31 ± 15.97	*179.24 ± 15.99
	Forskolin 1 µM	*0.82 ± 0.06	*34.26 ± 2.80	2.74 ± 0.48	155.75 ± 5.58	*96.89 ± 8.85
	Forskolin 10 µM	*1.17 ± 0.06	*77.17 ± 7.76	*3.42 ± 0.69	201.90 ± 22.20	87.34 ± 10.87

**Hormonal concentrations that were statistically significant different from the SC cells' hormone levels ($p \leq 0.05$).*

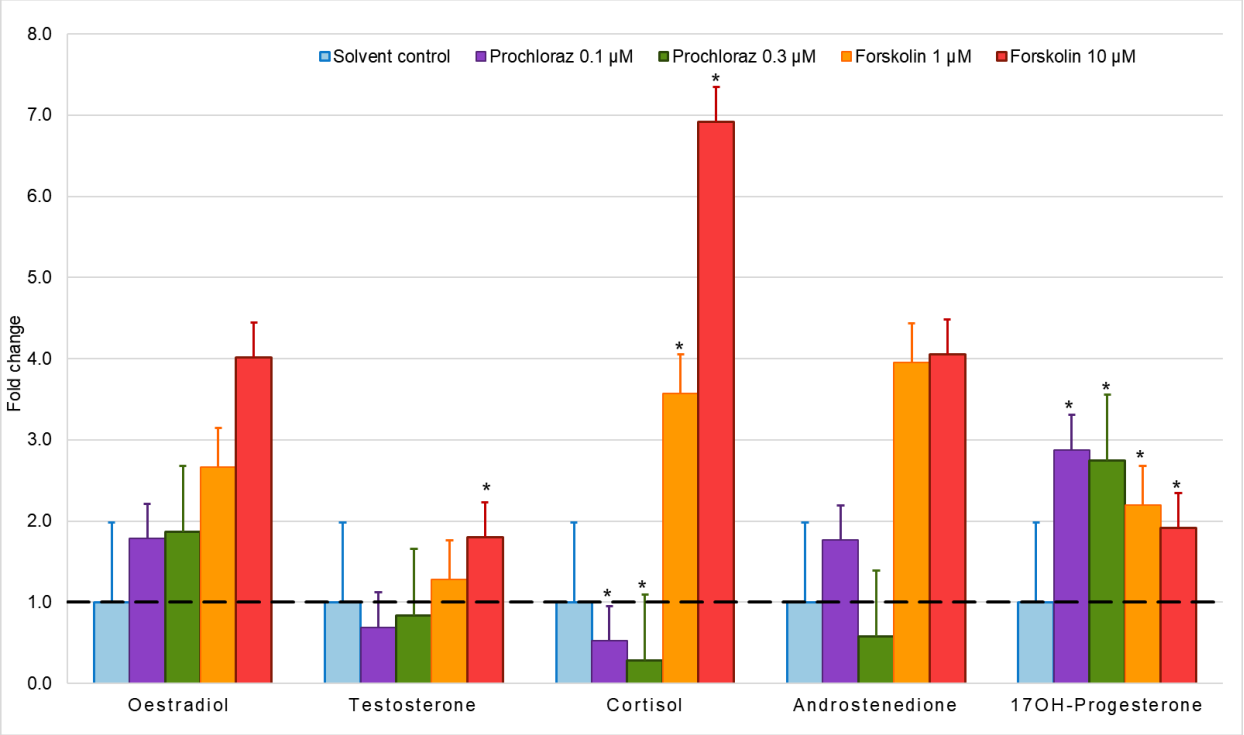

Figure 5-3: The fold changes of the steroid hormone concentrations for the control plate for day 1.

**Hormonal concentrations that were statistically significant different from the SC cells' hormone levels ($p \leq 0.05$). Dashed line is FC = 1. Standard deviations are indicated by error bars.*

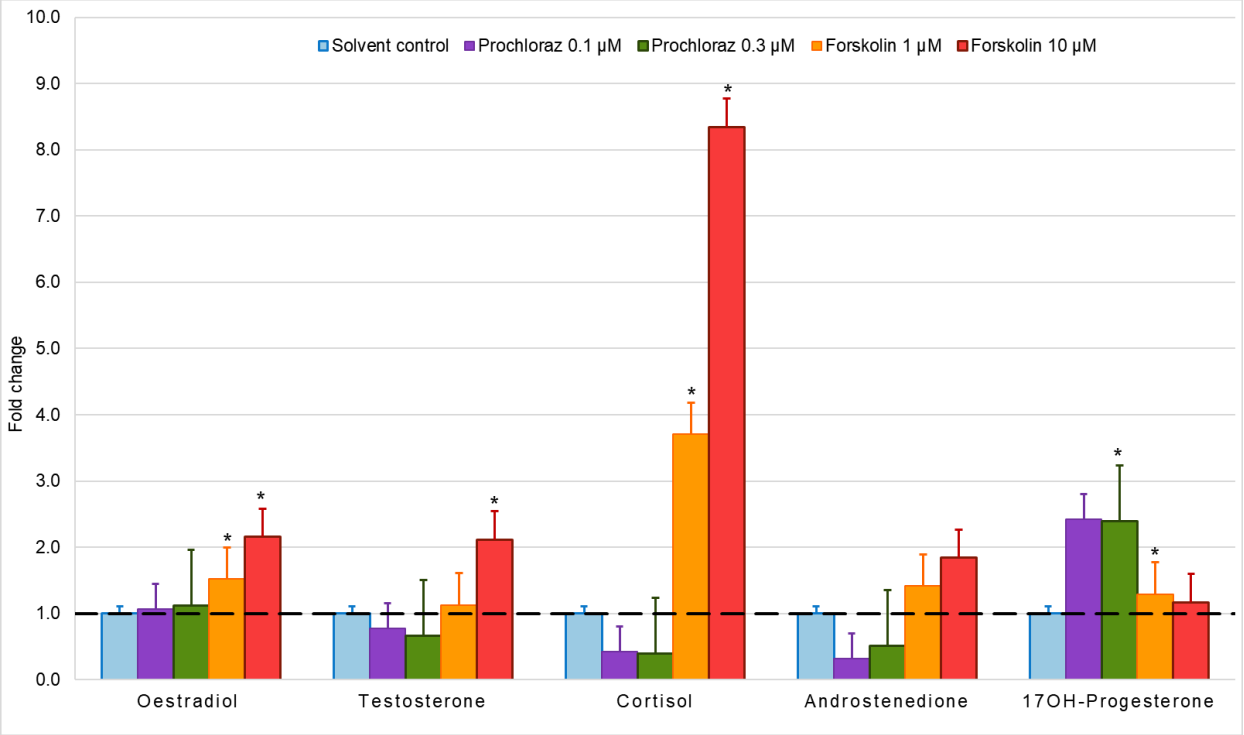

Figure 5-4: The fold changes of the steroid hormone concentrations for the control plate for day 2.

**Hormonal concentrations that were statistically significant different from the SC cells' hormone levels ($p \leq 0.05$). Dashed line is FC = 1. Standard deviations are indicated by error bars.*

5.3.1 Oestradiol

The mean oestradiol concentration across all SC cells from all of the plates, produced a mean of 0.68 ± 0.20 µg/L oestradiol (Supplementary Table 1), and the LOQ for oestradiol is 0.26 µg/L. According to the OECD guidelines, the SC cells should produce oestradiol concentrations of ≥ 2.5 times the LOQ (OECD, 2011). This study found that the SC cells produced 2.6 times the amount of oestradiol than the LOQ, and therefore met the requirements for basal hormone production in the SC cells. The concentration of oestradiol in this study's SC cells were similar to those reported by Hecker et al. (2006) (0.41 ± 0.05 µg/L) and Kjærstad et al. (2010) (0.13–0.64 µg/L), but were considerably higher than those reported by Nakano et al. (2016) (0.04 ± 0.02 µg/L), Zhang et al. (2011a) (0.05 µg/L), Nielsen et al. (2012) (0.07 ± 0.02 µg/L), and the OECD (2010) (0.02–0.155 µg/L).

Prochloraz is a known inhibitor of oestradiol activity, but contrary to what was predicted, in this study prochloraz caused an increase (FC > 1) in the concentrations of oestradiol on both days, at both exposure concentrations (OECD, 2011) (Figure 5-3 and Figure 5-4). According to the

OECD (2011) and USEPA (2011), the FC should be ≤ 0.5 times the SC for a 1 µM prochloraz dose, yet in this study the mean FC was 1.42 (at 0.1 µM) and 1.49 (at 0.3 µM) times. This unexpected effect was not statistically significant when compared to the SC cells. The prochloraz concentrations were too low to exert the desired inhibitory effect. The same effect of an induction instead of inhibition of oestradiol at 0.3 µM prochloraz, was found during the validation process of the OECD guidelines, for two of the participating laboratories OECD (2010). The increase in oestradiol could be due to the cells over compensating when exposed to lower doses of prochloraz, by means of negative feedback within the cells, due to the decreased oestradiol concentrations, thereby over producing oestradiol. Furthermore, the oestradiol concentrations throughout this study were low and close to its LOQ (0.26 µg/L) so that it would have been difficult to measure an inhibitory effect. There were many studies reporting similar responses of oestradiol inhibition to that of the OECD guidelines. The FCs reported for exposures to 1 µM prochloraz, included Zhang et al. (2011a) at 0.48 times, Nakano et al. (2016) at 0.49 times, Higley et al. (2010) at 0.43 times, and Hecker et al. (2006) at 0.18 times, but since this concentration was not used in this study (see section 4.4) it is not possible to guess if the cells used in this study would have responded in the same manner. Furthermore, Nielsen et al. (2012) were unable to detect oestradiol in any of their prochloraz-exposed cells.

Forskolin on the other hand, which is a known stimulant of oestradiol, responded as expected by causing an increase (FC > 1) in the concentrations of oestradiol on both days, at both exposure concentrations (OECD, 2011) (Figure 5-3 and Figure 5-4). According to the OECD (2011) and USEPA (2011), 10 µM forskolin should result in an oestradiol FC of ≥ 7.5 times the SC cells. In this study, however, the 10 µM forskolin only produced oestradiol at a FC of 3.09 times the SC cells. Although the forskolin exposed cells did not respond as prominently as the OECD guidelines suggest for oestradiol, the oestradiol concentrations in the forskolin-exposed quality controls were dependent on the dose of the control being administered. On both days in the H295R assays the higher dose (10 µM) of forskolin resulted in a higher production of oestradiol than the lower dose (1 µM). Furthermore, the oestradiol concentrations of the forskolin exposed cells at both doses, were statistically significant different from the SC cells on the second day of H295R assays ($p \leq 0.05$). Zhang et al. (2011a) had a FC of 16.9 times, with Nakano et al. (2016) reporting a similar change at 17.77 times. However, Higley et al. (2010) reported double the FC (35 times) of Zhang et al. (2011a) and Nakano et al. (2016). Furthermore, Nielsen et al. (2012) reported an even lower FC than these other studies, in oestradiol concentrations, at 6.6–11 times when their H295R cells were exposed to 10 µM forskolin, while Hecker et al. (2006) found only a 7 times FC when they exposed their cells to 30 µM forskolin doses. The 3.09 times FC in this study for oestradiol

production was therefore much lower than those reported by other studies that dosed with 10 µM forskolin.

Therefore, these control results for oestradiol confirm that a stimulant of oestradiol would be able to be identified in this study although not as intensely as those of other laboratories, however, any inhibitory effects on oestradiol may not have been picked up. Therefore, it cannot be ruled out that any of the ARVs might have caused inhibition of oestradiol.

5.3.2 Testosterone

The LOQ for testosterone in this study was 0.07 ± 0.2 µg/L, while the SC cells from all of the assay plates, produced a mean of 2.93 ± 1.53 µg/L of testosterone (Supplementary table 1). According to the OECD guidelines, the SC should produce ≥ 5 times the amount of testosterone than the LOQ (OECD, 2011). This study found that SC cells produced testosterone that was 41.86 times the LOQ, and therefore met the requirements for basal hormone production in SC cells. Testosterone concentrations produced in the SC cells of this study were double that reported by Nakano et al. (2016) (1.42 ± 0.34 µg/L), Strajhar et al. (2017) (1.09 µg/L), and Kjærstad et al. (2010) (0.56–2.50 µg/L), and 4 times that reported by Zhang et al. (2011a) (0.47 µg/L), Liu et al. (2010) (0.43 ± 0.01) and Nielsen et al. (2012) (0.57 ± 0.29 µg/L). This study found similar concentrations of testosterone to those reported by Hecker et al. (2006) (1.7–6.1 µg/L) and OECD (2010) (1.877–5.512 µg/L). On the other hand, Schloms et al. (2012) reported much higher basal SC testosterone concentrations of 11.38 µg/L, while Hasegawa et al. (2013) was unable to detect any testosterone.

The H295R cells performed on both assay days as expected when exposed to either doses of prochloraz, with the testosterone concentrations decreasing compared to the SC cells (Figure 5-3 and Figure 5-4). However, only on day 2 was there a dose dependent response (Figure 5-4). According to the OECD (2011) and the USEPA (2011), the use of 1 µM prochloraz should result in a FC of ≤ 0.5 compared with the SC cells for testosterone. Because the advised prochloraz concentrations were not used in this study, the cells were not inhibited as much. The FC for testosterone in the 0.3 µM prochloraz exposed cells of this study were 0.75 times that of the SC cells. Moreover, the observed lower testosterone levels in the prochloraz-exposed cells were not statistically significantly different from those observed in the SC cells due to their low exposure doses. Most studies from literature used a dose of 1 µM for prochloraz, and therefore they were able to satisfy the OECD guidelines of a FC ≤ 0.5 (0.05 times (Nakano et al., 2016), 0.28 times (Zhang et al., 2011a), 0.1 times (Higley et al., 2010), 0.33 times (Hecker et al., 2006), and 0.66–0.16 times (Nielsen et al., 2012). Conversely, the study by Strajhar et al. (2017) reported a FC of 1.23 ± 0.16 times.

Nonetheless, they also reported high levels of testosterone in the Nu serum ingredient of the nutrient medium, which is the probable cause of this stimulatory result.

Testosterone concentrations increased in a dose dependent manner when the cells were exposed to the known agonist forskolin. Furthermore, for the 10 µM forskolin on both days, the testosterone concentrations were statistically significantly higher than in the SC cells ($p \leq 0.05$) (Figure 5-3 and Figure 5-4). The OECD (2011) guidelines require that the FC in testosterone levels should be ≥ 1.5 times for the 10 µM forskolin dose, and the FC of 1.96 times in this study met those requirements. The USEPA (2011), however, suggests that a FC > 2 times for testosterone at 10 µM forskolin be used as the cut-off criteria, to which the FC of this study is just short of. This study's FC for testosterone concentrations was similar to those reported in other studies (2.9 times (Zhang et al., 2011a), 2.84 times (Nakano et al., 2016), 3 times (Hecker et al., 2006), 2.5 times (Higley et al., 2010), and 1.41 times (Schloms et al., 2012)). Nielsen et al. (2012), however, reported a very broad range of FCs for testosterone, ranging from FCs similar to this study (1.8 times) to those more than double that which we reported (5.1 times). Strajhar et al. (2017), however, reported a testosterone FC of 0.97 ± 0.15 times after exposure to 50 µM forskolin.

The repeated lower testosterone concentrations in the prochloraz inhibitor exposed cells, confirms that the cells were responding adequately when exposed to inhibitors. Furthermore, the satisfaction of the OECD guidelines for the forskolin exposures confirms that the cells were responding adequately to stimulators of testosterone as well.

5.3.3 Cortisol

The OECD (2011) and USEPA (2011) guidelines do not provide any recommendations on minimum basal SC cortisol concentrations or how cortisol concentrations produced by the cells exposed to the reference compounds (prochloraz and forskolin) should react compared to the SC cells. This study's SC cells' mean cortisol concentration was 7.89 ± 1.41 µg/L (Supplementary Table 1), which was similar to other studies, which reported 8.26 µg/L (Strajhar et al., 2017), 6.02 ± 1.03 µg/L (Nakano et al., 2016), and 9.72 ± 4.82 µg/L (Winther et al., 2013). However, the cortisol results were 1.5 times greater than the reported cortisol concentrations of 4.93 ± 0.20 µg/L by Liu et al. (2010) and nearly 5 times that of the 1.7 µg/L reported by Mangelis et al. (2016). Schloms et al. (2012), however, reported the highest SC cortisol concentrations of all the studies, at 208.87 µg/L.

The cortisol concentrations were found to be highly dependent on the doses of the controls, for both prochloraz and forskolin (Figure 5-3 and Figure 5-4). Both doses of prochloraz

resulted in cortisol concentrations that were statistically significantly different from the SC cells only on day 1 (Figure 5-3). The FC of cortisol production of prochloraz-exposed cells compared to SC cells was 0.48 and 0.34 times for the 0.1 and 0.3 µM prochloraz doses respectively. The FC in cortisol production for 1 µM prochloraz (0.34 times) was not as drastic as the 0.08 times FC reported by Winther et al. (2013) or the 0.01 times reported by Nakano et al. (2016). The cortisol FC that was achieved for the 0.3 µM prochloraz exposures in this study was however similar to the 0.26 times FC for 1 µM prochloraz exposure observed by Strajhar et al. (2017). Once again, it should be noted that the doses of prochloraz in this study were not as high as other studies, and therefore is the probable reason that our FC results were not as prominent as theirs were.

The cortisol concentrations were statistically significantly greater for forskolin exposure cells (both doses) compared to the SC cells, on both control days (Figure 5-3 and Figure 5-4). The FC of cortisol production of forskolin-exposed cells compared to SC cells was 3.64 and 7.63 times for the 1 and 10 µM forskolin doses, respectively in this study. The FC for cortisol at 10 µM forskolin exposure (7.63 times) matched the 7.2 times reported by Schloms et al. (2012), but was slightly higher than the 5.1 times reported by Winther et al. (2013), and substantially higher than the 2.38 times reported by Strajhar et al. (2017) (50 µM Forskolin). However, the FC for 10 µM forskolin was half of the 13.74 times FC reported by Nakano et al. (2016) and a quarter of the 28 times FC reported by Mangelis et al. (2016) for cortisol production.

Therefore, we can conclude that our findings for cortisol concentrations would be similar to those of other studies, if they were to test for the same compounds of interest.

5.3.4 Androstenedione

The OECD (2011) and USEPA (2011) guidelines do not provide any recommendations on minimum basal SC androstenedione concentrations, or how androstenedione concentrations produced by the cells exposed to the reference compounds should react compared to the SC cells. The mean androstenedione concentrations that we detected in the SC cells was 73.53 ± 44.59 µg/L (Supplementary Table 1). The androstenedione concentrations in the SC cells in the current study were higher than the concentrations reported as 12.43 µg/L by Strajhar et al. (2017), 13.11 ± 3.20 µg/L by Nakano et al. (2016), 17.9 ± 4.42 µg/L by Nielsen et al. (2012), 35.2 ± 2.6 µg/L by Liu et al. (2010), and 48.7 µg/L by Mangelis et al. (2016). However, the 231.75 µg/L androstenedione reported in the SC cells of Schloms et al. (2012) were three times the levels detected in the SC cells of this study.

The changes in androstenedione in response to the various doses of controls, were different for the 2 days of H295R assays, but none of the results for control exposure cells were statistically significant different from the SC cells. On day 1, 0.1 µM prochloraz caused a FC of 1.77, and 0.3 µM a FC of 0.58 (Figure 5-3). On day 2, prochloraz caused inhibition of androstenedione production at both doses, with a FC of 0.32 and 0.51 times for the 0.1 µM and 0.3 µM exposures respectively (Figure 5-4). This study's FC for androstenedione at 0.3 µM prochloraz exposure was not as pronounced at the FCs of 0.02 times (Nakano et al., 2016) and 0.05 times (Strajhar et al., 2017) reported in the other studies at 1 µM prochloraz exposure, but as stated prior, the reference compound doses were significantly lower.

This study found that androstenedione concentrations increased with the dosing of forskolin on both days (Figure 5-3 and Figure 5-4). Furthermore, androstenedione production was dose dependent on the forskolin doses, with the mean FC greater at the higher 10 µM forskolin dose (3 times), than at the lower 1 µM forskolin dose (2.7 times). These FCs in androstenedione at 10 µM forskolin exposures was similar to those reported by other studies (1.9 times (Schloms et al., 2012), 3.0 times (Mangelis et al., 2016) and 3.6 times (Nakano et al., 2016)). Strajhar et al. (2017) found a similar FC of 1.7 times, but they used a dose five times higher than what was used in this study (50 µM).

The FC of androstenedione concentrations in this study is similar to those of other studies for the reference compound exposures.

5.3.5 17-OH progesterone

The mean 17-OH progesterone concentrations for the SC H295R cells was 46.35 ± 26.98 µg/L (Supplementary Table 1). Although the OECD (2011) and USEPA (2011) do not provide any recommendations on 17-OH progesterone minimum basal SC concentrations, some other studies reported values considerably lower than those that were achieved in this study. Strajhar et al. (2017) and Nakano et al. (2016) reported the lowest 17-OH progesterone concentrations from SC cells of 2.62 µg/L and 2.33 ± 0.35 µg/L respectively, with Liu et al. (2010) reporting double those concentrations(6.12 ± 0.09 µg/L). In the available literature, Mangelis et al. (2016) and Schloms et al. (2012) reported the highest concentrations of 17-OH progesterone in SC cells, at 13.2 and 19.54 µg/L respectively, yet it was still less than half the levels that were found in this study.

Both prochloraz and forskolin stimulated the 17-OH progesterone production in this study, but with prochloraz producing the larger FCs (Figure 5-3 and Figure 5-4). Prochloraz was found to cause a lesser FC (2.57 times) for 17-OH progesterone concentration with the higher 0.3 µM

prochloraz dose, compared to the 2.66 times FC for the lower 0.1 µM dose. The concentration of 17-OH progesterone was also statistically significant greater for prochloraz-exposed cells (both doses), compared to the SC cells on day 1, but only for the 0.3 µM prochloraz dose on day 2. The 17-OH progesterone FC findings at 0.3 µM prochloraz exposures were similar to the 2.16 ± 0.37 µg/L reported by Strajhar et al. (2017) at 1 µM prochloraz exposures. Nakano et al. (2016), however, found contrary results, with an inhibitory FC of 0.6 times being reported for the 1 µM prochloraz exposures.

Although forskolin exposure caused an increase in the concentrations of 17-OH progesterone, it did so inversely to the exposure dose of forskolin. The mean FC for 17-OH progesterone was 1.54 times in 10 µM forskolin exposed cells, but 1.75 times in 1 µM forskolin exposures when compared to the SC cells (Figure 5-3 and Figure 5-4). Even though the 17-OH progesterone concentrations were statistically greater for both doses of forskolin compared to the SCs on day 1, they were only statistically greater for the 1 µM forskolin dose on day 2. This study had lower FC results for the 10 µM forskolin doses compared to Nakano et al. (2016) (3.77 times), but greater FCs compared to Mangelis et al. (2016) (1.3 times) and Schloms et al. (2012) (0.56 times).

This study's FC in 17-OH progesterone concentrations is similar to those of other studies for the reference compound exposures.

5.3.6 Aldosterone

All the aldosterone concentrations were below our LOQ of 0.3 µg/L, and therefore no FCs were calculated. These low level findings for aldosterone are common, with Strajhar et al. (2017) reporting 0.01 µg/L, Nakano et al. (2016) reporting 0.33 ± 0.10 µg/L, and Mangelis et al. (2016) reporting 0.13 µg/L in their SC cells. Schloms et al. (2012), however, was the only study that reported concentrations of aldosterone at levels as high as 1.42 µg/L.

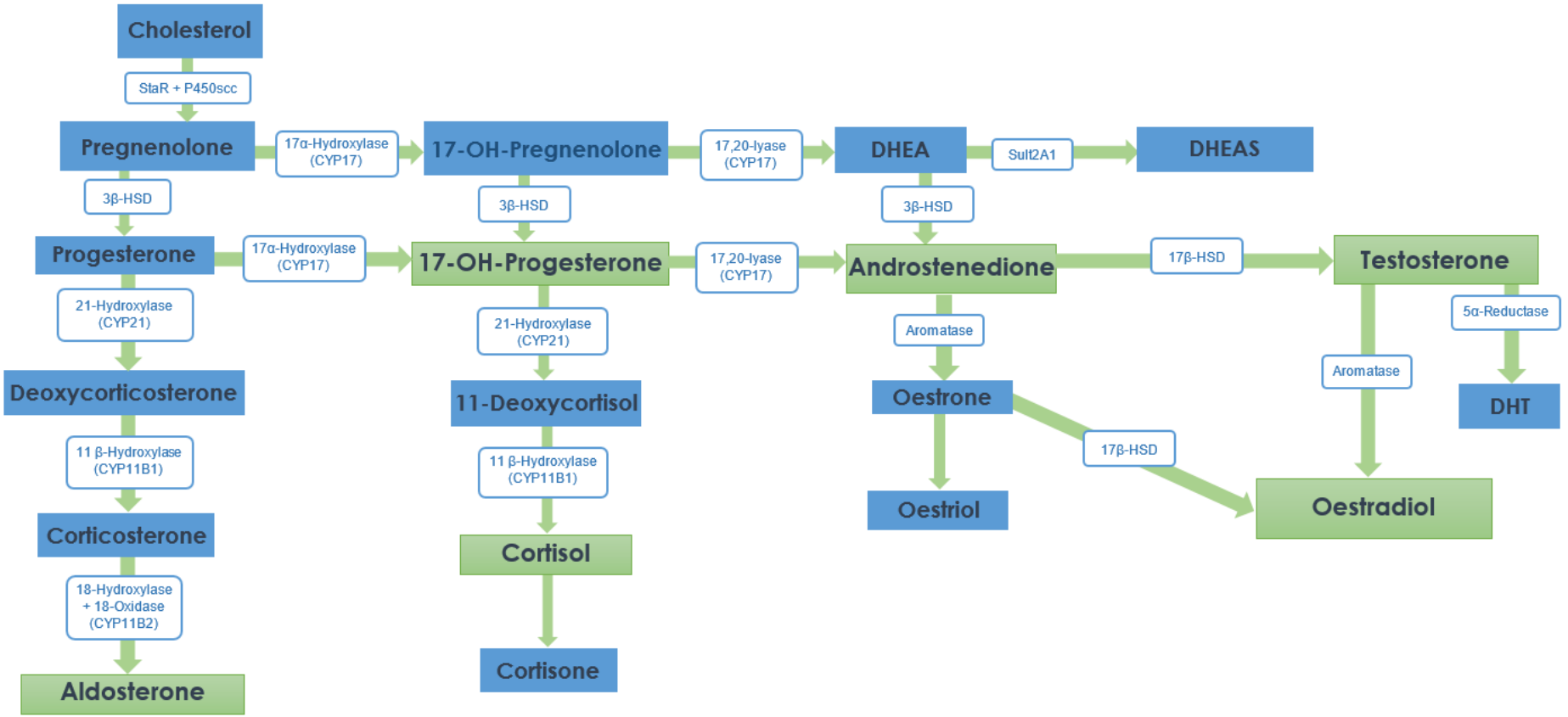

Figure 5-5: Schematic diagram of the steroidogenesis pathway, including the key steroid hormones and the respective steroidogenesis enzymes.

The green blocked hormones are the hormones of the pathway that were analysed in this study, whereas the blue blocked hormones are the other hormones not analysed. The white blocks are the enzymes involved in each relevant hormone conversion in the pathway.

(Adapted from Xu et al. (2006)) *(This figure is a copy of Figure 2 1 but was re-inserted here for ease of reference)*

5.4 Protease inhibitors

The class of ARVs that block the HIV protease enzyme, which thereby prevents the multiplication of the HIV virus, is known as protease inhibitors. Protease inhibitors mimic the natural substrate of viral protease, thereby competitively prohibiting cleavage of the proteolytic precursors by the HIV protease. When the HIV protease enzyme is blocked by an inhibitor, it is unable to breakdown its proteins, and in that way cannot multiply or spread.

Protease inhibitors cause various side effects in those people taking them. These side effects include nausea, vomiting, lipodystrophy, hyperlipidaemia, type 2 diabetes mellitus, kidney stones, liver damage, heart problems, and gallbladder problems (Ncube et al., 2018). However, the most detrimental possible effect would be when HIV becomes resistant to protease inhibitors. It is therefore important to use protease inhibitors in combination with the other classes of ARVs.

5.4.1 Ritonavir

Ritonavir (trade name is Norvir) was first approved by the FDA as a protease inhibitor in 1996. Monotherapy of ritonavir has been associated with ritonavir resistance, as well as numerous severe side effects, and is therefore no longer prescribed on its own. Ritonavir is restricted from being used as a monotherapy and therefore is rather used as an enhancer of other ARVs.

In this study, when the H295R cells were exposed to ritonavir (Figure 5-6), oestradiol production increased as the ritonavir doses increased. All the ritonavir doses decreased testosterone and cortisol production, except for the dose of 0.8 ng/L. The 0.0008 and 0.008 ng/L ritonavir concentrations resulted in a statistically significant decrease in testosterone and cortisol production, compared to the SC cells. Ritonavir at doses of 0.0008, 0.8, and 8 ng/L resulted in an increased FC for androstenedione. Although the FC for androstenedione at 0.8 ng/L is almost double (1.91 times) the SC, it is not a point of statistical significance. This could be due to the large standard deviations at that point, or that there were too few data points for accurate statistical analysis. The production of 17-OH progesterone increased as the ritonavir exposure doses increased, with an exception of 0.8 and 80 ng/L.

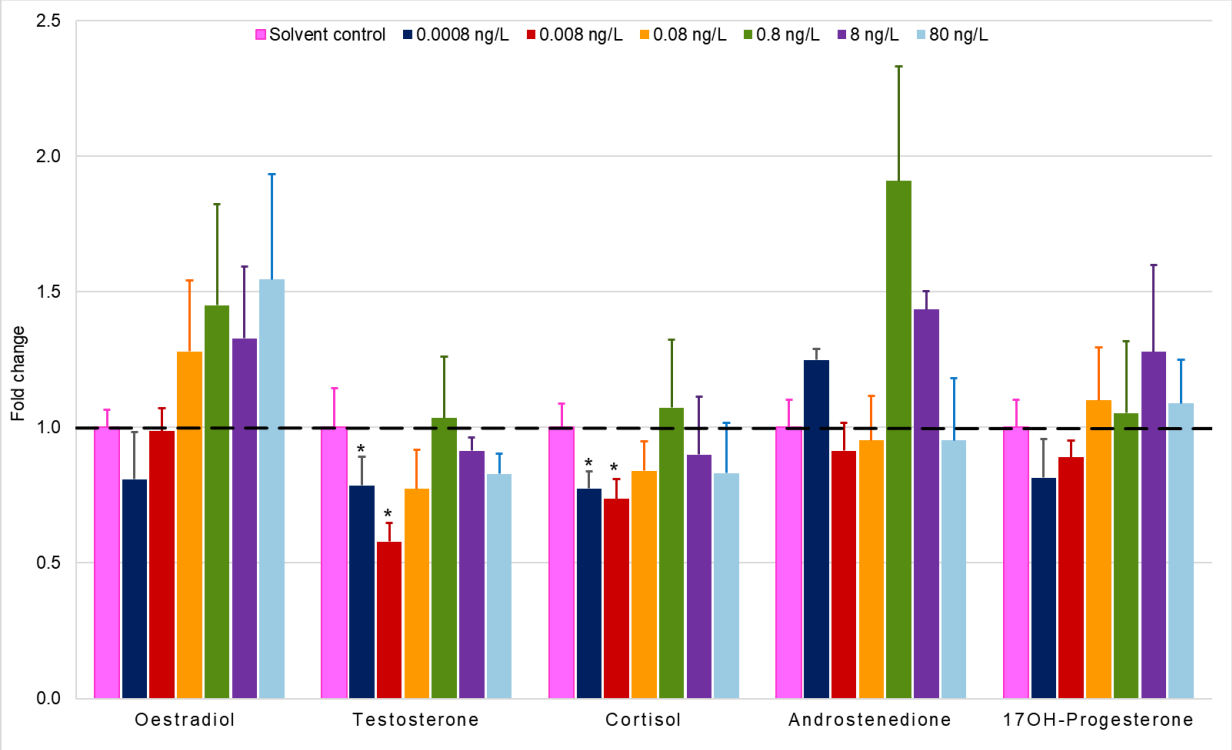

Figure 5-6: The FC of the steroid hormone concentrations for the ritonavir exposure plate.

**Hormonal concentrations that were statistically significant different from the SC cells' hormone levels ($p \leq 0.05$). Dashed line is FC = 1. Standard deviations are indicated by error bars.*

5.4.2 Lopinavir

Lopinavir (trade name Kaletra) is a protease inhibitor that was approved for use as an ARV by the FDA in 2000. It is used as part of a fixed dose combination with ritonavir. The FCs of the five quantifiable steroid hormone concentrations affected by the various doses of lopinavir in this study is summarized in Figure 5-7. All the doses of lopinavir resulted in a decreased FC for oestradiol, with the three lowest doses (0.08–0.0008 ng/L) resulting in FCs nearly half that of the SC cells. Testosterone was not significantly affected by the lopinavir doses, except for 0.8 ng/L which had an increased FC. The cortisol concentrations were barely affected at the different doses of lopinavir, except for the 8 ng/L dose, which resulted in a drastic decrease in cortisol production but was not statistically significant. The lopinavir doses at 0.0008, 0.8 and 80 ng/L caused androstenedione to increase drastically, while the remaining doses decreased the production of androstenedione. The 17-OH progesterone concentrations were dependent on the exposure doses of lopinavir. Furthermore, 17-OH progesterone is the only steroid hormone that showed statistically significant changes (at 80, 8 and 0.0008 ng/L lopinavir doses) between the lopinavir exposed cells and the SC cells.

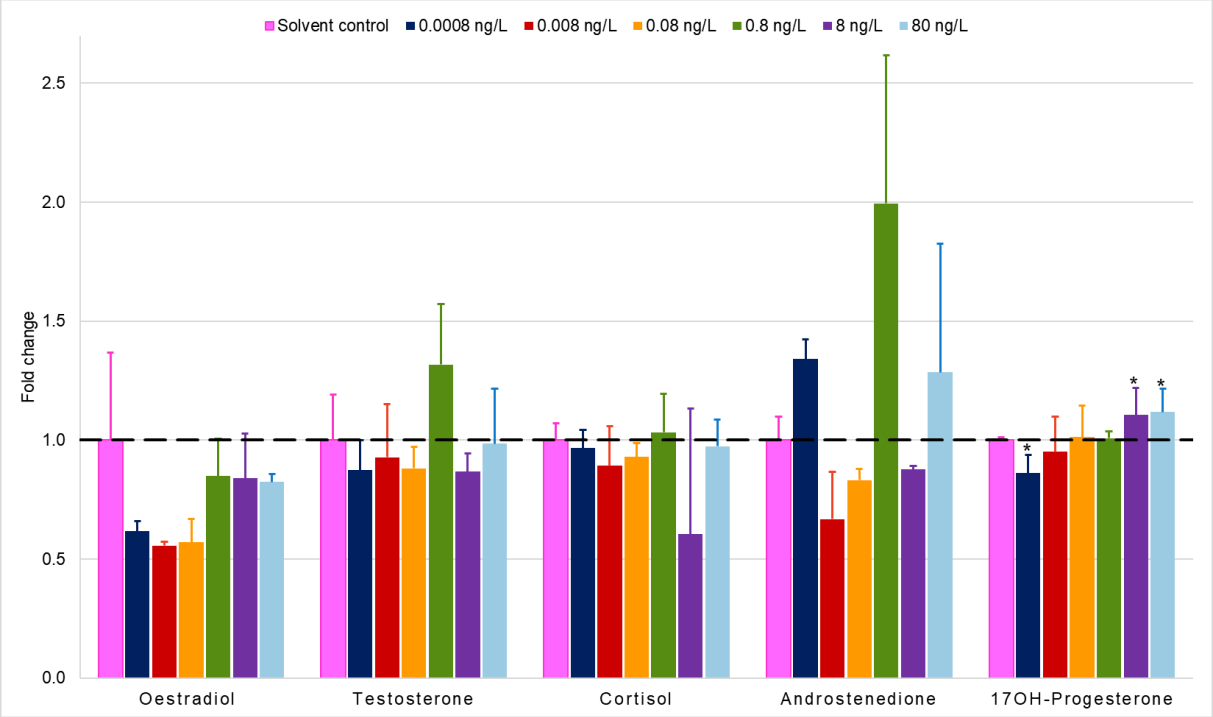

Figure 5-7: The FC of the steroid hormone concentrations for the lopinavir exposure plate. **Hormonal concentrations that were statistically significant different from the SC cells' hormone levels ($p \leq 0.05$). Dashed line is FC = 1. Standard deviations are indicated by error bars.*

5.4.3 Comparison of protease inhibitors

For both ritonavir and lopinavir, 17-OH progesterone appears to be dependent on the doses of the ARVs administered to the H295R cells. At 0.0008 ng/L of protease inhibitor exposure, there was a decrease in the FC of 17-OH progesterone, while at 8 and 80 ng/L there was an increased FC (Figure 5-6 and Figure 5-7). Furthermore, ritonavir caused decreased concentrations of cortisol (other than at the 0.8 ng/L dose). The increase in 17-OH progesterone and decrease in cortisol production, indicates that ritonavir causes some inhibition of the 21-hydroxylase (CYP21A2) enzyme of the steroidogenesis process (Figure 5-5).

Furthermore, androstenedione responds very similar when the H295R cells are exposed to either ritonavir or lopinavir: the fingerprint pattern caused by the bars are similar (Figure 5-6 and Figure 5-7), but none of the responses were statistical significant. Despite this fact, both protease inhibitors caused an increased FC in androstenedione concentrations for 0.0008 and 0.8 ng/L exposures. However, ritonavir caused an increase in androstenedione at 8 ng/L as well, while lopinavir caused an increase at 80 ng/L. Testosterone concentrations decreased in both ritonavir and lopinavir exposures, especially at the lower doses and for ritonavir that

was statistically significant (Figure 5-6 and Figure 5-7). Oestradiol levels decreased in response to all lopinavir concentrations and the lowest concentration of ritonavir also seemed to have decreased oestradiol levels, but the higher concentrations caused an increase in oestradiol levels (Figure 5-6 and Figure 5-7). These two protease inhibitors seemed to have different effects on oestradiol levels and suggest different mechanisms of interference with the steroidogenesis pathway between the different protease inhibitors. Ritonavir appears to stimulate aromatase activity, thereby decreasing testosterone by converting it to oestradiol. Furthermore, the raised androstenedione with decreased testosterone suggests some inhibition of the 17β-HSD enzyme. However, for lopinavir, the low testosterone and oestradiol, with raised androstenedione, suggests inhibition of 17β-HSD enzyme, but possibly even inhibition of the aromatase enzyme as well (Figure 5-5).

Although lopinavir and ritonavir appear to have similar responses for most of the steroid hormones, the most obvious difference is the oestradiol production, with ritonavir stimulating oestradiol production, while lopinavir inhibits oestradiol production. Furthermore, the fold changes due to ritonavir exposure were more pronounced than those caused by lopinavir indicting that ritonavir is a greater endocrine disruptor than lopinavir.

The conclusion of ritonavir being the protease inhibitor with the more obvious endocrine disruptive effect in this study supports what is reported in literature for ritonavir. Various studies reported that ritonavir causes the most pronounced endocrine disruption effects of all the protease inhibitors, with reports of inhibition of various cytochrome P450 enzymes and adrenal suppression in PLWHIV that were administered this class of ARV (Collazos et al., 2004, Hall et al., 2013, Hesse et al., 2001). Furthermore, PLWHIV on various protease inhibitors, such as indinavir, nelfinavir and saquinavir, have reported sexual disturbances (decreased libido, infertility and impotence), which could be associated with the production of the sex steroid hormones in the steroidogenesis pathway (Collazos et al., 2002). Similar to the findings of the current research, but in human studies at much higher exposures, Hall et al. (2013) reported decreased cortisol, while Christeff et al. (1999) reported raised androstenedione with treatment by means of HAART including ritonavir. Furthermore, a study by (Simon et al., 2011) on lopinavir-ritonavir treatment of neonates, concluded various effects on the steroidogenesis pathway, including the raised 17-OH progesterone levels observed in this study. Simon et al. (2011) suggested *in vitro* analysis to evaluate the full effect of protease inhibitors on steroid production. Preliminary results reported by Malikova et al. (2019) found that although lopinavir inhibits CYP21A2, with some inhibition of CYP17A1, that ritonavir did not inhibit these enzymes in the H295R assay. However, these results by Malikova et al. (2019) were not supported by this study.

The effects at these doses of protease inhibitors of 80 ng/L and lower, is of significance to the untargeted population, who may be exposed to these chemicals from the environment. Due to the low water solubility of ritonavir (1.26 mg/L) and lopinavir (0.0077 mg/L), they can accumulate in biosolids and sediments, being released slowly and consistently back into the water over time (Ncube et al., 2018). In France, ritonavir was detected at levels as high as 1 372 ng/L in suspended solids and sediments, which is much higher than the doses this study tested for (Aminot et al., 2015). Abafe et al. (2018) reported higher concentrations of lopinavir (1 900–3 800 ng/L) in wastewater effluent than inflow (1 200–2 500 ng/L) at a wastewater treatment facility in KwaZulu-Natal, SA. They ascribed it to the hydrophobic properties of these protease inhibitors. Only 50% of ritonavir (slightly less hydrophobic than lopinavir) was effectively removed (1 600–3 200 ng/L in influent and 460–1 500 ng/L in effluent water) by the WWTP processes (Abafe et al., 2018). This physico-chemical characteristic would cause the protease inhibitors to bio-accumulate in the ecosystem. Furthermore, lopinavir was found in concentrations of 130–305 ng/L in various SA water sources (Wood et al., 2015), while ritonavir was found at concentrations of 90–108 ng/L in water sources in Switzerland (Kovalova et al., 2012, Margot et al., 2013), and 4.6 ng/L in urine fertilizers (Bischel et al., 2015). The ARV exposure concentrations used in this current study were lower than the environmental concentrations that the untargeted population would likely be exposed to. Therefore the effects at various doses (low and high concentration ranges) of ritonavir and lopinivir should be tested on H295R assays, to see the true effects at the possible environmental exposure levels, provided that these concentrations are not cytotoxic to the cells.

5.5 Nucleoside reverse transcriptase inhibitors (NNRTIs)

NRTIs are ARVs that are designed to disrupt the replication cycle of HIV by competitively inhibiting the HIV reverse transcriptase enzyme. Known side effects of NRTIs' usage include nausea, lipodystrophy, lactic acidosis, heart disease, and dyslipidemia (Ncube et al., 2018).

5.5.1 Didanosine:

Didanosine (trade name is Videx EC) is a NRTI that was approved by the FDA in 1991 for the treatment of HIV. The FC in steroid hormone concentrations caused by the different doses of didanosine in this study is summarised in Figure 5-8. The majority of the hormones had FCs of around 1 for most doses of didanosine. The 8 ng/L didanosine dose caused a considerable increased FC for oestradiol (not statistically significant), while the 0.08 ng/L dose decreased oestradiol concentrations statistically significantly from the SC cells. Androstenedione levels at 8 and 80 ng/L didanosine doses had a decreased FC, while the 0.0008 ng/L dose caused

a drastic increased FC of 2.8 for androstenedione; yet no FCs were statistically significant. There were no considerable changes in the 17-OH progesterone concentrations due to didanosine exposure, other than the statistically significant increase in 17-OH progesterone concentration at the 0.0008 ng/L dose.

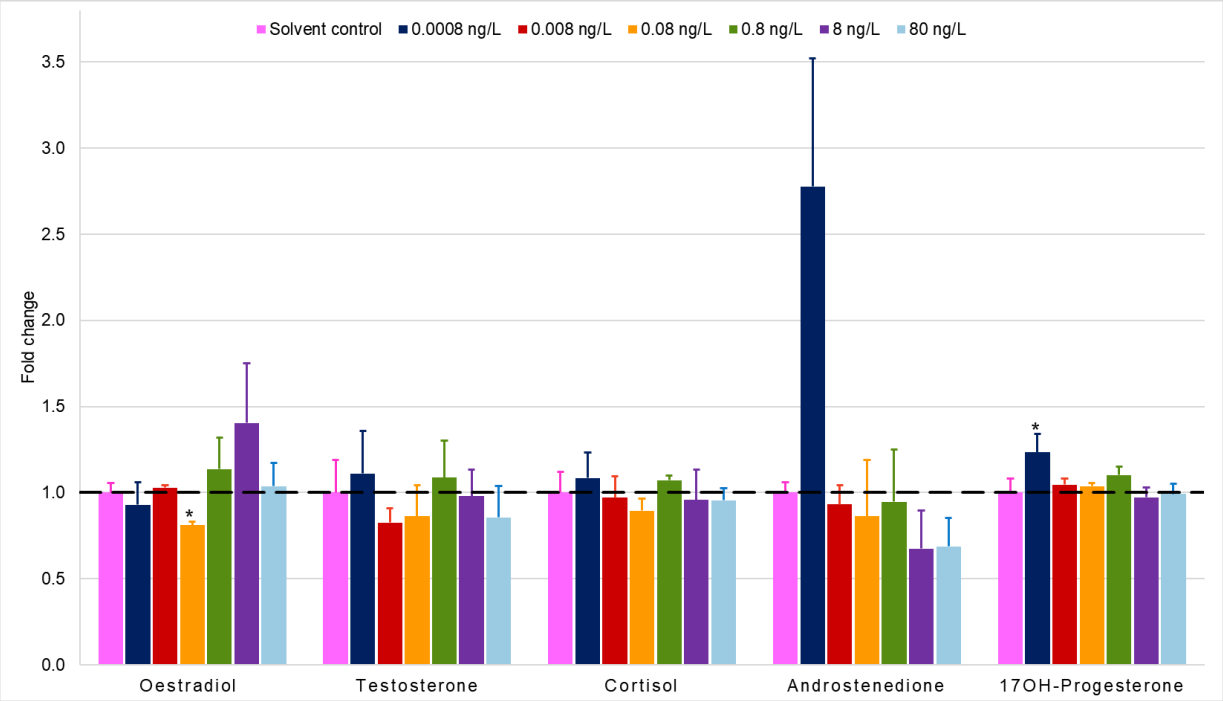

Figure 5-8: The FC of the steroid hormone concentrations for the didanosine exposure plate. **Hormonal concentrations that were statistically significant different from the SC cells' hormone levels ($p \leq 0.05$). Dashed line is FC = 1. Standard deviations are indicated by error bars*.

5.5.2 Lamivudine

Lamivudine (trade name Epivir) is an NRTI that was approved to treat HIV in 1995 by the FDA. In this study, lamivudine caused the most statistically significant changes in the steroid hormone concentration data ($p \leq 0.05$) of all the ARVs, compared to its' SC cells (Figure 5-9). Oestradiol production was decreased by lamivudine exposure, at all doses, other than 0.8 ng/L. The 0.008 ng/L dose of lamivudine caused a statistically significant decrease in oestradiol concentrations. The testosterone concentrations all decreased with exposures of lamivudine, with statistically significant lower concentrations at both the 80 ng/L and 0.008 ng/L doses. Although the FC of testosterone at the 8n g/L lamivudine dose was considerably lower than the SC cells, it was not statistically significant. The cortisol concentrations had no noteworthy changes. Androstenedione concentrations increased as the doses of lamivudine increased, but was also elevated with the 0.0008 ng/L dose. There

appears to be a hormetic effect of lamivudine on androstenedione concentrations. Hormesis is an occurrence of a dose-response relationship, whereby low-doses cause stimulation, while high-doses cause inhibition, resulting in a U-shaped effect (Calabrese and Baldwin, 2003). The 17-OH progesterone concentrations did not change with the lamivudine exposures, except for the statistically significant increased levels at 0.8 ng/L and 0.0008 ng/L lamivudine.

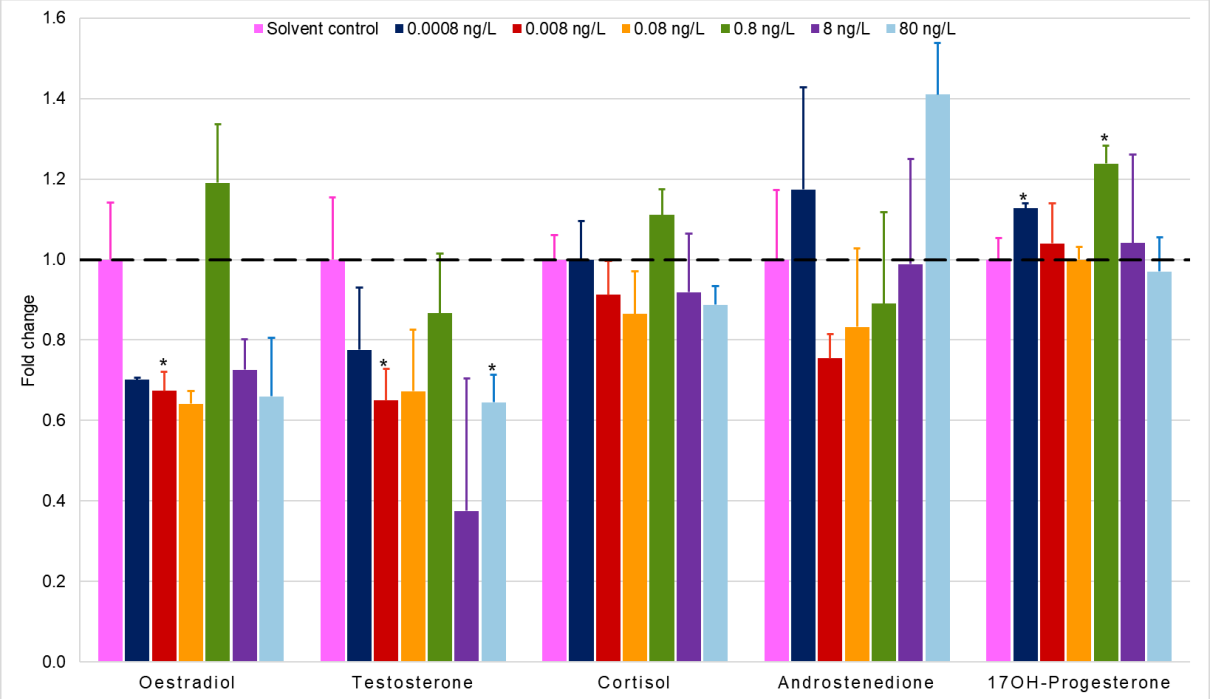

Figure 5-9: The FC of the steroid hormone concentrations for the lamivudine exposure plate. **Hormonal concentrations that were statistically significant different from the SC cells' hormone levels ($p \leq 0.05$). Dashed line is FC = 1. Standard deviations are indicated by error bars.*

5.5.3 Stavudine

Stavudine (trade name Zerit) is an ARV that falls into the NRTI class. It was approved by the FDA in 1994 and is administered to PLWHIV at relatively low dosages (of 40 mg/L) compared to the other ARVs.

All of the steroid hormone concentrations decreased at all stavudine exposure doses in comparison to the SC cells. 17-OH progesterone concentrations were the least influenced (Figure 5-10). The most drastic decrease in steroid concentrations was the testosterone production, which was less than half that of the SC cells. Although the decreased steroid levels were drastic, the only statistically significant results for stavudine were the 17-OH

progesterone concentration at the 80 ng/L stavudine dose, and androstenedione at the 8 ng/L dose (Figure 5-10).

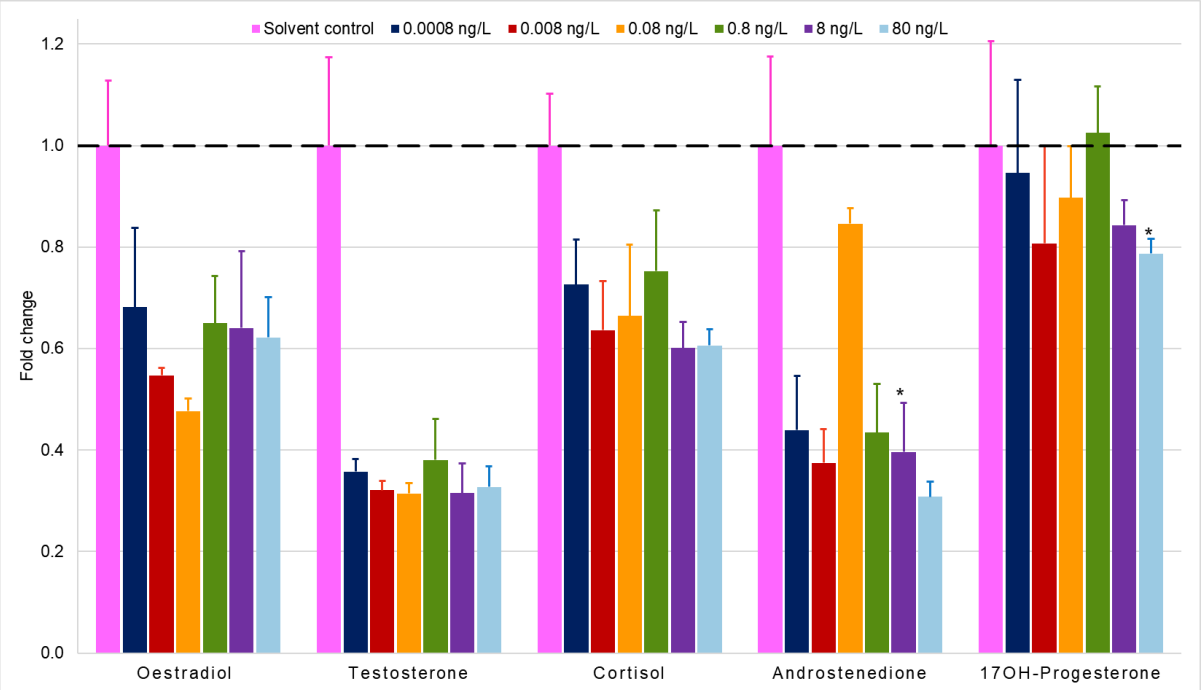

Figure 5-10: The FC of the steroid hormone concentrations for the stavudine exposure plate. **Hormonal concentrations that were statistically significant different from the SC cells' hormone levels ($p \leq 0.05$). Dashed line is FC = 1. Standard deviations are indicated by error bars.*

5.5.4 Comparison of NRTIs

When comparing the results obtained in this study for the NRTIs class of ARVs, it is clear that didanosine affects the steroidogenesis pathway the least of all three NRTI ARVs investigated (Figure 5-8, Figure 5-9 and Figure 5-10). However, although stavudine and lamivudine both caused a decrease in the testosterone and oestradiol productions, stavudine appeared to have a stronger effect. Cortisol production was considerably inhibited with stavudine exposures, but not with lamivudine or didanosine. The 17-OH progesterone concentrations were statistically increased with exposures to lamivudine (0.0008 and 0.8 ng/L) and didanosine (0.0008 ng/L), while statistically decreased with stavudine (80 ng/L). At the higher NRTI exposure doses, both didanosine and stavudine appeared to decrease androstenedione production, whereas lamivudine increased the androstenedione production. These steroid hormone profiles suggest that lamivudine inhibits 17β-HSD, stavudine inhibits 17α-hydroxylase, and didanosine stimulates aromatase activity. Therefore, although these ARVs

belong to the same class, they seem to have very different modes of action on the steroidogenesis pathway, but they all commonly decrease the testosterone levels.

Previous studies conducted on humans receiving NRTI ARVs, showed that PLWHIV presented with various symptoms resulting from possible endocrine disruption. Wong et al. (2017) noted a connection between decreased libido, erectile dysfunction and loss of muscle strength in patients on combinations of ARVs including lamivudine. Furthermore, gynecomastia was reported in multiple studies on patients being administered stavudine or lamivudine (Manfredi et al., 2001, Melbourne et al., 1998, Peyriere et al., 1999). These side effects seen in PLWHIV on NRTIs (stavudine, lamivudine or didanosine), have been linked to a decrease in testosterone levels, due to possible raised aromatase activity, although the oestradiol concentrations were not reported in these studies to confirm their speculation (Javanbakht et al., 2000, Manfredi et al., 2001, Melbourne et al., 1998, Peyriere et al., 1999). These studies correspond with the current study's findings with decreased testosterone with stavudine and lamivudine exposures. However, the present study did not find evidence of increased aromatase activity (oestradiol concentrations were decreased). However, partial raised aromatase activity appeared in the exposures with didanosine (slight oestradiol increase and testosterone decrease at the 8 and 80 ng/L doses) (Figure 5-5).

Another study that exposed H295R cells to NRTI ARV doses, found that zidovudine doses of 10 µM caused no significant effects on hormone production (Strajhar et al., 2017). However, they found an increased FC (1.06–1.29) for all steroid hormones tested, other than aldosterone, when exposing the H295R cells to zidovudine. The lack of any significant effects in the study by Strajhar et al. (2017) is similar to what was found in the present study for didanosine. Results from the present study show that some NRTIs, such as stavudine and lamivudine, have a much greater influence on the steroidogenesis pathway, than others such as didanosine and zidovudine.

All the NRTIs that were tested in the present study were highly water-soluble (70 000 mg/L for lamivudine, 83 000 mg/L for stavudine and 15 800 mg/L for didanosine) (Ncube et al., 2018). The high water solubility of these NRTIs could result in them not being efficiently removed during the WWTP process, thereby decreasing their concentration in the various environmental water sources (Abafe et al., 2018). Lamivudine was detected in various water sources in SA (94.5–242 ng/L) (Wood et al., 2015), France (44 ng/L) (Aminot et al., 2015), USA (27.73 ng/L) (Furlong et al., 2017) and Kenya (2 985–167 100 ng/L) (K'Oreje K et al., 2016, Ngumba et al., 2016). Stavudine was reported in water sources at concentrations of 102–778 ng/L in SA (Wood et al., 2015) and 2.9 ng/L in Germany (Prasse et al., 2010).

However, didanosine was only detected in SA water at a concentration of 54 ng/L (Wood et al., 2015).

Therefore, based on the findings of the alterations in FC of the steroid hormones in this study, some endocrine disruptive effects can be expected in aquatic wildlife.

5.6 Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

NNRTIs are ARVs that are designed to disrupt the replication cycle of HIV by non-competitively binding to the HIV reverse transcriptase enzyme, and thereby changing the reverse transcriptase's conformational structure. Common side effects of the administration of NNRTIs include rash, central nervous system adverse effects, lipoatrophy, hypertriglyceridemia, hepatotoxicity, and teratogenicity (Ncube et al., 2018).

5.6.1 Efavirenz

Efavirenz (trade name Sustiva) was the only NNRTI ARV that was used in this study. There was a general trend of efavirenz decreasing the concentrations of all of the hormones (except 0.008 and 0.08 ng/L for 17-OH progesterone) investigated in this study (Figure 5-11). Oestradiol was affected by efavirenz, with higher doses of efavirenz producing lower concentrations of oestradiol (80 ng/L caused a statistical significant decrease in oestradiol production). Although there was a decrease in testosterone concentrations, it was not statistical significant and its FC was not as low as those of oestradiol. Cortisol was affected by the different doses (dose-dependent) of efavirenz, with higher doses of efavirenz causing lower concentrations of cortisol. Androstenedione seemed to have been influenced the most negatively by efavirenz exposure. The androstenedione concentrations were so low that FCs as low as 0.25 (for 0.008 ng/L efavirenz) and 0.28 (for 8 ng/L) were determined. The 17-OH progesterone concentrations are only considerably effected at doses of 0.8 ng/L efavirenz and higher, as the lower doses of efavirenz used in this study could be inadequate to influence the 17-OH progesterone production. The higher doses (0.8, 8 and 80 ng/L) of efavirenz caused statistically significant decreases in cortisol and 17-OH progesterone concentrations, compared to the SC cells.

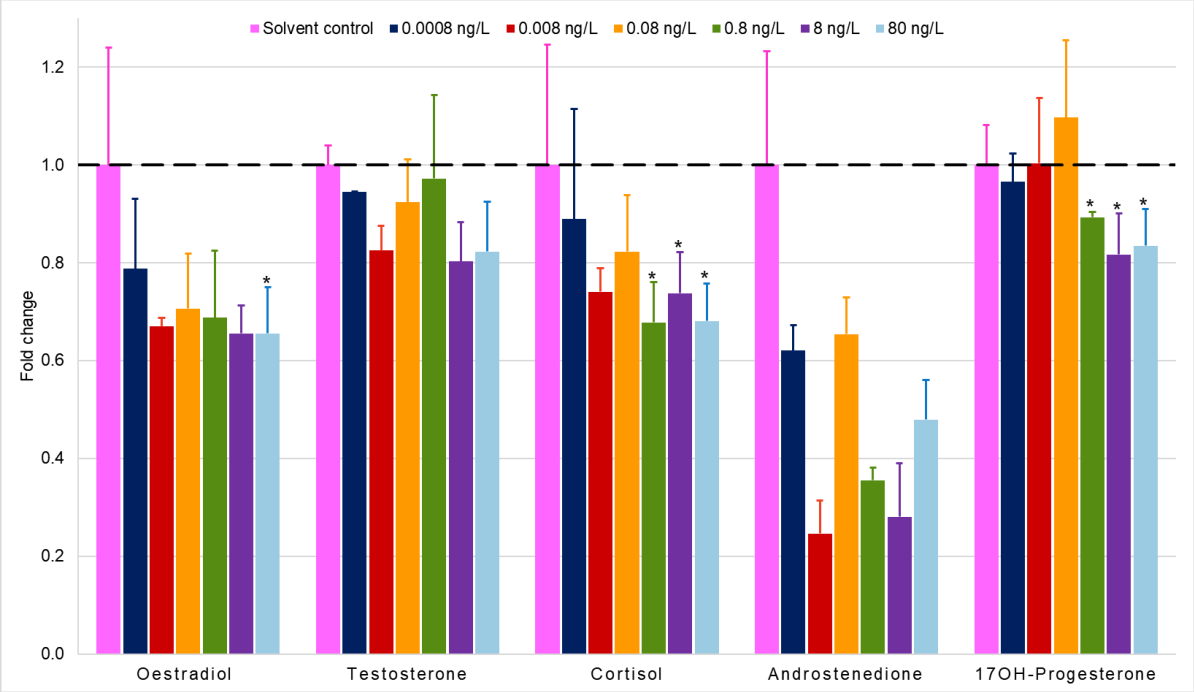

Figure 5-11: The FC of the steroid hormone concentrations for the efavirenz exposure plate. **Hormonal concentrations that were statistically significant different from the SC cells' hormone levels ($p \leq 0.05$). Dashed line is FC = 1. Standard deviations are indicated by error bars.*

5.6.2 NNRTI comparisons

The low 17-OH progesterone and very low androstenedione concentrations that resulted from exposing the H295R cells to efavirenz in this study, suggest an inhibition of the CYP17 enzymes (specifically 17,20-lyase) (Figure 5-5). Furthermore, there also appears to be some 21-hydroxylase inhibition, due to the further decreased cortisol levels. Moreover, the very low androstenedione concentrations resulted in limited availability to produce testosterone and oestradiol further along the steroidogenesis pathway, thereby decreasing all the androgen sex hormones.

Malikova et al. (2019) also studied the effects of efavirenz (1 578 375 ng/L) on H295R cells and found that it inhibited the 21-hydroxylase enzyme, which converts 17-OH progesterone to 11-deoxycortisol. Insufficiency of this enzyme would cause an increase in 17-OH progesterone production and a decrease in cortisol concentrations (Figure 5-5). This, however, was only the case for 0.08 and 0.008 ng/L efavirenz (very slightly) in this study. A more drastic, statistically significant decrease in 17-OH progesterone production was more prevalent at the higher concentrations. However, the cortisol levels decreased with the increased efavirenz doses, which does demonstrate inhibition of 21-hydroxylase.

Efavirenz is a hydrophobic molecule (93 mg/L water solubility) and it is probably for this reason that it is not removed from water sources during the WWTP processes (Abafe et al., 2018). Studies performed to detect efavirenz in water sources found 5–14 000 ng/L in SA (Rimayi et al., 2018, Schoeman et al., 2017, Wooding et al., 2017) and 110–560 ng/L in Kenya (K'Oreje K et al., 2016). Furthermore, efavirenz was detected at 17.7–43.6 ng/L in WWTP sludge in SA (Schoeman et al., 2017). Due to these levels in the environment, untargeted exposure to efavirenz is highly possible and the findings of endocrine disrupting effects at doses as low as 0.0008 ng/L add to the need to do repeat studies.

5.7 Conclusion of results

All the ARVs studied had effects on the steroidogenesis pathway. Although some effects were seemingly random, there was evidence of dose-dependent responses to the ARVs. A decrease in oestradiol was observed with exposures to lopinavir, lamivudine, stavudine and efavirenz, while an increase occurred with ritonavir exposures. Furthermore, a decrease in testosterone occurred with exposures to ritonavir, lamivudine, stavudine and efavirenz. Efavirenz and stavudine exposures cause a decrease in both cortisol and androstenedione levels. Although, ritonavir decreased cortisol, and didanosine decreased androstenedione. The efavirenz and stavudine exposures decreased 17-OH progesterone, while ritonavir, lopinavir, didanosine and lamivudine increased concentrations.

The changes in the steroid hormone concentrations can be used to predict the enzyme activity effected in the steroidogenesis pathway, by the relevant ARVs exposed to the H295R cells. Both protease inhibitors, ritonavir and lopinavir, inhibited 17β-HSD activity. However, ritonavir stimulated aromatase activity, while lopinavir possibly inhibited it. Furthermore, ritonavir also inhibited 21-hydrosylase. For the NRTIs, didanosine only stimulated aromatase activity, while stavudine inhibited 17α-hydroxylase activity, and lamivudine inhibited 3β-HSD activity. Efavirenz (the NNRTI) inhibited 21-hydroxylase, as well as 17,20-lyase.

In this study, although the exact mechanism(s) by which the ARVs changed the steroid hormone concentrations is unknown, it can be concluded that all the ARVs tested caused a change *in vitro*. The ARVs may have been competitors of the enzyme at the receptors, resulting in an inhibitory or stimulatory response, or they may have altered the genetic makeup of the enzymes in the steroidogenesis pathway. Further studies would need to be completed to come to a more thorough conclusion on the mechanism of effects of the ARVs in the steroidogenesis pathway. However, the fact that the ARVs tested influenced the steroidogenesis process in the H295R cells to some extent, demonstrates that they are EDCs.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

Our planet is being polluted more and more each day, not only by car fumes or industrial air pollution, but by rubbish disposal (landfills and sewage) as well. The chemicals and PPCPs that are not used or metabolised, are being discarded in household waste, or flushed down the drains, and therefore end up in the environment, causing a series of issues for the ecosystem. PPCPs have been reported in water, soil and sediment globally (Al-Rajab et al., 2010, Funke et al., 2016, Grund et al., 2011, Ngumba et al., 2016, Wood et al., 2017). PPCPs in the environment are not only pseudo-persistent, but also have the potential to bio-accumulate, causing detrimental effects, not only in humans, but in other organisms too. One of the detrimental effects is the ability to interfere with the endocrine system. With the rise of ARV use globally, but mainly in SA, to curb the AIDS pandemic, more ARVs are ending up in the environment. Little is known of the effects of ARVs on non-target organisms and humans from environmental exposures. However, chronic exposure to the high concentrations of ARVs found in the environment suggests endocrine disruption. Therefore, it is important to study the effects that these commonly used ARVs may have on the steroidogenesis pathway of HIV-negative people, and other organisms that could possibly be exposed.

The H295R cell line is commonly used to test for xenobiotics that are affecting the steroidogenesis pathway. H295R cells contain all the necessary adrenocortical enzymes, to produce a large variety of steroid hormones. Previous studies using the H295R cells demonstrated that various PPCPs and other chemicals found in the environment are in fact endocrine disruptive. A few examples of those chemicals include ibuprofen (Han et al., 2010), bisphenol A (Zhang et al., 2011b), antihistamines (Munkboel et al., 2018b), triptolide (Xu et al., 2019), atrazine (Tinfo et al., 2011), and naproxen (Kwak et al., 2018). These chemicals are very different from each other in many ways, yet they all affect the steroidogenesis pathway. In contrast, studies done on certain classes of chemicals such as phthalates found that the compounds within this class actually had different effects on the steroidogenesis pathway (Lee et al., 2019). Furthermore, the present study found that three ARVs within the same class (NRTIs) had very different effects on the steroidogenesis pathway. Therefore, one cannot predict the effects that a chemical will have on the steroidogenesis pathway based on similar compounds responses, without exposing H295R cells to the compound, and measuring its various effects.

The main aim of this study was to determine the effects that six ARVs have on the steroidogenesis pathway *in vitro*. The resultant steroid hormone production was evaluated by

quantifying six steroid hormones, using a method that was developed and validated in this study, on an UHPLC-QTOF.

The changes in the concentration and FC profile of the different steroid hormones were used to determine which of the metabolising enzymes in the steroidogenesis pathway are likely to be affected by the ARV. The exact mechanisms by which the ARVs caused a change in the FC of hormones in this study is unknown. It could be genetic alteration of the enzyme, inhibitor competition or competitive stimulation of the receptors, or a combination of effects expressed at different concentrations. Some ARV effects were dose-dependent, while some had a random effect on the hormones. The protease inhibitor ritonavir was a more prominent endocrine disruptor than lopinavir. Ritonavir resulted in the inhibition of 21-hydroxylase and 17β-HSD activity, while stimulating aromatase activity. Lopinavir resulted in the inhibition of 17β-HSD and possibly some aromatase activity as well. The NRTI didanosine did not cause many effects on the steroidogenesis pathway, with only aromatase activity being stimulated. However, stavudine decreased 17α-hydroxylase activity, while lamivudine decreased 3β-HSD activity. The NNRTI efavirenz, however, caused a decrease in all five quantifiable steroid hormones, while demonstrating inhibition of 17,20-lyase and 21-hydroxylase.

The H295R assay results in this study indicate that several of the ARVs tested influenced the steroidogenesis process in the H295R cells, possibly causing endocrine disrupting effects in vertebrate organisms. Due to the variation in the ARVs' chemical structures and sizes, their pharmacokinetic properties, and their modes of action to name a few, their steroidogenic effects on the H295R cells in this study were varied. Majority of the drastic changes in steroid hormone concentrations were not statistically significant, whereas many of the smaller changes in steroid concentrations were. This is most likely due to the small sample size and large standard deviations for certain measurements. Therefore, although a relatively general assumption can be made from the concentrations and FCs of the steroid hormones in ARV exposed cells, compared to the SC cells, the lack of statistical significance is the main limitation of this study and these results should be confirmed by repeating the exposures.

Future studies should also include higher concentrations of the various ARVs, to include the higher levels that have been found in environmental samples, as well as to indicate the bio-accumulative concentration effects. Care should be taken that the reference compounds, prochloraz and forskolin be dosed at the levels recommended by the OECD guidelines for the steroidogenesis assay. There is also further scope for optimisation of the UHPLC-QTOF method for steroid hormone analysis: negative ionisation mode should be investigated for aldosterone. The steroid extraction method from the cell media can also be improved on, by possibly using a double extraction technique, to decrease the LOQs, especially for oestradiol.

The changes in the steroid hormone levels from the H295R cells in response to the various ARVs in this study show where in the steroidogenesis pathway ARVs are likely to contribute to endocrine disruptive effects in humans and other organisms in the environment.

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