Emerging Biomarkers for Monitoring of Glucocorticoid Replacement Therapy in Congenital Adrenal Hyperplasia (CAH)

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Abstract

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder involving an enzyme defect which affects adrenal steroid synthesis. The incidence is approximately 1 in every 20,000 births, and early intervention and treatment is crucial in severe cases. It is currently screened for as part of the national newborn metabolic screening program at LabPlus, Auckland Hospital.

Reduced enzyme function leads to cortisol and aldosterone deficiency, in addition to androgen excess. There is a wide spectrum of disease severity; it causes virilisation of external genitalia, and the most severe forms can be life-threatening due to risk of salt wasting in the neonatal period. Patients are given lifelong replacement steroid therapy to counteract these effects and must undergo regular monitoring to maintain treatment balance. The main laboratory biomarker currently used for treatment monitoring, 17-hydroxyprogesterone (17-OHP), is not ideal as it is a steroid metabolite, not an active androgen, which may be influenced by several factors.

This study aims to investigate new androgenic steroids which have emerged in the last few years as being of importance in CAH patients and determine whether they have the potential to provide further information about the clinical status of CAH patients undergoing glucocorticoid therapy.

Method development and validation was performed using liquid chromatography mass spectrometry to investigate the viability of measuring 11-ketoandrostenedione, 11-hydroxyandrostenedione, 11-ketotestosterone, and 11-hydroxytestosterone in dried blood spots. Dried blood spots are the current sample type for newborn screening and monitoring of CAH patients in New Zealand. All four steroids demonstrated suitable intra-batch precision and linearity, but 11-ketoandrostenedione proved less reproducible than the others across the course of validation. The results of the validation indicate that at least three of the four steroids show promise for future work on the assessment of treatment monitoring for patients with CAH who are undergoing glucocorticoid therapy. In addition, there are other areas of clinical interest where this analysis could be adopted, for example polycystic ovary syndrome, adrenal tumours, and the doping industry.

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Attestation of Authorship

“I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly acknowledged), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.”

Lauren Grace Bresnahan
28/04/2023

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Ethics Approval

Full Health and Disability Ethics Committee (HDEC) and AUTEC review were deemed out of scope for the purpose of this thesis; no patient information or samples were used in this piece of research, and no other people were involved.

LabPlus, Auckland City Hospital holds an approval from the New Zealand Blood Service to use discarded expired donor blood for method development purposes and this was used to obtain blood for the laboratory method (2019/14).

Chapter 1

1. Background/Literature Review

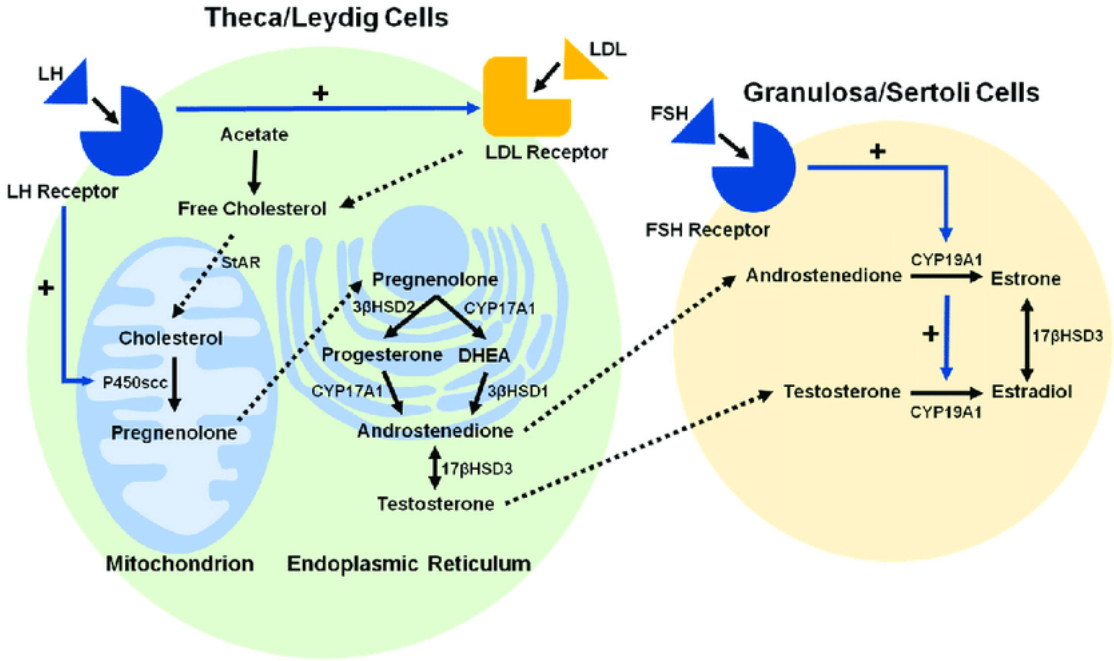
1.1 Introduction

Steroidogenesis, the production of steroids, involves the conversion of cholesterol into derivatives that are synthesised by a variety of tissues using a multitude of enzymes and cofactors. It is primarily performed in the adrenal glands, the gonads, and the placenta, with other organs having a variety of participating roles. The enzymes utilised in this process can be divided into two main groups: cytochrome P450 (CYP), and hydroxysteroid dehydrogenase/ketosteroid reductase (HSD/KSR). There are six types of CYP enzymes involved in steroidogenesis, and they contain a prosthetic heme group allowing them to activate the oxygen necessary to perform their functions, which is to catalyse oxidative reactions to produce steroids e.g., cortisol and dehydroepiandrosterone (DHEA), and these reactions are irreversible. HSD/KSR enzymes use either NAD+ to oxidise hydroxysteroids to ketosteroids, or NADPH to reduce ketosteroids to hydroxysteroids, and these reactions are reversible (Kater et al., 2022).

Cholesterol is a starting material for the biosynthesis of steroid hormones; these fat soluble, low molecular weight substances play a role in a diverse range of important physiological functions such as metabolism, growth, development, homeostasis, and reproduction. There is a large family of steroid hormones produced, but of particular importance are the following five biologically active steroid hormones: testosterone (androgen), oestradiol (oestrogen), progesterone (progestin), cortisol/corticosterone (glucocorticoid), and aldosterone (mineralocorticoid). Stimulation of the steroid pathway initiates uptake of cholesterol from circulation, resulting in intracellular movement of steroid precursors between the cytosol and mitochondria (see Figure 1). This in turn generates hydroxylation and oxy-reduction reactions to convert cholesterol into pregnenolone, and then form biologically active steroids from their precursors. In addition to the steroidogenesis formation pathway, there are also steroid hormones that are metabolised or activated peripherally for example, once testosterone is released into circulation it is enzymatically activated to become dihydrotestosterone, a potent circulating androgen (Kater et al., 2022).

Figure 1

Schematic illustrating the conversion of cholesterol into steroid hormones



Note: Free cholesterol is deposited into Theca and Leydig cells by low density lipoprotein (LDL), receptor-mediated LDL endocytosis. The conversion of cholesterol to pregnenolone is initiated by the binding of luteinising hormone (LH) to the luteinising hormone receptor (LHR), and conversion of androgens to oestradiol (E2) is initiated by the binding of follicle-stimulating hormone (FSH) to the follicle-stimulating hormone receptor (FSHR). While the steroidogenic pathways in female and male reproductive organs are nearly identical, the principal hormone products of steroidogenesis differ in females and males.

Sourced from: *https://doi.org/10.3390/ijms19071842*

1.2 Regulation of Steroidogenesis

Steroidogenesis is often discussed in a gland-specific fashion but is better understood as a single process that is repeated in each gland with cell type-specific variations. Thus, the regulation of steroidogenesis occurs with the transcription and post-translational modification of the steroidogenic enzymes and co-factors, in a tissue-specific fashion. Understanding steroidogenesis permits understanding of disorders of sexual differentiation, reproduction, fertility, hypertension, obesity, and physiologic homeostasis, and is essential for rational steroid therapies.

Adrenocorticotropic hormone (ACTH) is produced by the pituitary gland in the brain and acts on the adrenal gland to regulate the production of these steroids. An increase in ACTH activity initiates uptake of LDL cholesterol from circulation, which

triggers the steroidogenic pathway as seen in Figure 1. The adrenal glands synthesise three classes of steroids:

- Mineralocorticoids – predominantly aldosterone – produced in the outermost layer of the adrenal cortex (zona glomerulosa)
- Glucocorticoids – predominantly cortisol – produced in the middle layer of the adrenal cortex (zona fasciculata)
- Adrenal androgens – sex hormones – produced in the inner layer of the adrenal cortex (zona reticularis)

Aldosterone is responsible for sodium retention and water reabsorption, which controls blood pressure and electrolyte balance. Cortisol plays a role in regulating stress by decreasing inflammation, helping regulate blood pressure, and increasing blood glucose. It is an important hormone for maintaining homeostasis, and is responsible for the negative feedback pathway which shuts off ACTH secretion (Kater et al., 2022). Androgens contribute towards reproductive activity and the development of male characteristics, and are primarily produced by the gonads, with some contribution from the adrenals (Bacila et al., 2019), which has relevance when in the context of steroid disorders with androgen excess (Turcu et al., 2020).

DHEA and its sulphated version (DHEAS) are the major adrenal androgen precursors, and although they have little androgenic activity themselves, they are precursors in the pathway to testosterone and the most potent androgen, dihydrotestosterone. Foetal development is a time when adrenal contribution of DHEA and DHEAS is high – at birth the adrenal glands are almost the same size as the kidneys. Following birth however the foetal zone shrinks, and levels of DHEA and DHEAS rapidly decline and stay low until adrenarche. They then continue to rise throughout puberty, peak in the 20-30 age bracket, and then decline again with age (Turcu et al., 2020).

ACTH release, and therefore adrenal steroid production, follows a circadian rhythm, meaning steroid levels will fluctuate throughout the day.

There is a variety of steroidogenic disorders which can disrupt this pathway, leading to a range of problems. The factors discussed above are all important to consider when diagnosing and treating patients with steroid disorders, as levels of circulating steroids in the body can be greatly influenced by gender, age, and sample collection times; therefore, caution must be taken when interpreting laboratory results (Eisenhofer et al., 2017).

1.3 Congenital Adrenal Hyperplasia

Patients with congenital adrenal hyperplasia (CAH) have an autosomal recessive enzyme defect in cortisol synthesis (Rege et al., 2019). Over 90% of cases are caused by a deficiency in the 21-hydroxylase enzyme (see Figure 2), with a smaller percentage caused by a deficiency in the 11β-hydroxylase enzyme. There is a broad spectrum of disease severity depending on the extent of the underlying gene mutation (Turcu et al., 2015). In fact, Balsamo et al. (2010) have demonstrated a strong correlation between genotype and phenotype. The relevant genes have all been identified and therefore may be of clinical importance.

CAH cases are then further subdivided into classic and non-classic.

- Classical presentation – this can be either salt-wasting (SW-CAH) with marked glucocorticoid and mineralocorticoid deficiencies, and overproduction of adrenal androgens; or simple virilising (SV-CAH) with sufficient enzyme to maintain salt balance, but with cortisol deficiency and overproduction of androgens. In New Zealand and most developed countries, the severest SW-CAH is usually diagnosed in the neonatal period by newborn screening before severe clinical symptoms appear. In developing countries with no newborn screening facilities, it can be a cause of sudden neonatal death. Patients with SV-CAH can be detected by screening, but some will present with the symptoms of androgen excess in early childhood, with most cases showing before a patient is 5 years of age.
- Non-classical presentation – this is the mildest form of CAH and may sometimes remain undetected, or present later in life. It is diagnosed more often in females, most likely due to the obvious symptoms of androgen excess, such as hirsutism and menstrual irregularities.

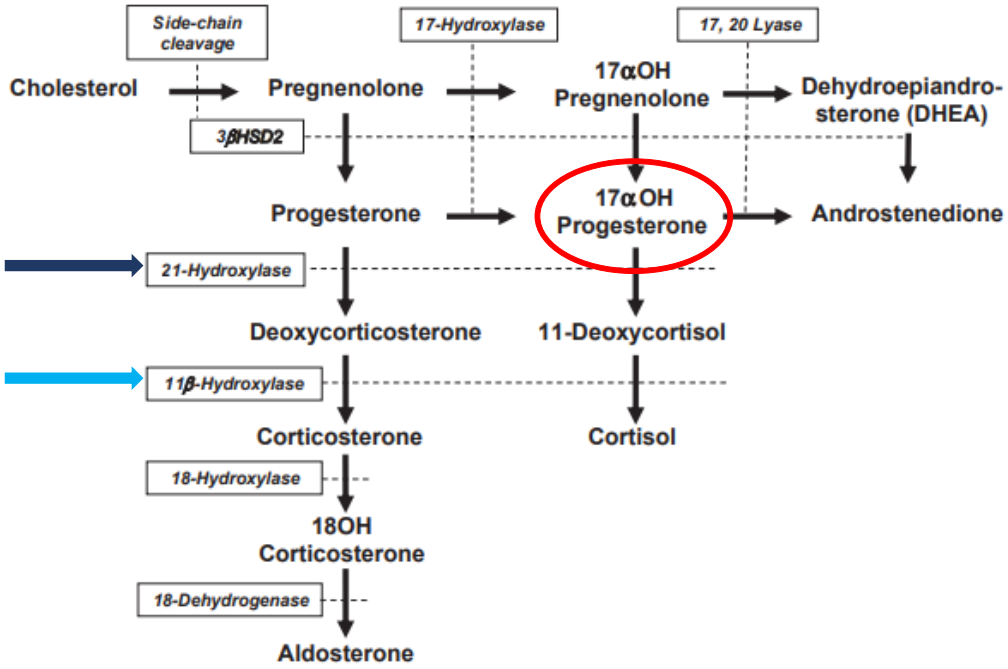
The CYP21A2 gene codes for the 21-hydroxylase enzyme, which catalyses the conversion of 17-hydroxyprogesterone (17-OHP) to 11-deoxycortisol (11-DOC) in the cortisol production pathway and the conversion of progesterone to 11-deoxycorticosterone in the aldosterone production pathway. The conversion of 11-DOC to cortisol and 11-deoxycorticosterone to corticosterone are catalysed by 11β-hydroxylase coded by CYP11B1 (Kater et al., 2022). Because CYP enzymes cause irreversible reactions, with no downstream feedback mechanism and they can act on multiple substrates (Bacila et al., 2019), that contributes to the variability of the disease presentation. A gene mutation in the CYP21A2 or CYP11B1 gene leads to these patients having variable enzyme activity. Decreased levels of 21-hydroxylase, or occasionally 11β-hydroxylase, results in low levels of cortisol and aldosterone production, and consequentially high levels of cortisol precursors such as 17-OHP.

The metabolic block in CAH diverts adrenal steroid synthesis towards excessive androgen production (see Figure 2). 11β-hydroxylase deficiency can be differentiated from 21-hydroxylase deficiency in the laboratory by measuring specific steroid metabolites such as 11-DOC, 17-OHP and 21-deoxycortisol (21-DOC). In 21-hydroxylase deficiency 17-OHP will be elevated, while 11-DOC will be elevated in 11β-hydroxylase deficiency. Elevations in 21-DOC, caused by the conversion of 17-OHP by 11β-hydroxylase, is a highly specific and sensitive marker of 21-hydroxylase deficiency as 11-hydroxylase is an adrenal specific enzyme.

Figure 2 below shows the adrenal steroid pathway of mineralocorticoids, glucocorticoids, and androgens. The dark blue arrow indicates where the enzyme defect occurs in approximately 90% of CAH cases. The light blue arrow is where the enzyme defect occurs in the cases of 11β-hydroxylase deficiency. 17-OHP is circled in red.

Figure 2

Adrenal cortical steroidogenic pathway



Note: The boxes include the names of the steroidogenic enzymes, with dashed lines indicating the reactions catalysed by each enzyme. The dark arrows indicate the direction of the pathway and products arising from the enzyme reactions.
Key: 3bHSD2 = 3b-hydroxysteroid dehydrogenase type 2; 17-OHP = 17αOH Progesterone; 11-DOC = 11-Deoxycortisol.
Sourced from: https://doi.org/10.1373/clinchem.2010.146035

1.4 Clinical Significance of CAH

The clinical symptoms of CAH result directly from the abnormal steroid levels mentioned above. Excess androgens lead to virilisation in both sexes (development of male characteristics) in utero which can present as ambiguous genitalia in females at birth, sometimes causing confusion about the gender of the child. In both sexes, if left untreated SW-CAH, named in reference to the sodium loss seen due to aldosterone deficiency, can lead to salt wasting, dehydration, and hypovolemic shock due to mineralocorticoid imbalance; sometimes resulting in death (Honour, 2014). This 'classic' form of the disorder is generally picked up clinically at birth or by the newborn screening results. Non-classic forms of the disease are often picked up later in life when patients show symptoms of androgen excess.

Without careful treatment male and female patients may be susceptible to premature pubarche, advanced bone age, and subfertility (Honour, 2014). These symptoms are all largely resulting from an excess of circulating androgens due to adrenal hyperactivity, which can accelerate their growth leading to premature maturation of the growth plates and ultimately a shorter stature overall (Dauber et al., 2010). The Tanner scale is often used when classifying symptoms related to physical development. This scale is a description of the expected physical development based on external primary and secondary sex characteristics, described for both males and females across five stages and can be correlated with androgen levels (Turcu, Mallappa, et al., 2017). Young patients may also experience signs of premature puberty such as early breast development for females, or facial hair, penis growth, and muscular development in males. These symptoms are resulting purely from androgen excess, and the correlating pubertal signs seen in the ovaries and testes will be absent (Honour, 2014).

Long-term complications from poorly controlled disease, or over-treatment with steroids, can also lead to a higher risk of developing adrenal mass or testicular adrenal rest tumours (TART) (Turcu, Mallappa, et al., 2017). Females can experience prolonged issues with fertility, such as anovulation (no egg is released from the ovary), and irregular periods (Honour, 2014).

Another potential complication has been described by Balsamo et al. (2010) who have also shown a correlation between CAH genotypes and free metanephrine concentrations. Metanephrines are the circulating by-products of epinephrine (adrenaline) and a deficiency can contribute to the development of hypoglycaemia in an adrenal crisis. Cortisol deficiency, as seen in CAH patients, contributes to

abnormal functioning of the adrenals, where epinephrine is produced, and this can therefore trigger adrenal crisis.

1.5 Prevalence of CAH

The worldwide incidence of CAH is approximately 1 in 18,850 babies (Heather et al., 2015). In New Zealand it is thought to be about 1 in 20,000 babies, meaning up to 3 cases per year are diagnosed (National Screening Unit, 2017). Balsamo et al. (2010) suggest that for the non-classic form the incidence could be much higher than this. Since all forms of CAH have an autosomal recessive pattern of inheritance, both sexes can be equally affected. However, because of the accumulated testosterone or precursor hormones, the phenotypic expression may be different in both genders. New Zealand has been screening for CAH since 1984, and a review of CAH in New Zealand by Heather et al. (2015) shows that just under 50% of them are diagnosed by the newborn screening programme. The review covered the period of 1994-2013 and during this time 44 cases of CAH were identified. Within these cases 73% were New Zealand Europeans, 16% Pacific Islanders, 7% Maori, and 4% other ethnicities.

1.6 Diagnosis and Treatment of CAH

The aim of treatment for CAH is to replace lost glucocorticoids, and mineralocorticoids, if necessary, which in turn suppresses ACTH and therefore suppresses excess adrenal androgens (Kamrath et al., 2017) (Dauber et al., 2010). However, in practice this is quite a difficult balance to achieve. The persisting enzyme blockage in these patients means adrenal steroid precursors accumulate despite normalisation of ACTH. Additionally, the glucocorticoids used for treatment can also have growth suppressive properties if over-prescribed. Clinical parameters, e.g., height and growth velocity, have always been the gold standard for optimising adequate control in treatment (Dauber et al., 2010) however it is helpful to have a laboratory test to compliment this and assist the clinicians as to whether the treatment is working, particularly as clinical assessment on its' own may not identify subtle under/over treatment (Dahl et al., 2018).

Historically the cortisol precursor 17-OHP has been used for diagnosis and treatment monitoring and is currently the target analyte for most newborn screening programmes. Additional laboratory investigations would likely reveal low sodium and glucose, with elevated renin and potassium, and metabolic acidosis (Sharma & Seth, 2014), however these are not specific diagnostic indicators in themselves. 17-OHP is not an active androgen itself, and, as we know that many of the symptoms arise from an excess of androgens, this analyte alone may not be an accurate indication of the

patient's clinical status (Honour, 2014). It also fluctuates rapidly in response to glucocorticoid therapy, which can make it difficult to monitor disease control using this marker alone (Turcu et al., 2018).
Another common problem with measurement of 17-OHP is that traditional methods have always been some form of immunoassay, which are widely recognised as having problems with interferences and therefore falsely elevated results can occur, particularly in the newborn period. The foetal zone of the adrenal glands produces large amounts of steroid sulphates, and these steroids, particularly 17α-hydroxypregnenolone (17OH-PREG seen in Figure 3), can interfere with 17-OHP immunoassays (Wong et al., 1992). Several attempts have been made to improve the specificity of the antibodies used, or to reduce interferences by performing solvent extraction prior to analysis of samples, but there continues to be problems with these assays. Alongside these interferences, 17-OHP can also be elevated in infants due to a variety of causes that are not CAH related such as stress, poor kidney function, other illnesses, or delayed enzyme expression in premature babies (Minutti et al., 2004).

The issues associated with this marker can mean that for a small percentage of babies, several follow-ups are required to confirm or rule out a diagnosis. This causes anxiety for the families and increases the cost of the screening programme (Balsamo et al., 2010).

Genetic analysis can be used to confirm a CAH diagnosis, however in truly urgent cases the results will not be available fast enough, and additionally in cases where genetic testing does not confirm a diagnosis of CAH this is not confirmatory enough to stop the follow-up of the patient. However, it can be useful in helping to understand the severity of the disease. It is even suggested that the genotype is a better predictor of how serious the salt-wasting condition could get than the degree of genital virilisation which is often one of the first clinical signs observed. Genetic testing can also play a part in genetic counselling of affected families, and prenatal testing of high-risk patients. Genetic testing should be used in conjunction with other diagnostic tests and clinical assessments to provide broader understanding of the condition (Balsamo et al., 2010).

Common practice in newborn screening programmes is to use immunoassay techniques for an initial screen, due to their affordability, availability, and ease-of-use. However, second tier newborn screening is becoming more widespread to assist with diagnosis of those conditions for which immunoassay interferences are a common

problem, such as CAH. One option for second tier screening for CAH is to use mass spectrometry as it has the potential to reduce the false positives seen in screening due to the specificity of the technique. Several methods have been described for the measurement of 17-OHP using liquid chromatography mass spectrometry (LCMS) (Dahl et al., 2018; De Hora et al., 2020; Salter et al., 2015) however, ideally it should be a steroid profile rather than testing for 17-OHP alone due to the issues with using this as a stand-alone marker as discussed above (Balsamo et al., 2010). Due to the complicated nature of the adrenal steroid pathway, and the wide spectrum of disease associated with CAH, steroid profiling may have the ability to provide better diagnostic specificity, particularly when looking at ratios against other steroids in comparison with a control group (Turcu, Ketha, et al., 2017).

Kamrath et al. (2016) looked at using metabolites of 17-OHP to improve the diagnostic criteria as many 17-OHP assays have problems with antibody specificity and cross-reactivity from other steroids. However, these steroid metabolites also are not active androgens, so probably wouldn't improve the clinical picture in terms of treatment monitoring. Kamrath et al. (2017) and Turcu et al. (2016) both discuss the lack of reliable biomarkers for monitoring. Traditionally A4, T, 17-OHP and DHEAS have been used as markers of androgen excess in CAH treatment monitoring. DHEAS is paradoxically suppressed in treated CAH patients (Turcu et al., 2016). T and A4 show poor correlation with clinical evidence of androgen excess (Turcu, Ketha, et al., 2017) which suggests that there are other active androgens present in these patients (Rege et al., 2019). Generally, 17-OHP is used particularly as blood spot assays are available through newborn screening laboratories.

A review by Pretorius et al. 2017 showed that four 11-oxygenated steroids, metabolites of androstenedione and testosterone, have been known since the 1950's, but were until recently considered dead end metabolites with no real biological function. Metabolites include:

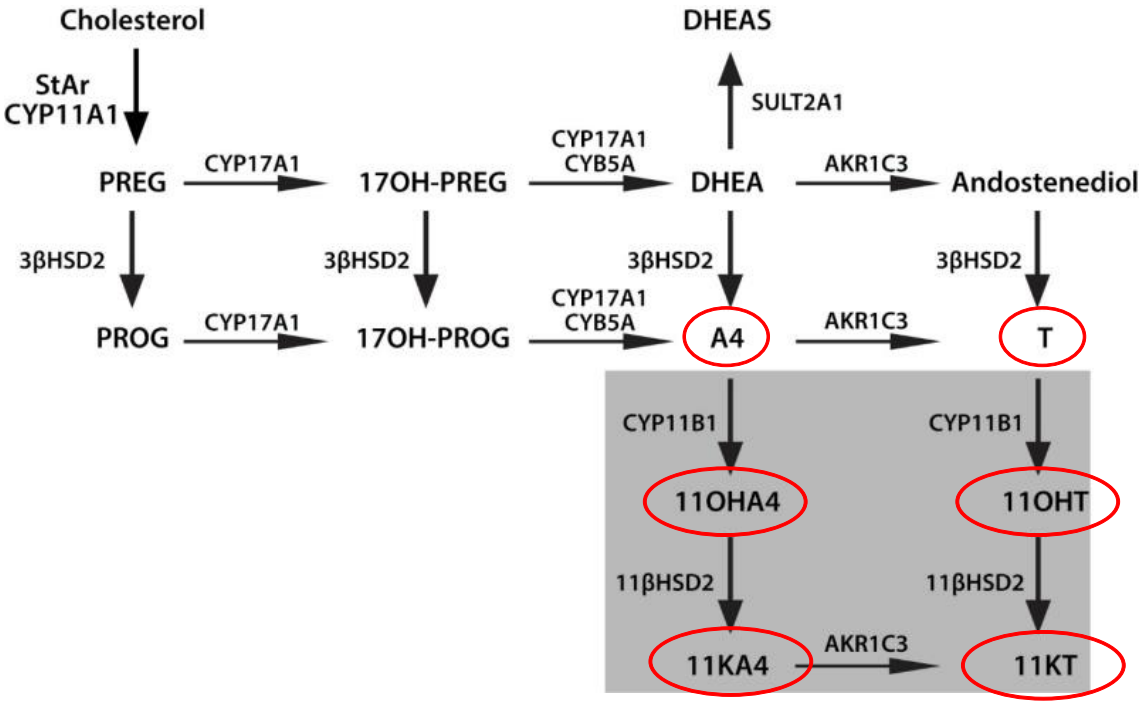
- 11-hydroxyandrostenedione
- 11-ketoandrostenedione
- 11-hydroxytestosterone
- 11-ketotestosterone

Figure 3 below shows the additional steroid pathway including the four 11-oxygenated steroids, the analytes of interest, which are circled in red along with their precursors, androstenedione, and testosterone. They have been collectively termed

the 11-oxygenated steroids due to the shared characteristic of containing an oxygen atom on carbon 11.

Figure 3

Pathway showing novel adrenal androgens



Note: Novel adrenal androgens. In this figure the enzymes are pictured next to the arrows of the reaction they are catalysing.

Key: 3bHSD2 = 3b-hydroxysteroid dehydrogenase type 2; 11KA4 = 11-ketoandrostenedione; 11KT = 11-ketotestosterone; 11OHA4 = 11-hydroxyandrostenedione; 11OHT = 11-hydroxytestosterone; 17OH-PREG = 17a-hydroxypregnenolone; 17OH-PROG = 17a-hydroxyprogesterone; A4 = androstenedione; AKR1C3 = aldo-keto reductase 1C3; CYB5A = cytochrome b5; CYP11A1 = cytochrome P450 cholesterol side-chain cleavage; CYP11B1 = cytochrome P450 11b-hydroxylase; CYP17A1 = cytochrome P450 17a-hydroxylase/17,-20-lyase; DHEA = dehydroepiandrosterone; DHEAS = DHEA sulfate; PREG = pregnenolone; StAR = steroidogenic acute regulatory protein; T = testosterone; SULT2A1 = Sulfotransferase Family 2A Member 1; PROG = progesterone; CYP17A2 = cytochrome P450 family 17 polypeptide 2; 11βHSD2 = 11-β-hydroxysteroid dehydrogenase type 2.

Sourced from: https://www.ncbi.nlm.nih.gov/sites/books/NBK278929/

At first discovery they were thought to have negligible activity, but since 2013 they have been shown to have some androgenic activity (Pretorius et al., 2017). Recent advances in technology, such as the introduction of mass spectrometry, has meant

that measurement of these steroids is more readily available and, as discussed above, looking at panels of steroids, instead of just one specific marker, may give a better picture of the patient's clinical status (Honour, 2014). Turcu et al. (2016); Turcu, Ketha, et al. (2017); and Turcu, Mallappa, et al. (2017) have all shown that the 11-oxygenated steroids are elevated in the serum of CAH patients by using methyl-t-butyl ether (MTBE) liquid extraction and mass spectrometry analysis, and as they are derived primarily from the adrenal glands they therefore show the same pattern in males and females, as opposed to gonadal androgens. Supporting the theory behind the adrenal origin of 11-KT, are the correlations seen between T and 11-KT across the five tanner stages in both males and females (Turcu, Mallappa, et al., 2017). Kamrath et al. (2017), and Kamrath et al. (2018) have investigated the presence of these steroids in urine of CAH patients however, as mentioned in a review by Turcu, Ketha, et al. (2017), measuring steroid metabolites in urine requires lengthy sample preparation and it is often difficult for patients, particularly children, to comply with the requirements of a 24-hour urine collection. A study by Schiffer et al. (2019) has also included the 11-oxygenated steroids in their panels, and they were the first to consider them in saliva samples as this provides an easy option for monitored patients to collect non-invasive samples frequently. This study also used liquid extraction to extract the steroids from the saliva samples, but by using dichloromethane as the extraction solvent.

It has been shown already in New Zealand that it is possible to extract steroids from a dried blood spot collection for mass spectrometry analysis. A study by de Hora et al. (2020) demonstrated successful extraction using acetonitrile, but the panel was limited to 17-OHP, cortisol, androstenedione, 11-DOC, and 21-DOC.

As discussed previously, immunoassay techniques can be subject to interferences when trying to analyse steroids, one of the reasons for choosing a more specific chromatographic method. As steroids are usually present in low concentrations in the blood, and some can be difficult to ionise for mass spectrometry, an alternative strategy to improve sensitivity when measuring by mass spectrometry is derivatisation, where a compound is transformed by altering one of its functional groups, rendering it more stable. Häkkinen et al. (2019) have published a successful method of steroid derivatisation with hydroxylamine which covers all the steroids looked at in this research. A solvent extraction is performed, the sample is concentrated by drying under a stream of nitrogen, and then reconstituted in 30% acetonitrile containing 100 mmol/L hydroxylamine hydrochloride. The derivatisation reaction then takes place by heating the samples at 60^{o}C for 30 minutes. The

hydroxylamine forms oximes (nitrogen containing compounds) with carbonyl or aldehyde functional groups, and this in turn increases the sensitivity of them during the electrospray ionisation technique due to the stability of nitrogen bonds (Häkkinen et al., 2019).

All patients with CAH, classic or non-classic, will have some degree of androgen excess with, or without, cortisol deficiency (Turcu, Ketha, et al., 2017), and this needs to be taken into consideration when monitoring these patients. It is known that 17-OHP is not specifically correlated with symptoms of androgen excess, and the introduction of mass spectrometry, which allows for faster, more specific analysis of these compounds, has further highlighted the importance of other steroids in the cortisol pathway. A review by Turcu and Auchus (2017) postulated a few research questions regarding the dynamic changes in 11-oxygenated steroids in glucocorticoid replacement therapy and whether measurements correlate with clinical evidence of androgen excess.

Of the 11-oxygenated steroids discussed, in-vitro studies have revealed that 11-KT has been shown to be a potent circulating androgen with a similar potency to testosterone (Turcu et al., 2018). However, all four of the 11-oxygenated steroids have been seen in high concentrations in CAH patients (Häkkinen et al., 2019) and could be useful for laboratory monitoring in CAH.

Turcu et al. (2016) compared serum concentrations of the 11-oxygenated steroids between a control group and a group of CAH patients with 21-hydroxylase deficiency. The ranges they observed can be seen in Table 1 below, testosterone and androstenedione have been included for reference.

Table 1

Serum steroid concentrations in nmol/L

Steroid	CAH group (n=38)	Control group (n=38)
11-OHA4	6.2 – 26.2	2.3 – 5.1
11-KA4	1.9 – 4.8	0.7 – 1.4
11-OHT	0.7 – 3.4	0.3 – 0.7
11-KT	3.5 – 12.2	1.0 – 2.6
T	1.3 – 5.6	0.4 – 10.7
A4	2.5 – 13.6	0.8 – 2.2

Note: 11-OHA4 is generally the most elevated of the four 11-oxygenated steroids, however, is not as potent as 11-KT.

1.7 Testing in New Zealand

Newborn screening in New Zealand currently screens for over 20 rare metabolic conditions, with the goal of early detection and treatment for conditions that could otherwise become life-threatening or detrimental to quality of life. Specimen collection for newborn screening involves a blood sample collected by heel prick spotted onto a Guthrie card composed of special adsorbent paper designed for specimen collection (Whatman 903). This is designed to be as minimally invasive as possible and uses a small volume of blood (see Figure 4 below). Screening takes place 24 - 72 hours after birth to ensure positive results can be followed up on within the first few days of life. Early treatment with electrolyte and hormone replacement is effective and lifesaving for babies with CAH (Lacey et al., 2004).

Figure 4

Image of a Guthrie card

Sourced from: https://www.health.govt.nz/your-health/pregnancy-and-kids/first-year/first-6-weeks/health-checks-first-6-weeks/newborn-screening-tests/newborn-metabolic-screening

The current testing protocol in New Zealand is that CAH monitored patients can collect blood spots onto “Guthrie cards” at home and send them to the laboratory for testing. Due to the diurnal nature of these steroids, timed samples overnight or across the day are required for monitoring of treatment. Patients and families benefit from being able to collect blood spot samples at home and post directly to the laboratory for analysis, rather than travel to specialist centres for repeated venous puncture.

Initial screening for CAH involves an immunoassay technique to measure 17-OHP in the blood spots of newborns. Further work has already been done to improve the diagnostics of CAH in New Zealand by adding in second tier testing using LCMS technology (De Hora et al., 2020). Improvements in monitoring 11-oxygenated

steroids in blood spots by LCMSMS may be beneficial for CAH patients undergoing glucocorticoid therapy.

1.8 Liquid Chromatography Mass Spectrometry

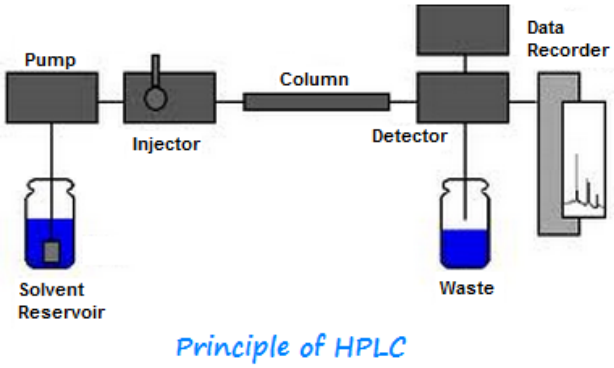
In recent years LCMS has become a routine analytical technique in clinical laboratories. It allows for more sensitive and specific simultaneous analysis of multiple compounds in clinical samples, and the Clinical Practice Guidelines for CAH recommend that it is used for second-tier newborn screening to reduce the number of falsely elevated results encountered using immunoassay (Speiser et al., 2018).

Liquid chromatography (LC), or more commonly known these days as high-pressure liquid chromatography (HPLC), is a separation technique for chemical mixtures, and is commonly used in clinical laboratories. In its simplest form LC has existed since the early 1900's, with HPLC then arriving in the 1960's, allowing for faster separations of analytes due to higher pressure pumps capable of more efficient mobile phase delivery (Snyder et al., 2010).

The basic principle of HPLC is that a liquid mobile phase will carry the sample through a stationary phase for separation, based on different molecule sizes and properties, into a detector for analysis. The stationary phase is generally a column which can be of varying size and chemistries depending on what compounds are of interest to retain (Snyder et al., 2010). The size can vary in the length of the column, generally ranging from 50mm to 150mm (occasionally longer), and in the particle size inside the column, which usually ranges from 1.8µm to 3.5µm, and both factors need to be considered as they will have an impact on the separation of compounds. A high-pressure pump will deliver a continuous flow of mobile phase, then an autosampler/injector will introduce the sample into the instrument. The sample will then travel in the mobile phase through the column into the detector, of which there are various types such as ultraviolet, fluorescent, electrochemical, and mass spectrometers. The diagram in Figure 5 below shows the basic components of an HPLC system (CLSI C62-A, 2014).

Figure 5

Schematic showing the principle of HPLC



Sourced from: https://www.pharmaguideline.com/2013/07/principle-of-hplc-liquid-chromatography.html

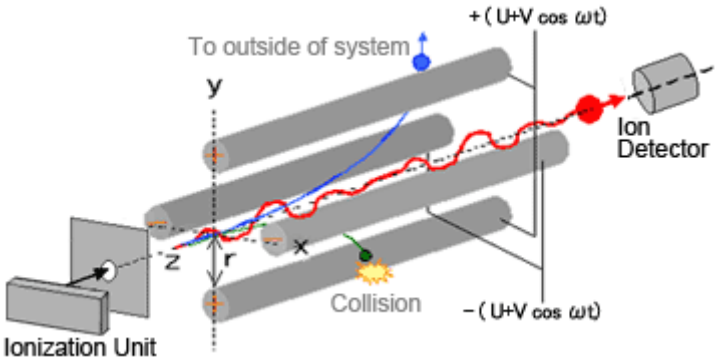
Mobile phases can be run in isocratic mode, meaning the chromatographic conditions do not change across the run, or in a gradient elution mode. The gradient elution mode uses a combination of organic solvents and aqueous solutions, with the proportions changing across the chromatographic run. Gradient elution is especially useful in methods with multiple analytes of interest to separate as it can slow down or speed up the rate at which compounds elute off the column into the detector.

A commonly used method of elution is reverse phase chromatography where the stationary phase (column) is less polar, and the mobile phase is more polar. Initial mobile phase conditions will use a higher percentage of a more polar solvent (usually a water-based phase), moving to a higher percentage of a more nonpolar solvent (commonly acetonitrile or methanol) to elute the compounds off the column (CLSI C62-A, 2014).

Once the compounds have been separated within the HPLC system they enter the detector for analysis. As mentioned above, a mass spectrometer is one option for a detector. This functions as an ion detector, therefore focusing on the molecular weight of the compounds. Common types are Quadrupole, Time-of-Flight, and Ion Trap (CLSI C62-A, 2014). For the purposes of this study, focus will be on the Quadrupole MS, the basic components of which can be seen in Figure 6 below.

Figure 6

Schematic showing the components of a quadrupole MS



Note: The sample is introduced into the ionisation unit, and ions of interest follow the pathway of the red arrow, whilst other ions fly out of the instrument.

Sourced from: https://www.shimadzu.com/an/service-support/technical-support/analysis-basics/lcms-intro/61intro.html

Quadrupole mass spectrometers contain four conducting rods (as seen in Figure 6). Compounds will travel from the HPLC to the ion source/unit where they are ionised and selected based on their mass-to-charge ratio (m/z). The quadrupole rods then apply a voltage to the ions, and only ions of a certain m/z will have a stable path through to the detector. Other ions have an unstable path and are lost.
A tandem quadrupole will have two sets of rods with a collision cell in between. Precursor ions can be selected by the first quadrupole based on your method settings, then enter the collision cell, where they are fragmented. This is usually carried out using an inert gas such as argon. The product ions of interest are then selected by the second quadrupole and reach the detector. This greatly improves the specificity of the method; hence this is a popular technique. The data acquisition method can be setup to contain information about the analytes of interest. This is referred to as either selected reaction monitoring (SRM), or multiple reaction monitoring (MRM) when there are multiple analytes, and greatly improves the sensitivity of the method as the mass spectrometer focuses on monitoring these transitions only (CLSI C62-A, 2014).

Commonly used ionisation techniques are electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). ESI applies a high voltage to create a solvent spray, and the ions are subsequently contained in the droplets. These are then evaporated in the source of the mass spectrometer, allowing the ions

to be analysed. This technique can occasionally be subject to ion suppression, but also has a wide range of applicability, and therefore is more commonly used. APCI uses heat and gas to form an aerosol and subsequently an electrical discharge ionises the analytes in the aerosol, before releasing them to the mass spectrometer. The heat can result in greater fragmentation of analytes, however they must be heat stable. This technique is commonly used for more non-polar analytes (CLSI C62-A, 2014).

Heated ESI is a technology which combines benefits from both techniques. There is a vaporiser temperature control available which can increase the ionisation efficiency. Although most steroids are non-polar the ESI ionisation technique is more robust, and there is plenty of evidence to suggest that in combination with the right mobile phases to enhance ionisation, this technique can be successfully applied for steroid analysis (Dahl et al., 2018; De Hora et al., 2020). Heated ESI methodology is the source that was available on the Thermo fisher LCMS in the LabPlus laboratory and was therefore used for this method.

1.9 Aims and Objectives

The aim of this research is to develop a method to measure four 11-oxygenated steroids, 11-OHT, 11-OHA4, 11-KT and 11-KA4 in dried blood spots. There are currently no reports of these potentially clinically important compounds being measured in dried blood spots. LabPlus provides all testing for the national newborn screening program so at this stage there is little New Zealand research into extended steroid panels for CAH.

The research aims are as follows:

1. Can 11-oxygenated steroids be reliably measured in dried blood spots?

- Develop a method for extracting these steroids from dried blood spots, and analysing levels by liquid chromatography tandem mass spectrometry (LCMSMS)
    - Use reference steroids to determine LCMSMS method parameters.
    - Prepare in-house blood spot calibrators using washed packed red cells and reference standards. Then use these calibrators to determine whether all four steroids are measurable in blood spots. Data will only be collected for steroids at measurable levels that can give relevant clinical information.
    - Validate the method to determine whether it is fit-for-purpose.

2. Discuss whether measurement of these potent androgens could help inform clinical decisions in CAH treatment.

Chapter 2

2. Materials

2.1 Reagents and Standards

Optima Acetonitrile, Methanol, Ammonium Formate, and Formic Acid, all LCMS grade, were obtained from Thermo Fisher (Auckland, NZ).

Reference material was obtained from Steraloids Inc (NY, USA):

- 4-ANDROSTEN-11β-OL-3, 17-DIONE (11-OHA4, 10mg)
- 4-ANDROSTEN-3, 11, 17-TRIONE (11-KA4, 10mg)
- 4-ANDROSTEN-11β, 17β-DIOL-3-ONE (11-OHT, 10mg)
- 4-ANDROSTEN-17β-OL-3, 11-DIONE (11-KT, 10mg)

Reference material was weighed, dissolved in methanol, and diluted to a concentration of 1000 nmol/L, and subsequently further diluted as required for each experiment.

The internal standard used for the method was testosterone-d2 obtained from Cambridge isotopes (Cambridge, USA).

2.2 Instrumentation

The instrument consisted of a Thermo Fisher Transcend TLX1 HPLC, coupled with a TSQ Vantage tandem mass spectrometer.

The Transcend HPLC system consists of two 1250 Accela pumps, an autosampler unit, a valve interface module, and a column manager which can hold up to six columns and has heating capability. It contains an online turbulent flow sample preparation module that assists with sample clean-up prior to analysis. Turbulent flow chromatography makes use of a column prior to the analytical column, with a combination of high flow rate, diffusion, and chemistry to retain the analytes of interest on the turboflow column while flushing away unwanted molecules. The turboflow column typically has a larger particle size to allow for the high flow of mobile phase, the one in use on this instrument is the Turboflow Cyclone-P 1 x 50mm.
The sample is then transferred to the analytical column where a change in mobile phase results in further separation before mass spectral analysis. The sample is sprayed through a heated ESI probe to vaporise and charge the droplets of sample. Not all ESI probes are heated but, applying temperature does make this process more effective. The ions are then collected and transported through an ion transfer tube into what is referred to as an S-lens. The TSQ vantage also has a sweep cone covering the

entry to this tube to help concentrate the ions and reduce build-up of unwanted sample. S-lens is Thermo Fisher technology that uses its “S” design to capture and focus the ions into a tight beam. Equally spaced electrodes have a voltage applied to them, and as the rf amplitude increases, ions of higher mass to charge ratio pass through. These settings can be optimised when tuning the instrument. Ions that have passed out of the S-lens then move into the first quadrupole of the vantage mass spectrometer, where those that match the molecular weight of the compounds of interest (as determined in your method MRM settings) will be collected and passed into the collision cell for fragmentation, and finally the product ions of interest will be collected in the next quadrupole and the strength of signal read by the detector.

2.3 Calibrator and Control Preparation

Calibrators were prepared using whole blood to replicate the matrix of patient blood spot samples. The whole blood, with plasma run off, was first washed with saline to remove the naturally occurring steroids, then mixed with a steroid free serum substitute (Serasub) to bring it to normal blood haematocrit (Hct) levels. Haematocrit is a measure of the proportion of red blood cells, and 45% was picked as a mid-range target. This was performed as follows: 25 mL of red cells were placed into 4x 50 mL polypropylene tubes and 25 mL of 0.9% saline was added to the red cells. The tubes were carefully mixed by inversion and then centrifuged at 3400rpm for 10 minutes at 4^{o}C for separation. The supernatant was removed off the top using a suction device and was discarded. This process was repeated three more times and then the washed red cells were pooled together in a glass beaker giving a total volume of approximately 100 mL. The Hct of the pooled red cells was measured and found to be 75%. Then the volume of serasub required to obtain the desired Hct was calculated as described below:

There is 100mL of packed red cells with a Hct of 75% which means the sample has: 100 x 75 / 100 = 75mL red cells and
100 x 25 / 100 = 25mL plasma
The desired Hct is 45%, therefore 55% plasma:
75 x 55 / 45 = 91mL plasma
There is already 25mL of plasma so 91 – 25 = 66mL to be added

66 mL of serasub was added to the pooled red cells and mixed well using a magnetic flea and mixer. The Hct level was measured again for confirmation and found to be 43%, which was deemed acceptable.

The washed blood was then spiked with steroids to the desired concentrations. A 10mL stock standard was prepared in ethanol with the following concentrations: 11KA4 – 0.5 mmol/L, 11KT – 0.5 mmol/L, 11OHT – 0.5 mmol/L, 11OHA4 – 1.25 mmol/L.

1.0mL of stock solution was then made up to 100mL with saline to produce a spiking standard with the following concentrations: 11KA4 – 5.0 µmol/L, 11KT – 5.0 µmol/L, 11OHT – 5.0 µmol/L, 11OHA4 – 12.5 µmol/L.

10mL of blood was added to 5 plastic tubes and spiked as follows to obtain working calibrators in the desired nmol/L concentrations:

Table 2

Calibrator preparation

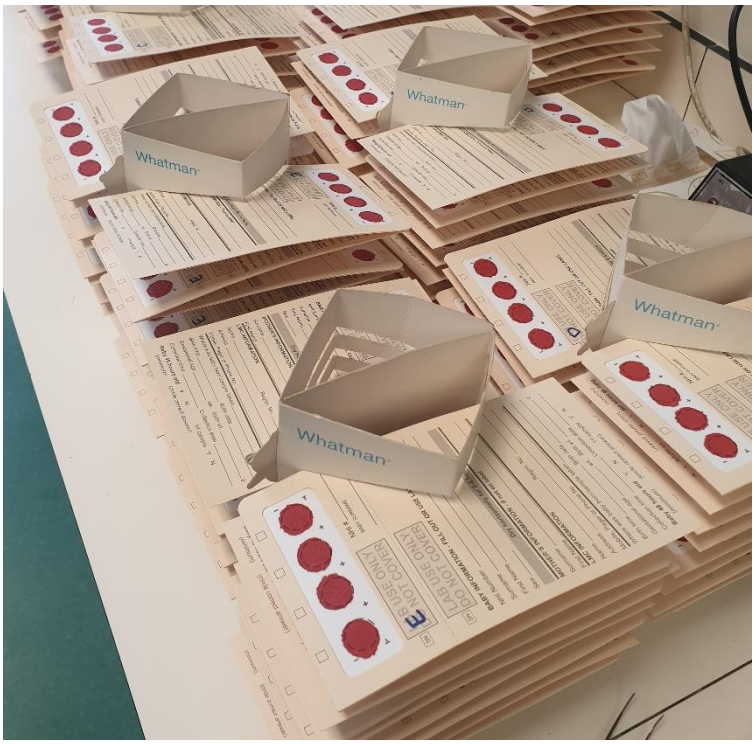
Std	Blood Vol (mL)	Remove blood (µL)	Vol Std (µL)	11KA4 (nmol/L)	11KT (nmol/L)	11OHT (nmol/L)	11OHA4 (nmol/L)
A	10	0	0	0	0	0	0
B	10	30	30	15	15	15	37.5
C	10	60	60	30	30	30	75
D	10	120	120	60	60	60	150
E	10	240	240	120	120	120	300

Note: These calibrator concentrations were chosen to obtain a working test method and may need to be adjusted for a live patient method based on the data presented in Table 1.

Whatman 903 filter paper cards were used to contain the spots. These are made from special adsorbent paper designed for collection of biological fluids and contain four circles in the sample collection area that can each hold 75-80 µL of fluid. The cards were labelled according to calibrator level, and 75 µL of calibrator was pipetted onto each spot (four spots on each card). The cards were then left to dry overnight, and then stored in a sealed bag at -20^{o}C for the duration of the research.

Figure 7

Image of calibrator and control preparation



Note: Image shows preparation of calibrators and controls using Guthrie cards in the newborn screening section at LabPlus, Te Whatu Ora NZ. Own image.

The quality control (QC) materials were prepared in the same way, as described above, and spiked according to the following table:

Table 3

Control preparation

QC	Blood Vol (mL)	Remove blood (µL)	Vol Std (µL)	11KA4 (nmol/L)	11KT (nmol/L)	11OHT (nmol/L)	11OHA4 (nmol/L)
Low	10	90	90	45	45	45	112.5
High	10	180	180	90	90	90	225

2.4 Internal Standard Preparation

Internal standards help to account for variance in recovery and matrix effects during sample preparation and mass spectrometry analysis (CLSI C62-A, 2014). For mass spectrometry, internal standards should have identical physical properties. Cold isotopic standards are universally used for this purpose. They must not be compounds that are biologically present in your samples.

Deuterated Testosterone (testosterone-d2) was available in the laboratory in powder form with a molecular weight of 290.44 g/mol. Internal standard (3 mg) was weighed

and dissolved in a small volume of methanol and made up to 50 mL volumetrically (concentration 206.5 µmol/L). This was further diluted in methanol to obtain an intermediate stock of 516.3 nmol/L. Finally, this intermediate stock was further diluted to obtain a working solution with a concentration of 51.6 nmol/L. The concentration of an internal standard must be consistently reproducible, and therefore often sits in the middle of the calibration range, or approximately ten times the lower measuring limit of the assay (CLSI C62-A, 2014).

Chapter 3

3. Method Development

The first step to answering the research questions is to develop the LCMS method. The parameters that need to be included in this are tuning of the mass spectrometer and selection of appropriate precursor and product ions, and evaluation of reagents, mobile phases, columns, and sample preparation techniques.

3.1 Mass Spectrometry Settings

Optimisation of instrument settings and methodology was the first step in method development. Reference standards were used to tune the mass spectrometer settings. A 500 nmol/L solution of 11-KT was chosen to identify the ionization probe settings in the initial tuning. The solution was infused via syringe directly into the mass spectrometer at a rate of 5 µL per minute, while the instrument checks a range of temperature, voltage, and gas settings to obtain maximum sensitivity. The parameters available for optimisation on the TSQ Vantage are as follows:

- Spray voltage: this is applied to spray the liquid from the probe and charge the droplets prior to entering the mass spectrometer.
- Sheath gas pressure: this helps to nebulise the sample into a fine mist as it leaves the probe.
- Ion sweep gas pressure: this is used to carry the ions to the ion transfer tube and into the mass spectrometer.
- Auxiliary gas pressure: this helps with evaporation of the sample solution in the ion source.
- Vaporiser temperature: the temperature of the probe that is spraying the sample.
- Capillary temperature: the temperature of the ion transfer tube.

The optimised results for these instrument settings can be seen below in Table 4.

Table 4

Mass spectrometry method settings

Spray voltage	5000V
Sheath gas flow rate	60
Ion sweep gas flow rate	2.0
Auxiliary gas flow rate	15
Vaporiser temperature	420^{o}C
Capillary temperature	200^{o}C

Note: The sheath, ion sweep, and auxiliary gas flow rate units are arbitrary.

Once the initial method parameters have been set, there are further settings which can be optimised for each individual compound. These are as follows; identification of the precursor and product ions to be used for analysis, the collision energy applied to the mass spectrometer collision chamber for fragmentation of the compound, and the S-lens RF amplitude to maximise analyte signal.

In this instance, each compound was used to determine the optimum parameters for precursor and product ions. For this experiment the steroids were infused at a concentration of 500 nmol/L straight from the stock standard, and additionally after undergoing derivatisation with hydroxylamine to see if this improved signal level. As per Häkkinen et al. (2019) the standard was dried down under nitrogen and then reconstituted with 30% acetonitrile containing 100mmol/L of hydroxylamine and heated for 30 minutes at 60^{o}C.

The possible options for steroid derivatives once oximated (converted to an oxime) were calculated and are as follows:

Table 5

Candidate precursor ions when derivatised

Compound	**$[M+H]^+$ Underivatised**	**$[M+NH+H]^+$ Derivatised**	**$[M+2(NH)+H]^+$ Derivatised**	**$[M+3(NH)+H]^+$ Derivatised**
Testosterone D2	291.2	306.2		
11-OHA4	303.1	318.2	333.2	
11-KA4	301.1	316.2	331.2	346.2
11-OHT	305.1	320.2		
11-KT	303.1	318.2	333.2	

Note: The $[M+H]^+$ is the underivatised molecular weight of each compound that would be used for mass spectral analysis. The other columns represent the possible derivatisation products.

Key: M = molecular weight, H = hydrogen ion, NH = ammonium ion

The products formed will depend on the derivatisation reaction and the sensitivity of the ions seen in the mass spectrometer. Based on the results seen during method development, the chosen tuning settings for the 11-oxygenated steroids and the internal standard can be seen below in Tables 6 and 7.

Table 6

Compound tuning parameters

Compound	Parent ion	Product ion	Collision energy (V)	S-lens RF
Testosterone D2	291.2	99.1	21	105
		111.1	23	
11-OHA4	303.1	91.1	49	105
		121.1	25	
11-KA4	301.1	91.1	48	116
		121.1	23	
11-OHT	305.1	91.0	43	110
		269.2	13	
11-KT	303.1	91.1	49	105
		121.1	25	

Table 7

Compound tuning parameters – derivatised with hydroxylamine

Compound	Parent ion	Product ion	Collision energy (V)	S-lens RF
Testosterone D2	306.2	114.0	30	105
		126.0	33	
11-OHA4	333.2	79.0	57	105
		138.0	29	
11-KA4	331.2	121.0	33	105
		178.0	37	
11-OHT	320.2	105.0	45	105
		138.0	29	
11-KT	318.2	120.0	33	105
		121.0	35	

Product ions provide greater specificity as it narrows down the chances of interference from other compounds. The ratio of product ions from the same parent ion against each other should be consistent throughout analysis, helping to confirm you are seeing the correct compound. The product ion with the largest presence should be used as the main peak in quantitative LCMS methods, with other product ions used as confirmatory peaks.

3.2 Liquid Chromatography

When developing a method using HPLC there are several parameters that need to be taken into consideration:

- Column (stationary phase)
- Mobile phase composition
- Mobile phase gradient and flow rate
- Temperature

3.2.1 Column

When selecting HPLC columns, the following should be considered; the chemistry of your compound, how many compounds you are trying to separate, how long you are prepared for your run time to be, and the pressure that can be withstood by the system you are using – these factors will impact the length and pore size of the column you choose.

A common choice for separation of steroids, using reverse-phase chromatography, is a C18 column. Octadecanoyl chain lengths of carbon compounds are covalently bound to the base silica beads packed inside the column. They have been proven suitable for a wide range of applications (CLSI C62-A, 2014). An alternative for steroid analysis is a C18 biphenyl column. This has been shown helpful in resolving isomeric compounds (those which have the same molecular formula as each other). A biphenyl column uses the phenyl groups attached to the silica to promote van der waals interactions between the atoms, leading to greater separation between compounds. Therefore, these two types of columns were chosen to evaluate for this method:

- Phenomenex Kinetex 2.6µm XB-C18 100A, 150x2.1mm
- Phenomenex Kinetex 1.7µm Biphenyl 100A, 100x2.1mm

A mixed standard containing all four compounds of interest, plus internal standard, was injected into both columns during the method development phase to check peak shape and size. Although the Biphenyl column did give better peak resolution, injections using the C18 column resulted in 1-35% larger peak areas for each of the four analytes (Table 8). As baseline separation of all the compounds was the same using both columns, the C18 column was chosen for the method to improve analytical sensitivity.

Table 8

Column comparison results

11-OHA4	C18 Peak area	Biphenyl Peak area	% Difference
Cal B	40056	36184	9.7
Cal C	69348	73398	-5.8
Cal D	132362	132311	0.0
Cal E	293690	204544	30.4
11-KA4			
Cal B	17550	19827	-13.0
Cal C	26438	34449	-30.3
Cal D	44175	53167	-20.4
Cal E	94694	69991	26.1
11-OHT			
Cal B	58644	44078	24.8
Cal C	101723	86999	14.5
Cal D	196938	185387	5.9
Cal E	434337	281214	35.3
11-KT			
Cal B	39794	39281	1.3
Cal C	76219	72412	5.0
Cal D	137038	169828	-23.9
Cal E	324773	218327	32.8

3.2.2 Mobile Phase Selection

The choice of mobile phase will have an impact on the separation of compounds, pump pressure, and success of ionisation. Aqueous mobile phases commonly use additives such as acids and buffers to stabilise the pH and assist with ionisation. Selection of LCMS grade solvents and high purity water is an important consideration, as lower grade reagents can contain impurities that can affect the analyte signal, and dirty the instrument.

de Hora et al. (2020) have already shown successful analysis of 17-OHP using LCMS with a combination of 2 mmol/L ammonium formate in 0.05% aqueous formic acid for mobile phase A, and pure LCMS grade methanol for mobile phase B. Turcu et al. (2015) used 0.25 mmol/L ammonium fluoride for mobile phase A, and pure LCMS grade methanol for mobile phase B, for a steroid panel not including the 11-oxygenated steroids. And Schiffer et al. (2019) used 2 mmol/L ammonium acetate in 0.1% aqueous formic acid for mobile phase A, and 0.1% formic acid in LCMS grade methanol for mobile phase B for a steroid panel that did include the 11-oxygenated steroids. Keevil (2019) found that using acetonitrile instead of methanol for mobile phase B gave better

resolution between the isobaric steroids (same molecular weight), in this case 11-KT and 11-OHA4.

A mixed standard containing all four compounds of interest, plus internal standard, was used to evaluate different mobile phase options.

1. A = 2 mmol/L ammonium formate in 0.05% aqueous formic acid
   B = Methanol
2. A = 2 mmol/L ammonium formate in 0.05% aqueous formic acid
   B = Acetonitrile
3. A = x50 dilution of 2 mmol/L ammonium formate in 0.05% aqueous formic acid
   B = Methanol

The first comparison between methanol and acetonitrile showed no difference between peak areas, and marginally better peak shapes with methanol. This factor, combined with the knowledge that acetonitrile has a higher toxicity level, confirmed the choice of keeping methanol for mobile phase B. A further comparison was done using a diluted version of mobile phase A, and this showed improved sensitivity with larger peak areas. This is possibly due to some ion suppression with higher concentrations of mobile phase buffer. Therefore, the chosen combination for the analytical column was option 3 listed above.

Mobile phases used for the turboflow system were the same for A and B, with the addition of a solvent wash (Isopropanol: Acetone: Acetonitrile in a ratio of 4.5:4.5:1) as mobile phase C to be used for column clean-up in between samples.

3.2.3 Mobile Phase Gradient and Flow Rate

As discussed in section 1.8, the starting mobile phase conditions will typically be a higher percentage of mobile phase A (aqueous phase) used for loading of the sample, then moving to a higher percentage of mobile phase B (methanol) to elute the compounds off the column.

The turboflow starting conditions are 95% A, 5% B, held for just over 2 minutes. This includes transfer of sample onto the analytical column using solvent stored in the sample loop at a concentration of 20% A, 80% B. The column is then washed for 2 minutes with C, and then the sample loop is refilled, and finally it is returned to the starting conditions.

The analytical mobile phase gradient starts at 95% A, 5% B, held for just over 2 minutes to equilibrate the column, moving to 90% A, 10% B, over a period of 4 minutes to elute the steroids off the column, where it's held for 3 minutes, and then finally moving back to the starting conditions to wash and re-equilibrate the column. A summary of this can be seen in Figure 8 below.

Figure 8

Image of table depicting mobile phase conditions

Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B	%C	%D
0.00	60	1.50	Step	95.0	-	-	5.0	====	out	0.30	Step	95.0	-	-	5.0
1.00	15	0.10	Step	95.0	-	-	5.0	====	out	0.20	Step	95.0	-	-	5.0
1.25	60	0.10	Step	95.0	-	-	5.0	T	in	0.20	Ramp	95.0	-	-	5.0
2.25	120	1.00	Step	-	-	100.0	-	====	in	0.30	Ramp	20.0	-	-	80.0
4.25	120	1.50	Step	20.0	-	-	80.0	====	in	0.30	Ramp	10.0	-	-	90.0
6.25	180	1.50	Step	95.0	-	-	5.0	====	out	0.30	Step	-	10.0	-	90.0
9.25	60	1.50	Step	95.0	-	-	5.0	====	out	0.30	Ramp	95.0	-	-	5.0
10.25	30	1.50	Step	95.0	-	-	5.0	====	out	0.30	Step	95.0	-	-	5.0

Note: Time is depicted in the grey columns on the left. Flow is in mL/min. Step refers to a sudden change or hold in mobile phases and ramp a gradual change to the desired % of mobile phase. "T" is when the sample is transferred from turboflow to analytical column, and the loop column indicates when the sample loop is in or out of the flow path. Blue = Turboflow flow and mobile phase %, Pink = Analytical flow and mobile phase %

The typical flow rate for turboflow is quite high, at 1.5mL per minute, to assist with sample clean up. This drops down to 0.1mL per minute only during the period where the connection is open to transfer compounds onto the analytical column. The flow rate on the analytical column needs to be much lower to reduce the back pressure on the column as it typically has a smaller particle size to enhance separation. The flow rate for this method was optimised at 0.3mL per minute, as a compromise between column pressure and peak separation.

3.2.4 Temperature

Temperature in the column oven can help with elution of compounds, stabilisation of the retention time, and speeding up the run time. A common temperature for steroid analysis is around 40-50^{o}C, as demonstrated by Häkkinen et al. (2019) and Schiffer et al. (2019). 40^{o}C was chosen for this method and used for all validation work.

3.3 Sample Preparation

To achieve the maximum sensitivity benefits of using mass spectrometry, samples should be cleaned up and concentrated prior to analysis to try and minimise the

introduction of contaminants which could interfere with analysis or cause potential suppression of signal. This process also prolongs the life of the instrument as the ion source and quadrupoles are not being contaminated.

Turboflow technology assists with this sample clean-up process, but some common types of manual sample preparation used with chromatographic techniques are:

- Protein precipitation: organic solvents are used to precipitate the proteins in a blood sample out. This is then separated from the organic supernatant which will contain the analyte of interest. This is one of the quickest and easiest forms of sample preparation.
- Liquid-liquid extraction: a combination of immiscible organic solvents is used to extract compounds from a sample. The clean extracted portion is then used for analysis.
- Solid phase extraction: this is the most complex form of sample extraction and uses a type of solid material in either cartridge, or 96-well plate format. The solid phase can have different composition depending on the properties of the analyte of interest, and a combination of washing steps and extraction steps using solvents are performed to concentrate and elute the analytes through the packing material. It is a time-consuming process but usually results in the cleanest extracts.

Blood spots present different challenges than working with liquid whole blood or serum samples, as they come as dried material. However, this could prove beneficial during a solvent extraction if some of the unwanted proteins and other compounds stay stuck to the paper whilst the steroids are released. de Hora et al. (2020) and Salter et al. (2015) have both shown successful extraction of steroids from dried blood spots using organic solvents. This technique was therefore chosen as the focus for the development of optimal sample preparation.

3.3.1 Selection of Extraction Solvent

No published method exists for extraction of the 11-oxygenated steroids from dried blood spots, but as discussed in section 1.6, there are several organic solvents available which have been used successfully across a range of methods for analysing various steroids in liquid blood or dried blood spots. Based on this review the solvents that were chosen for comparison were acetonitrile, dichloromethane, methyl tert-butyl ether (MTBE), and diethyl ether. Peak areas of underivatised 11-OHT were used for comparison of solvent extraction and results can be seen in Table 9 below.

Table 9

Comparison of different solvents for sample extraction

Calibrator Level	Acetonitrile	Dichloromethane	MTBE	Diethyl ether
B	6930	14697	4475	4631
C	16097	30356	15279	10464
D	30324	53791	23358	25723
E	59441	123691	54004	46991

MTBE and Diethyl ether gave the lowest peak areas but are both quite volatile solvents. Dichloromethane gave good peak areas; however, it is also quite a volatile solvent, and this made it difficult to work with due to hazardous vapours, and rapid evaporation if care is not taken. Acetonitrile ultimately was selected as it is the least volatile and provided favourable peak shapes and reasonable peak areas.
Note: all solvents were evaluated as 80% solutions in water to assist with extraction of compounds from the dried blood spots and reduce solvent evaporation during sample preparation.

3 x 3mm blood spots, equating to 9.6 µL of sample, were punched out of the card into a deep well plate. The method used by de Hora et al. (2020) used 2 x 3mm blood spots and so a comparison of peak areas between two and three spots for the QC material was performed and the results can be seen in Table 10.
Internal standard and organic solvent are added to the wells with the dried blood spots. Plates were mixed gently for 1 hour. Following this there is a centrifugation step to separate out any potential debris. The supernatant is then moved to a standard 96-well plate for concentrating under a constant gentle flow of nitrogen and reconstitution. This process can also be performed in microcentrifuge tubes for the solvent extraction, transferring the samples into glass vials for analysis.

Table 10

Data showing comparison between two blood spots versus three

Compound	Low QC Peak Area 2 spots	Low QC Peak Area 3 spots	High QC Peak Area 2 spots	High QC Peak Area 3 spots
11-OHA4	7176	9752	16295	20783
11-OHT	27073	35356	56855	78661
11-KT	30098	44290	62289	88196

Blood spot samples, as with any other patient specimen, should be treated as precious, and used as sparingly as possible. However, as is to be expected, the peak areas were greater using three blood spots, rather than two, for sample extraction. During the

experiment, peak areas for 11-KA4 were unable to be calculated when using two blood spots due to lack of sensitivity, further cementing the decision to go with three blood spots.

3.3.2 Derivatisation conditions evaluation

A comparison was carried out between underivatised standard, and derivatised standard, to discover if there was a difference in sensitivity. Stock standard was used to prepare a 200 nmol/L solution. Two aliquots were prepared in duplicate; both were dried down with nitrogen gas to concentrate the samples. One set was then reconstituted with 40% methanol to mimic mobile phase conditions, and the other set was reconstituted with 30% acetonitrile containing 100mmol/L of hydroxylamine and heated for 30 minutes at 60^{o}C.

Table 11

Comparison of derivatised versus underivatised compounds

Compound	Underivatised Peak Area	Derivatised Peak Area	% Difference
11-OHA4	385818	728752	47
11-KA4	139583	178118	22
11-OHT	445540	633236	30
11-KT	441204	347299	-27

For three out of four compounds, derivatisation improved the peak size, and therefore was chosen as part of the sample preparation to optimise the chances of obtaining useable results. Derivatisation of the compounds in this method also resulted in all four compounds having different molecular weights, making the chromatographic part of the method easier as the compounds will all be separated from one another.

3.3.3 Sample Mixing Time

A review of methods by de Hora et al. (2020), Janzen et al. (2007), Salter et al. (2015), and Schwarz et al. (2009) demonstrated that samples were commonly mixed for between 30 – 60 minutes during the sample extraction phase. The preference was to keep the plate shaker on a low setting to minimise the risk of splashing and cross-contamination between wells during the mixing period. Comparisons were performed between a 30-minute mixing period, and a 60-minute mixing period. Results from the QC in each batch can be viewed in the tables below.

Table 12 A-D

Comparison of different mixing times

Table 12A

11-KA4					
Low QC	**30 minutes**	**60 minutes**	**High QC**	**30 minutes**	**60 minutes**
Conc (nmol/L)	44.2	45.0	**Conc (nmol/L)**	70.8	64.3
Peak Area	15809	21331	**Peak Area**	31634	35974
Int Std Peak Area	503549	491223	**Int Std Peak Area**	530467	625807
Response Ratio	0.031	0.043	**Response Ratio**	0.060	0.057

Table 12B

11-KT					
Low QC	**30 minutes**	**60 minutes**	**High QC**	**30 minutes**	**60 minutes**
Conc (nmol/L)	48.9	51.6	**Conc (nmol/L)**	84.5	99.5
Peak Area	71527	58805	**Peak Area**	128328	132898
Int Std Peak Area	503549	491223	**Int Std Peak Area**	530467	625807
Response Ratio	0.142	0.120	**Response Ratio**	0.242	0.212

Table 12C

11-OHT					
Low QC	**30 minutes**	**60 minutes**	**High QC**	**30 minutes**	**60 minutes**
Conc (nmol/L)	48.0	48.5	**Conc (nmol/L)**	85.5	93.2
Peak Area	85840	76931	**Peak Area**	160278	180991
Int Std Peak Area	503549	491223	**Int Std Peak Area**	530467	625807
Response Ratio	0.170	0.157	**Response Ratio**	0.302	0.289

Table 12D

11-OHA4					
Low QC	**30 minutes**	**60 minutes**	**High QC**	**30 minutes**	**60 minutes**
Conc (nmol/L)	125.2	116.1	**Conc (nmol/L)**	218.9	230.5
Peak Area	50189	49235	**Peak Area**	99933	114282
Int Std Peak Area	503549	491223	**Int Std Peak Area**	530467	625807
Response Ratio	0.100	0.100	**Response Ratio**	0.188	0.183

For 11-KA4, peak areas were larger for both the low and high QC, using a 60-minute mixing time. For 11-KT, 11-OHT and 11-OHA4 the peak area for the low QC was larger

at 30 minutes, and the peak area for the high QC was larger at 60 minutes. The decision was made to carry out all further validation batches using a 60-minute mix time on the lowest setting of the plate mixer.

3.4 Summary of LCMS method

In summary, here is a description of the confirmed LCMS method used for validation. Optimised mass spectrometer parameters are spray voltage 5000, sheath gas flow rate, 60, ion sweep gas flow rate 2.0, auxiliary gas flow rate 15, vaporiser temperature 420^{o}C, and capillary temperature 200^{o}C.

For the liquid chromatography analysis the following column was chosen; Phenomenex Kinetex 2.6µm XB-C18 100A, 150x2.1mm, using a mobile phase combination of x50 dilution of 2 mmol/L ammonium formate in 0.05% aqueous formic acid (mobile phase A) and methanol (mobile phase B) with gradient elution starting at 95% A, 5% B, held for just over 2 minutes to equilibrate the column, moving to 90% A, 10% B, over a period of 4 minutes to elute the steroids off the column, where it's held for 3 minutes, and then finally moving back to the starting conditions to wash and re-equilibrate the column. The flow rate was 0.3mL per minute, with a temperature of 40^{o}C, and a total run time was just over 10 minutes.

Sample preparation is as follows; 3 x 3mm blood spots were punched for each sample into the designated well of a deep-well microtiter plate (holds 1mL per well). 20 µL of working internal standard solution was added to each well, followed by 200 uL of 80% acetonitrile. The plate was sealed with foil and mixed on the plate mixer for 1 hour at the lowest setting. Following this was centrifugation for 5 minutes at 3000rpm and 200 µL of supernatant was transferred to a clean 96-well microtiter plate or glass LCMS vials. The samples were dried down under a constant gentle flow of nitrogen at approximately 40^{o}C and reconstituted with 80 µL of 30% acetonitrile containing 100 mmol/L of hydroxylamine. The plate was sealed again, or vials capped, and heated for 30 minutes at 60^{o}C. Samples were left to cool to room temperature prior to analysis and 30 µL of each sample was injected into the LCMS for analysis.

Chapter 4

4. Method Validation Process and Results

Once the method parameters had been confirmed, the working method then underwent validation to ensure robustness and final testing that it was fit for purpose. CLSI guideline C62-A for Liquid Chromatography-Mass Spectrometry Methods was followed whenever possible whilst carrying out validation experiments.

4.1 Precision

Precision tests the reproducibility of a method, and should be looked at within a run, and between runs; often referred to as intra-batch imprecision and inter-batch imprecision. Precision is demonstrated by calculating the %CV (coefficient of variation), and the recommendation in the CLSI guidelines is that up to 15% variation is acceptable at each concentration, with 20% being acceptable at the lower limit of the method.

For this validation intra-batch imprecision was evaluated using 6 replicates of the prepared QC material, and the inter-batch imprecision was evaluated using both the calibration material and QC material from throughout the validation.

Parameters that were monitored during the precision experiments were the calculated concentration (conc), peak area of the compound, peak area of the internal standard, the response ratio, and the retention time of the compound.

The response ratio is calculated as follows:

$$\frac{\text{Peak area compound}}{\text{Peak area internal standard}}$$

Response ratios are plotted against the theoretical concentrations of the calibrators in each batch to generate a calibration curve. The response ratio for each sample is then plotted on the curve to determine the calculated concentration.

Table 13 A-D

Intra-batch imprecision of QC material for the four 11-oxygenated steroids

Table 13A

11-OHA4					
Low QC	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	118.7	63432	553335	0.115	6.63
2	120.9	67035	575009	0.117	6.63
3	119.0	65813	572556	0.115	6.64
4	127.1	67639	554195	0.122	6.63
5	118.9	62931	547970	0.115	6.63
6	130.4	66326	531021	0.125	6.64
Average	**122.5**	**65529**	**555681**	**0.118**	**6.63**
SD	**5.0**	**1928**	**16332**	**0.004**	**0.01**
CV %	**4.1**	**2.9**	**2.9**	**3.6**	**0.1**
High QC	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	247.5	126059	553571	0.228	6.64
2	241.6	121454	545784	0.223	6.64
3	222.1	115290	561217	0.205	6.64
4	236.3	119019	546195	0.218	6.63
5	243.2	123999	553734	0.224	6.64
6	240.8	121988	549882	0.222	6.64
Average	**238.6**	**121302**	**551731**	**0.220**	**6.64**
SD	**8.8**	**3788**	**5776**	**0.008**	**0.00**
CV %	**3.7**	**3.1**	**1.0**	**3.6**	**0.1**

Table 13B

11-KA4					
Low QC	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	47.5	26214	553335	0.047	6.59
2	39.7	23298	575009	0.041	6.59
3	41.6	24615	572556	0.043	6.57
4	50.1	25747	554195	0.046	6.61
5	51.9	27500	547970	0.050	6.62
6	49.6	28262	531021	0.053	6.58
Average	**46.7**	**25939**	**555681**	**0.047**	**6.59**
SD	**4.9**	**1826**	**16331**	**0.004**	**0.02**
CV %	**10.6**	**7.0**	**2.9**	**9.5**	**0.3**
High QC	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	79.9	48169	553571	0.087	6.58
2	73.5	42585	545784	0.078	6.59
3	75.5	42406	561217	0.076	6.60
4	69.1	39241	546195	0.072	6.59
5	77.1	40271	553734	0.073	6.56
6	78.1	45487	549882	0.083	6.58
Average	**75.5**	**43027**	**551731**	**0.078**	**6.58**
SD	**3.8**	**3317**	**5776**	**0.006**	**0.01**
CV %	**5.1**	**7.7**	**1.0**	**7.5**	**0.2**

Table 13C

11-OHT					
Low QC	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	51.0	92954	553335	0.168	6.68
2	48.7	92512	575009	0.161	6.61
3	44.8	85453	572556	0.149	6.61
4	52.1	94976	554195	0.171	6.6
5	47.8	86723	547970	0.158	6.68
6	48.7	85553	531021	0.161	6.61
Average	**48.9**	**89695**	**555681**	**0.161**	**6.63**
SD	**2.6**	**4253**	**16332**	**0.008**	**0.04**
CV %	**5.3**	**4.7**	**2.9**	**4.8**	**0.6**
High QC	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	99.4	173860	553571	0.314	6.62
2	103.1	177531	545784	0.325	6.69
3	85.6	152895	561217	0.272	6.61
4	96.0	165932	546195	0.304	6.61
5	102.5	179114	553734	0.323	6.69
6	101.6	176390	549882	0.321	6.61
Average	**98.0**	**170954**	**551731**	**0.310**	**6.64**
SD	**6.6**	**9992**	**5776**	**0.020**	**0.04**
CV %	**6.8**	**5.8**	**1.0**	**6.5**	**0.6**

Table 13D

11-KT					
Low QC	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	51.1	70728	553335	0.128	6.57
2	49.2	70820	575009	0.123	6.57
3	47.5	68252	572556	0.119	6.57
4	49.2	68364	554195	0.123	6.56
5	49.8	68329	547970	0.125	6.57
6	47.0	62688	531021	0.118	6.56
Average	**49.0**	**68197**	**555681**	**0.123**	**6.57**
SD	**1.5**	**2956**	**16332**	**0.004**	**0.01**
CV %	**3.0**	**4.3**	**2.9**	**3.0**	**0.1**
High QC	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	102.1	145498	553571	0.263	6.58
2	97.8	131567	545784	0.241	6.58
3	92.9	131512	561217	0.234	6.56
4	94.6	128879	546195	0.236	6.57
5	99.6	135823	553734	0.245	6.57
6	99.2	134328	549882	0.244	6.57
Average	**97.7**	**134601**	**551731**	**0.244**	**6.57**
SD	**3.4**	**5862**	**5776**	**0.010**	**0.01**
CV %	**3.5**	**4.4**	**1.0**	**4.2**	**0.1**

As shown in Tables 13A-D, the CV percentages for the calculated concentrations recorded during the intra-batch imprecision experiment were less than 15% for all compounds, indicating good within batch repeatability. Stable peak areas show that the sample preparation, column, and instrument are performing as expected. CLSI C62-A, (2014) recommends 5-10% variation of peak area within a batch, which has been achieved for all compounds (for this parameter specifically, the intra-batch measurement is more important than the inter-batch).

Table 14 A-D

Inter-batch imprecision of QC material for the four 11-oxygenated steroids

Table 14A

11-OHA4					
Low QC	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	119.0	65813	572556	0.115	6.64
2	116.1	61395	533605	0.115	6.66
3	125.1	50189	503549	0.100	6.64
4	116.1	49235	491223	0.100	6.67
5	121.9	9752	170621	0.057	6.75
6	124.0	9392	161569	0.058	6.65
7	114.2	9646	179872	0.054	6.67
Average	**119.5**	**36489**	**373285**	**0.086**	**6.67**
SD	**4.3**	**25820**	**191309**	**0.028**	**0.04**
CV %	**3.6**	**70.8**	**51.3**	**32.8**	**0.6**
High QC	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	222.1	115290	561217	0.205	6.64
2	248.6	117789	541204	0.218	6.66
3	218.9	99933	530467	0.188	6.64
4	230.5	114282	625807	0.183	6.67
5	238.5	20783	188285	0.11	6.64
6	244.5	20248	179007	0.113	6.65
7	234.7	18041	166072	0.109	6.76
Average	**234.0**	**72338**	**398866**	**0.161**	**6.67**
SD	**11.0**	**49583**	**209091**	**0.048**	**0.04**
CV %	**4.7**	**68.5**	**52.4**	**30.0**	**0.6**

Table 14B

11-KA4					
Low QC	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	41.6	24615	572556	0.043	6.57
2	44.1	22149	533605	0.042	6.58
3	44.2	15809	503549	0.031	6.59
4	45.0	21331	491223	0.043	6.66
Average	**43.7**	**20976**	**525233**	**0.040**	**6.60**
SD	**1.5**	**3717**	**36224**	**0.006**	**0.04**
CV %	**3.4**	**17.7**	**6.9**	**14.7**	**0.6**
High QC	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	75.5	42406	561217	0.076	6.60
2	70.1	33804	541204	0.062	6.60
3	70.8	31634	530467	0.060	6.58
4	64.3	35974	625807	0.057	6.63
Average	**70.2**	**35955**	**564674**	**0.064**	**6.60**
SD	**4.6**	**4652**	**42701**	**0.008**	**0.02**
CV %	**6.5**	**12.9**	**7.6**	**13.2**	**0.3**

Table 14C

11-OHT					
Low QC	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	44.8	85453	572556	0.149	6.61
2	48.8	82612	533605	0.155	6.71
3	48.0	85840	503549	0.170	6.61
4	48.5	76931	491223	0.157	6.72
5	44.6	35356	170621	0.207	6.61
6	43.5	32700	161569	0.202	6.61
7	34.1	28963	179872	0.161	6.61
Average	**44.6**	**61122**	**373285**	**0.172**	**6.64**
SD	**5.1**	**27144**	**191309**	**0.023**	**0.05**
CV %	**11.4**	**44.4**	**51.3**	**13.7**	**0.8**
High QC	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	85.6	152895	561217	0.272	6.61
2	100.1	166081	541204	0.307	6.64
3	85.5	160278	530467	0.302	6.62
4	93.2	180991	625807	0.289	6.73
5	92.6	78661	188285	0.418	6.61
6	92.8	74926	179007	0.419	6.71
7	94.8	70975	166072	0.427	6.62
Average	**92.1**	**126401**	**398866**	**0.348**	**6.65**
SD	**5.2**	**48998**	**209091**	**0.070**	**0.05**
CV %	**5.6**	**38.8**	**52.4**	**20.1**	**0.8**

Table 14D

11-KT					
Low QC	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	47.5	68252	572556	0.119	6.57
2	47.1	62774	533605	0.118	6.59
3	48.9	71527	503549	0.142	6.57
4	51.6	58805	491223	0.120	6.58
5	51.3	44290	170621	0.260	6.56
6	52.0	42490	161569	0.263	6.57
7	37.4	34046	179872	0.189	6.57
Average	**48.0**	**54598**	**373285**	**0.173**	**6.57**
SD	**5.1**	**14334**	**191309**	**0.065**	**0.01**
CV %	**10.6**	**26.3**	**51.3**	**37.8**	**0.1**
High QC	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	92.9	131512	561217	0.234	6.56
2	100.9	132226	541204	0.244	6.62
3	84.5	128328	530467	0.242	6.57
4	99.5	132898	625807	0.212	6.59
5	92.6	88196	188285	0.468	6.56
6	92.8	84043	179007	0.469	6.57
7	100.0	83938	166072	0.505	6.58
Average	**94.7**	**111592**	**398866**	**0.339**	**6.58**
SD	**5.8**	**24589**	**209091**	**0.133**	**0.02**
CV %	**6.1**	**22.0**	**52.4**	**39.3**	**0.3**

Table 15 A-D

Inter-batch imprecision of calibrator material for the four 11-oxygenated steroids

Table 15A

11-OHA4					
Cal B (37.5nmol/L)	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	34.3	22500	554391	0.041	6.63
2	42.9	33507	573501	0.058	6.67
3	41.0	14250	435592	0.033	6.64
4	36.8	18377	502968	0.037	6.68
5	39.0	3131	162107	0.019	6.64
Average	**38.8**	**18353**	**445712**	**0.038**	**6.65**
SD	**3.4**	**11129**	**167297**	**0.014**	**0.02**
CV %	**8.7**	**60.6**	**37.5**	**37.5**	**0.3**
Cal C (75nmol/L)	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	82.7	46652	561814	0.083	6.64
2	65.0	45312	600197	0.075	6.66
3	70.5	26705	447307	0.060	6.62
4	70.6	38987	586932	0.066	6.67
5	78.1	6300	169550	0.037	6.65
Average	**73.4**	**32791**	**473160**	**0.064**	**6.65**
SD	**7.0**	**16779**	**180131**	**0.018**	**0.02**
CV %	**9.5**	**51.2**	**38.1**	**27.3**	**0.3**
Cal D (150nmol/L)	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	149.2	81045	573026	0.141	6.64
2	142.2	75883	561098	0.135	6.66
3	140.4	58911	476872	0.124	6.63
4	166.8	57261	377999	0.151	6.67
5	149.5	13400	192048	0.070	6.76
Average	**149.6**	**57300**	**436209**	**0.124**	**6.67**
SD	**10.4**	**26641**	**157300**	**0.032**	**0.05**
CV %	**7.0**	**46.5**	**36.1**	**25.6**	**0.8**
Cal E (300nmol/L)	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	296.3	156320	577671	0.271	6.63
2	312.5	155039	580502	0.267	6.67
3	305.5	140453	511810	0.274	6.64
4	292.8	88561	336801	0.263	6.77
5	299.3	22956	166211	0.138	6.64
Average	**301.3**	**112666**	**434599**	**0.243**	**6.67**
SD	**7.8**	**57232**	**179802**	**0.059**	**0.06**
CV %	**2.6**	**50.8**	**41.4**	**24.2**	**0.9**

Table 15B

11-KA4					
Cal B (15nmol/L)	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	19.3	11841	554391	0.021	6.65
2	22.8	13996	573501	0.024	6.66
3	16.5	6393	435592	0.015	6.55
4	16.9	10286	502968	0.020	6.66
5	N/A	N/A	N/A	N/A	N/A
Average	**18.9**	**10629**	**516613**	**0.020**	**6.63**
SD	**2.9**	**3208**	**61682**	**0.004**	**0.05**
CV %	**15.3**	**30.2**	**11.9**	**18.7**	**0.8**
Cal C (30nmol/L)	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	32.0	18226	561814	0.032	6.61
2	31.2	18715	600197	0.031	6.62
3	30.4	11530	447307	0.026	6.59
4	32.1	20043	586932	0.034	6.69
5	N/A	N/A	N/A	N/A	N/A
Average	**31.4**	**17129**	**549063**	**0.031**	**6.63**
SD	**0.8**	**3810**	**69679**	**0.003**	**0.04**
CV %	**2.5**	**22.2**	**12.7**	**11.1**	**0.7**
Cal D (60nmol/L)	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	59.8	32550	573026	0.057	6.61
2	59.4	30228	561098	0.054	6.60
3	60.2	23709	476872	0.050	6.58
4	65.2	24160	377999	0.064	6.62
5	N/A	N/A	N/A	N/A	N/A
Average	**61.2**	**27662**	**497249**	**0.056**	**6.60**
SD	**2.7**	**4411**	**90286**	**0.006**	**0.02**
CV %	**4.4**	**15.9**	**18.2**	**10.5**	**0.3**
Cal E (120nmol/L)	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	119.1	62743	577671	0.109	6.58
2	119.0	59151	580502	0.102	6.60
3	119.6	49812	511810	0.097	6.59
4	116.6	37098	336801	0.110	6.64
5	N/A	N/A	N/A	N/A	N/A
Average	**118.6**	**52201.0**	**501696**	**0.105**	**6.603**
SD	**1.3**	**11449.1**	**114419**	**0.006**	**0.026**
CV %	**1.1**	**21.9**	**22.8**	**5.9**	**0.4**

Table 15C

11-OHT					
Cal B (15nmol/L)	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	14.7	32271	554391	0.058	6.69
2	15.5	32268	573501	0.056	6.63
3	14.8	22750	435592	0.052	6.61
4	15.5	27864	502968	0.055	6.64
5	16.9	13817	162107	0.085	6.62
Average	**15.5**	**25794**	**445712**	**0.061**	**6.64**
SD	**0.9**	**7760**	**167297**	**0.013**	**0.03**
CV %	**5.7**	**30.1**	**37.5**	**22.0**	**0.5**
Cal C (30nmol/L)	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	31.4	61043	561814	0.109	6.62
2	28.6	56992	600197	0.095	6.71
3	33.0	52347	447307	0.117	6.60
4	30.3	60881	586932	0.104	6.63
5	32.6	26154	169550	0.154	6.70
Average	**31.2**	**51483**	**473160**	**0.116**	**6.65**
SD	**1.8**	**14598**	**180131**	**0.023**	**0.05**
CV %	**5.8**	**28.4**	**38.1**	**19.7**	**0.7**
Cal D (60nmol/L)	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	58.2	108650	573026	0.190	6.62
2	60.7	106633	561098	0.190	6.63
3	55.8	94496	476872	0.198	6.61
4	61.3	77716	377999	0.206	6.73
5	58.0	51084	192048	0.266	6.61
Average	**58.8**	**87716**	**436209**	**0.210**	**6.64**
SD	**2.2**	**23895**	**157300**	**0.032**	**0.05**
CV %	**3.8**	**27.2**	**36.1**	**15.2**	**0.8**
Cal E (120nmol/L)	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	120.8	218774	577671	0.379	6.67
2	120.2	212750	580502	0.366	6.64
3	121.4	220640	511810	0.431	6.61
4	119.2	133133	336801	0.395	6.73
5	120.1	89533	166211	0.539	6.70
Average	**120.3**	**174966**	**434599**	**0.422**	**6.67**
SD	**0.8**	**60170**	**179802**	**0.070**	**0.05**
CV %	**0.7**	**34.4**	**41.4**	**16.5**	**0.7**

Table 15D

11-KT					
Cal B (15nmol/L)	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	16.2	24053	554391	0.043	6.58
2	18.6	28858	573501	0.050	6.58
3	15.1	18795	435592	0.043	6.61
4	14.8	18583	502968	0.037	6.58
5	15.0	12342	162107	0.076	6.56
Average	**15.9**	**20526**	**445712**	**0.050**	**6.58**
SD	**1.6**	**6237**	**167297**	**0.015**	**0.02**
CV %	**9.9**	**30.4**	**37.5**	**30.8**	**0.3**
Cal C (30nmol/L)	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	30.4	43739	561814	0.078	6.57
2	30.1	46472	600197	0.077	6.58
3	33.5	43333	447307	0.097	6.58
4	31.5	43699	586932	0.074	6.59
5	29.8	25633	169550	0.151	6.57
Average	**31.1**	**40575**	**473160**	**0.095**	**6.58**
SD	**1.5**	**8447**	**180131**	**0.032**	**0.01**
CV %	**4.8**	**20.8**	**38.1**	**33.9**	**0.1**
Cal D (60nmol/L)	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	60.6	86472	573026	0.151	6.57
2	59.3	82043	561098	0.146	6.60
3	53.8	74485	476872	0.156	6.56
4	61.6	53693	377999	0.142	6.58
5	60.5	58823	192048	0.306	6.57
Average	**59.2**	**71103**	**436209**	**0.180**	**6.58**
SD	**3.1**	**14329**	**157300**	**0.071**	**0.02**
CV %	**5.3**	**20.2**	**36.1**	**39.1**	**0.2**
Cal E (120nmol/L)	**Calculated Conc (nmol/L)**	**Peak Area**	**Int Std Peak Area**	**Response Ratio**	**Retention Time**
1	119.4	169494	577671	0.293	6.59
2	119.9	167763	580502	0.289	6.60
3	122.2	182526	511810	0.357	6.57
4	118.9	91140	336801	0.271	6.59
5	119.8	100657	166211	0.606	6.63
Average	**120.0**	**142316**	**434599**	**0.363**	**6.60**
SD	**1.3**	**42888**	**179802**	**0.140**	**0.02**
CV %	**1.1**	**30.1**	**41.4**	**38.4**	**0.3**

Note. 11-KA4 did not have measurable peaks in all experiments, hence there is less recorded data for this compound than the others.

Peak areas for the internal standard were monitored and analysed across four experiments, as seen in Table 16 below, to determine stability and suitability for monitoring sample extraction, machine status, and calculating concentrations.

Table 16

Internal standard imprecision

Peak area					
569149	531021	473883	369399	506744	
554391	553571	443473	502968	479860	
561814	545784	533605	586932	534220	
573026	561217	541204	377999	532833	
577671	546195	583225	336801	559206	
564298	553734	405723	472676	531651	
495345	549882	435592	584121	493234	
553335	516252	447307	563768	546934	
575009	573501	476872	481458		
572556	600197	511810	503564	**522979**	**Average**
554195	561098	503549	570383	**57759**	**SD**
547970	580502	530467	493647	**11.0**	**CV %**

The percentage CV was 11% across the batches monitored, indicating its stability and suitability as a choice for internal standard.

4.2 Linearity

CLSI C62-A, (2014) guidelines recommend 9-11 points to evaluate linearity using 2-4 replicates. However, for the purpose of this validation, 5 points were chosen across the desired measuring range and tested using stock standard made up to the desired concentrations and performed in duplicate. The results were averaged and are shown in the graphs below using polynomial regression. Polynomial regression, like linear regression, is a type of regression that looks at the relationship between the two variables, and the best way to draw a line through the data points but does not require there to be a linear relationship.

Figure 9A-D

Linearity assessed using the stock standard

Figure 9A: 11-OHA4 Linearity

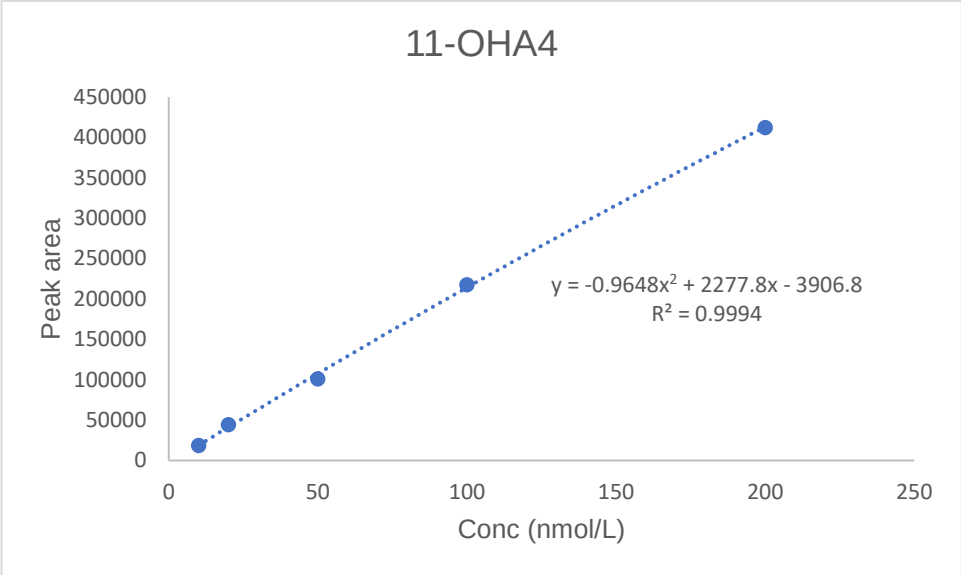


Figure 9B: 11-KA4 Linearity

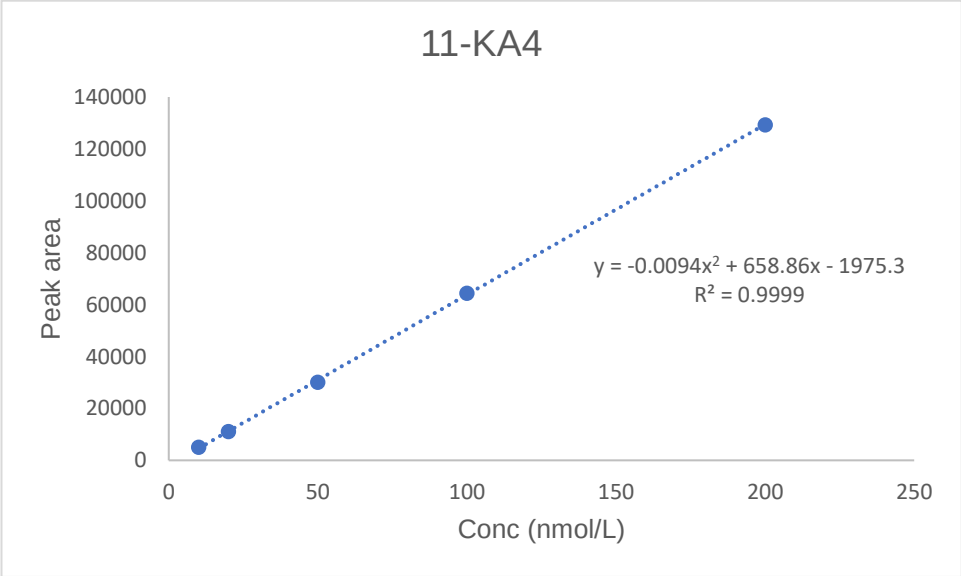


Figure 9C: 11-OHT Linearity

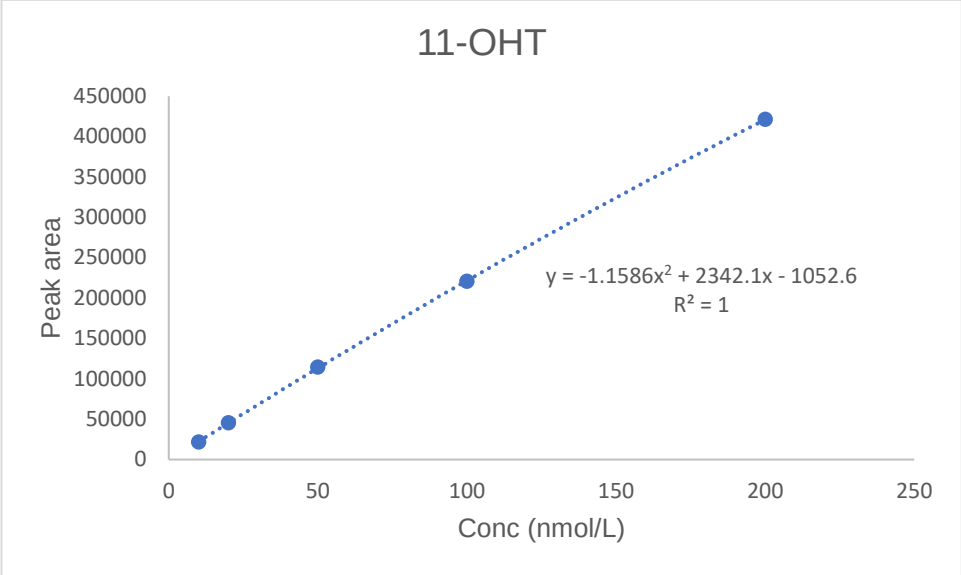
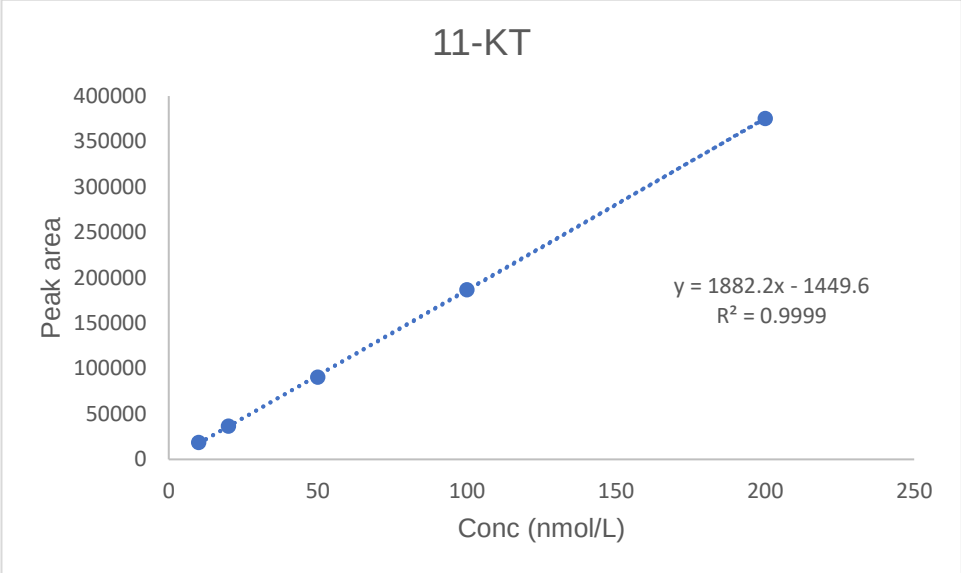


Figure 9D: 11-KT Linearity



The R^2 value looks at the scatter of the points around the regression line, or in other words how well the line fits the data and is represented as a value between 0-1. In this experiment, all R^2 values were 0.99-1.0 indicating a good fit of the data around the regression line.

Linearity was also demonstrated by obtaining calibration curves using the extracted calibrator material from dried blood spots, as shown in Figures 10A-D.

Figure 10A-D

Calibration curves for each compound

Figure 10A: 11-OHA4 Calibration curve

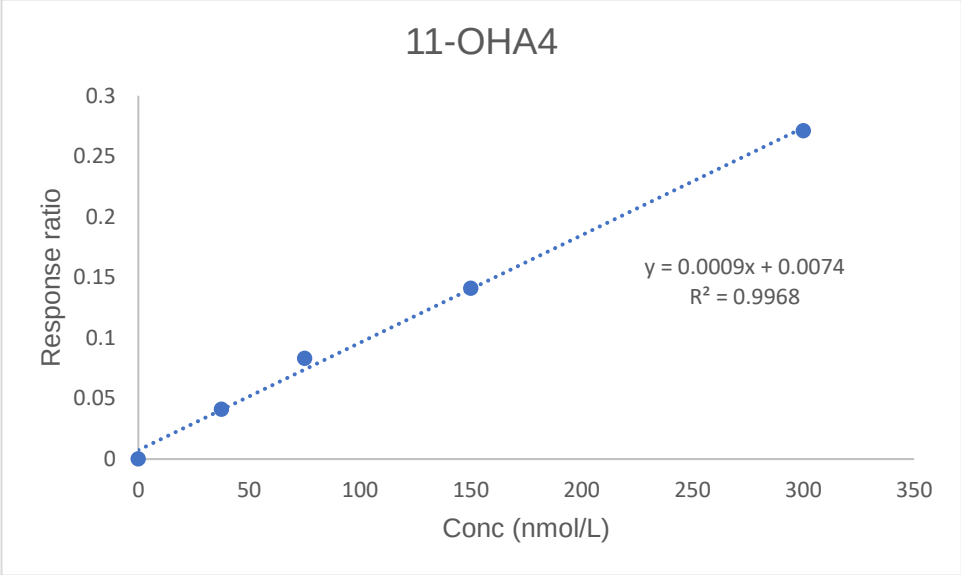


Figure 10B: 11-KA4 Calibration curve

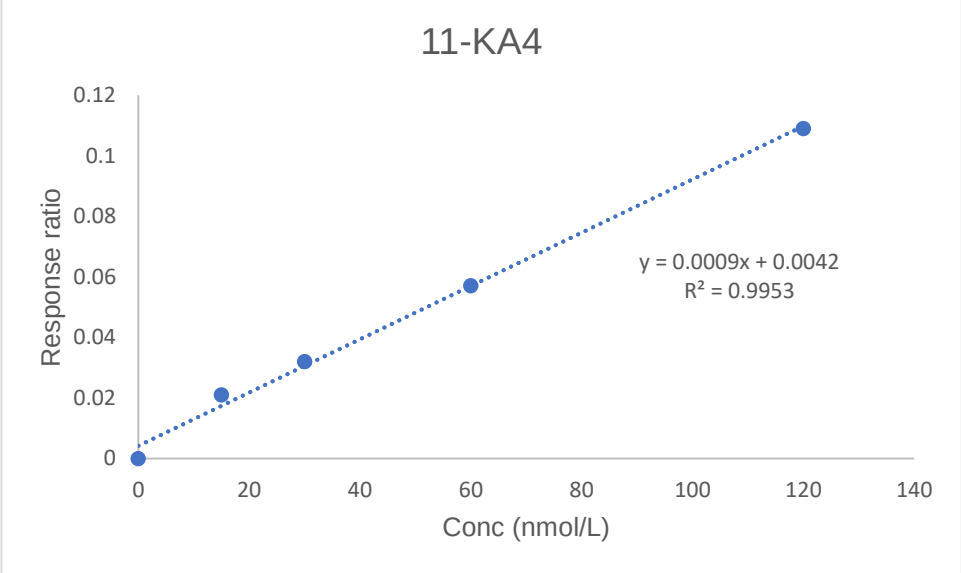


Figure 10C: 11-OHT Calibration curve

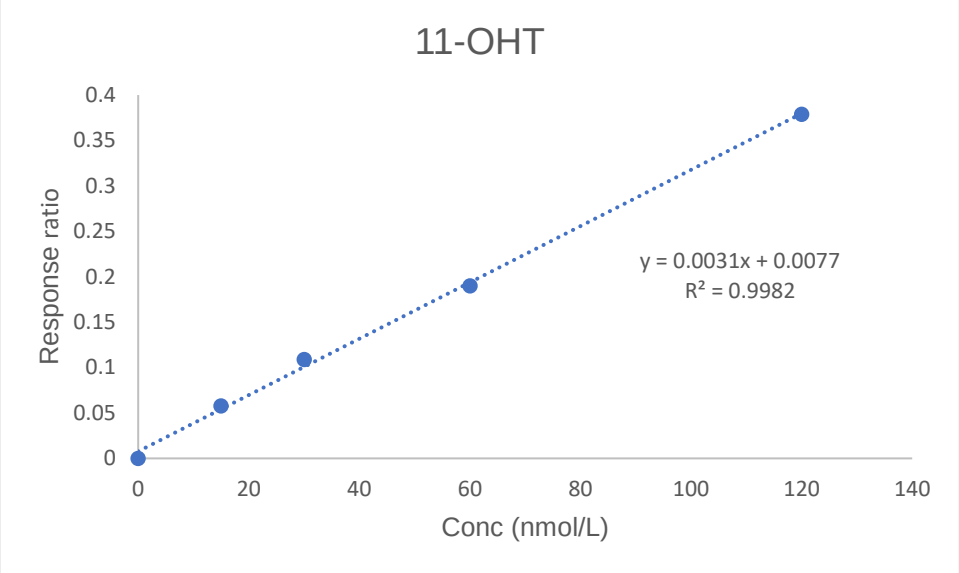
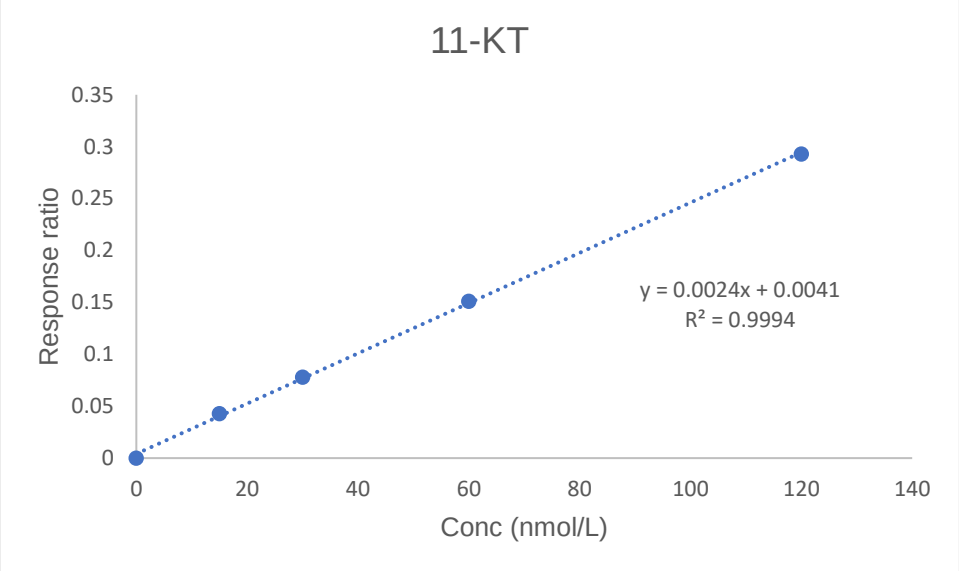


Figure 10D: 11-KT Calibration curve



Note: As linearity had already been demonstrated using the stock standard, the calibration curves were generated using linear regression. Once again, all R^2 values are >0.99 demonstrating excellent linearity of extracted calibrators.

4.3 Interferences

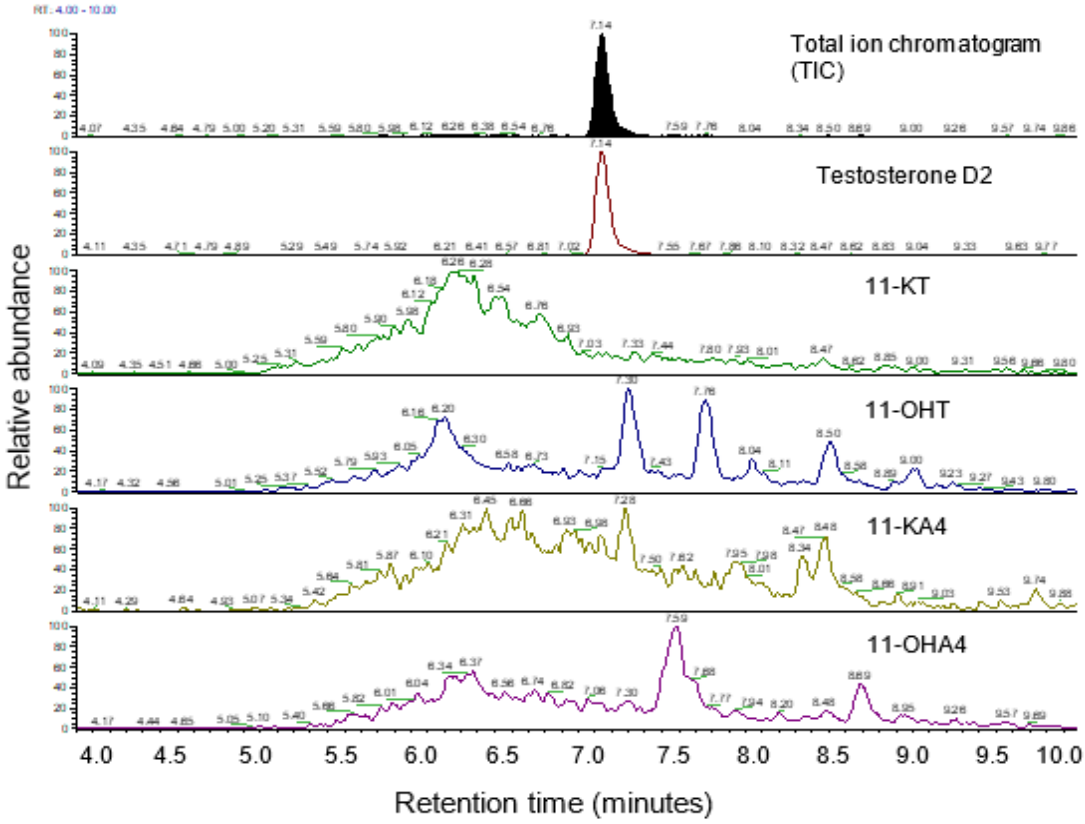
Although the use of mass spectrometry reduces the chance of interferences due to the use of specific molecular weights and fragmentation patterns, the method should still be assessed for potential interferences. This is particularly the case when analysing steroids, many of which have the same molecular weights and are present in blood spots. Using different product ions for identification, and separation of compounds on

the chromatographic column are some of the ways you can work around these interferences.

For this method validation a high level of laboratory control material was available containing some common steroids: 17-hydroxyprogesterone, cortisol, 11-deoxycortisol, 21-deoxycortisol, and androstenedione. The control material was processed with the same extraction as the samples and analysed in duplicate using the finalised LCMS method. There were no peaks seen at the retention times of the compounds of interest, meaning no interference from these compounds, and this is demonstrated in Figure 11 below.

Figure 11

Interference chromatogram



Note: The chromatogram is a snapshot of mass spectral analysis between 4 – 10 minutes. The relative abundance is a representation of the signal obtained by the mass spectrometer. The four 11-oxygenated steroids are all seen at a retention time of 6.58 – 6.67 minutes and there are no quantifiable peaks present in the chromatogram during this period.

There is still further opportunity to investigate potential interferences from other compounds that were not available as part of this project e.g., drugs or other steroids.

The ion ratios discussed in section 3.1 are another way of identifying whether an interference is present in your sample. Recommendations from CLSI C62-A, (2014) suggest that the ratio of product ions seen in samples should be within ± 20% of the average ion ratios seen across the calibration set run with the samples. Indicating another reason that it is important for the accuracy of your results to run a calibrator set with each batch of samples.

4.4 Carryover

As LCMS is a system with a continual flow of liquid, versus a random-access automated instrument, there is potential for carryover from compound getting stuck in the system either at the point of introduction (injection using the autosampler), or on the path through the HPLC column to the mass spectrometer. The LCMS instrument is set up to use two different wash solutions to clean out the syringe and injection port between samples to minimise the risk of carryover; three wash cycles are performed with wash solution A: 80% methanol, followed by two wash cycles with wash solution B: Acetonitrile: Isopropanol: Acetone in a ratio of 7:2:1, and a final wash cycle with the 80% methanol to flush out the strong solvents from wash B.

The CLSI guidelines discuss using a double blank to check for carryover. This is the analysis of two blank samples that have gone through the same sample preparation process, but do not contain the compounds of interest or the internal standard and, are analysed consecutively after a high sample. In this case the experiment was performed by extracting and running two blank samples after the high calibrator, and results can be seen in Table 17 and Figure 12 below.

Table 17

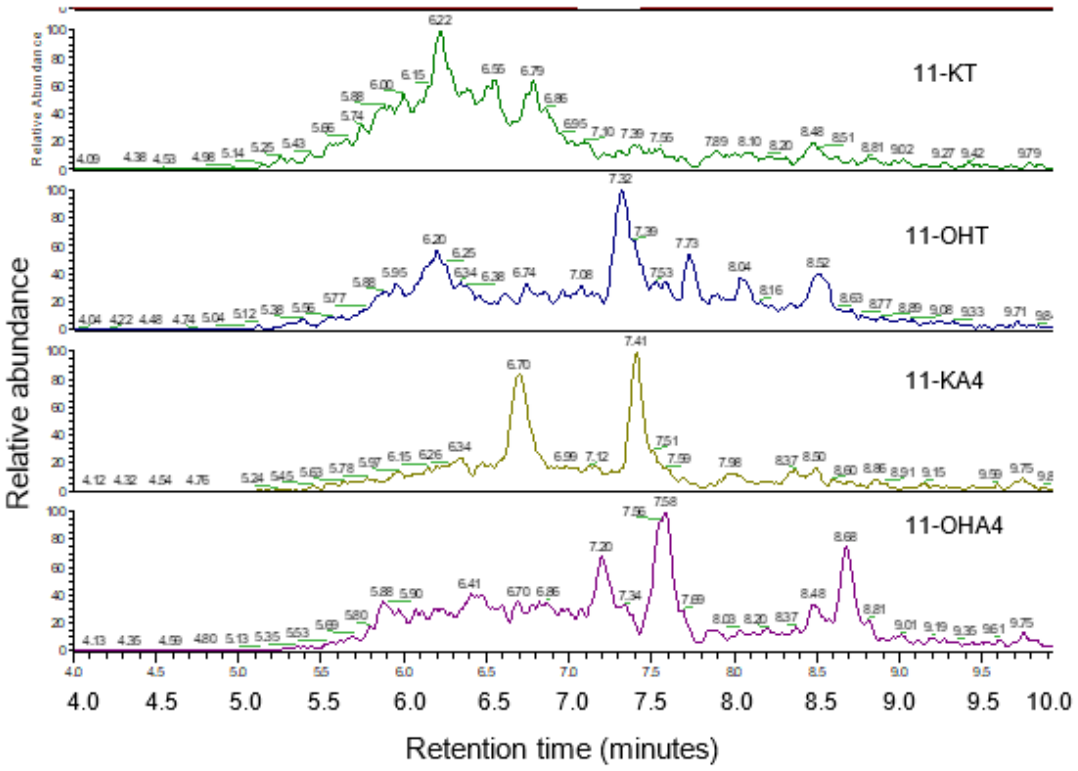
Carryover results

11-KA4	**Peak Area**	**Calculated Conc (nmol/L)**	**11-KT**	**Peak Area**	**Calculated Conc (nmol/L)**
High Calibrator	59151	119.0	**High Calibrator**	167763	119.9
Blk	N/F	N/F	**Blk**	N/F	N/F
Blk1	N/F	N/F	**Blk1**	N/F	N/F
11-OHA4	**Peak Area**	**Calculated Conc (nmol/L)**	**11-OHT**	**Peak Area**	**Calculated Conc (nmol/L)**
High Calibrator	155039	312.5	**High Calibrator**	212750	120.2
Blk	N/F	N/F	**Blk**	N/F	N/F
Blk1	N/F	N/F	**Blk1**	N/F	N/F

Key: N/F = Not found

Figure 12

Carryover chromatogram



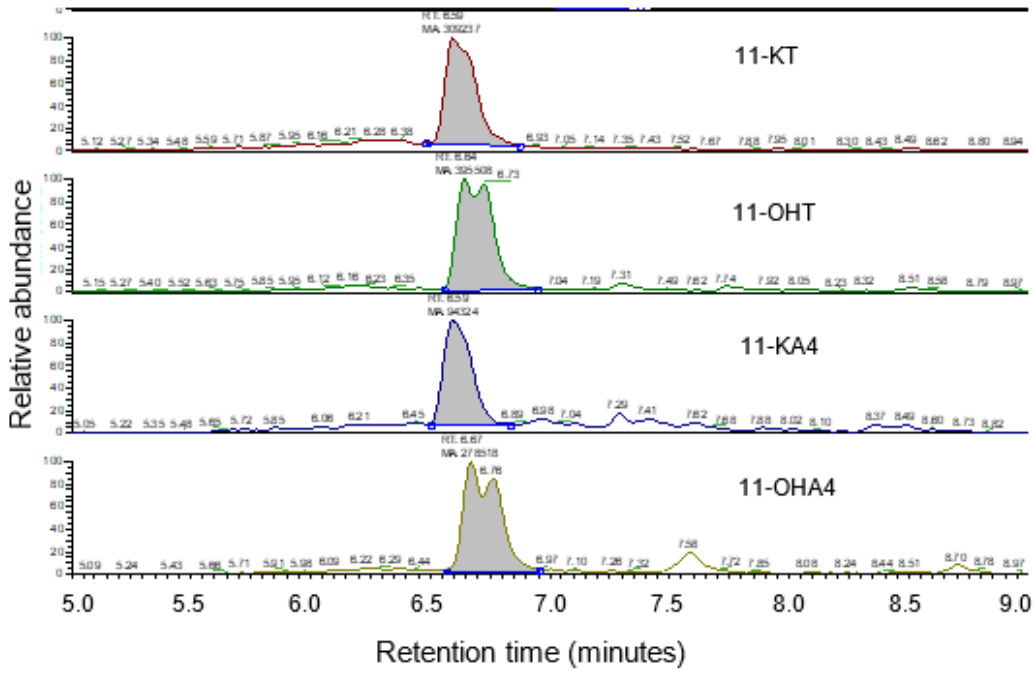
Note: The chromatogram is a snapshot of mass spectral analysis between 4 – 10 minutes. The relative abundance is a representation of the signal obtained by the mass spectrometer. The four 11-oxygenated steroids are all seen at a retention time of 6.58 – 6.67 minutes and there are no quantifiable peaks present in the chromatogram during this period, in either of the blank samples run after the high calibrator. Therefore, it was concluded that the wash settings in the autosampler method and column method were sufficient. However, it would also be good to confirm this after running some abnormally high patient samples.

4.5 Chromatogram and Retention Times

Figure 13 below shows an example of a final chromatogram and the compounds retention times using an extracted high calibrator.

Figure 13

Final chromatogram of high calibrator



Note: The chromatogram is a snapshot of mass spectral analysis between 5 – 9 minutes from an extracted calibrator. The relative abundance is a representation of the signal obtained by the mass spectrometer. The four 11-oxygenated steroids are seen at retention times of 6.58 – 6.67 minutes.

The retention times of the four compounds are overlapping, however because derivatised compounds were used, different ions are being monitored for each compound and therefore chromatographic separation becomes less of a priority. If you are trying to measure compounds with the same ion transitions, then it becomes important to use chromatographic conditions such as column chemistry, mobile phase, and temperature, to try and separate your compounds from each other to avoid interference.

The derivatisation process changes the three-dimensional structure of the steroids and depending at which point the hydroxylamine group attaches to the steroid, you can have one, two, or three resulting peaks seen on your stationary phase (column). These steroids were all observed as double peaks merged, and therefore the total combined peak area was used in the calculations.

Chapter 5

5. Discussion

The purpose of this research was to determine firstly if the 11-oxygenated steroids can be extracted and analysed from dried blood spot samples, and to discuss whether this had the potential to provide further clinical information for CAH patients undergoing glucocorticoid therapy.

5.1 First Research Aim

"Can 11-oxygenated steroids be reliably measured in dried blood spots?"

- Develop a method for extracting these steroids from dried blood spots, and analysing levels by liquid chromatography tandem mass spectrometry (LCMSMS)
    - Use reference steroids to determine LCMSMS method parameters.
    - Prepare in-house blood spot calibrators using washed packed red cells and reference standards and use these to determine whether all four steroids are measurable in blood spots. Data will only be collected for steroids at measurable levels that can give relevant clinical information.

This question was answered throughout the method development and validation stages. The project has demonstrated firstly that the 11-oxygenated steroids can be successfully extracted from blood spots. Good linearity and precision have been shown across all four compounds of interest. However, 11-KA4 has not performed as reliably as the other three compounds and in some instances was not measurable, as seen in Table 15B. Further work would be required to determine whether this was due to inefficient extraction of it from the blood spot sample, or whether the LCMS method requires improvement. This project did not attempt any other forms of sample preparation and extraction, therefore perhaps a more intricate technique, such as solid phase extraction, would assist in obtaining a higher quality sample for these low concentration steroids.

In saying that however, the emphasis in the literature (Turcu et al., 2018) is on measurement of 11-KT and 11-OHA4, due to their higher levels of androgenic activity, and higher concentrations present in blood. Therefore, a valid argument could potentially be made for the method to be useful even without the measurement of 11-KA4.

Secondly, the project has shown that these compounds can be successfully measured using liquid chromatography tandem mass spectrometry. All four compounds of interest

were successfully retained on the column, ionised, and detected. Except, as mentioned previously, 11-KA4 was less reproducible than the other steroids. The method showed no signs of interference or carryover and had a run time of approximately ten minutes to detect all four analytes. Retention time is also important to monitor if you are concerned about interferences from similar compounds. CLSI C62-A, (2014) recommends variation of <2.5% for retention time shift and this has been achieved across the validation. Stable retention times are also a good indication of column and pump performance. If a chromatography column is becoming blocked or damaged, is near the end of its life, or a pump is not delivering the expected mobile phase due to a blockage or seal issue, then your peak areas and retention time would highlight these problems.

5.2 Second Research Aim

"Discuss whether measurement of these potent androgens can help inform clinical decisions in CAH treatment."

This research area cannot be fully answered without the analysis of patient samples; however, the project demonstrates that the potential is there for these steroids to be utilised as part of CAH treatment monitoring. In New Zealand these blood spot samples are already being collected from monitored patients to check their 17-OHP levels, therefore the addition of these analytes that can be measured on the same sample could be beneficial. Literature has already shown that 17-OHP is not a good marker for measuring androgen control as it is not an androgen itself (Honour, 2014). These 11-oxygenated steroids have been shown to have androgenic activity, and, as androgen excess is one of the primary problems for these CAH patients, these markers have the potential to provide clinical information that could allow for treatment to be tweaked in a way that can give these patients better quality of life, possibly before they even start to develop serious side effects as a result of androgen excess.

One of the biggest limitations to answering this question, or any time that new compounds of interest are identified, is the lack of clinically useful reference ranges available. Generally, hundreds of patient samples would need to be analysed to determine these reference ranges, and additionally, to be able to successfully develop reference ranges, non-diseased samples are required.

5.3 Study Limitations

Unfortunately, there were several limitations to the study design that should be discussed. Accuracy of results is an important aspect of laboratory method validation,

to confirm true values. However, it has not been assessed in this project for several reasons. Firstly, because these are new compounds there was no available commercial quality control material, external quality assurance material with set values that is used to assess a laboratory's performance of a test, or inter-laboratory comparisons, as no one else in New Zealand is performing this testing. Additionally, there was only one source of standard material available for the evaluation, consequently this was used to prepare the calibrator and control material. Potentially this means there is an increased chance of error – if there is a problem with the concentration of the reference standard material it will not be identified throughout the validation as there is no external checks on the concentrations. This is something that would most definitely require further validation work prior to the method being used in a patient scenario.

Calibrator values were set at the desired levels to test this as potential method, but in practice it is likely that there will be lower concentrations of calibrators required. The sensitivity of the method may need to be improved to be able to detect these. This could be done by improving the sample extraction technique, using more sample, further optimisation of the LCMS method, or use of a more sensitive LCMS instrument. Because of the lack of suitable sensitivity in the test method, there was also no limit of quantitation studies performed. This would be essential to determine the reporting limit of a method for patient testing.

LCMS method development would also normally include testing the extraction efficiency, or recovery of analytes. This is because, compared with neat standard solutions used for method development, patient samples will contain a variety of other compounds such as proteins, lipids, and drugs that may affect the extraction process. This is usually tested by comparing samples that are spiked with the compounds of interest prior to extraction, with samples that are spiked post the extraction process. However, because this method was extracting analytes from dried blood spots, this type of experiment could not be performed.

Another parameter that ideally should be evaluated during LCMS method development, is checking for ion suppression/enhancement. The usual methodology to perform this type of experiment is to use infusion of the compound of interest directly into the mass spectrometer, whilst simultaneously injecting a blank sample that has been through the extraction process (Salter et al., 2015). Following this a check for any change in signal at the retention time of the compound of interest as this could indicate either suppression or enhancement. Unfortunately, this type of experiment did not work on

the Thermo Fisher Vantage LCMS, so the precision results must be relied upon to assume a lack of significant ion suppression or enhancement.
To ensure the robustness of the method, the inter-batch precision experiments could be carried on for a longer period. Ideally for at least 21 batches, but unfortunately the instrument availability was a limiting factor here. The last few experiments were run during a period when the mass spectrometer was having issues with the vacuum pump. This led to a decrease in sensitivity and smaller peak areas for all compounds. This in turn has affected the percentage CVs for the inter-batch imprecision monitoring. However, except for calibrator B for 11-KA4, the percentage CV for the calculated concentration of all calibrators and controls for all compounds is still less than 15%, as shown in Tables 14 A-D and Tables 15 A-D. This indicates that there is still good assay reproducibility, regardless of instrument issues, providing there is a calibration run with each batch that is used to calculate the concentrations of samples in that batch. The internal standard areas were also affected by this issue and when including the batches with sensitivity issues due to the instrument vacuum, the CV percentage increases from 11% (as shown in Table 16) to 32% variation across the experiments. However, when assessing those low sensitivity batches on their own, the CV percentage is 6% (data not shown), confirming that it is still a stable compound and can be used for calculation and assessment of batches, again provided there is a calibration included with the batch.

A further consideration when working with dried blood spot samples, is the effect that the quality of these samples can have on measurement. George and Moat (2016) looked at this in detail for newborn screening dried blood spot samples and found that there are several factors which can have an impact on results. Firstly, the size of the blood spot that is spotted onto the card is significant. They identified that spots should be ≥50 µL as smaller volumes than this can lower the results significantly. 75 µL was used during preparation of the calibrators and controls for this method, which should be enough, however care needs to be taken when analysing patient samples. Secondly, they investigated the effect on concentration that the location of the punch can have and suggested that a central location for the punching of the blood spot is best. This recommendation was followed throughout the course of the validation for punching of calibrator and control samples, but it can have an impact on the availability of sample when they are coming from patients who are unwell and are having to regularly provide blood samples. Finally, they looked at the factors that can affect quality of the sample, such as double layering of the blood (particularly if left to dry in between), applying blood to both sides of the card, multi-spotting (again, particularly an issue if the spots are drying in between), and compressing the sample after applying it in order to make it

spread further across the spot area. The most significant finding was compression of the sample, and they noted that this can result in falsely low measurements and therefore the potential for missing a diagnosis or referral.

And finally, as mentioned in section 5.2, the lack of real patient samples is a drawback for this piece of research. These will be required for full validation of the method to establish suitable reference ranges in a control group, determine what values are seen in patients with CAH, and how these values change across the day, and at different stages of treatment, and lastly to confirm that the chosen sample extraction and LCMS method is suitable for real patient samples.

5.4 Future Direction

As discussed by Turcu and Auchus (2017), there are other factors that should be looked at when considering the suitability of these analytes for use in treatment monitoring of CAH patients.

"Do these 11oxy steroids have a circadian rhythm?" (Turcu & Auchus, 2017, p.258)
We know that adrenal steroids show a pattern of circadian rhythm, and therefore this must be considered when collecting samples from patients and guidelines should be provided for what time the samples should be collected, with careful recording of what time they are collected (Kamrath et al., 2017). A recent study by Nowotny et al. (2021) used salivary steroid profiles to attempt to demonstrate the diurnal variation of these steroids. They measured two of the 11-oxygenated steroids in their profile, 11-OHA4 and 11-KT, and collected five samples across the day from CAH patients on steroid replacement therapy. Their findings indicated that the 11-oxygenated steroids showed a similar pattern to 17-OHP, with a peak in the morning, in the patient and control groups, followed by a steady decline over the course of the day. Interestingly they found that female patients with CAH had a slight rise in the 11-oxygenated steroids in the evening again, which was not seen in male patients although they were on similar medication schedules. This study supports the idea of a circadian rhythm however, it was a small-scale study and there should be further investigation into the circadian pattern of these 11-oxygenated steroids, and therefore consequently whether this will affect timing of sample collection and interpretation of data.

"What are their dynamic changes with glucocorticoid therapy as compared with established biomarkers?" (Turcu & Auchus, 2017, p.258)
We know that 17-OHP levels in blood are elevated when disease is present and uncontrolled, and that they should come back under control once patients have

achieved a steady state with their glucocorticoid therapy. Additionally, cortisol levels are very low in some patients prior to treatment, and this should also resolve itself when treatment is optimal. However, we currently don't have a full understanding of the effect that glucocorticoid treatment has on these 11-oxygenated steroids. How long does it take them to respond to treatment? Do they all respond at the same level? Are they more sensitive than traditional biomarkers such as 17-OHP, cortisol and androstenedione in the fact that they may respond more quickly to treatment or, may rise more quickly in cases of under-treatment?
Balsamo et al. (2010) have shown a strong relationship between 17-OHP levels and severe genetic mutations, so there may also be future opportunities to explore whether any of the other biomarkers show correlation with the underlying genetic mutation.

"How do they correlate with clinical evidence of androgen excess?" (Turcu & Auchus, 2017, p.258)
To answer this question would require a greater level of clinical information concerning CAH patients, to involve them in a research study that looks at all aspects of their disease. What is their age and biological sex, when were they were diagnosed, what symptoms did they have at the time, when did treatment start and what dose of glucocorticoid was required, did symptoms resolve after treatment started, and, most importantly for this question, what were the levels of 11-oxygenated steroids when they had symptoms versus the levels when the disease was under control.

As discussed, the primary purpose of this method is to assist in improving treatment outcomes for congenital adrenal hyperplasia patients on steroid therapy. However, there are also several other areas in which measurement of these steroids could potentially be of help.

Firstly, there are further medical conditions where these results could be used to assist with diagnosis or treatment. The primary one being in patients with polycystic ovary syndrome (PCOS). This is a common ovarian disorder affecting women of reproductive age, that can present itself in a variety of different ways, and therefore is often referred to as a group of disorders (Pretorius et al., 2017).
Diagnostic criteria are ovarian dysfunction and biochemical or clinical evidence of hyperandrogenism (excess androgens), with or without polycystic appearance of the ovaries (Turcu et al., 2018). The ovaries have historically been considered the primary source for the androgen excess, however studies going back to the 1990's have shown that 11-OHA4 is also elevated in these patients and there is a growing body of evidence to support the idea that adrenal androgens may also contribute to the excess

level of androgens, as other adrenal androgens, such as DHEA, have been seen to be elevated in these patients (Turcu et al., 2018) (Keevil, 2019). These patients are also at risk for metabolic complications, and a review by Turcu et al. (2018) talked about a study showing correlation between 11-OHA4 and 11-KA4, and markers of metabolic risk such as BMI and insulin levels. It is still in its infancy, but the potential is there for these 11-oxygenated steroids to assist with diagnosis of PCOS, monitoring of these patients, and the ability to distinguish between different forms of PCOS (Pretorius et al., 2017).

A further medical area where measurement of these steroids shows promise is in the evaluation of prostate cancer, particularly when it has become castration resistant. Prostate cancer is androgen driven, and androgen dependent, and therefore surgical or chemical castration is a common treatment. However, the cancer can sometimes re-emerge and therefore reduction of circulating androgen levels will be an important consideration in treatment. Testosterone is an obvious one, but 11-KT and 11-OHT have also been implicated as active androgens of potential concern (Pretorius et al., 2017).
Correlation has also been shown between the 11-oxygenated steroids, and CAH patients who develop complications such as increased adrenal volume (overactive adrenals) and testicular adrenal rest tumours (TART). These are generally patients who have poor disease control or overtreatment, therefore the addition of these markers in treatment monitoring could potentially be beneficial in preventing these outcomes. Additionally, the sex steroids and steroid treatment play a part in growth, therefore, for those patients who start treatment during their adolescent years, the monitoring of treatment is critical (Turcu, Mallappa, et al., 2017).

A recent case study by Kitamura et al. (2022) discussed a patient with a rare case of an adrenocortical adenoma causing hyperandrogenism. Steroid profiling and genetic analysis both played a large role in the diagnosis and follow-up of this patient. The patient was female and presented with hypertension and menstrual irregularities but had no cushingoid features or hirsutism. The adrenal tumour was surgically removed, and pre-operative and post-operative levels of 11-oxygenated steroids showed a substantial decrease after surgery, and her menstrual cycle returned to normal indicating effects from androgen excess were playing a role in her symptoms. Genetic analysis of the tumour showed presence of CYP enzymes required for cortisol synthesis, and for conversion of androstenedione and testosterone into 11-OHA4 and 11-OHT. This ability to produce androgens is something that is rarely seen in adrenal

tumours, and the extended steroid profiles may assist with understanding in these rare cases.

Another interesting case involving 11-oxygenated steroids was discussed by Nagasaki et al. (2020). A female infant presented with virilisation, but newborn screening for CAH was negative. The mother was discovered to have an adrenal tumour, with high serum levels of two of the 11-oxygenated steroids, 11-KT and 11-OHT, but normal T and A4. We know that testosterone and androstenedione can cross the placenta but are normally enzymatically converted to oestrogens. This study indicates that the 11-oxygenated steroids have passed through the placenta, not converted to oestrogens, and contributed to virilisation of the infant due to hyperandrogenism. This raises a new area of focus for these androgens and their pathogenicity that could be considered in further areas of research or clinical utility. There is a chance steroid treatment for the mother could have reduced the virilisation, however exposure of a foetus to steroids during development has not been widely studied, and treatment would be required from early in the pregnancy during the period of genitalia development. Sharma & Seth, (2014), and McCann-Crosby et al., (2018) also discuss steroid treatment during pregnancy, but for mothers who are known carriers of CAH. This is controversial, as the baby cannot be confirmed CAH until the end of the first trimester but is a potential area for future discussion.

Secondly, there is discussion around steroid analysis being used in doping cases where people may have taken endogenous steroids from exogenous sources, and further evidence is required around the impact it is having on their steroid levels and pathway. The traditional sample used in doping analysis has always been urine, due to the ease of collection and ability to detect metabolites in addition to parent drugs. However, the World Anti-Doping Agency (WADA) now accepts blood samples as a suitable sample type as well. Blood samples, paired with urine samples, can give a clearer indication of the impact on the athlete, as blood concentrations provide an accurate real-time picture. Blood spots, as mentioned previously, are an easier alternative to regular drawing of venous blood samples, and in 2021 WADA accepted these as suitable and published guidelines for their use (Brockbals et al., 2023).

Piper and Thevis (2023) have developed an LCMS method for the detection of 11-KT in urine as it is recognised as having anabolic properties, and is readily available for purchase over the internet, therefore implicating it for use in athlete doping scenarios. Kojima et al. (2016) have published a comparison between urine analysis and dried blood spot analysis in the doping world. They were looking specifically at ephedrine and methylephedrine, but the concept is still relevant. The conclusions drawn were that

blood analysis provides more information indicating whether doping agents were used during competition periods, or outside competition periods, and that the collection and storage of dried blood spots is much more convenient than urine.

However, one point to note is that as these 11-oxygenated steroids are produced endogenously, care must be taken in developing solid reference ranges to be able to confirm whether doping using these substances has or has not occurred.

In summary, there is a range of scenarios where this assay could be utilised, and it could be a useful expansion to the New Zealand laboratory testing service.

Chapter 6

6. Conclusion

The aim of this study was to determine whether new biomarkers, 11-KT, 11-OHT, 11-KA4, and 11-OHA4, could be extracted from dried blood spots and measured by LCMS. They are now recognised as active androgens in the adrenal steroid pathway and could be used as additional markers of treatment success in CAH patients undergoing glucocorticoid therapy. A successful sample extraction and LCMS method has been created for measurement of the 11-oxygenated steroids, however 11-KA4 gave less reliable results than the other three steroids. However, the potential is available for these compounds to provide another layer of clinical information regarding androgen control in CAH patients undergoing glucocorticoid therapy, and to therefore assist in improving their treatment outcomes. There is also a variety of other clinical scenarios where this analysis could prove useful, such as polycystic ovary syndrome, adrenal tumours, and the doping industry. However, the LCMS method would benefit from further improvements, as discussed, and obtaining patient samples is an essential step to performing a thorough method validation procedure and inform of clinical suitability.

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