Determination of eleven steroid hormones and bioavailable testosterone in human serum using salting-out assisted liquid-liquid extraction with UHPLC-MS/MS

Marliet Ramos

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Supervisors: Cato Brede, Ph.D. & Øyvind Skadberg, M.D.

University of Stavanger – Faculty of Science and Technology

Stavanger University Hospital – Department of Medical Biochemistry

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Acknowledgements

I was first introduced to the fascinating world of endocrinology combined with analytical chemistry during my BSc thesis at the University of Oslo. I initially thought that my work in these fields had ceased when I chose to move back to Stavanger. I cannot begin to express my deepest and most immense gratitude to Cato Brede for giving me the opportunity to spend the past year working on an incredibly exciting topic and, most especially, the chance to further expand my knowledge in bioanalytical chemistry. Not only has he been a wonderful supervisor, but also a great mentor and role model. I must also express my sincere appreciation to my supervisor Øyvind Skadberg and Grete Jonsson for their valuable contributions and insights into my work.

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It is my pleasure to present the work we have done during the past year for my MSc thesis. I hope that every single reader learns something new from my paper and hopefully begins associating steroid hormones with their essential biological functionality and not only sports doping scandals. Thank you again to everyone who has contributed to this work!

Table of contents

Abbreviations and definitions

11DF	11-deoxycortisol
11KT	11-ketotestosterone
11OHA4	11β-hydroxyandrostenedione
17OHP	17α-hydroxyprogesterone
21DF	21-deoxycortisol
A4	Androstenedione
ACN	Acetonitrile
ACTH	Adrenocorticotrophic hormone
AMS	Ammonium sulphate
B	Corticosterone
BT	Bioavailable testosterone; non-SHBG bound
CAH	Congenital adrenal hyperplasia
cFT	Calculated free testosterone
CMIA	Chemiluminescent magnetic microparticle immunoassay
CV	Coefficient of variation
DOC	Deoxycorticosterone
E	Cortisone
ED	Equilibrium dialysis
ESI (+)	Electrospray ionization in positive mode
F	Cortisol
FA	Formic acid
FAI	Free androgen index
FSH	Follicle-stimulating hormone
FT	Free testosterone; unbound testosterone
GnRH	Gonadotrophic releasing hormone
HPA	Hypothalamic-pituitary adrenal axis
HPG	Hypothalamic-pituitary gonadal axis
IA	Immunoassay
ISTD	Internal standard
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LDL	Low-density lipoprotein
LH	Luteinizing hormone
LLE	Liquid-liquid extraction
MeOH	Methanol
MP	Mobile phase
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NBCS	New-born calf serum
NH_4OH	Ammonium hydroxide
nM	Nanomolar, nmol/L
P	Progesterone
PCOS	Polycystic ovarian syndrome
RP	Reversed-phase; a separation principle in chromatography
SALLE	Salting-out assisted liquid-liquid extraction
sFT	Free testosterone in saliva; unbound to carrier proteins
SHBG	Sex-hormone binding globulin
SP	Stationary phase
T	Testosterone
TT	Total testosterone; protein-bound and free testosterone
UF	Ultrafiltration or centrifugal ultrafiltration
UPLC-MS/MS	Ultra-performance liquid chromatography with tandem mass spectrometry

List of analytes

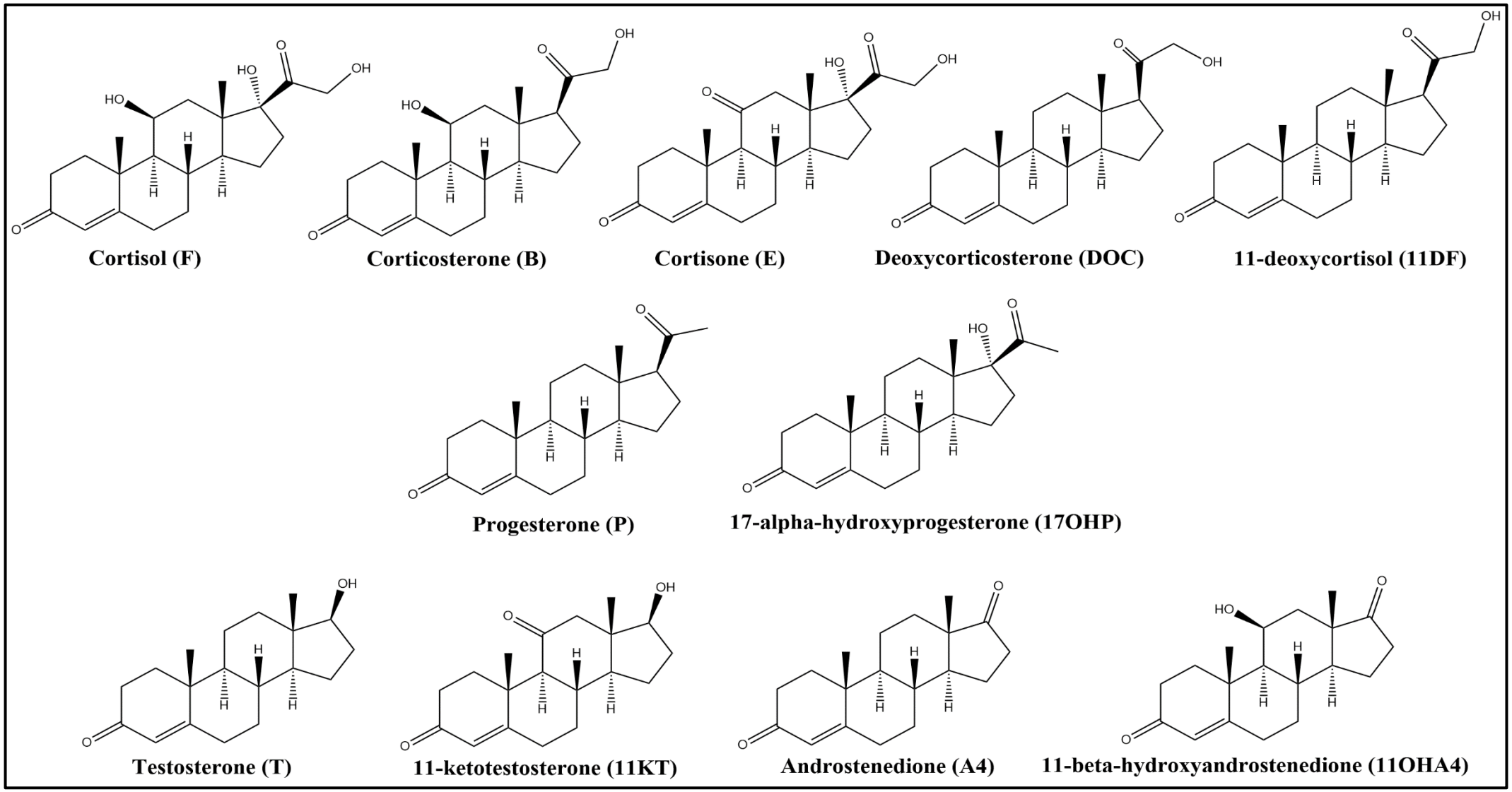


Figure 1: Overview of analytes with their molecular structure, name, and abbreviations used in parenthesis. Glucocorticoids grouped in the first row, progestogens in the second and androgens in the last row.

Definition of the total and bioavailable fraction of testosterone

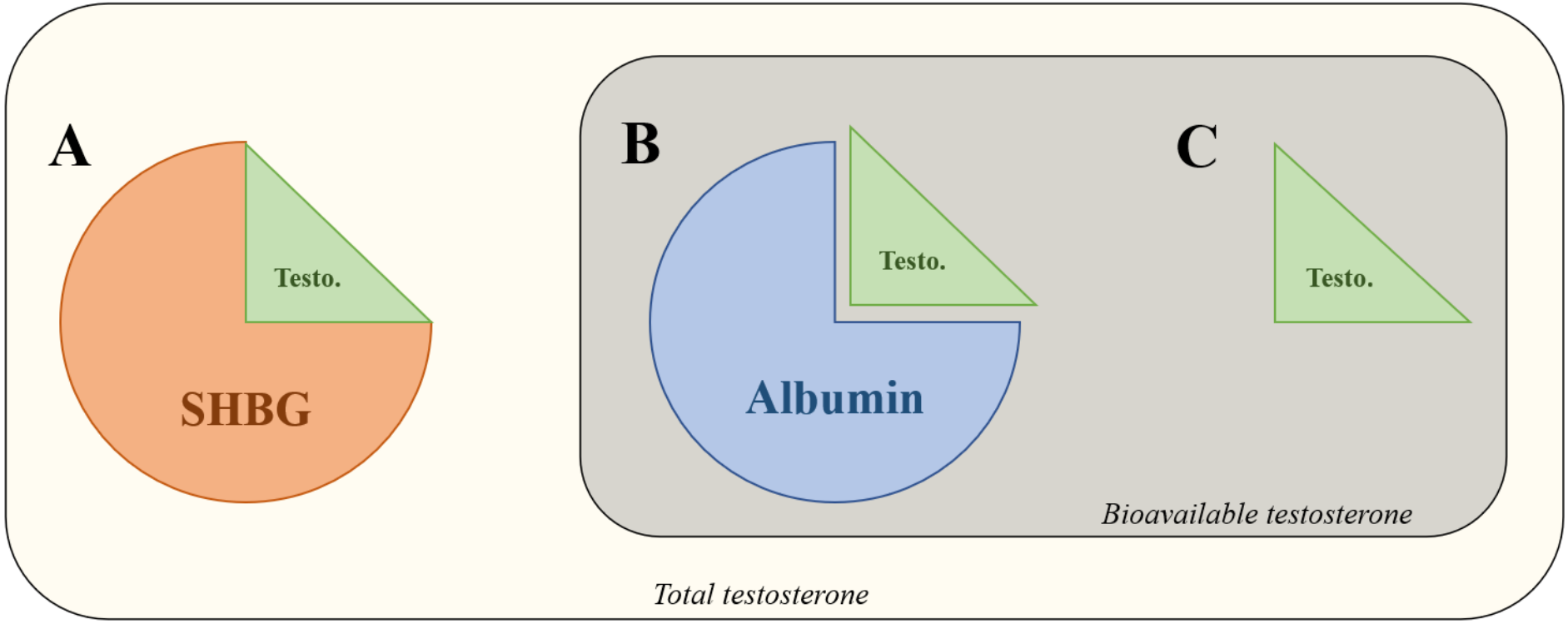


Figure 2: Simplified definition of total testosterone (TT) and bioavailable testosterone (BT). (**A**) Testosterone specifically bound to SHBG (SHBG-bound T) with high binding affinity. (**B**) Testosterone bound non-specifically to albumin with low binding affinity resulting in increased protein disassociation. (**C**) Free testosterone (FT) unbound to transport proteins and circulates freely. *Small fraction of T binds to various proteins such as orosomucoid and corticosteroid-binding globulin (not depicted in the figure).

1. Abstract

Steroid hormones are essential in various biological roles such as the regulation of sexual maturation, fertility, bone and muscle growth, stress-mediated responses, and inflammation, to name a few examples. Impaired steroidogenesis leads to anomalous steroid hormone concentrations and develops into endocrinological disorders, such as polycystic ovarian syndrome (PCOS) and hypogonadism, with physical and mental manifestations affecting life quality and possibly longevity. The detection of aberrant steroid concentrations is crucial for diagnosis and requires adequate biochemical assays to provide correct and reliable quantitation. Ultra-high-performance liquid chromatography (UHPLC-MS/MS) provides greater selectivity and sensitivity for endocrine analysis compared to modern direct immunoassays.

This study optimized and validated an UHPLC-MS/MS method using salting-out assisted liquid-liquid extraction (SALLE) for sample clean-up and analyte extraction of 11 steroid hormones from human serum. The analyte panel includes glucocorticoids (cortisol, cortisone, corticosterone, deoxycorticosterone, 11-deoxycortisol), progestogens (progesterone, 17α-hydroxyprogesterone) and androgens (total testosterone, bioavailable testosterone, androstenedione, 11-ketotestosterone, 11β-hydroxyandrostenedione). Additionally, the study involved the analysis of 38 sera samples from healthy individuals through the project "Blood Reference in Stavanger (BRIS)". The method exhibited its ability as a diagnostic tool by identifying three individuals with anomalous androgen concentrations, including the 11-oxygenated androgens, from the small population cohort studied.

The proposed multiplex method demonstrated high-throughput performance with a 2.67-minute cycle time per injection and a fully automated sample preparation with large sample capacity and high time and cost efficiency. Sensitive detection for all analytes was achieved with detection limits (LOD) and quantification limits (LOQ) at 0.01 – 0.12 nmol/L and 0.03 – 0.29 nmol/L, respectively. Satisfactory intermediate precision was found for total testosterone (6%), cortisol (11%), cortisone (4%), progesterone (5%), 17α-hydroxyprogesterone (7%), androstenedione (11%), and corticosterone (6%) in the upper range of endogenous hormone concentrations in real human serum. Additionally, the imprecision of total testosterone in the lower concentration range, corresponding to female endogenous levels, was 8%. Higher imprecisions were observed for deoxycorticosterone (23%), 11-deoxycortisol (16%), 11-

ketotestosterone (17%), 11β-hydroxyandrostenedione (11%) due to limitations in the endogenous levels of these steroid hormones, and bioavailable testosterone (21%).

The method can simultaneously determine 11 steroid hormones from one single analysis, enabling the exploration of hormone patterns and the intracrine relationship in endocrinological conditions. Multiplex steroid analysis by UHPLC-MS/MS is advantageous in the exclusion of differential diagnoses, replacing multiple single-analyte assays, and expanding the analyte panel to include other steroid hormones such as 21-deoxycortisol and oestrogens.

2. Introduction

2.1 Steroid hormones

2.1.1 Molecular structure and chemical properties

The importance of steroid hormones is evident when considering their biological functions in the human physiology. They affect fluid and salt retention, sexual maturation and fertility, muscle and bone growth and even mental health conditions, such as depression, to name a few examples (Brown, 2008; Lösel & Wehling, 2003; Stetler & Miller, 2011; Wehling, 1997). Steroid hormones, alike other classification of hormones, are considered signalling molecules responsible for inducing biochemical responses upon internal and external triggers to regulate the homeostasis in the human body through genomic and non-genomic actions (Wilkenfeld et al., 2018). The collective characteristic of steroid hormones is their closely related chemical structure with a fused ring system consisting of three cyclohexenes and one cyclopentane derived from cholesterol (**Figure 3**).

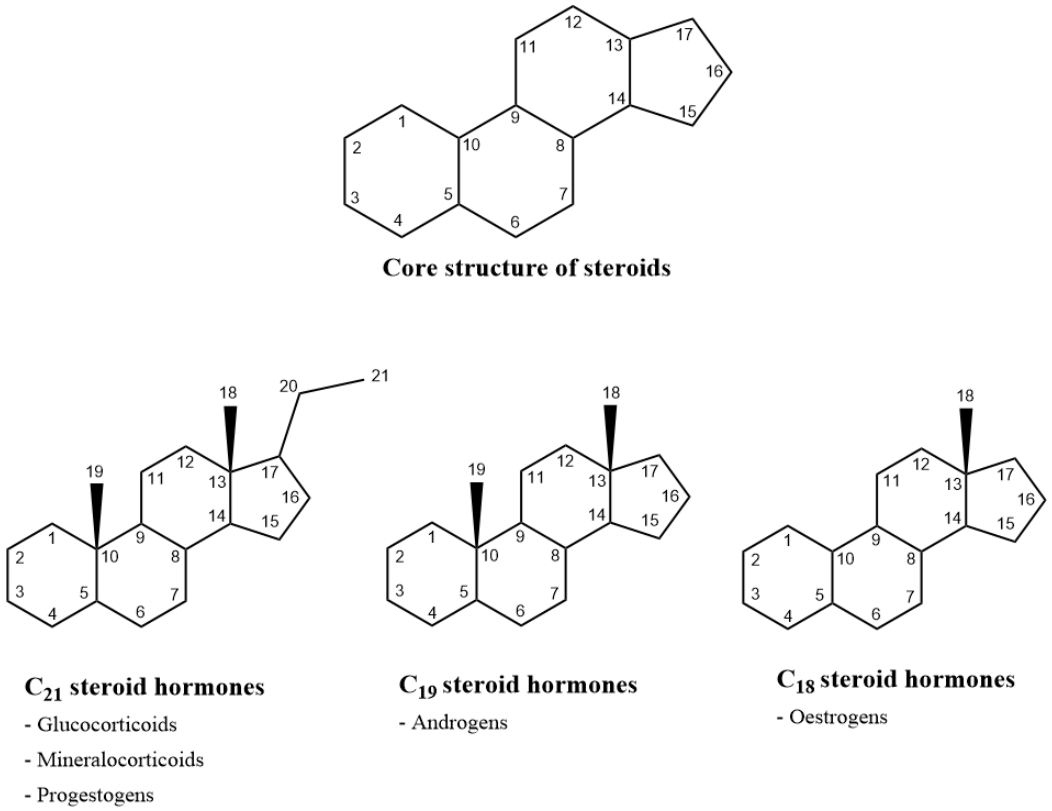


Figure 3: The general core structure of steroids and the molecular structures of steroid hormone classes. Steroid hormones belong to a single class of hormones based on their general steroidal core structure differing from peptide and amino-acid derived hormones. Illustration modified from Jeanneret et al. (2016).

Steroid hormones differ from peptide and amino acid-derived hormones due to their non-soluble steroidal core structure derived from cholesterol, thus making them lipid soluble with the ability to diffuse across the cell membrane. The hormone receptors responsible for inducing genomic effects are located intracellularly and binds to the bioactive fraction of the steroid hormones upon cell diffusion, then further undergoes transcription to ultimately express hormone-induced genes (Beato & Klug, 2000). Moreover, varying functional groups attached to the core steroidal skeleton results in different polarities of the steroid hormones although they are generally classified as lipophilic compounds. The specific binding of steroid hormones to respective hormone receptors is partly due to their varying degree of polarity (Beato et al., 1995; Whitfield et al., 1999). Their chemical properties of steroid hormones is particularly important in biochemical assays such as liquid chromatography for their separation, determination and quantification (French, 2016).

2.1.2 The hypothalamic-pituitary complex

The activation and regulation of the steroid hormone biosynthesis is governed by the hypothalamic-pituitary (HP) complex in the brain. The secretion of steroid hormones from the endocrine glands activated by the HP complex functions as responses to external and internal triggers (Tsigos & Chrousos, 2002). The interactions between the HP complex and the endocrine glands can be described as a signalling cascade with feedback mechanisms promoting the inhibition or stimulation of steroid hormone biosynthesis (Chrousos, 1992). Steroidogenesis can have origins from the adrenal glands or the gonads through the hypothalamic-pituitary adrenal (HPA) axis and the hypothalamic-pituitary gonadal (HPG) axis, respectively. An illustrative description of the HPA- and HPG-axes are given in **Figure 4**.

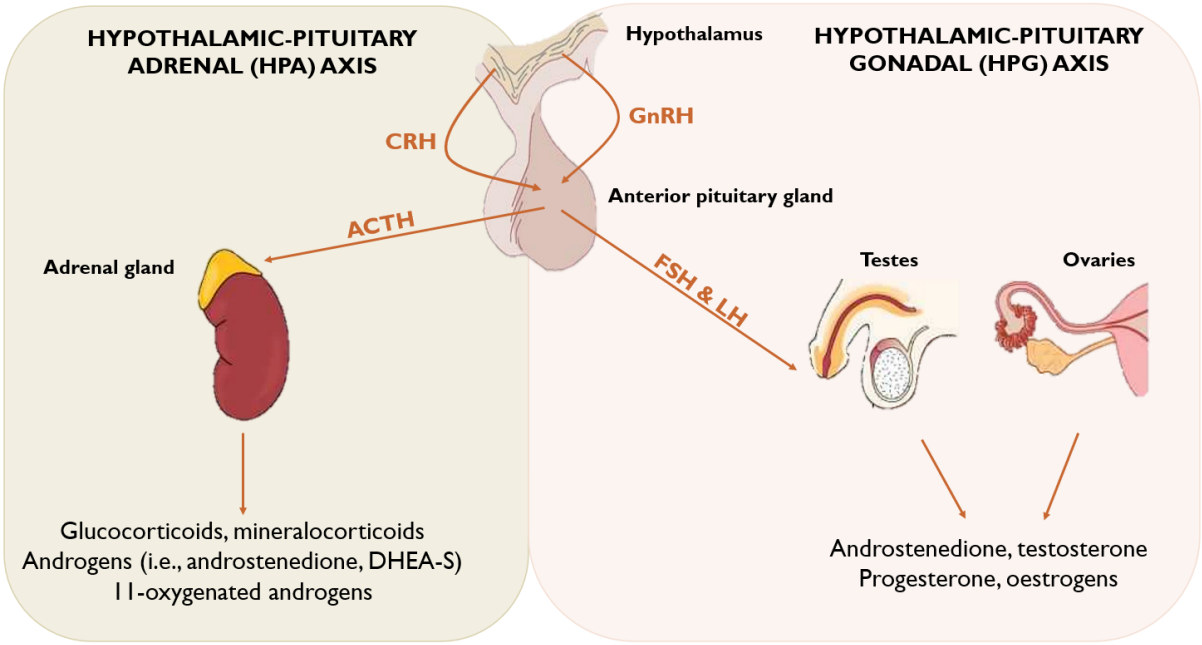


Figure 4: The signalling cascade of the hypothalamic-pituitary adrenal (HPA) axis and the hypothalamic-pituitary gonadal (HPG) axis. Hypothalamus secretes gonadotrophic releasing hormones (GnRH) to induce the secretion of the follicle-stimulating hormone (FSH) and luteinizing hormones (LH) from the anterior pituitary gland to target the gonads, testes in men and ovaries in women. Likewise, adrenocorticotrophic hormone (ACTH) is secreted by the anterior pituitary gland upon secretion of the corticotropic-releasing hormone (CRH) from the hypothalamus. The outer cortex of the adrenal gland, located on top of the kidneys, secretes glucocorticoids, mineralocorticoids, and androgens upon stimulation from ACTH. The gonads primarily produce androgens, as well as progestogens and oestrogens, when induced by FSH and LH.

The biosynthesis of steroid hormones from the adrenal glands (i.e., mineralocorticoids, glucocorticoids, and adrenal androgens) is initiated by the secretion of the corticotropic-releasing hormone (CRH) from the hypothalamus directed to the pituitary gland (Vale et al., 1981). The release of adrenocorticotropic hormone (ACTH) from the anterior lobe of the pituitary gland is activated by hypothalamic CRH, and the direct transport of ACTH in the circulatory system to the adrenal glands will stimulate the steroidogenesis of adrenal steroid hormones (Ghayee & Auchus, 2007).

The primary mineralocorticoid (MC) produced from the adrenal gland is aldosterone; crucial for the regulation of salt and fluid retention and hypertension (Connell & Davies, 2005). Cortisol is the main glucocorticoid (GC) in humans, whereas corticosterone is in animals due to the lack of the enzyme 17α-hydrolase (Jacobson, 2005). GCs are important for the stress response upon environmental triggers, in addition to the feedback regulation of the HPA-axis amongst other biological functions of GCs (Tsigos & Chrousos, 2002). Furthermore,

androstenedione (A4), dehydroepiandrosterone (DHEA) and its sulphated form dehydroepiandrosterone sulphate (DHEA-S) are the prime adrenal androgens and subjected to further activation in peripheral cells through conversion into bioactive steroid hormones (Labrie, 2004; Rainey & Nakamura, 2008). The produced steroid hormones will undergo transport through the circulatory system aided by carrier proteins to be attained by target issues and reach their nuclear receptors upon diffusion (Willnow & Nykjaer, 2010).

The gonads serve as the primary source of androgens which are important sex hormones mainly affecting sexual maturation and fertility (Buvat et al., 2010). The gonads are classified as the testes in men and the ovaries in women. The HPG-axis is comparable to the HPA-axis regarding the initiation of steroidogenesis by the hypothalamus in the brain upon the secretion of gonadotrophic-releasing hormone (GnRH) to the anterior pituitary lobe (Crowley et al., 1985). Furthermore, stimulation of the gonadal steroid hormone biosynthesis is triggered by the delivery of the follicle-stimulating hormone (FSH) and luteinizing hormone (LH) to the gonads (Burns & Matzuk, 2002). Androgens and oestrogens are the end-products of the HPG-axis with testosterone (T) as the dominant androgen in men in the same manner as oestradiol (E2) and progesterone (P) are the main sex hormones in women (Peper et al., 2010). While produced at lower amounts, androgens also have certain physiological effects in females (Davison & Davis, 2003; Hammes & Levin, 2019).

A prime example of a feedback mechanism regulating the HPA-axis involves the direct inhibition of CRH release from the pituitary gland by the synthesized glucocorticoids to decrease the ACTH concentrations (Watts, 2005). Likewise, there is constant control of GnRH production and FSH/LH release by the peripheral gonadal androgens through negative feedback effects (Tilbrook & Clarke, 2001). The negative feedback mechanism of the HPA-axis may also alter the steroidogenesis of androgens in the HPG-axis, thus having direct consequences regarding bone mass development, lipolysis and cardiovascular diseases (Chrousos, 2000). Furthermore, the HPA-axis and HPG-axis are tightly linked as stress exposure (i.e., increased secretion of GCs) may inhibit GnRH, thus lowering LH and FSH responsible for androgen steroidogenesis (Acevedo-Rodriguez et al., 2018; Mastorakos et al., 2006). Coordination between axes can therefore give rise to endocrinological intracrine conditions such as hyperandrogenism seen in non-classical congenital adrenal hyperplasia (CAH) and in Cushing's syndrome (Unluhizarci et al., 2012).

The absence of storage mechanisms for steroid hormones indicates that circulating hormones may be further metabolized and transformed in peripheral organs. For example, the adrenal androgen DHEA-S undergoes conversion into testosterone in peripheral cells in young women (Baulieu, 1996). Inactivation may occur carried out by the enzymes 11β-hydroxysteroid dehydrogenase (HSD11B) for C_{21} and C_{19} hormones and 17β-hydroxysteroid dehydrogenase (HSD17B) and specific sulfotransferases for C_{18} oestrogens (Roy, 1992). As the HPA- and HPG-axes are tightly regulated by feedback mechanisms, most of the synthesized steroid hormones will be utilized by the target organs for genomic transcription or rapid non-genomic actions (Wilkenfeld et al., 2018).

2.1.3 Steroidogenesis

Steroid hormones are signalling molecules responsible for converting external and internal stimuli received by the brain into physiological responses through the expression by hormone-induced genes. The biosynthesis of steroid hormones is stimulated by the secretion of pituitary ACTH, FSH and LH upon activation from the hypothalamic CRH and GnRH. Signals from the HP complex reaches the target endocrine glands and initiates steroidogenesis. Steroidogenesis involves numerous enzymes and co-factors aiding the generation and conversion of precursor hormones into the final steroid hormones through distinct pathways depending on the type of endocrinological gland, as well as the type of enzymes and co-factors expressed in the target cells.

The synthesis of steroid hormones is initiated by the translocation of cholesterol, derived from low-density lipoproteins (LDL), from the outer mitochondrial membrane to the inner mitochondrial membrane by the steroid acute regulatory protein (StAR) (Halkerston et al., 1961). Upon entry of the inner mitochondrial membrane, the sidechain of cholesterol is cleaved and further converted into the precursor hormone pregnenolone by the cholesterol side-chain cleavage enzyme (CYP11A1). The presence of CYP11A1 is crucial for the initiation of cholesterol cleavage and steroid hormone biosynthesis and determines whether target cells are steroidogenic (Miller & Auchus, 2011). The transport, cleavage and conversion of cholesterol to pregnenolone is the first and rate-limiting step in the biosynthesis of every steroid hormone regardless of the cell type in the target endocrine gland (Tsujishita & Hurley, 2000), see **Figure 5**.

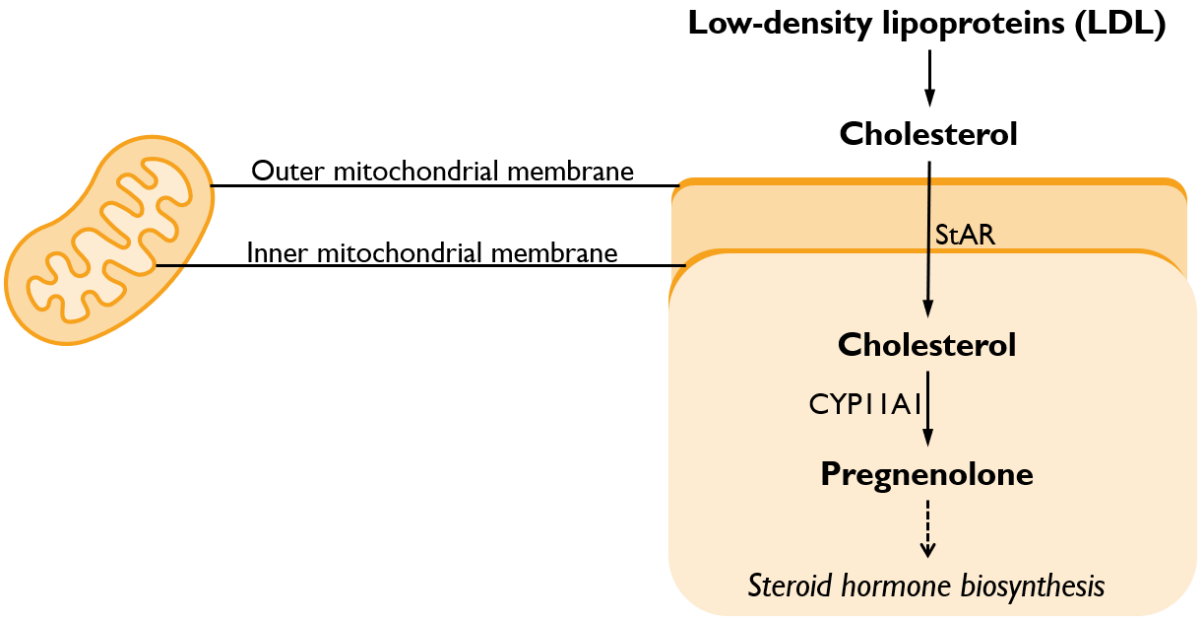


Figure 5: Transport of cholesterol into the mitochondria for steroidogenesis. Low-density lipoproteins (LDL) releases cholesterol which is transported to the mitochondria by the steroid acute regulatory protein (StAR). The sidechain of cholesterol is cleaved by the cholesterol side-chain cleavage enzyme (CYP11A1) to obtain the core nucleus structure of steroid hormones.

2.1.3a Adrenal pathways

Steroidogenic cells are found in the adrenal glands located on top of the kidneys and are capable of synthesizing glucocorticoids, mineralocorticoids, and adrenal androgens upon activation of ACTH secretion from the anterior pituitary gland stimulating the adrenal steroidogenesis through the HPA-axis (Jacobson, 2005). The center of the adrenal gland consists of the medulla where catecholamines such as dopamine, adrenaline and noradrenalin are produced (Perlman & Chalfie, 1977). Following the medulla, the adrenal cortex is the section of the adrenal gland responsible for the steroidogenesis of adrenal steroid hormones. Lastly, the adrenal gland is encapsulated by a connective tissue called the capsule (**Figure 6**).

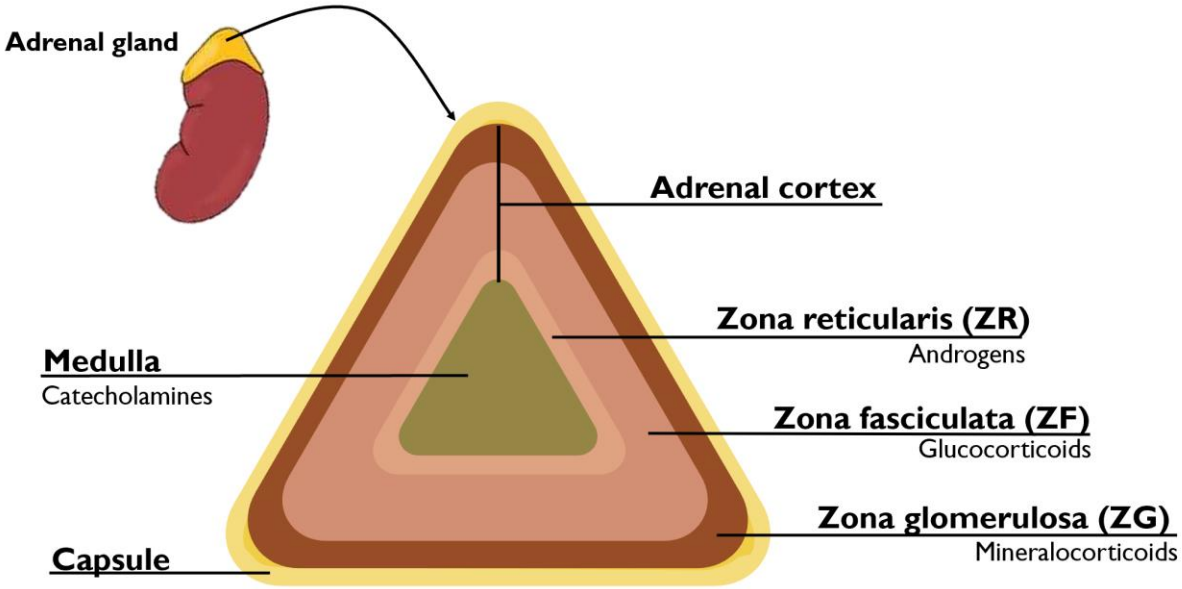


Figure 6: Simplified overview of the adrenal gland. The medulla is colored in green, the adrenal cortex in red nuances and the capsule in yellow. Steroid hormones derived from the adrenal glands are produced in the adrenal cortex. The adrenal cortex is divided into three sections: the zona glomerulosa (ZG), zona fasciculate (ZF) and zona reticularis (ZR). Biosynthesis of mineralocorticoids, glucocorticoids and androgens occurs in ZG, ZF and ZR, respectively.

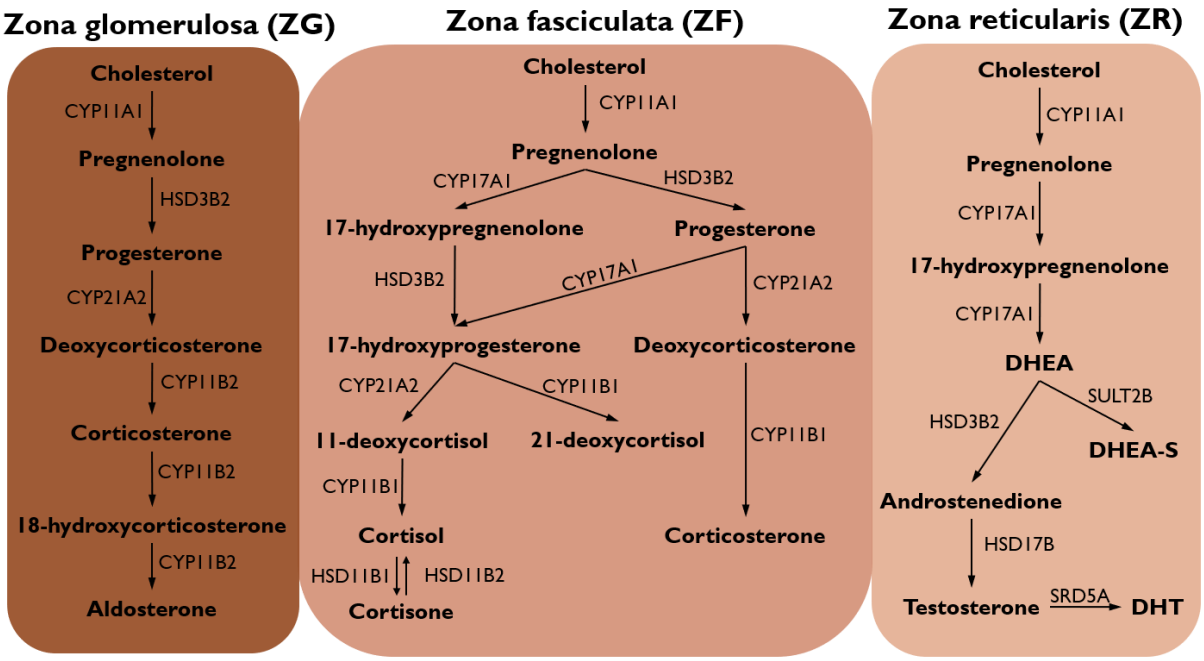
The biosynthesis of adrenal androgens occurs in the Zona Reticularis (ZR) and is characterized by the conversion of 17α-hydroxypregnenolone (17OHPreg) to DHEA catalyzed by 17α-hydroxylase (CYP17A1) (Conley & Bird, 1997). DHEA functions as the precursors of A4 catalyzed by 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2) and DHEA-S by DHEA sulfotransferase (SULT2B) (Conley & Bird, 1997). The latter chemical reaction enables the inactivation of DHEA (Rainey & Nakamura, 2008). The synthesized A4 and DHEA-S are exported to peripheral cells to be converted to bioactive steroid hormones such as T, which can be further converted to the more potent dihydrotestosterone (DHT) by 5α-reductase (SRD5A) and oestradiol by aromatases (Schiffer et al., 2018). The contribution of adrenal DHEA and T is significant for children and women exhibiting the importance of adrenal androgens (Couzinet et al., 2001; Labrie et al., 2017; Nakamura et al., 2009)

Furthermore, the biosynthesis of GCs occurs in the Zona Fasciculata (ZF) situated in the middle section of the cortex and constitutes the largest region of the adrenal cortex (Neville & Mackay, 1972). Two pathways are involved in the steroidogenesis of the primary GCs, cortisol (F) and corticosterone (B). Firstly, the 17-hydroxylation pathway is initiated by the conversion of pregnenolone to 17OHPreg and P by HSD3B2 and CYP17A1, respectively, producing the precursor 17α-hydroxyprogesterone (17OHP) (Conley & Bird, 1997). The generation of F is

obtained through the conversion of 17OHP to 11-deoxycortisol (11DF) by 11β-hydroxylase (CYP11B1) and 21-deoxycortisol (21DF) by 21α-hydroxylase (CYP21A2) (Kater et al., 2022). The precursors of cortisol (i.e., 17OHP, 11DF and 21DF) are important biomarkers for CAH regarding deficiencies in the enzymes CYP21A2 and CYP11B1 (Tonetto-Fernandes et al., 2006). F may further undergo a reversible reduction into cortisone (E) by 11β-hydroxysteroid dehydrogenase types 1 and 2 (HSD11B1 and HSD11B2) (Stewart & Mason, 1995) .

Secondly, the steroidogenesis of corticosterone is referred to as the 17-deoxy pathway. Pregnenolone is converted to P by HSD3B2 followed by the 21-hydroxylation of P by CYP21A2 resulting in the generation of deoxycorticosterone (DOC) (Lieberman et al., 1984). The end-product corticosterone (B) is then produced upon the conversion of DOC by the enzyme CYP11B1 (Biglieri & Kater, 1991). Corticosterone is the main glucocorticoids in rodents and birds due to lack of CYP17A1, thus excluding the synthesis of cortisol via the 17-hydroxylation pathway (Koren et al., 2012). Nevertheless, B is an important precursor for aldosterone synthesis in humans.

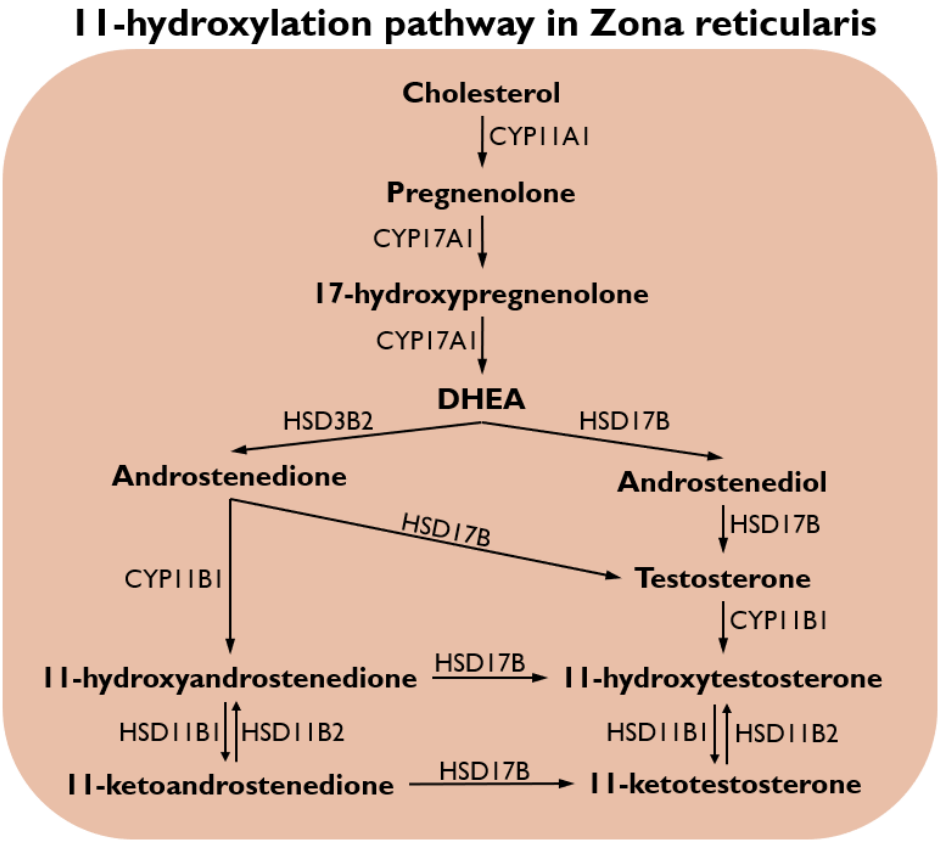
The steroidogenesis of MCs with aldosterone as the end-product occurs in the outer layer of the adrenal cortex, the Zona Glomerulosa (ZG). It follows similar biosynthesis patterns as the 17-deoxy pathway as seen for the corticosterone steroidogenesis in ZF (Seccia et al., 2018). However, DOC is further hydroxylated by the ZG specific enzyme aldosterone synthase (CYP11B2) into 18-hydroxycorticosterone (Biglieri et al., 1989; Lieberman et al., 1984). 18-hydroxycorticosterone is once again subjected to hydroxylation of the same enzyme to generate aldosterone. Aldosterone is essential for the regulation of salt and fluid retention, in addition to hypertension and cardiovascular system (Connell et al., 2008). This pathway for the generation of mineralocorticoids is therefore required by humans to obtain normal biological functionality throughout the whole lifetime (Kater et al., 1989). A schematic overview of the adrenal steroidogenesis is illustrated in **Figure 7**.



***DHEA**: dehydroepiandrosterone, **DHEA-S**: dehydroepiandrosterone sulphate, **DHT**: dihydrotestosterone. **CYP11A1**: cholesterol side-chain cleavage enzyme, **CYP11B1**: 11β-hydroxylase, **CYP11B2**: aldosterone synthase, **CYP17A1**: 17α-hydroxylase, **CYP21A2**: 21α-hydroxylase. **HSD3B2**: 3β-hydroxysteroid dehydrogenase type 2, **HSD11B1/B2**: 11β-hydroxysteroid dehydrogenase types 1 and 2, **HSD17B**: 17β-hydroxysteroid dehydrogenase. **SULT2B**: DHEA sulfotransferase. **SRD5A**: 5α-reductase.*

Figure 7: The biosynthesis pathways of steroid hormones with adrenal origins. The steroidogenesis of mineralocorticoids in ZG, glucocorticoids in ZF and androgen in ZR are placed on color-coded boxes corresponding their respective zones in the adrenal cortex seen in Figure 6. Modified from Kater et al. (2022) and Kegg Pathway "Steroid hormone biosynthesis" available at https://www.genome.jp/pathway/map00140.

The ZR in the adrenal cortex is the source of 11-oxygenated androgens through the 11-hydroxylation pathway. Recent studies show their significance as potent androgens in humans (Rege et al., 2018). Unlike the adrenal androgens DHEA-S and A4, the 11-oxygenated androgen 11-ketotestosterone (11KT) possess androgenic functionality in the same degree as testosterone and dihydrotestosterone (DHT) with the ability to bind to the androgen receptors (Pretorius et al., 2017). The steroidogenesis of 11-oxyandrogens begins with the conversion of A4 to T by HSD3B2 where A4 and T functions as the precursors. The 11-oxyandrogens 11-hydroxyandrostenedione (11OHA4) and 11-hydroxytestosterone (11OHT) is generated upon the reduction of A4 and T by the adrenal specific enzyme CYP11B1, respectively (Swart et al., 2013). Furthermore, 11OHA4 and 11OHT undergoes oxidation from secondary alcohols to ketone by HSD11B1 to obtain 11-ketotestosterone (11KT) and 11-ketoandrostenedione (11KA4) (Storbeck et al., 2013). The 11-hydroxylation pathway is illustrated in **Figure 8**.



***DHEA**: dehydroepiandrosterone, **CYP11A1**: cholesterol side-chain cleavage enzyme, **CYP11B1**: 11β-hydroxylase, **CYP17A1**: 17α-hydroxylase, **HSD3B2**: 3β-hydroxysteroid dehydrogenase type 2, **HSD11B1/B2**: 11β-hydroxysteroid dehydrogenase types 1 and 2, **HSD17B**: 17β-hydroxysteroid dehydrogenase.*

Figure 8: The 11-hydroxylation pathway for the biosynthesis of 11-oxygenated androgens occurring in the ZR of the adrenal cortex. Modified from Storbeck et al. (2019).

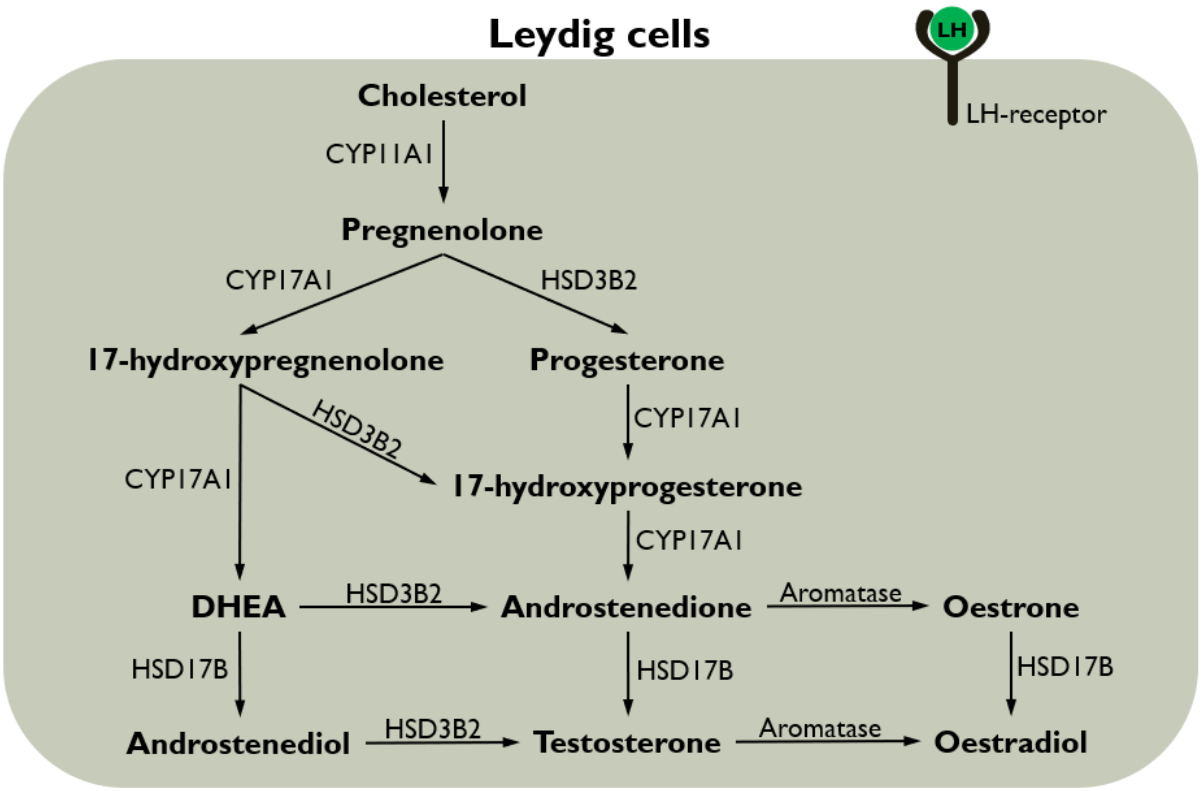
2.1.3b Gonadal pathways

The gonadal pathways are engaged in the biosynthesis of the steroid hormones responsible for sexual maturation, development of sexual characteristics and fertility. They are often referred to as sex hormones because of their significance in the male and female reproductive systems (Kuiri-Hänninen et al., 2014). Additionally, androgens are essential for bone and muscle growth (Notelovitz, 2002). The secretion of the gonadotrophic hormones FSH and LH from the anterior pituitary gland stimulated by the hypothalamic GnRH promotes the steroidogenesis of the hormones with gonadal origins (Burns & Matzuk, 2002).

Biosynthesis of androgens (i.e., T, A4 and DHEA) in men occurs almost exclusively in the Leydig cells of the testes, the male reproductive glands (Hall et al., 1969). It is stimulated and regulated by LH secretion by the anterior pituitary gland upon stimulation of GnRH from the hypothalamus upon binding to the LH-receptor (Zirkin et al., 1980). The synthesized T from

the Leydig cells and the pituitary FSH is then transported to the Sertoli cells and utilized to initiate spermatogenesis in men (Griswold, 1998).

The presence of CYP11A1 indicates that the Leydig cells are steroidogenic cells capable of converting cholesterol, transported through the mitochondrial membrane by StAR, into pregnenolone (Papadopoulos & Miller, 2012). The generation of androgens in the testes follow a similar pathway occurring in the adrenal cortex. However, the pathway converting 17OHPreg to DHEA by CYP17A1 is the preferred pathway to obtain testosterone as low progesterone levels are produced in the male testes (Flück et al., 2003). Furthermore, A4 and T are further aromatized by aromatases for the conversion to oestrone (E1) and E2, respectively (Carreau & Hess, 2010). **Figure 9** illustrates the testicular pathway for the generation of androgens and oestrogens.

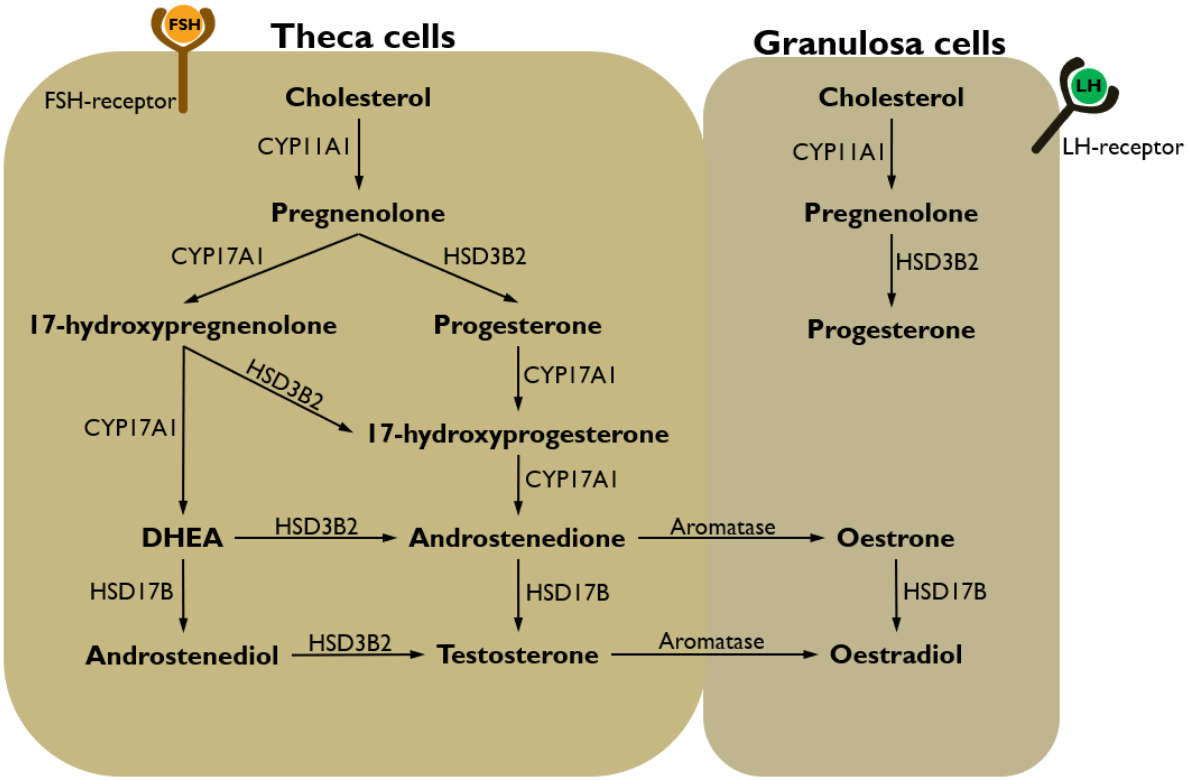


***DHEA**: dehydroepiandrosterone, **CYP11A1**: cholesterol side-chain cleavage enzyme, **CYP11B2**: aldosterone synthase, **CYP17A1**: 17α-hydroxylase, **HSD3B2**: 3β-hydroxysteroid dehydrogenase type 2, **HSD17B**: 17β-hydroxysteroid dehydrogenase.*

Figure 9: Biosynthesis of androgens and oestrogens in the testicular Leydig cells. The LH-receptor is drawn for clarification on which pituitary hormone stimulates the testicular steroidogenesis. Modified from Miller and Auchus (2011).

The female androgen steroidogenesis occurs in ovaries, more specifically androgen synthesis (mainly A4) in the theca cells and androgen metabolism to oestradiol in the granulosa cells (Tsang et al., 1979). According to the 'two-cell mechanism', the granulosa cells have LH receptors to induce androgen synthesis whilst the FSH receptors on the theca cells stimulates androgen conversion to oestrogens (Hillier et al., 1994). Androgens, progestogens and oestrogens are essential steroid hormones for the regulation of the female menstruation cycle, and the abundance of the final hormones synthesized depends on the cycle length and phase (Brailly et al., 1981). The ovarian hormone concentration fluctuates throughout the menstrual cycle, but there is a surged increase of progesterone in the early and mid-luteal phase while oestradiol maintains a relatively high concentration during the mid- and late follicular phase as well as mid-lutheal phase (McNatty et al., 1975).

The theca cells are responsible for the androgen synthesis, and share the same biosynthesis pathway of DHEA, A4 and T seen in the Leydig cells in the testes of males. The comparatively small concentration fluctuations of FSH throughout the menstrual cycle indicates that the theca cells produces A4 and T to complement the stable production and secretion of estradiol (McNatty et al., 1975). The conversion of androgens to oestrogens is mediated by aromatases upon the diffusion of A4 and T to the granulosa cells during the follicular phase (Havelock et al., 2004). Moreover, the increased LH concentrations during the luteal phase promotes progesterone steroidogenesis due the absence of CYP17A1 in the granulosa cells (Wood & Strauss, 2002). **Figure 10** illustrates the ovarian steroidogenesis for androgens, progestogens, and oestrogens.



***DHEA**: dehydroepiandrosterone, **CYP11A1**: cholesterol side-chain cleavage enzyme, **CYP11B2**: aldosterone synthase, **CYP17A1**: 17α-hydroxylase, **HSD3B2**: 3β-hydroxysteroid dehydrogenase type 2, **HSD17B**: 17β-hydroxysteroid dehydrogenase.*

Figure 10: Biosynthesis of progestogens, androgens and oestrogens in the granulosa and theca cells. The LH- and FSH-receptors are drawn for clarification on which pituitary hormone stimulates the ovarian steroidogenesis. Modified from Miller and Auchus (2011) and Kegg Pathway "Ovarian Steroidogenesis" available at https://www.kegg.jp/kegg-bin/show_pathway?hsa04913

2.1.4 Transport proteins

Transport proteins controls the regulation of hormone bioavailability by limiting the intracellular entry of hormones, in addition to aiding the delivery of steroid hormones to target cells. Binding to transport proteins is an additional mechanism regulating steroid hormone activity, and concentration anomalies of such proteins can affect the overall endocrine homeostasis. Three carrier proteins are known bind steroid hormones with varying binding affinity: corticosteroid-binding globulin (CBG), sex hormone-binding globulin (SHBG) and albumin.

The binding of steroid hormones (SH) to their carrier proteins (Prot) is in equilibrium described as $SH + Prot \rightleftharpoons SHProt$. The disassociation constant (K_D) reflects the ratio of the binding association and disassociation rate, thus the binding affinity of a ligand to a protein. Simply put, low K_D values indicate slower disassociation between the hormone and the protein (i.e.,

high affinity) whereas high K_D represents low binding affinity with more rapid disassociation (Södergard et al., 1982).

CBG, also referred to as transcortin, is the primary transport protein for corticoid steroid hormones including glucocorticoids, mineralocorticoids, and progestogens. The binding affinity of CBG to corticoids can be ranked as follows: deoxycortisol ≅ corticosterone ≅ cortisol > 17OHP > deoxycorticosterone > progesterone with K_D values in 10^{-6} magnitude (Dunn et al., 1981). Moreover, the data provided by Dunn et al. shows that GCs are not exclusively bound to CBG and exhibits comparatively low binding to the androgen transport protein (Dunn et al., 1981). It is known that 80% of cortisol is bound to CBG with the remaining 15% bound to albumin and 5% as the free cortisol fraction (Lewis et al., 2005). CBG regulate the bioavailability of GCs, thus the HPA-axis and ACTH secretion upon adrenal stimulation (i.e., stress-mediated response) (Kumsta et al., 2007). Moreover, CBG is also involved in rapid and non-genomic functions of GCs involving inflammation responses (Henley et al., 2016).

The carrier protein responsible for the transport of androgens (i.e., T, A4 and DHEA) and oestrogens (i.e., oestrone and oestradiol) is SHBG. The binding affinity of SHBG to various sex hormones are as follows: dihydrotestosterone > testosterone > androstenediol > androstanediol > oestradiol > estrone (Dunn et al., 1981). SHBG binds to T and oestradiol with great affinity with disassociation constants in the 10^{-9} magnitude, indicating the high binding specificity (Södergard et al., 1982). Directly comparing the obtained K_D values of CBG for GCs and SHBG to androgens and oestrogens, SHBG binds to its respective ligands with a greater affinity in 1000-fold (Dunn et al., 1981). This may reflect the difference in the biological functions of the carrier proteins as CBG is described to be a circulating cortisol storage enabling the rapid disassociation of F (Henley et al., 2016), whereas SHBG inhibits the bioactivity of androgens and oestrogens during childhood and adulthood as regulation of the bioavailability of sex hormones (Hammond, 2011). Elevated SHBG concentrations are especially important during pregnancy to limit the bioactivity of circulating androgens protecting the mother from foetal androgen production and the foetus from maternal androgens (Carlsen et al., 2006). Moreover, decreased SHBG reflects metabolic syndromes such as insulin resistance and is an additional biomarker for polycystic ovarian syndrome (PCOS) further illustrating its importance aside from the reproductive health (Pugeat et al., 2010).

Albumin is the most abundant protein found in human serum and binds to lipophilic compounds such as steroid hormones (Baker, 2002). Contrary to SHBG and CBG, albumin binds to steroid hormones with relatively low binding affinity with comparatively larger K_D values (Dunn et al., 1981). Thus, steroid hormones can rapidly disassociate from albumin becoming readily bioavailable for receptor binding. For example, the K_D value for albumin-bound T is in the magnitude of 10^{-4} whilst SHBG-bound T has K_D values in 10^{-9} indicating the large difference in binding affinity of T to various carrier proteins (Zheng et al., 2015). The albumin-bound fraction of T is considered bioavailable and determination of albumin concentrations is an important factor for the calculation the bioactive concentrations of T in serum (Emadi-Konjin et al., 2003; Södergard et al., 1982; Vermeulen et al., 1999). Additionally, a small fraction of T binds to orosomucoid, but its functional role on the bioavailability of T is less understood (Goldman et al., 2017).

2.2 Free Hormone Hypothesis

The core structure of steroid hormones is non-polar enabling them to freely diffuse across the lipid membrane and bind to their respective receptor when unbound to carrier proteins. This biochemical mechanism is the central dogma in endocrinology called as the Free Hormone Hypothesis depicting the bioavailability of steroid hormones (Mendel, 1989). The lipid-solubility of the steroid hormones prevents them from being circulated to target organs and glands through the blood circulatory system. Each class of steroid hormones is carried by specialized transport proteins with specific binding sites, as demonstrated by the binding of SHBG to androgens and CBG to glucocorticoids and progestogens for hormone transport in the water-soluble blood plasma (Heyns & De Moor, 1971; Slaunwhite & Sandberg, 1959). The binding affinity of the transport proteins varies and is a crucial factor on the bioavailability of steroid hormones. For example, androgens bind specifically with high affinity to SHBG and non-specifically with lower binding affinity to albumin (Goldman et al., 2017). To illustrate the Free Hormone Hypothesis, the binding of T to albumin and SHBG and the cell diffusion of free is used as example (**Figure 11**).

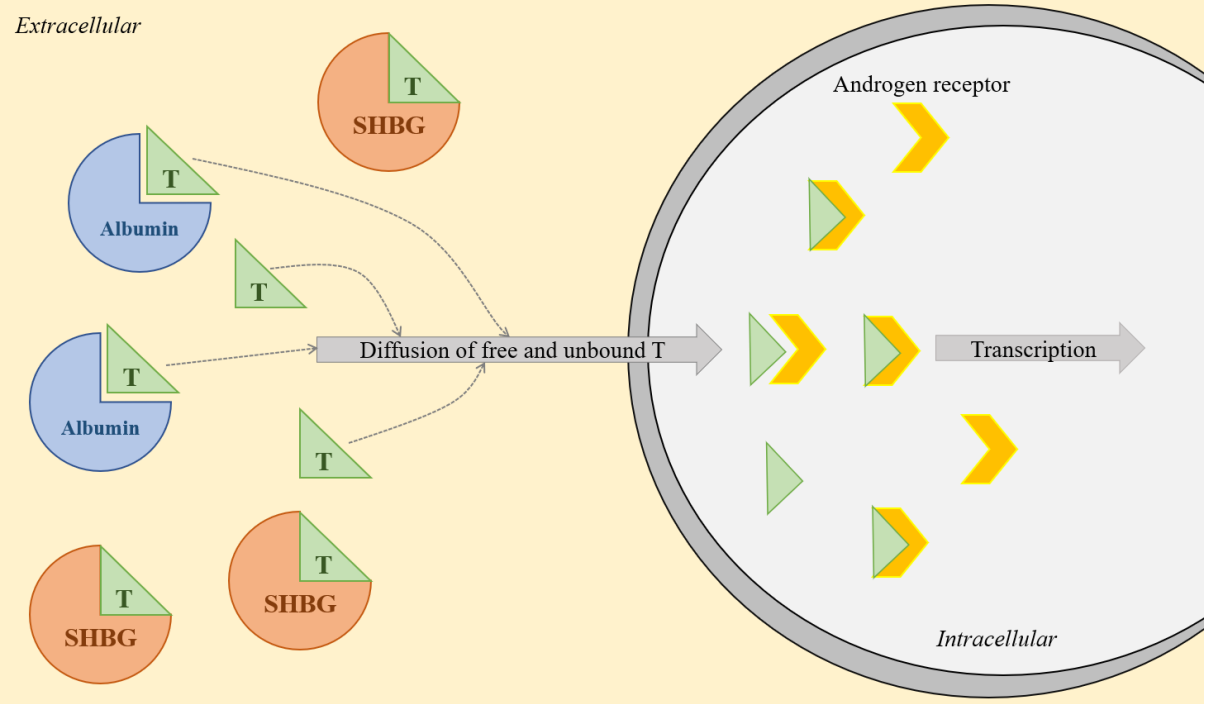


Figure 11: Illustrative description of the Free Hormone Hypothesis using testosterone as the example hormone. The bioavailable T, the free T, and the albumin-bound T can diffuse through the lipid membrane and bind to the androgen receptors located intracellular to engage protein transcription.

According to the Free Hormone Hypothesis, the bioavailable fraction of T, the free fraction and the loosely bound T to albumin, will further undergo protein transcription in the nucleus upon entering the cell and binding to the androgen receptor (AR) (Goldman et al., 2017). The portion of T tightly bound to SHBG will remain inaccessible to cells for transcription, thus inactivated, whilst the albumin-bound T will dissociate as albumin cannot diffuse across tissue capillaries (Manni et al., 1985).

Vermeulen et al. has shown that approximately 58% of T is specifically bound to SHBG, 40% is bound to albumin with lower binding affinity and the remaining 2% circulates freely in adult males. Additionally, the study explored how gender plays a crucial role on the binding specificity of T as the increasing SHBG levels in women resulted in 81% of T tightly bound to SHBG (Vermeulen et al., 1971). Furthermore, the use of oral contraceptives consists of exogenous synthetic hormones which causes elevated levels of oestrogens and progestogens. The concentrations of SHBG will therefore increase accordingly to regulate the excess sex hormones in circulation (Panzer et al., 2006). Oral contraceptives functions as antiandrogens by suppressing androgen production or by increasing SHBG to bind and inactivate excess androgens (Zimmerman et al., 2013). Consequently, the concentrations of total testosterone

(TT), bioavailable testosterone (BT) and free testosterone (FT) will decrease upon use of oral contraceptives (van der Vange et al., 1990; Wiegratz et al., 1995)

Diagnosis of endocrinological disorders with anomalous levels of androgens, such as hyperandrogenism and hypoandrogenism, involves the biochemical measurement of TT. Quantification of BT or FT may provide a more appropriate representation of actual androgen concentrations responsible for the physical manifestations of hyperandrogenism and hypoandrogenism. Multiple methods are used to obtain serum BT and FT, for example by ultracentrifugation and equilibrium dialysis (Morley et al., 2002). Moreover, saliva as an alternative diagnostic medium has been found to mostly consist of free T as transport proteins are unable to diffuse across cell membranes in the salivary glands (Vining et al., 1983). Thus, sensitive and specific assays capable of measuring extremely low concentrations of salivary T can be used as diagnostic tools to determine FT (Keevil & Adaway, 2019).

2.3 Steroid hormones as biomarkers

Steroid hormones are involved in a wide range of crucial biological functions such as stress response, electrolyte balance and sexual maturity to name a few. The human body does not store steroid hormones in the same manner as glucose is converted to glycogen, but the steroid biosynthesis is rather escalated to supply the increased demand and delivery to target cells by the carrier proteins CBG, SHBG and albumin (Willnow & Nykjaer, 2010). Steroidogenesis is regulated by feedback mechanisms to maintain homeostatic levels of steroid hormones in the circulatory system. However, any flaw or lapses in the signalling cascade of the feedback mechanism may result in erroneous amounts of hormones being produced and secreted (Ghayee & Auchus, 2007; Schiffer et al., 2017).

This current study will focus on endocrinological conditions associated with irregular production and of androgens, also called sex hormones, derived from the gonads (i.e., T and A4) and the adrenal glands (i.e., 11-oxygenated androgens). The term **hypo**androgenism refers to the decrease in androgen secretion, and an increase is referred to as **hyper**androgenism. This section will briefly introduce clinical endocrinological conditions where the proposed UPLC-MS/MS method in this study can serve as a relevant tool for diagnostic and drug monitoring purposes.

2.3.1 Hypoandrogenism

Hypoandrogenism is characterized by the diminished production of androgens and arises in endocrinological conditions such as hypogonadism in men and hypoestrogenism in women. Hypogonadism is defined as impairment in the HPG-axis and is classified between primary and secondary hypogonadism depending on which stage of the hormone signalling cascade is disrupted (Basaria, 2014). Primary hypogonadism is characterized by defective T secretion from the testes and increased production of the hypothalamic GnRH and pituitary LH in an attempt to increase T production. Furthermore, secondary hypogonadism is caused by diminished concentrations of gonadotrophic hormones from the hypothalamus-pituitary complex resulting in decreased stimulation of the gonads for T secretion ultimately affecting spermatogenesis (Nachtigall et al., 1997). An illustrative description of primary and secondary hypogonadism is given in **Figure 12**.

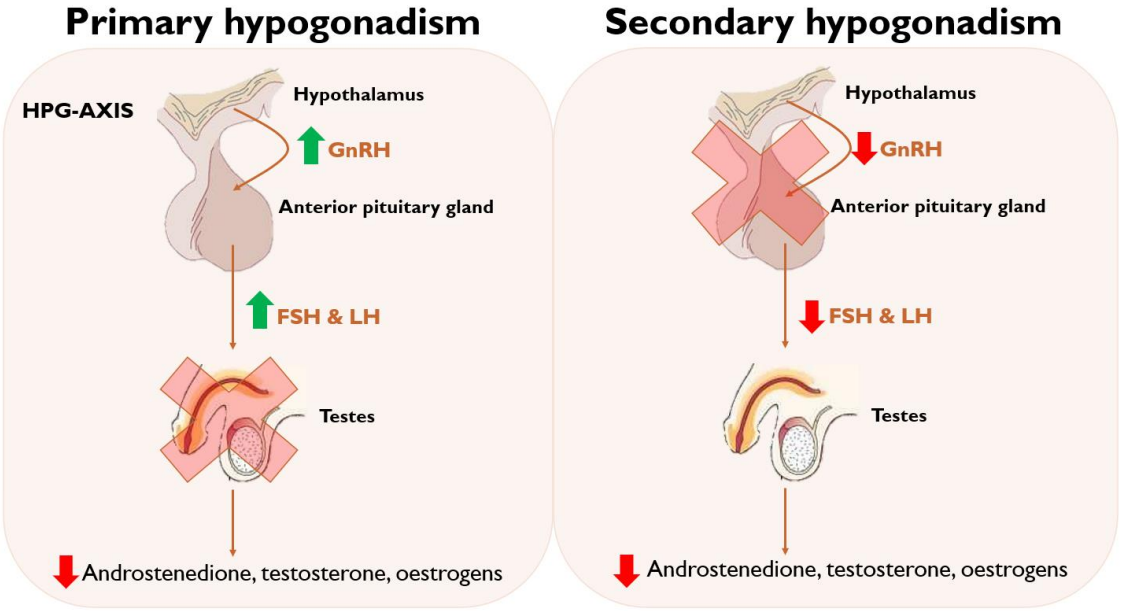


Figure 12: Illustrative description of the disruption in the hypothalamic pituitary gonadal (HPG)-axis associated with hypogonadism. Increased hormone secretion is illustrated by green arrows and decrease by red arrows. **GnRH**: gonadotrophic releasing hormone, **FSH**: follicle stimulating hormone and **LH**: luteinizing hormone.

Testosterone and its conversion to oestradiol by aromatases are essential for spermatogenesis, muscle and bone growth and the development of physical male characteristics (Grumbach, 2000). Men with hypogonadism, thus with substantial decrease of T concentrations, have greater risk of osteoporosis, infertility, cardiovascular issues and decline in overall vitality (Oldenburg, 2015). Moreover, decreased SHBG concentrations are seen in hypogonadal men

associated to metabolic syndromes such as diabetes type II and obesity due to insulin resistance (Kapoor et al., 2005).

The characteristic diminished androgen concentration in hypoandrogenism is also consequential to women as low T and A4 concentrations results in decreased oestrogen levels associated with hypoestrogenism. Oestrogens are produced upon the conversion of testosterone to oestradiol and A4 to estrone by P450 oxidoreductase and the placental P450 aromatase, respectively (Storbeck et al., 2019). Alike testosterone for men, oestrogens are essential for bone mass density and fertility in women (Meczekalski et al., 2010). Increased risk for osteopenia and osteoporosis are therefore associated by hypoandrogenism in young pre-menopausal women (Gordon, 2000). However, concentrations of testosterone in women are naturally low and commercially available assays measures inaccurately in the lower A4 ranges (Herold & Fitzgerald, 2003). Biochemical assays measuring A4 and oestrogens could potentially provide more accurate biomarkers for hypoandrogenism in women. The method presented in the current study do not include oestrogens, but provides sensitive quantifications of T (i.e., TT) and A4 to determine potential hypoandrogenism in women, nevertheless.

Aside from clinical symptoms, biochemical assays measuring the androgen concentrations are useful as diagnostic and drug monitoring tools for determining and treating hypogonadism. There are readily available established reference ranges for the androgens most used as biomarkers (i.e., TT and A4). However, the measurement of the BT or FT may provide clearer insight of the concentration of the circulating bioactive T subjected to cell uptake. According to Kapoor et al., the Free Androgen Index (FAI), a measure of T bioavailability, is not suitable for determination of hypogonadism in men due to the relatively lower SHBG levels resulting in false results (Kapoor et al., 1993).

2.3.2 Hyperandrogenism – polycystic ovarian syndrome

Polycystic ovarian syndrome (PCOS) is a complex endocrinological condition affecting reproductive-aged women associated with increased risk of fertility, insulin resistance, cardiovascular diseases, endometrial cancer, and general decrease in life quality (Hoeger et al., 2020). Approximately 1 out 10 women are affected by PCOS (March et al., 2009), but PCOS phenotypes may differ between individuals (Guastella et al., 2010). Clinical manifestations of

PCOS arise during puberty in adolescent girls, but similar symptoms is associated with the physiological activation of the HPG-axis in non-PCOS prepubertal girls (Franks, 2008). Thus, PCOS can remain undetected until adulthood resulting in delayed treatment. PCOS is diagnosed according to the Rotterdam criteria where two out of three criteria must be met: chronic oligo-/amenorrhea, clinical and biochemical assessment of hyperandrogenism and polycystic ovaries (Teede et al., 2018).

One of the characteristics of PCOS is excess androgen concentrations, most importantly T and A4, from the hyperstimulation of the HPG-axis. The increased LH secretion from the HP-complex combined with the low-to-normal FSH levels stimulates of the ovaries for elevated androgen biosynthesis as an impaired negative feedback mechanism (Yoo et al., 2006). High LH/FSH ratios are observed in PCOS, but does not suffice to become an exclusive biomarker (Cho et al., 2006). However, the observed elevation of 11-oxygenated androgens indicates an intracrine relationship between the ovaries and adrenal glands with hypersecretion of androgens from both origins suggesting the potential of 11-oxygenated androgens as PCOS biomarkers (O'Reilly et al., 2016). An illustrative description of hormonal levels in PCOS is given in **Figure 13**.

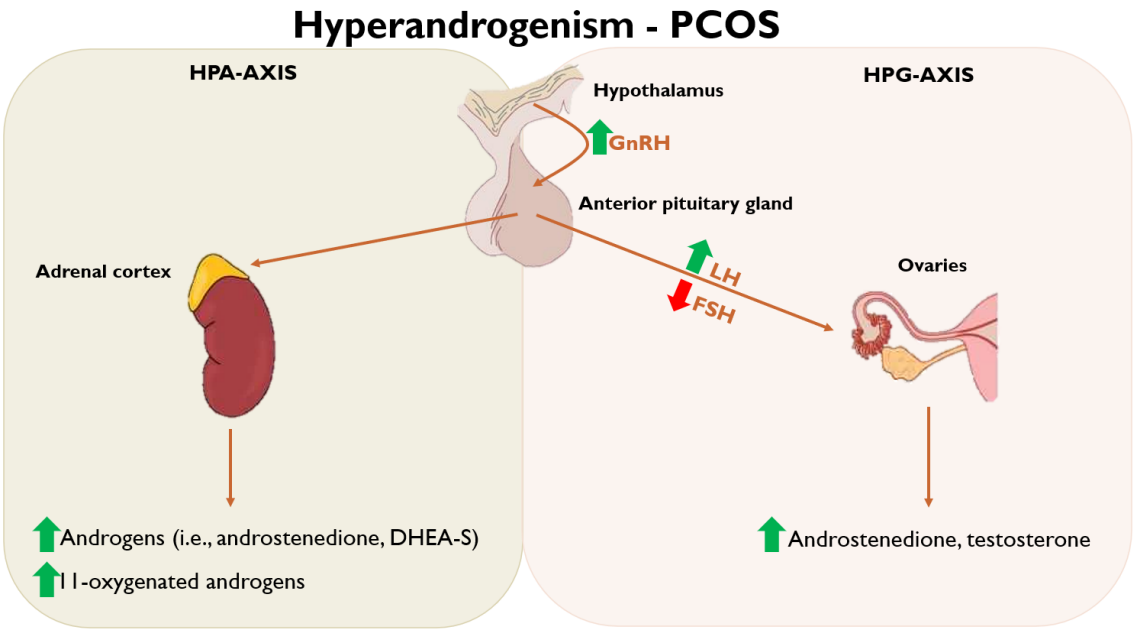


Figure 13: Illustrative description of the disruption in the hypothalamic pituitary gonadal (HPG)-axis associated with PCOS, and the intracrine relationship leading to the increase of adrenal steroid hormones. Increased hormone secretion is illustrated by green arrows and decrease by red arrows. **GnRH**: gonadotrophic releasing hormone, **FSH**: follicle stimulating hormone and **LH**: luteinizing hormone.

Women with PCOS are shown to have increased insulin sensitivity which in turn decreases SHBG levels and elevates the risk for metabolic and cardiovascular diseases (Baldani et al., 2015; Moran et al., 2015). The significant decrease in the carrier protein SHBG consequently develops into elevation of bioactive androgens in circulation (Simó et al., 2015). Moreover, maternal androgen excess affects foetal hormone levels potentially leading to the transgenerational progression of PCOS (Sir-Petermann et al., 2009).

The assessment of PCOS is crucial for the overall long-term physical and mental health for women affected by this endocrinological disorder. The PCOS International Guidelines recommends the biochemical quantification of TT and BT to be carried out upon clinical manifestations of PCOS (Rotterdam, 2004). Additionally, A4 is a biomarker with increased sensitivity than T regarding detection of PCOS phenotype with normal T levels (O'Reilly et al., 2014). Moreover, 11-oxygenated androgens can function as additional biomarkers with the possibility of identifying PCOS phenotypes without symptoms of hyperandrogenism caused by classical androgens (Carmina et al., 1992; Holownia et al., 1992; O'Reilly et al., 2016). However, conflicting results were expressed by Owen et al., and Taylor et al., indicating that more studies on the utility of 11-oxyandrogens as PCOS biomarkers must be conducted (Owen et al., 1992; Taylor et al., 2022).

Differential diagnoses with hyperandrogenism must be distinguished and excluded upon the diagnosis of PCOS (Elhassan et al., 2018). The intracrine relationship between the HPA- and HPG-axes is expressed in endocrinological conditions with abnormal secretion of adrenal steroid hormones further affecting the production of androgens. Non-classical CAH (Auer et al., 2021; Ueland et al., 2022), Cushing's syndrome (Hána et al., 2019; Nowotny et al., 2022), and androgen-secreting adrenal tumours (Cordera et al., 2003) are prime examples of conditions with disruption in the adrenal steroidogenesis and hypersecretion of androgens. Moreover, sex-cord stromal tumours affecting the gonads (i.e., the ovaries and testes) can induce steroidal overproduction of testosterone and oestrogen (Acar et al., 2009; Schultz et al., 2016).

2.4 Measurement of steroid hormones

Quantification of steroid hormones are most commonly carried out with immunoassays (IA) for the assessment of endocrinological conditions. However, detection and quantitation

methods based on mass spectrometry, such as LC-MS/MS, provides a more sensitive and specific detection of small and complex compounds (Soldin & Soldin, 2009). The reference methods for determining the bioactive fraction (i.e., non-protein bound) of steroid hormones are equilibrium dialysis (ED) and ultrafiltration (UF) coupled with mass spectrometry-based methods capable of efficiently separating the protein-bound and unbound forms of steroid hormones and detecting with great specificity (Faix, 2013). Moreover, calculations to determine the bioactive fraction of T such as the Free Androgen Index (FAI), calculated bioavailable T (cBT) and calculated free T (cFT) serve as alternatives T when ED or UF and biochemical assays of BT and FT are absent (Mueller et al., 2006).

2.4.1 Immunoassay

The principle of IA is based on the binding interactions between the target analyte and their respective specific antibody with known binding capacity, and quantitation of the antibody-bound analyte reflects the measured hormone concentration in a sample. The most employed forms of direct IAs are radioimmunoassay (RIA) and chemiluminescent immunoassay (CMIA) (Rosner et al., 2007).

The foundation of RIA is based upon the competitive binding to the antibody between the target analyte and primary antigen labelled with radioactive isotope (**Figure 14A**). Analyte detection and quantitation is conducted by measuring the radioactivity (Gosling, 1990). The negative linear relationship between the analyte concentration and measured radioactivity determines the analyte concentration (Zettner, 1973). On the other hand, CMIA utilizes antibodies bound to a luminescing agent for the detection and quantitation of hormone concentration upon analyte binding to the primary antigen (**Figure 14B**). Luminescence is achieved through initiation of chemical reactions, such as oxidation reaction, catalysed by enzymes to induce light emission from the chemiluminescent antibody bound to the target analyte (Thorpe et al., 1985). The relative analyte concentration is reflected by the measured light intensity (Schroeder et al., 1976).

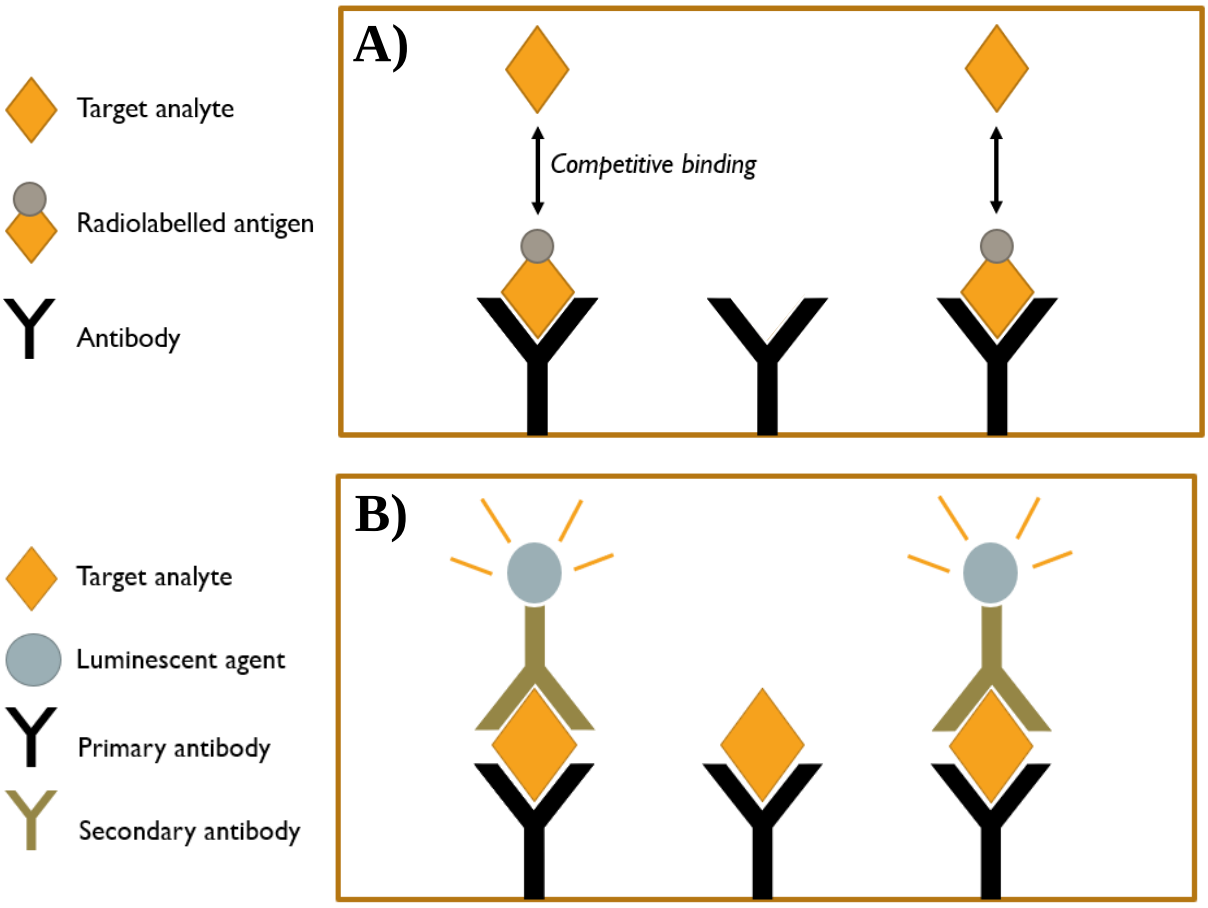


Figure 14: Schematic illustration of the basic principles of (**A**) radioimmunoassay (RIA) and (**B**) chemiluminescent immunoassay (CMIA). Yellow rays on figure B illustrates chemiluminescence.

Steroid measurements in modern clinical laboratories employs commercially available IAs without the use of radioisotope labelling (e.g., CMIA) to achieve rapid analysis for large sample quantities at low cost with minimal operator knowledge. However, steroid hormones and their metabolites are structurally similar in their chemical nature, thus making their determination challenging upon the use of immunological-based methods. The primary challenge that all IAs face is analyte cross-reactivity and poor antibody specificity affecting their reliability in providing correct steroid concentration measurements (Herold & Fitzgerald, 2003; Krasowski et al., 2014). Additionally, direct IAs performs directly on the sample matrix, thus lacking an essential step involving sample clean-up and removal of possible interferences to avoid further cross-reactivity and matrix effects (Stanczyk et al., 2003).

Correct determination of T is crucial to provide proper diagnosis of endocrinological conditions such as hyperandrogenism and hypogonadism, but measurement variability in direct IAs can undermine their eligibility as clinical diagnostic tools (Sikaris et al., 2005). Taieb et. al., concluded in their study that T concentrations measured with IAs were overestimated in women

and underestimated in men when compared to measurements conducted by gas chromatography coupled with mass spectrometry (Taieb et al., 2003). High intraassay variability has also been reported by Wang et al., and establishment of local reference ranges may be required to compensate for the variability in different IA kits (Wang et al., 2004). Moreover, variable SHBG levels can affect the biochemical analysis of T conducted on IAs implying that multiple parameters can potentially lead to the variable T concentrations (Boots et al., 1998).

The Journal of Clinical Endocrinology and Metabolism published an editorial in 2013 stating the requirements of using LC-MS/MS for the determination of androgen steroid hormones indicating the widespread obstacle of poor accuracy between IAs (Handelsman & Wartofsky, 2013). However, this recommendation was understandably retracted upon meeting push-back ("Letter of concern," 2014). It should be considered that mass spectrometry (MS)-based methods are not necessarily available and accessible in every laboratory, and IAs are sufficient in most cases (Wang et al., 2004). Taylor et al. states that the flaw is not necessarily in the methodology itself, but rather the quality of assays as well as use of proper quality controls and intraassay standardization (Taylor et al., 2015). However, commercial direct IAs still suffer from poor sensitivity and specificity, particularly in the lower hormone concentration range, and MS-based methods should be considered when possible (Auchus, 2014).

2.4.2 Equilibrium dialysis and ultrafiltration

The free fraction of steroid hormones is capable of cell diffusion and binding to the nuclear steroid receptors initiates transcription of hormone-induced genes. The carrier proteins SHBG and CBG binds to steroid hormones with great binding affinity and specificity, thus making protein-bound hormones biologically inactive through the inhibition of cell uptake which is essential for the circulation and regulation of hormone activity (Willnow & Nykjaer, 2010). For example, measuring the biologically active portion of T in serum is more representative of the circulating concentrations associated with hyperandrogenism seen in PCOS (Pinola et al., 2015). The reference method for the determination of free steroids includes equilibrium dialysis (ED) or ultrafiltration (UF).

ED is considered the “gold” standard for determination of FT (i.e., non-protein bound T). The primary mechanism of ED involves the membrane diffusion of unbound hormone through a

semipermeable membrane, further separating the free fraction from the protein-bound, see **Figure 15** (Kley et al., 1977). UF is based upon the same principle as ED but differs in the speed of diffusion. Centrifugation is used to aid the diffusion of the free hormone fraction in the dialysate in UF (Hammond et al., 1980). Both ED and UF are separation techniques and further quantification of hormone concentration must be conducted by IAs or LC-MS/MS upon achieving equilibration in the dialysate chamber (Chen et al., 2010).

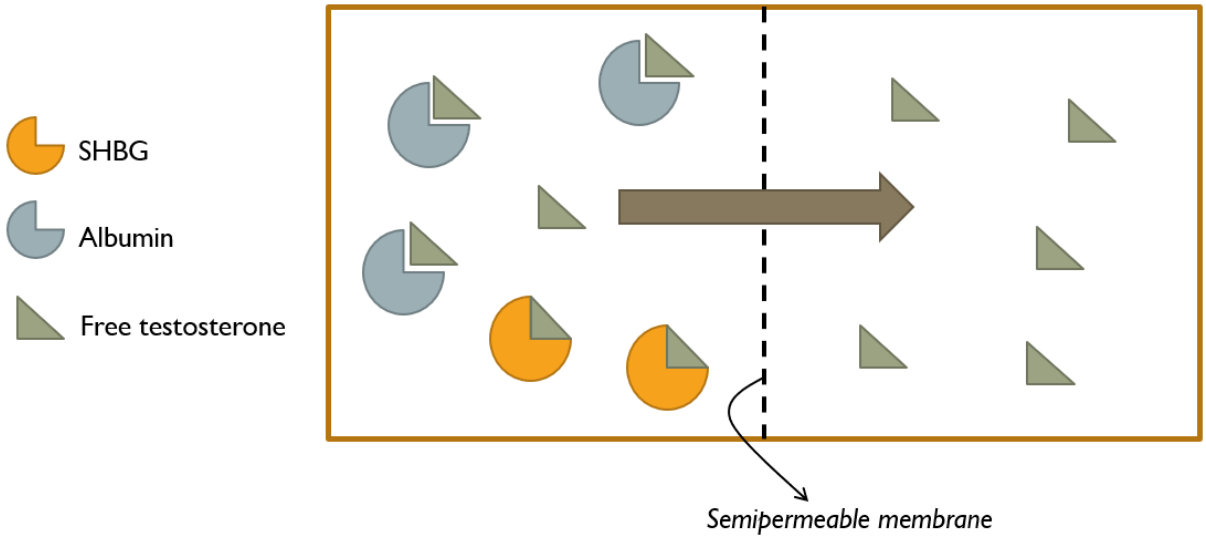


Figure 15: Schematic overview of the basic principle of equilibrium dialysis (ED) and ultrafiltration (UF). Dashed lines indicate the semipermeable membrane dividing the dialysis chamber into two separation compartments.

Although ED and UF are the reference methods for FT measurements, they are not applicable to routine analyses due to their challenges in time efficiency and sample capacity. Routine analyses used in clinical laboratories must have the capability of analysing large amounts of samples in a relatively short time requiring high-throughput methods. The analysis protocol is time intensive in regards of reaching equilibrium with ED requiring longer time (Mathor & Wajchenberg, 1985) without the aid of centrifugal force as in UF (Vlahos et al., 1982). Moreover, ED and UF requires an additional assay (e.g., LC-MS/MS) for quantification prolonging the analysis procedure, thus requiring more time, personnel and instrumentation (Chen et al., 2010). The labour-intensive analysis for the golden methods is the primary pitfall making it unfeasible for clinical use. The use of LC-MS/MS for separation, detection and quantification is therefore becoming more applicable in clinical laboratories due to its capabilities to analyse large number of samples with high time-efficiency whilst offering a sensitive and selective method (Vogeser & Parhofer, 2007).

The determination of the TT and the transport proteins albumin and SHBG are comparatively easier and more common in clinical routine using directs IAs than the reference methods. Due to this reason, mathematical approaches are used for the calculated bioavailable testosterone (cBT) and calculated free testosterone (cFT) using parameter values (i.e., total T, SHBG and albumin) obtained by IAs as alternatives to the tedious ED/UF procedures (Keevil & Adaway, 2019). The Free Androgen Index (FAI) is also used to determine the bioactive portion of T by calculating the ratio between the total T and SHBG, and is included in the PCOS international Guideline as an alternative biomarker when FAI > 0.6 (Teede et al., 2018).

2.4.3 Extraction of bioavailable testosterone with ammonium sulphate

Measurement of the BT (i.e., albumin-bound T and free T) is recommended as a viable alternative in cases when TT is found to be subnormal or when there is suspected altered SHBG levels (Bhasin et al., 2010). Extraction of BT presents a challenge in protein precipitation as it expects full precipitation of SHBG to remove SHBG-bound T and minimal loss of albumin to retain the albumin-bound T in solution.

The commonly employed method for SHBG precipitation for BT extraction is utilization of ammonium sulphate (AMS) (Manni et al., 1985; O'Connor et al., 1973). The advantage of using AMS is the selective protein precipitation at a certain salt saturation. Loric et. al. have shown that AMS with 50% salt saturation enables the precipitation of SHBG whilst leaving albumin in solution (Loric et al., 1988). Their findings implies that the albumin-bound T and free T remains in the supernatant free from SHBG-bound T. Earlier studies coupled SHBG precipitation by AMS with RIA (Déchaud et al., 1989) and isotope-labelled T (Cumming & Wali, 1985; Tremblay & Dube, 1974). Alternative methods for BT extraction can also be employed by the use of Concanavalin A (Yamamoto et al., 2009) and SHBG immunocapture (Raverot et al., 2010). Moreover, cBT obtained by mathematical equations can serve as an alternative to the tedious extraction procedures (Vermeulen et al., 1999).

2.4.4 Mathematical equations

Estimation of the free hormone concentration is evident for the determination of the free and biologically active hormones. Vermeulen et. al. first described an algorithm for measuring cBT and cFT concentrations based on the law of mass action, and is the most widely applied

equation (Vermeulen et al., 1999). The proposed algorithm uses the values for albumin, SHBG and TT for the determination of cBT and cFT concentrations. Fiers et. al., have compared FT measurements conducted on ED coupled with LS-MS/MS to conclude that cFT by the Vermeulen-algorithm showed satisfactory linear correlation with the ED-based analysis. However, a systematic overestimation has been observed for cFT indicating that mathematical equations are mere approximations of bioactive portion of T (Fiers et al., 2018; Ly & Handelsman, 2005).

Multiple calculations are available with different approaches to determine the biologically active portion of T (i.e., BT and FT). Alongside the Vermuelen-algorithm, an alternative equation for FT and BT based on the mass of law action is proposed by Södergard et. al. upon experimentally obtaining the association constants by ED (Södergard et al., 1982). The mathematical equations introduced by Morris et. al. and Emadi-Konjin et. al. are established on the SHBG-precipitation by AMS to attain BT and requires TT and SHBG concentrations for cBT (Emadi-Konjin et al., 2003; Morris et al., 2004). Moreover, Ly et al. developed an algorithm for determining FT based on centrifugal UF (Ly & Handelsman, 2005).

Limitations of algorithms include difference in the analysis methods to obtain BT, FT and/or the association constants, and the equation performance may be affected in different degrees by extreme values of SHBG and TT concentrations (Hackbarth et al., 2011). It should be noted that the association constants between T and SHBG or albumin utilized in the mathematical equations are measured *in vitro*, and the observed deviations from the true FT concentrations may derive from biologically distinct binding affinity *in vivo* (Vermeulen et al., 1971).

The Free Androgen Index (FAI = 100T/SHBG) is recognized to be less reliable than the alternative equations for the determination of BT and FT in cases with low SHBG concentrations (Rivera-Woll et al., 2004). Kapoor et. al., have expressed the invalidity of FAI in male samples with high T concentrations and lower SHBG levels further confirming the weakness of FAI (Kapoor et al., 1993). Likewise, Keevil et al. confirms the unreliableness of FAI in women with hyperandrogenism due to low SHBG levels (Keevil et al., 2018). SHBG levels differs in age, gender and various metabolic and endocrinological conditions (Aribas et al., 2021; Vermeulen et al., 1971). Thus, implying that the FAI-formula, alike some of the forementioned equations is vulnerable to extreme SHBG values (Hackbarth et al., 2011; Ho et al., 2006).

cBT and cFT are sufficient alternatives to FAI and the time-consuming ED. However, parameters used in the proposed algorithms are commonly done with measurements of TT, SHBG and/or albumin conducted by IAs (De Ronde et al., 2006). Thus, the use of mathematical equations must equip reliable assays to minimize variables and errors. However, MS-based methods can eliminate time-consuming analysis procedures (e.g., with ED) for the determination of cFT by measuring FT in saliva samples (Keevil et al., 2014; Nadarajah et al., 2017). It is anticipated that T is not bound to proteins in saliva and may be referred to as FT (Gröschl, 2008).

2.4.5 Liquid chromatography with tandem mass spectrometry

In the recent years, liquid chromatography with tandem mass spectrometry (LC-MS/MS) is becoming more widespread in biochemical analysis for steroid hormone determination. In comparison with IAs, LC-MS/MS offers an array of advantages such as higher selectivity and sensitivity, lower detection limits and multi-analyte analysis (Soldin & Soldin, 2009). The general arrangement of an LC-MS/MS analysis can be divided into three main sections: 1) sample preparation, 2) chromatographic separation, and 3) analyte detection.

2.4.5a Sample preparation

Sample preparation is a crucial step prior to the chromatographic separation and essential in minimizing the amount of potential analyte interreferences and matrix components affecting the analysis performance and instrumentation, in addition to providing analyte extraction and enrichment. Phospholipids are abundant in serum and their removal is important to obtain neat sample extracts to avoid ion suppression and column clogging (Jemal et al., 2010). The following methods of sample preparation are often employed for steroid analysis from liquid samples (i.e., serum and plasma): protein precipitation (PP), solid phase extraction (SPE) and liquid-liquid extraction (LLE) (Stone, 2018). Furthermore, applying the principle of LLE by using salt complexes can be achieved in salting-out assisted liquid-liquid extraction (SALLE) as an alternative sample preparation method (Valente & Rodrigues, 2015).

The principle of PP involves the use of water-miscible organic solvents (e.g., MeOH and ACN) to generate phase separation with two phases (i.e., solvent and precipitate) in an aqueous

sample aided by vortex mixing and centrifugation. Analyte extraction and sample clean-up are achieved through protein precipitation with the solid precipitate containing protein-bound complexes separated from the sample supernatant. The supernatant can be directly injected to the LC-MS/MS system for analysis, see **Figure 16A** (Polson et al., 2003) As solvent-soluble matrix components, such as phospholipids and triglycerides, also resides in the supernatant together with the target analytes, direct injection makes the analysis susceptible to matrix effects resulting in ion suppression (Chambers et al., 2006).

Sample preparation using SPE have higher capacity in removal of interferences as it employs chromatographic separation principles. In the same manner as an LC-column, SPE-cartridges involves the use of solid sorbents functioning as the stationary phase (SP) and analyte elution with organic solvents (Zwir-Ferenc & Biziuk, 2006). As steroid hormones are non-polar in nature, it is employed SPE with the separation technique of reversed-phase (RP) with a non-polar SP with hydrocarbons and polar mobile phase (MP) with polar organic solvents (e.g., MeOH and ACN) (Travers et al., 2017). Analyte isolation is therefore enhanced compared to PP with the capacity to prepare large sample number with 96-wells, but SPE require more extraction steps and lacks the simplicity that PP offers (Koal et al., 2012). First, the solid sorbent must be conditioned as preparation for sample loading. Then, the sample is applied and washed to elute interferences. Lastly, target analytes are eluted from the column with solvent with higher elution strength (**Figure 16B**). SPE has proved to effectively remove phospholipids in SPE using RP principle with ACN as the eluent (Lahaie et al., 2010).

Immiscibility of solvents with differing polarities is foundation of the separation in LLE. Phase separation is achieved when non-polar solvents (e.g., ethyl acetate or ethyl-tert-butylether) are added to aqueous samples (e.g., serum) and applied to mixing and centrifugation. Steroids will reside in the solvent phase due to their non-polar property enabling high solubility (French, 2016). Thus, target analytes are separated from matrix components by analyte partition in the solvent. Removal of matrix components are comparatively improved in LLE than in PP resulting in decreased matrix effects and higher analyte signal (i.e., higher signal-to-noise ratio) (Aubry, 2011). However, the supernatant must be subjected to evaporation and reconstitution in LC-compatible solvents as non-polar solvents cannot be introduced in an RP-LC system (Turpeinen et al., 2008). During the step of evaporation and reconstitution, analyte enrichment can be obtained when reconstituted in less solvent volume than the initial sample volume (**Figure 16C**). Another advantage of LLE is the possibility of conducting multiple extraction

of the sample extract for further removal of interferences to attain cleaner extracts prior to injection in the LC-MS/MS system.

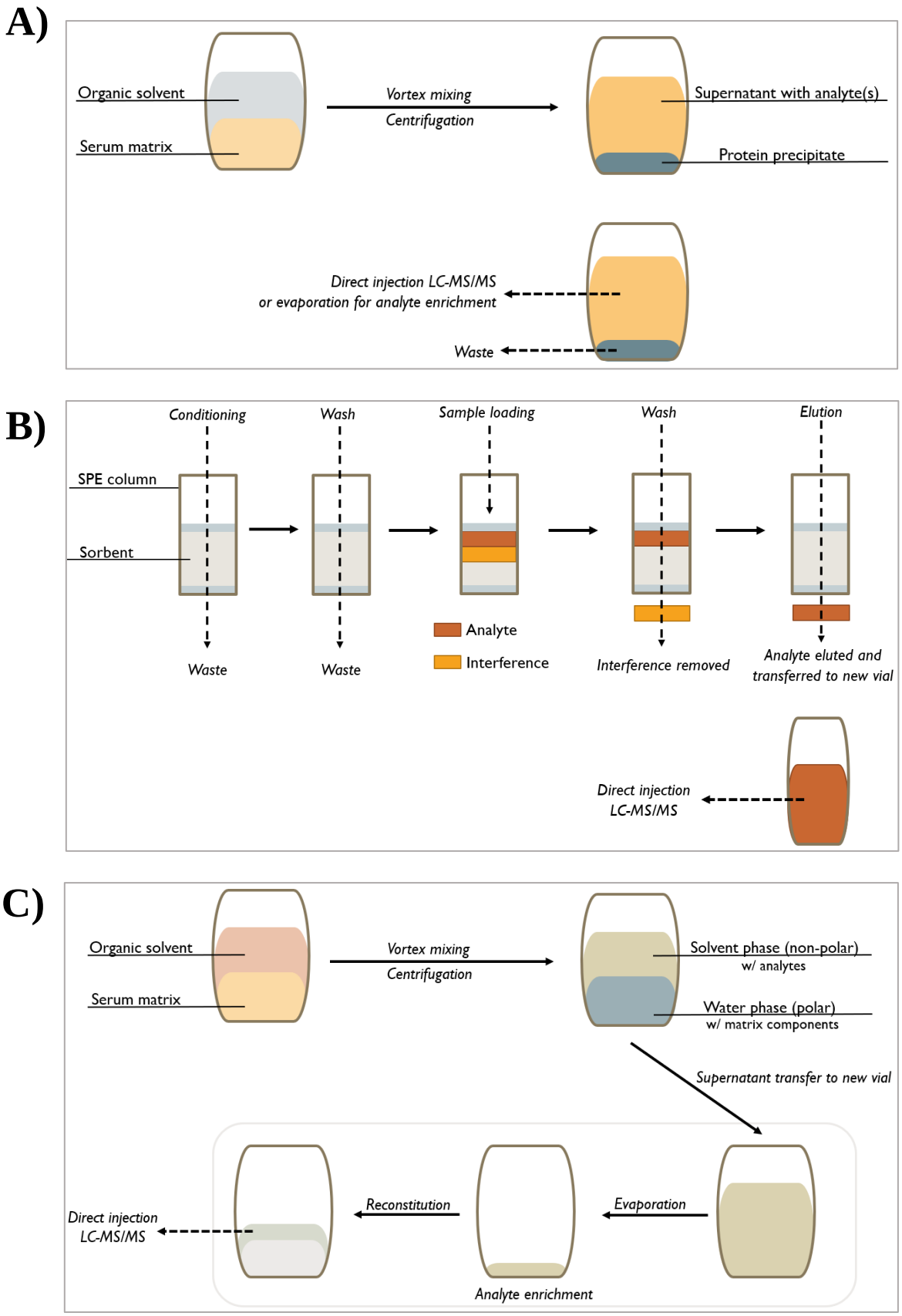


Figure 16: Descriptive illustration for the analysis procedure for (A) protein precipitation (PP), (B) solid phase extraction (SPE), (C) Liquid-liquid extraction (LLE).

The principle of SALLE is based on analyte partitioning to the organic solvent equivalent to LLE. However, SALLE utilizes water-miscible solvents (e.g., ACN and acetone) and concentrated salts (e.g., magnesium sulphate and ammonium sulphate) in solid or aqueous forms instead of non-polar solvents (Tang & Weng, 2013). The behaviour of certain anions in salt complexes affects the hydrogen bonding in water and the extent of their ability to alter the structure and dynamics of water is ranked in the Hofmann series (Collins & Washabaugh, 1985). The sulphate anion (SO_4^{2-}) is extremely effective in decreasing protein solubility in aqueous solutions (i.e., protein precipitation) promoting the salting-out effect (Zhang & Cremer, 2006). High salt concentrations initiate the salting-out effect by increasing the polarity of the aqueous phase, thus pushing the most non-polar compounds out of the water-phase ultimately inducing phase separation (Valente et al., 2013). The target analytes with non-polar properties will therefore be retained in the solvent phase in the SALLE system. The water-miscible solvent ACN, widely used in SALLE, offers an additional advantage by poorly dissolving phospholipids further minimizing potential matrix effects (Chambers et al., 2007), and efficiently precipitating proteins (Polson et al., 2003). Then, phase separation is induced upon mixing and centrifugation enabling direct injection of the supernatant to the LC-system (**Figure 17**). The final SALLE extract will have the solvent phase on top completely separated from the salt-saturated aqueous solution, but the water-miscible solvent will easily infuse with the aqueous MP with less salt content when injected. The procedure of SALLE is in many ways a combination of PP and LLE, where both an aqueous salt mixture and a water-miscible solvent is added to the sample matrix to precipitate proteins and induce phase separation (Valente & Rodrigues, 2015).

SALLE can be fully automated and carried out in 96-well formats in the same manner as LLE (Zhang et al., 2009). Advantages of SALLE includes the absence of toxic solvents to achieve solvent separation providing a green alternative (Tang & Weng, 2013), as well as being applicable to a wider range of analyte enabling simultaneous extraction due to the salting-out effect (Kole et al., 2011). Moreover, ammonium salts (e.g., ammonium acetate) used in SALLE can improve MS-performance by enhancing the formation of positive ions $[M+H]^+$ in ESI+ (Wu et al., 2008).

Interestingly, AMS is known to separate BT (i.e., albumin-bound T and free T) from serum by precipitation of the SHBG-bound T upon 50% salt saturation (Loric et al., 1988). Not only can AMS be applied for SALLE to offer an effective sample preparation with the simplicity of PP

and interference removal efficiency of LLE, but AMS can also be applied for BT extraction. This implies that after precipitating the SHBG-bound T from serum with 50% AMS saturation, the supernatant can be collected and ACN added for extracting the free- and albumin-bound T by SALLE. Thus, sample clean-up and analyte (including BT) extraction can be carried out in a simple and effective step with minimal labour compared to the conventional sample preparation (i.e., SPE and LLE) and methods for BT extraction (i.e., EF and UF). A recent study by Urge et. al. have shown the exceptional extraction efficiency and reduction of matrix effects by SALLE for the extraction of steroid hormones from serum compared to SPE and LLE (Urge et al., 2023).

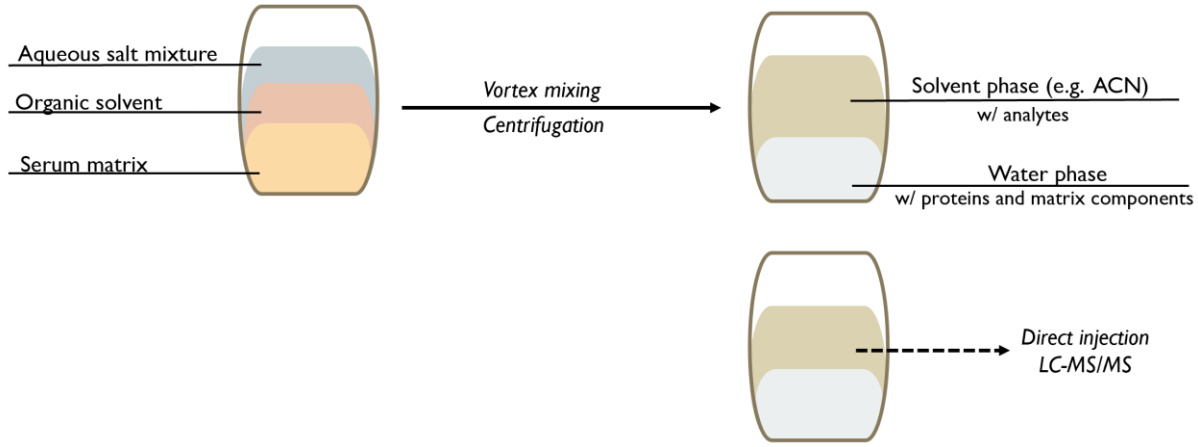


Figure 17: Simplified procedure overview of salting-out assisted liquid-liquid extraction (SALLE)

2.4.5b Chromatographic separation

The main concept of chromatography resides in the chemical interactions between the target analyte, SP and MP. The term “The same dissolves the same” explains the central theory in chemistry regarding the relationship between polarity and solubility. It explains the high solubility of polar compounds in polar solvents, and the same goes for non-polar compounds in non-polar solvents. The polarity of the SP and MP used in the chromatographic analysis can be adjusted to manipulate the interaction and solubility of the target analyte (van Deemter et al., 1956). Thus, molecules with increased solubility in the SP will have greater residing time and delayed elution. Likewise, molecules with less interactions with SP (i.e., more soluble in MP) will elute first with the least retention (**Figure 18A**). Several separation principles can be employed depending on the polarity of the compound of interest to achieve optimal separation of compounds in complex samples (Zhang et al., 2012).

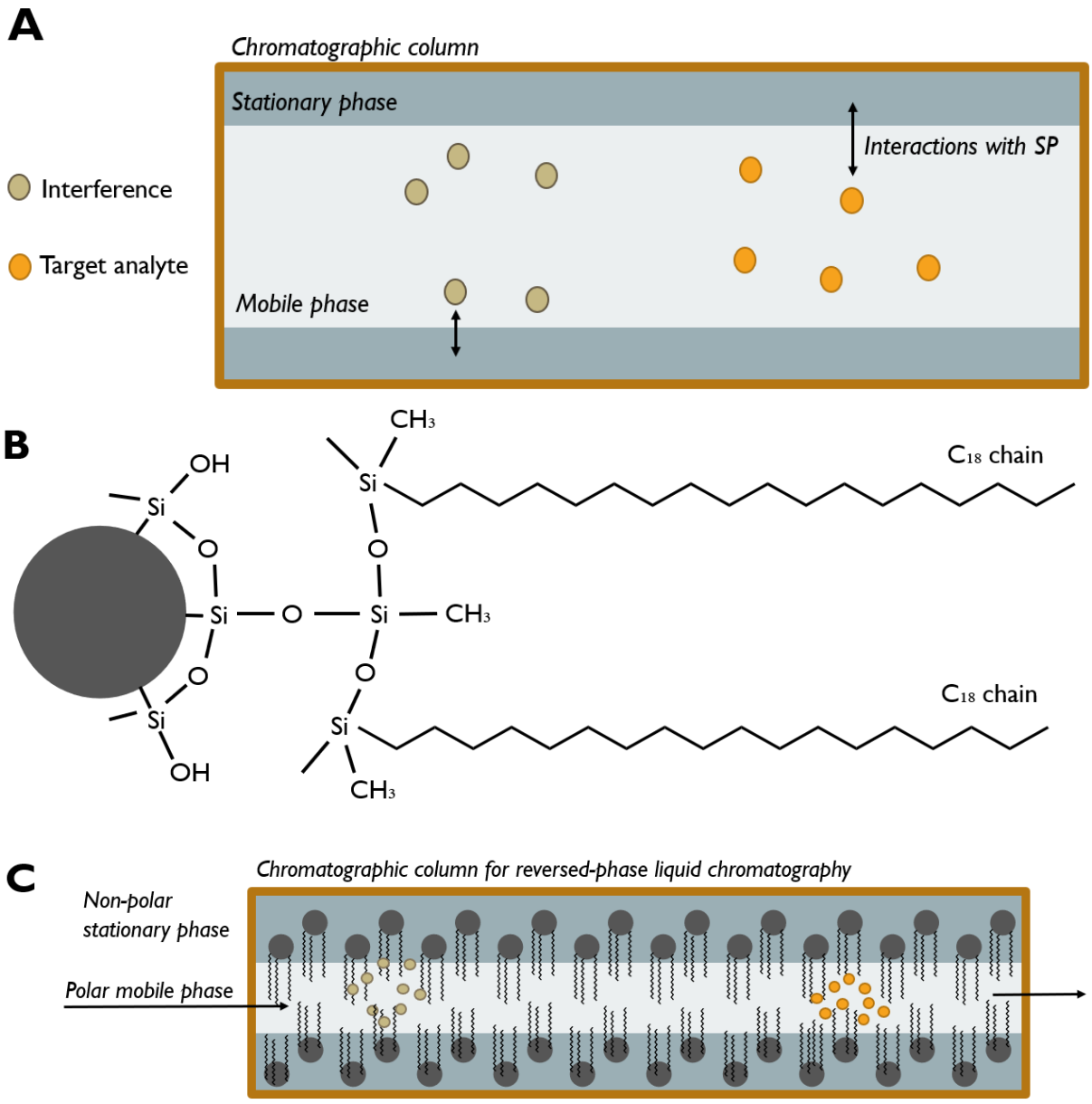


Figure 18: (**A**) Main concepts of separation theory in liquid chromatography regarding chemical interactions with the mobile phase (MP) and stationary phase (SP). The interfering compound, interference, (brown) has higher solubility in the SP resulting in longer residing period causing the increased retention and delayed elution. The target analyte (yellow) is eluted faster due to less interactions with the SP. (**B**) Spherical and porous silica particles covalently bound to non-polar hydrocarbons are common SP column particles in reversed-phase (RP) separation principle. (**C**) Simplified illustration of RP-LC column with C_{18} SP. The target analyte moves along the chromatographic column based on hydrophobic interactions with the SP and MP

Steroid hormones are non-polar molecules, and the most employed separation technique is the reversed-phase liquid chromatography (RP-LC) (Siggia & Dishman, 1970). In this technique, SP is the non-polar component and MP is the polar component in the chromatographic column affecting the analyte solubility based on hydrophobicity (Aguilar, 2004). The column is packed with spherical and porous silica particles where the surface has a covalently bound stationary

phase consisting of non-polar hydrocarbon functional groups (e.g. C_8 and C_{18}) to generate a non-polar SP (Zhou et al., 1991) (**Figure 18B and 18C**). Longer sidechains on the hydrocarbon will increase analyte retention due to decreased polarity, thus making the non-polar steroid hormones more soluble in the SP. Furthermore, the elution of steroid hormones from the column is accomplished by increased interaction with the polar MP (Vailaya & Horváth, 1997). For instance, by increasing the elution strength with more organic solvent in the MP in gradient elutions. The polar, water-miscible solvents MeOH and ACN are commonly used as MP.

The general principle of LC is applied in high-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC) in the same manner. Commercial instrumentation for high pressure mobile phase delivery was first introduced in 2004, together with new columns and the term "ultra-performance liquid chromatography™ (UPLC), by Waters Corporation (Swartz, 2005). The main difference between HPLC and UPLC/UHPLC lies in the chromatographic efficiency caused by differences in the particle dimensions used to support the column SP (Jerkovich et al., 2003). The particle size commonly used in HPLC is 3.5 - 5 µm, whereas UPLC utilizes particle dimensions < 2 µm (Swartz, 2005).

High column efficiency can be described as low values of the theoretical plate height (HETP) and the use of sub-2µm particle dimensions in UPLC enables higher linear velocity rates without the increase in theoretical plate height (**Figure 19**). The high chromatographic efficiency in UPLC-MS/MS is therefore maintained with faster MP flow rate, thus providing high-throughput analysis without sacrificing overall chromatographic performance affecting the separation resolution. Moreover, increased column temperature lowers the eluent viscosity and makes it possible to further increase linear velocity (Mazzeo & Kele, 2005). Reduced matrix effects are also observed in UPLC-systems compared to HPLC with narrower peaks and increased peak capacity for improved resolution (Chambers et al., 2007). However, smaller column particles will ultimately result in higher back-pressure and the use of more powerful pumps withstanding up to 20 000 psi is required in UPLC systems (Nováková et al., 2006). Thus, the cost of instrumentation is therefore higher with UPLC than HPLC.

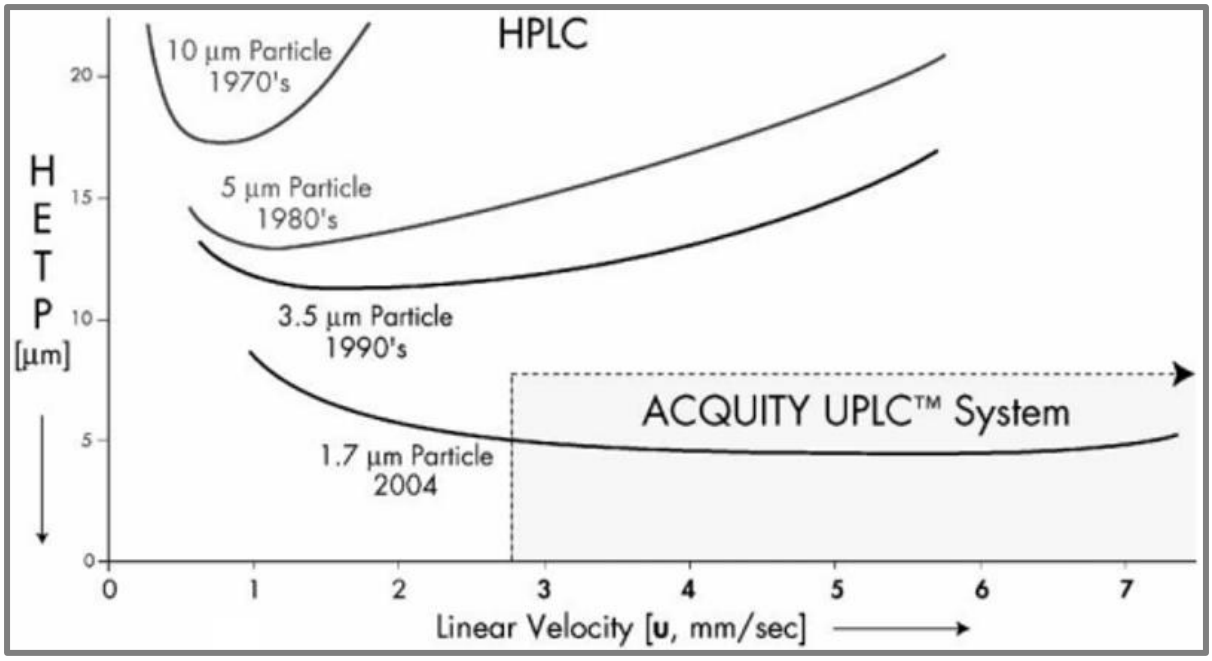


Figure 19: Van Deemter plot with theoretical plate height (HETP) in y-axis and linear velocity in x-axis describing the effects of particle downsizing on chromatographic efficiency through the years (70's – 00's). Figure reprinted from Swartz (2005)

2.4.5c Analyte detection

Ionization of the eluent from the chromatographic column in an ion source occurs following the chromatographic separation. Analyte ionization is crucial in MS-based methods for the formation of molecular ions. Soft ionization techniques are utilized in LC-MS resulting in minimal analyte fragmentation, whereas gas chromatography coupled with mass spectrometry (GC-MS) typically implements hard ionization with electron ionization (EI) for greater analyte fragmentation. Several ionization techniques can be applied in LC-MS/MS including atmospheric pressure chemical ionization (ACPI), atmospheric pressure photoionization (APPI) and electrospray ionization (ESI) (Zaikin & Halket, 2006). The latter ionization technique is commonly applied for steroid measurements and can be used in positive (ESI+) or negative (ESI-) mode for the generation of positive $[M+H]^+$ or negative $[M-H]^-$ molecular ions, respectively (Karashima & Osaka, 2022).

The primary task of the ion source is to function as an interface between the chromatographic column and the mass spectrometer involving the transformation of analytes dissolved in aqueous solutions into ions in gaseous phase. In ESI, the eluent (i.e., MP with analytes) from the analytical column enters a thin capillary with high voltage to assist the formation of charged ions. Towards the outlet of the capillary, a nebulizing gas (e.g., nitrogen) is utilized to aid

nebulization and the generation of electrically charged droplets. The formed droplets are then met with a drying gas (e.g., nitrogen) going against the flow to provoke solvent evaporation and analyte ion transition to gaseous phase upon entering the mass spectrometer (Bruins, 1998). Moreover, the pH of the mobile phase is essential in facilitating protonation and deprotonation of molecular ions in ESI (Liigand et al., 2017). MP additives are volatile acids and bases such as formic acid (FA) and ammonium hydroxide (NH_4OH) to promote low and high pH, respectively. Protonation of the molecular ion is favoured by low pH upon ionization generating a positively charged molecular ion $[M+H]^+$ to be detected in ESI+. Likewise, deprotonation is favoured by high pH to generate the negatively charged molecular ion $[M-H]^-$ detected in ESI- (Cech & Enke, 2001). The latter method is preferred for the detection of acids and phenols (e.g., oestrogens) (Bertelsen et al., 2020). Additionally, NH_4OH can be applied in ESI+ to aid protonation and high $[M+H]^+$ signals even with high MP pH. This is due to the generation of NH_4^+ which functions as the proton donor instead of H_3O^+ (Mess et al., 2009). However, silica-based analytical columns are limited to operate within pH 2-8 and greater pH can result in silica dissolution and ultimately liquifying the particles supporting the SP, thus limiting the use of bases such as NH_4OH for ESI (Nawrocki, 1997). Columns with hybrid silica particles, such as Waters™ Ethylene Bridged Hybrid (BEH) technology, can provide chemical stability with a wider pH range up to pH 12 enabling the use of high pH for ESI+ (Wyndham et al., 2003).

Tandem mass spectrometry (MS/MS) instruments that utilize quadrupole mass filters are known as triple quadrupole instruments. These are widely used for the detection of various analytes from large proteins and peptides to small molecules such as steroid hormones (Shushan, 2010). Quadrupoles are capable of scanning and selecting ions with specified mass-to-charge (m/z) ratios from a beam of ions generated by the ion source. A collision cell is located directly after the first mass analyser in MS/MS instruments responsible for the fragmentation of molecular ions (Yost & Enke, 1978). Then, the second mass analyser selects specific fragment ions which are detected by the ion transducer (i.e., the detector). The generation of charged molecular ions from the ion source is essential for the selectivity provided in MS/MS-systems prior to ion selection in the mass analyser (Matysik & Liebisch, 2017). During method development, the analytes are subjected to MS-tuning for the determination of their precursor ion (molecular ion) m/z and daughter ions (fragment ions) m/z.

The quadrupole consists of two rods with positive polarity and two with negative polarity, and high RF/DC frequency interchanges the polarity between the rods (Reuben et al., 1996). Thus, moving the ions with specified m/z through with potential energy in a stable trajectory until reaching the detector (**Figure 20A).** Molecular ions with unspecified m/z values will be prohibited from moving forward by unstable trajectories and ejection into the space between the quadrupole rods (Henchman & Steel, 1998). From the ion source, the ionized molecular ion enters the first quadrupole (Q1) and the analyte precursor ion with the specified m/z value moves to the second quadrupole (Q2) with a stable trajectory (Hopfgartner et al., 2004). Further collision of the precursor ion occurs in the Q2, often referred to as collision cell (Sleno & Volmer, 2004). The collision cell contains an inert gas (i.e., collision gas) and ions are accelerated here by a certain voltage corresponding to a certain energy level (i.e., collision energy). This results in collision induced dissociation (CID) of the precursor ion to produce the fragment ions. The specified m/z for the fragment ion is then selected in the third quadrupole (Q3) (**Figure 20B)**. The fragment ion is obtained from Q3 and further navigates its way to the ion transducer (i.e., detector) for measurement of the relative abundance of the fragment ions which is translated to a chromatogram (Hopfgartner et al., 2004). Today, triple quadrupole instruments often utilize a different type of ion guide for their collision cell. The beam of ions may be guided through the collision cell by a hexapole or by a stack of charged ring-shaped plates instead of the quadrupole (Q2) (Giles et al., 2004).

Multiple reaction monitoring (MRM) utilizes the unique transition of the precursor-to-daughter m/z values to detect target analytes. This increases the method selectivity and sensitivity upon analyte detection, thus deflecting the cross-reactivity problems associated with IAs (Janzen et al., 2008). Moreover, multiplex analysis is enabled by using MRM since the precursor and product ion transitions for multiple analytes can be simultaneously monitored and selected by the quadrupoles (Peti et al., 2018). Detection by MS/MS offers specificity and selectivity superior to IAs, however, isobaric compounds will result in equal fragmentation patterns and molecular ions. Column separation is therefore specially crucial for isobaric compounds for distinct column retention to avoid co-elution upon reaching the ion source (Couchman et al., 2011).

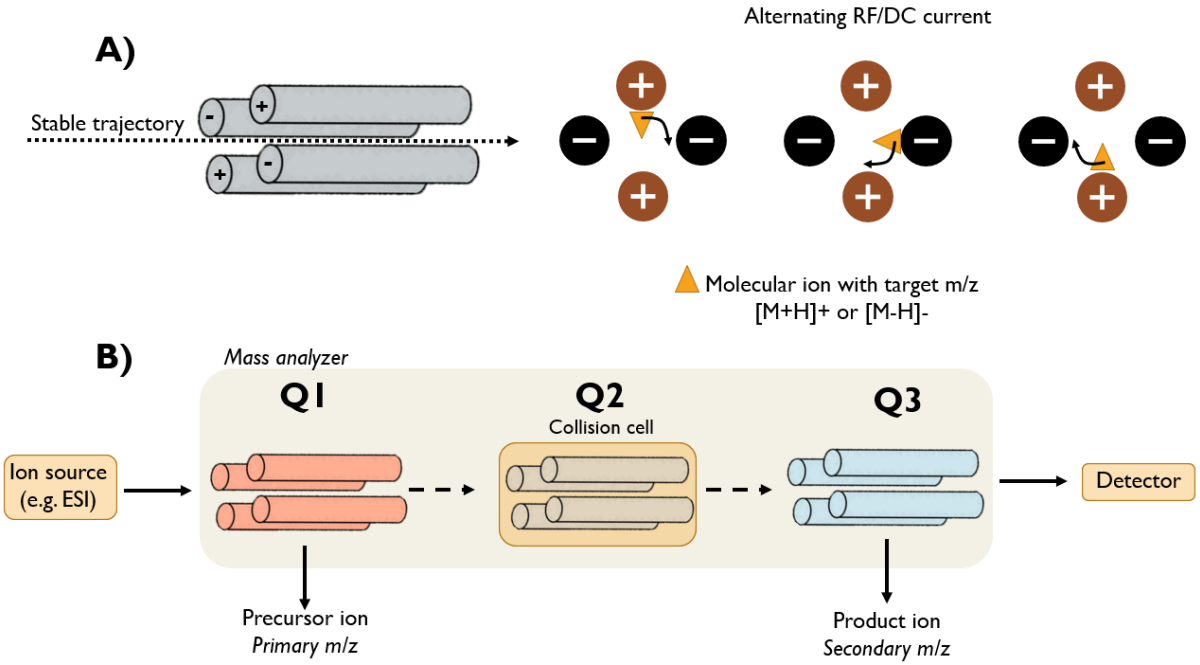


Figure 20: (**A**) Oscillation pattern of ions with stable trajectories in the alternating RF/DC current in the quadrupole. The molecular ions with target m/z pass through the quadrupoles with a stable trajectory, whereas molecular ions with unspecified m/z values are ejected upon unstable trajectory through the quadrupoles. (**B**) Illustrative description of the quadrupole mass analyser. Ionized molecule from the ion source enters the mass analyser. The precursor ion is separated in the first quadrupole (Q1) and further subjected to collision in the second quadrupole (Q2). The third quadrupole (Q3) produces the secondary fragment ion.

2.4.5d Multiplex analysis

One of the remarkable advantages of LC-MS/MS compared to the alternative assays for steroid measurements is the opportunity to conduct simultaneous analysis of multiple analytes. Obtaining steroid hormone profiles is advantageous for excluding differential diagnosis necessary for correct diagnosis and treatment plan (Keevil, 2019). Additionally, it may provide an overview of the hormone concentration pattern associated with endocrinological conditions for further understanding the steroidogenesis pathways and intracrine relationships. For instance, 11-oxyandrogens have shown their significance in endocrinological conditions with clinical hyperandrogenism such as PCOS and CAH with distinct cause of defect in steroidogenesis occurring in the ovaries and adrenal glands, respectively (Nowotny et al., 2022; O'Reilly et al., 2016; Turcu et al., 2016).

Measurement of TT concentrations conducted on IAs shows high variability between assays with samples with characteristic low SHBG levels seen in hyperandrogenism and hypogonadism (Boots et al., 1998). Standardized reference ranges are advantageous to properly

diagnose individuals with suspected endocrinological disorders, and their establishment must therefore be conducted on sensitive and specific bioanalysis assays with proper quality control programmes for standardization (Kushnir et al., 2010). Multiplex analysis also offers lower cost and higher time efficiency in the long run as multiple assays conducted on IAs may be replaced by a single analysis.

2.5 Aim of study

The current study intends to optimize and validate an in-house developed UPLC-MS/MS method at the Stavanger University Hospital for the multiplex measurement of 11 steroid hormones, including bioavailable T, from human serum. The analyte panel consists of glucocorticoids, progestogens, and androgens. SALLE will be employed using ammonium sulphate as the salting-out reagent for sample clean-up and analyte extraction. As to our knowledge, this will be the first study that explores the extraction of 11-oxygenated androgens using SALLE from human serum.

Method validation involves the assessment of limit of detection (LOD), limit of quantification (LOQ), intermediate precision, repeatability, working range and analyte carry-over for each analyte. Moreover, bias evaluation for determining method trueness was conducted by a series of experiments including spiked recovery experiments, inter-laboratory comparison with LC-MS/MS methods and method comparison with IAs and mathematical equations. Limitations in assay availability for every analyte resulted in inter-laboratory and method comparison for only for a handful of analytes.

The proposed method is designed as a routine analysis, thus the criteria of high analysis throughput, cost- and time efficiency, sample capacity and user-friendliness were considered during the optimization process. The benefit of multiplex analysis with UPLC-MS/MS enabled the generation of 38 hormone profiles of healthy volunteers obtained through the Blood Reference in Stavanger (BRIS) project. This study focuses on the application of the proposed UPLC-MS/MS method on endocrinological conditions with aberrant androgen concentrations such as PCOS in women and hypogonadism in men.

3. Experimental

3.1 Reagents and materials

The HPLC-MS grade ACN, MeOH, FA and NH_4OH used for the preparation of the mobile phases were purchased from VWR International (Radnor PA, United States). Ammonium sulphate was purchased from Merck (Darmstadt, Germany) with 99.5% purity. The glucocorticoids B (98%), DOC (97%), E (98.5), F (98%), 11DF (98%), androgens T (99%), 11KT (98%), 11OHA4 (98%), and progestogens P (99%) and 17OHP (99.6%) were purchased from Sigma-Aldrich (Burlington, MA, United States). A4 was purchased from Cerilliant (Round Rock, TX, United States). The deuterium (D) labelled internal standards (ISTD) D4-11OHA4, D4-F, D3-T and D7-E were obtained from Sigma-Aldrich. D8-A4 was purchased from Alsachim (Illkirch Graffenstaden, France), and the progestogen ISTDs D9-P and D8-17OHP were purchased from Cerilliant.

3.2 Stock solutions, calibrators, and quality control samples

The primary, individual stock solutions were prepared by weighing the solid compound then diluted in 25 mL MeOH, except for A4 which was diluted in ACN, to achieve concentrations ranging from 40.4 – 368 μg/mL. Furthermore, a mixed stock solution containing every target analyte was prepared by adding 50-500 μL of the primary stock solutions and further diluted with 25 mL MeOH to obtain analyte concentrations ranging from 177.6 – 7360 ng/mL. The prepared mixed stock solution was used for the preparation of the calibration solutions The prepared primary stock solutions and mixed stock solution were kept at 4°C until further use.

The stock solutions of the internal standards were prepared according to the same procedure as previously described obtaining concentrations ranging from 4.0 – 40 μg/mL. The individual ISTDs were pooled in a ISTD mixed solution and was prepared by diluting 100 – 1000 μL of the ISTD stock solutions in 500 mL deionized H_2O to achieve concentrations between 7.2 – 26.2 ng/mL. The ISTD mixed solution was kept at room temperature until further analysis. An overview of the parameters for the preparation of the stock solutions and final concentrations of the mixed solutions for the analytes and ISTD are given in **Table 1** and **2,** respectively.

Table 1: Concentrations of analytes and the final mixed stock solution given in ng/mL. Analyte primary and mixed stock solutions were diluted in 25 mL MeOH besides than A4 which was diluted in ACN.

	Primary stock solution		**Mixed stock solution**
Compound	Concentration, ng/mL	Volume added, µL	Concentration, ng/mL (nmol/L)
Androgens			
T	252 800	50	505.6 (1753.0)
A4	43 940	200	177.6 (492.7)
11KT	40 400	300	484.8 (1603.1)
11OHA4	45 200	300	
Progestogens			
P	234 800	50	469.6 (1493.4)
17OHP	223 600	50	447.2 (1353.3)
Glucocorticoids			
F	368 000	500	7360 (20 305.7)
E	351 600	100	1406.4 (3901.9)
B	182 800	100	731.2 (2110.5)
11DF	269 600	20	215.7 (622.5)
DOC	218 800	20	175.0 (529.7)

Table 2: Concentrations of internal standards in the primary stock solutions with concentrations given in ng/mL or nmol/L. ISTD primary stock solutions were diluted in 25 mL and mixed solution in 500 mL H_2O.

	Primary stock solution		**Mixed stock solution**
Compound	Concentration, ng/mL	Volume added, µL	Concentration, ng/mL (nmol/L)
Internal standard			
D3-T	36 000	100	7.2 (24.7)
D8-A4	40 000	100	8.0 (27.2)
D4-11OHA4	40 000	100	8.0 (26.1)
D9-P	4000	1000	8.0 (24.7)
D8-17OHP	4000	1000	8.0 (23.6)
D4-F	33 600	300	20.1 (55.0)
D7-E	43 600	300	26.1 (71.2)

The ammonium sulphate (AMS) solution utilized as the salting-out reagent in the sample preparation procedure was prepared by pouring crystalized AMS salt granules in a 500 mL graduated reagent bottle and filling up to the mark with deionized H_2O. The amount of AMS should be enough to leave a visual layer of precipitated AMS in the bottom of the bottle to ensure adequate saturation. No direct measurements of the salt amount were made as the aim was to prepare a saturated AMS solution. This saturated salt solution was mixed at a 1+1 ratio with serum to achieve 50% salt saturation.

The standard solutions (i.e., calibrators) were prepared in HyClone™ new-born calf serum (NBCS) from GE Life Sciences (Chicago, IL, United States). The NBCS was subjected to a 10-minute 4000xg centrifugation to remove any possible interferences from the sample matrix. The amount of 10 - 500 µL mixed stock solution were added to a 25 mL volumetric flask and filled to the line with room-tempered NBCS for each standard solution to achieve an appropriate concentration range for all analytes distributed between eight calibration solutions (**Table 3**). NBCS was chosen rather than bovine serum as an alternative sample matrix to human serum. This is to ensure that the sample matrix had little to no endogenous concentrations of the target analytes as infant calves will comparably have lower hormone concentrations, especially androgens, compared to adult cows. The standard solutions (STD 0 – STD 8) were aliquoted in polypropylene tubes (Sarstedt; Nümbredcht, Germany) and kept in -80°C until further analysis.

Table 3: Final added concentrations of target analytes in eight standard solutions (STD) prepared with new-born calf serum (NBCS). No analyte was added to STD 0 (not shown). Concentrations are given in nmol/L and volumes in uL.

	STD 1	**STD 2**	**STD 3**	**STD 4**	**STD 5**	**STD 6**	**STD 7**
From mixed stock solution Volume (uL)	10	25	50	75	100	250	500
Compound	**Concentration in 10 mL NBCS (nmol/L)**						
Androgens							
T	1.8	4.4	8.8	13.1	17.5	43.8	87.7
A4	1.2	3.1	6.1	9.2	12.2	30.7	61.4
11KT	1.6	4.0	8.0	12.0	16.0	40.1	80.2
11OHA4	1.8	4.5	9.0	13.4	17.9	44.8	89.7
Progestogens							
P	1.5	3.7	7.5	11.2	14.9	37.3	74.7
17OHP	1.4	3.4	6.8	10.1	13.5	33.8	67.7
Glucocorticoids							
F	20.3	50.8	102	152	203	508	1015
E	3.9	9.8	19.5	29.3	39.0	97.5	195
B	2.1	5.3	10.6	15.8	21.1	52.8	106
11DF	0.62	1.6	3.1	4.6	6.2	15.6	31.1
DOC	0.53	1.3	2.6	4.0	5.3	13.2	26.4

Moreover, the quality control (QC) samples used in this study reflected three concentration levels of testosterone. The labelled high and low QCs were obtained from healthy volunteers, a male and female, representing true high and low T concentrations. The commercially available reference material Autonorm™ Immunoassay Liquid-1 from Sero (Billingstad,

Norway) served as the mid-level QC. In general, the three QC samples covered a wide concentration range for most of the analytes. For a few analytes, it was not possible to prepare a QC with a high concentration level due to limitations in the endogenous levels of these steroid hormones (i.e., DOC, 11DF, 11KT and 11OHA4) with concentrations near or below the method limit of detection in addition to being absent in the commercially available QC. Upon the evaluation of method trueness of T, A4, P, 17OHP and F, external QCs from the External Quality Assessment (EQA) scheme of the accredited Labquality (Helsinki, Finland) were utilized. The Labquality QCs, 29 human serum samples, was sent to participating laboratories in the years 2019-2022. The sample had been stored at -20° prior to the presented analysis in the current study.

3.3 Collection, processing and analysis of serum and saliva samples

Sera and saliva samples were collected through the Blood Reference in Stavanger (BRIS) project at the Stavanger University Hospital and conducted in accordance with the Norwegian Data Protection Authority (Datatilsynet) and the Regional Ethics Board for Medical Research (REK). The volunteers filled out a questionnaire providing information about age, sex, weight, height, and medications. All volunteers were presumed healthy. The obtained test results were anonymized, except for age and gender due to their relevance in the hormonal levels, and securely stored in a research server under the supervision of the Stavanger University Hospital. Moreover, the Data Protection Impact Assessment (DPIA) for the BRIS data register has recently been conducted following the completion of this study.

The 38 healthy volunteers, 20 women and 18 men, provided 3 mL saliva and two 10 mL serum collected with Vacuette® tubes with CAT serum separator clot activator (Greiner Bio-One; Kremsmünster, Austria) between 07.00 and 09.00 in the morning. One sample was excluded from the data analysis due to pregnancy and heighted hormonal concentrations. Analysis of morning samples will ensure minimal day variations in hormone concentrations, most especially in T and F, between samples (Dickmeis, 2009; Gamble et al., 2014). The sera samples were kept at 4°C and room-tempered prior to analysis, if not subsequently analysed within the same day of serum collection. One of the two collected serum sample was subjected to the measurement of total T, F, P and SHBG using chemiluminescent immunoassay Alinity I from Abbott (Abbott Park, IL, United States) and albumin using photometry. The concentrations of the proteins SHBG and albumin were further used for the cFT and cBT by

the Vermeulen-algorithm (Vermeulen et al., 1999) using the calculator provided by the International Society for the Study of the Aging Male (https://www.issam.ch/freetesto.htm). The second serum sample was analysed for all the target analytes included in this study by the in-house developed UPLC-MS/MS multiplex method. The saliva samples were used for the determination of free testosterone using the established UPLC-MS/MS analysis at the Stavanger University Hospital as previously reported (Nadarajah et al., 2017). An illustrative overview of the three different analytical approaches is shown in **Figure 21**.

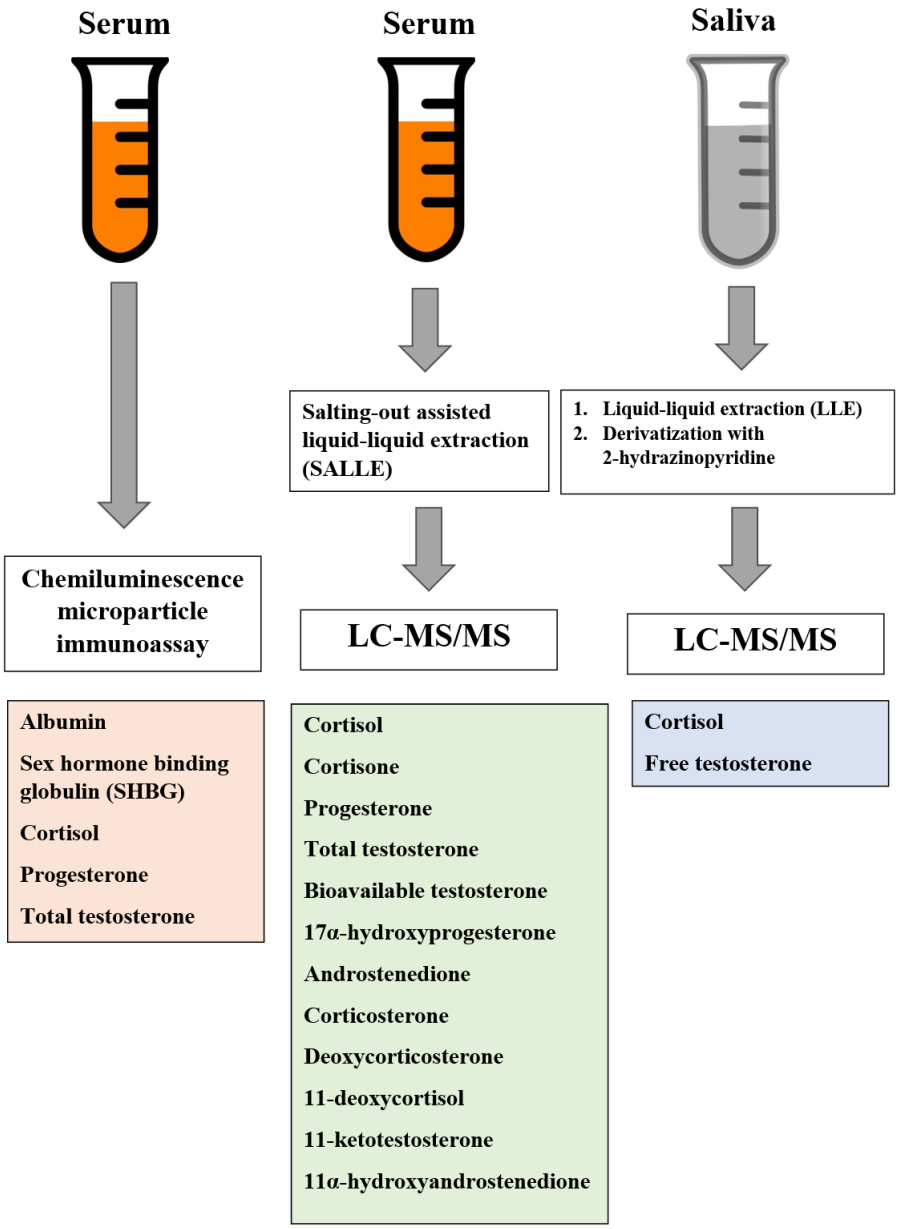


Figure 21: Overview of the distribution of the biological samples provided by healthy volunteers through the BRIS-project for the analysis by three different approaches. Information about the sample preparation of the LC-MS/MS methods are provided, as well as the analytes measured by each assay.

3.4 Analysis of serum by UPLC-MS/MS

3.4.1 Sample preparation

Frozen QCs and calibrators from -80°C were thawed at room temperature and vortex mixed for 30 seconds in addition to gently inverting the sample tubes prior to insertion in the automized liquid handling system ensuring complete homogenization. Furthermore, the AMS solution was taken out of the incubator set at 25°C and homogenized by manual shaking of the flask approximately 30 minutes prior to the sample preparation. The sample preparation was fully automized with the aid of Microlab STAR Liquid Handling System from Hamilton (Reno, NV, United States) using 96-well microplates from Thermo Scientific (Waltham, MA, United States). However, for the sake of simplification and clarity, the steps of the sample preparation will be illustrated using reagent glasses (**Figure 22).**

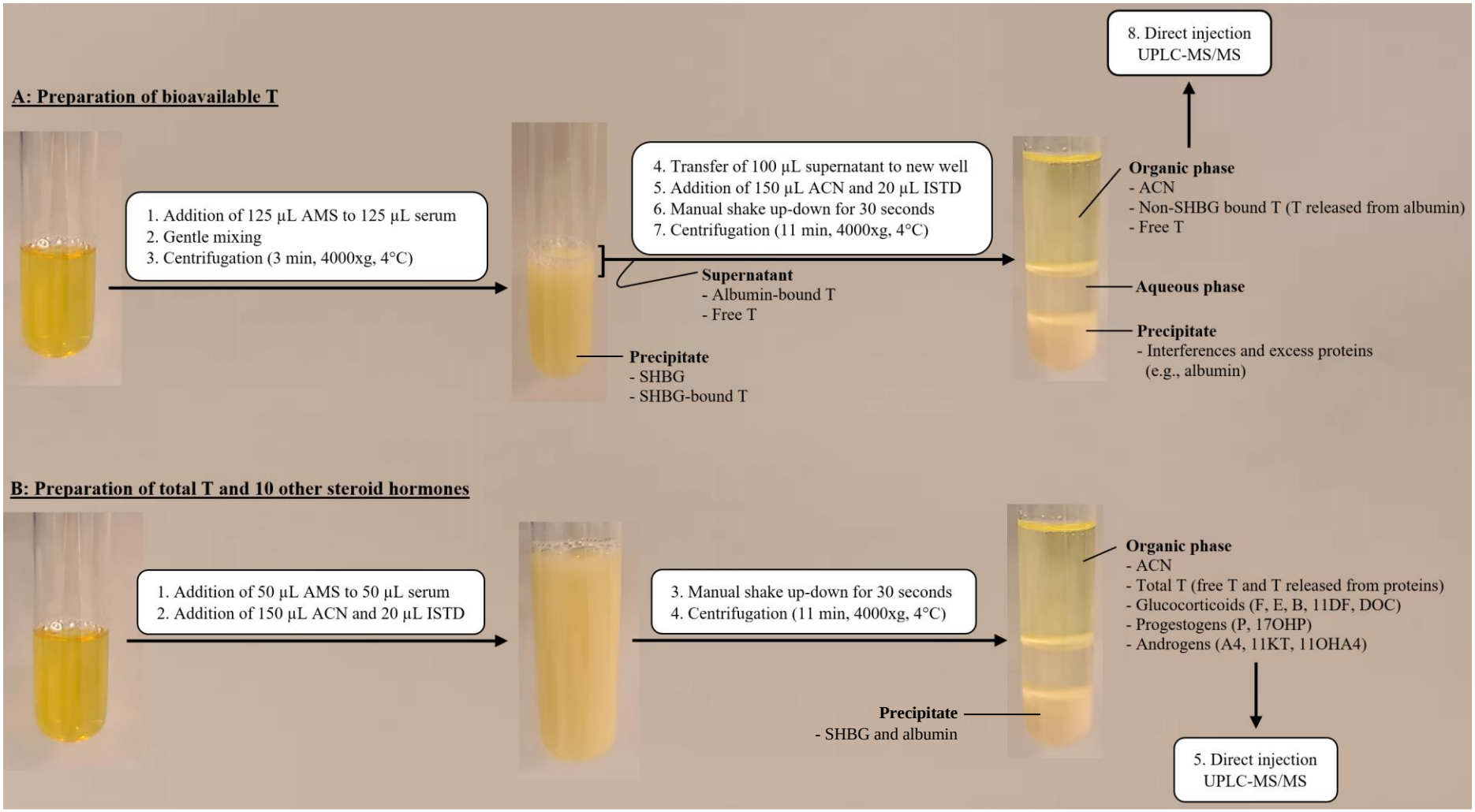


Figure 22: Sample preparation by SALLE for the LC-MS/MS measurement of (**A**) Bioavailable T and (**B**) Total T and 10 other steroid hormones. The figure illustrates one sample representing each well in the two 96-well plates utilized in the automized sample preparation for A and B, respectively. Note that A required a separate 96-well plate for precipitation of SHBG-bound T by mixing serum and AMS in 1+1 ratio. Experimental procedure is described as numbered bullet points in chronological order. For clarification, the 100 µL supernatant in step A is free from SHBG and SHBG-bound T. Analysis of 100 µL of this supernatant will be equivalent to the analysis of 50 µL serum and 50 µL AMS mixture in step B, but without the precipitated SHBG.

The sample preparation can be considered to have two separate preparations, one for the BT and the other for the multiplex steroid panel. To start, the extraction of BT (**Figure 22A**) was initiated by incorporating 125 µL AMS with 125 µL serum. The mixture was gently mixed by aspirating and dispensing from the pipette tips, and was further centrifuged for three minutes at 4 °C for 4000x g. This is the crucial step in extracting BT by precipitating maximum amount of SHBG and minimal amounts of albumin using 50% saturated AMS (Loric et al., 1988). The supernatant is assumed to only contain albumin-bound T and free T, as the SHBG-bound T would have been precipitated at the bottom, see **Figure 22A, step 4**. A volume of 100 µL supernatant was then transferred to a separate microplate for the SALLE which acts as the step for sample clean-up where 150 µL ACN and 20 µL ISTD was added. The mixture of the water-miscible solvent ACN and AMS saturated solution will induce phase separation with the aqueous layer in the bottom and the organic phase on top upon a manual and vigorous shake for 30 seconds in an up-down motion. To induce phase separation and aid the protein precipitation by the addition of ACN, the solution was centrifuged for 11 minutes in 4 °C at 4000 x g prior to UPLC-MS/MS analysis.

The sample preparation for the steroid panel, including the TT, follows the same procedure as described for the TT excluding the first step of SHBG precipitation (**Figure 22B**). Extraction and sample clean -up of the 11 steroid hormones was conducted by mixing 50 µL serum, 50 µL AMS, 150 µL ACN and 20 µL ISTD. Then, the solution was vigorously mixed for 30 seconds and further centrifuged for 11 minutes in 4 °C at 4000 x g prior to UPLC-MS/MS analysis.

3.4.2 UPLC-MS/MS instrumental analysis

The UPLC-MS/MS analysis was performed on ACQUITY UPLC system coupled with the Xevo TQ-S tandem mass spectrometry, both from Waters (Milford, MA, United States). The analytical column used was an ACQUITY UPLC C18 (2.1 mm x 50 mm, 1.7 µm) with 130Å Bridged Ethylene Hybrid (BEH) particles from Waters.

The chromatographic separation occurred in a flowrate of 0.800 mL/min with the mobile phase composition of 0.1% ammonium hydroxide (NH_4OH) in water (A) and acetonitrile (B). The gradient elution was arranged as follows: 20-45% B for 0-1.2 min, 45-65% B for 1.20-1.40 min, 65-95% B for 1.40-1.60 min, 95% B for 1.60-1.80, 95-20% B for 1.80-1.90 min, and

finally re-equilibration for 0.77 min. The total cycle time between injection was 2.67 min (2 min and 40 s). **Figure 23** shows the finalized gradient elution in both % (v/v) of the mobile phase compositions and an illustrative graph. The column temperature was set to 50°C, and the injection volume was 5 µL.

Time (min)	A (%)	B (%)
0.0	80	20
1.2	55	45
1.4	35	65
1.6	5	95
1.8	5	95
1.9	80	20

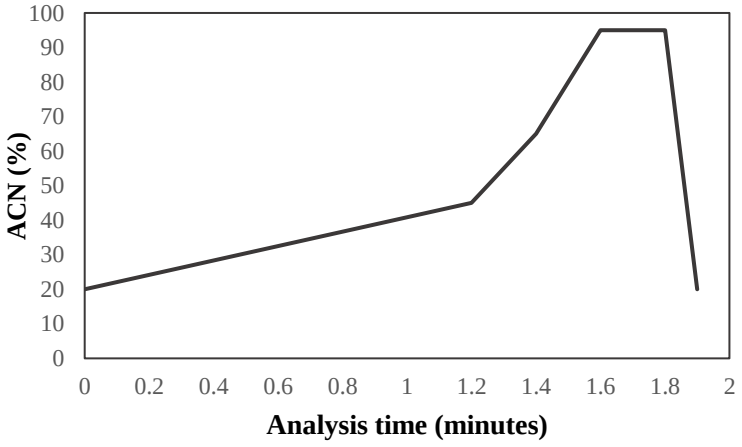


Figure 23: Description of the gradient elution using A: 0.1% NH_4OH in H_2O and B: ACN in the proposed method with constant flowrate at 0.800 mL/min. To the left, a table numerically describing the exact mobile phase composition at specific time periods in the analysis. To the right, a schematic illustration of the gradient elution following the volume (v/v %) of the organic solvent in the mobile phase ACN.

The compounds were subsequently ionized by electrospray ionization set in positive mode (ESI+) and detected through multiple reaction monitoring (MRM). The conditions for the mass spectrometry were set to have capillary voltage: 1.0 kV, cone voltages in the range: 25-40 V, ion source temperature: 150°C, and desolvation temperature: 600°C, desolvation gas flow: 1000 L/h, and cone gas flow: 150 L/h. A detailed overview of the MRM parameters for each analyte and their respective internal standards are given in **Table 4**.

Table 4: Overview of the MRM transitions, each with 6 ms dwell time, for analytes and their respective internal standards. Each analyte was granted two MRM transition; one for quantification (marked *) and one for additional confirmation.

	Parent ion > daughter ion (m/z)	Cone voltage (V)	Collision energy (kV)
Androgens			
T	289.2 > 109.1	40	22
	289.2 > 97.1*	40	20
A4	287.2 > 109.1*	40	22
	287.2 > 97.1	40	18
11KT	303.2 > 259.2*	25	20
	303.2 > 121.1	25	23
11OHA4	303.2 > 145.1*	25	23
	303.2 > 121.1	25	23
Progestogens			
P	315.2 > 109.1	40	20
	315.2 > 97.1*	40	21
17OHP	331.2 > 109.1	40	23
	331.2 > 97.1*	40	23
Glucocorticoids			
F	363.2 > 327.3	40	12
	363.2 > 121.1*	40	20
E	361.2 > 163.1*	40	22
	361.2 > 121.1	40	26
B	347.2 > 121.1*	40	22
	347.2 > 97.1	40	27
DOC	331.2 > 109.1	40	23
	331.2 > 97.1*	40	21
11DF	347.2 > 109.1*	40	25
	347.2 > 97.1	40	27
Internal standards			
D3-T	292.2 > 97.1	40	20
D8-A4	295.2 > 101.1	40	18
D4-11OHA4	307.2 > 148.1	25	23
D9-P	324.3 > 100.2	40	20
D8-17OHP	339.3 > 100.1	40	22
D4-F	367.2 > 121.1	40	20
D8-E	369.2 > 169.1	40	23

3.5 Method optimalization

3.5.1 Solvent mixtures in the salting-out assisted liquid-liquid extraction

The effect of various solvent mixtures for SALLE on ion suppression and extraction recovery was investigated by the addition of MeOH and Ace to neat ACN (**Table 5**). The sample matrix used in this experiment was the commercially available reference material Autonorm™ Immunoassay L1 human serum. All replicates consisted of 50 µL serum and 50 µL AMS with varying solvent mixtures. Volumes ranging from 0 to 50 µL (3 – 30%, v/v) MeOH and Ace in ACN were assessed as solvent additives. The total solvent volume was at a constant 170 µL where the volume increase of MeOH or Ace correspond to the decrease in ACN.

Table 5: Experimental parameters for the assessment of extraction recovery and ion suppression in the SALLE sample preparation. Solvent additives are methanol or acetone. The amount the solvent additive in the solvent mixture with ACN is given in percentage (%) as the volume-volume ratio (v/v).

Volume of solvent additive (µL)	Volume of ACN (µL)	Solvent additive (v/v, %)
0	170	0
5	165	3
10	160	6
15	155	9
20	150	12
25	145	15
30	140	18
35	135	21
40	130	24
45	125	27
50	120	30

3.5.2 SHBG precipitation and bioavailable testosterone extraction

The precipitation of SHBG from the sera samples for the extraction of BT was the only step in the sample preparation not corrected by ISTD. It was therefore feasible to observe how differences in temperature of the AMS solution affects the SHBG precipitation, thus the extraction of the BT with varying salt saturation. Two AMS solutions were prepared by pouring AMS salt crystals to a 500 mL graduated reagent bottle up to the 100 mL mark and further

filled with deionized H_2O. Salt concentration in the AMS solution was not regarded as the aim was to prepare a fully saturated AMS solution, and it was therefore unnecessary to the weigh the exact amount of AMS. However, there should be sufficient precipitated AMS in the bottom of the bottle when inspected visually to ensure complete saturation. One AMS solution was kept at room temperature, approximately 21°C, and the other incubated at 37°C. Upon analysis, the AMS solution kept at 37°C was introduced to the Hamilton Microlab STAR Liquid Handling System lastly to avoid decrease in solution temperature prior to analysis start.

3.6 Method validation

3.6.1 Linearity and working range

The method linearity and working range for all steroid hormones in the analyte panel was observed through the linear regression of the calibration curve. Eight calibrators were used for the establishment of the calibration curve. Further information on the calibrator concentrations can be found in *Section 3.2.* The linearity with a variation coefficient $R^2 > 0.996$ was deemed acceptable, and the working range was evaluated to be from the analytes' limit of quantification (LOQ) until the highest concentration within the linear range as defined by the Eurachem Guide "*The Fitness for Purpose of Analytical Methods"*(Magnusson & Ornemark, 2014)

3.6.2 Limit of detection and limit of quantification

The Eurachem Guide defines the limit of detection (LOD) as the minimum analyte concentration detected which can be distinguished from the background noise. The method LOD was evaluated by analysing blank phosphate-buffer-saline (PBS) samples (n = 10) through the whole method procedure including the automated sample preparation. The LOD was calculated as described by *Equation 1*. The LOD response was converted into concentration (given in nmol/L) was done by dividing the LOD by the slope of the generated linear curve.

$$\text{Limit of Detection} = \text{mean}_{\text{blank response}} + 3 * \text{SD}_{\text{blank response}} \qquad \textit{Equation 1}$$

Furthermore, the limit of quantification (LOQ) is defined as the lowest analyte concentration detected measured at a specified level of confidence with acceptable repeatability and accuracy. The method LOQ was evaluated in the same manner as LOD using the same blank PBS samples (n = 10). The LOQ calculated as described by *Equation 2*. The LOQ concentration in nmol/L was further determined using the linear calibration equation by dividing the LOQ response with the calibration slope.

$$\text{Limit of Quantification} = \text{mean}_{\text{blank response}} + 10 * \text{SD}_{\text{blank response}} \qquad \textit{Equation 2}$$

3.6.3 Repeatability and intermediate precision

The method repeatability and intermediate precision was evaluated by measuring quality control samples (QCs) in low, mid, and high T concentration levels for each level. Intra- and inter-assay variation for TT and BT were evaluated with an additional high level. The QCs used for the low- and high-level were obtained from healthy individuals, representing a true low and high T level. The mid-level and the additional high-level QCs were commercially available reference material Autonorm™ Immunoassay L1 and Autonorm™ Human Liquid with human serum as sample matrix.

The intra-assay variation illustrates the method repeatability using the standard deviation of the QC samples at each level (n = 10) from an analysis performed by a single operator within the same day. Moreover, the inter-assay variation, also called the intermediate precision, depicts the method reproducibility by analysing a single replicate of the QC samples at each level for 16 separate analyses performed by a single operator conducted in the time span of five months. The coefficient of variation (CV, %) for describes the relative variance of imprecision for both the method repeatability and intermediate precision.

3.6.4 Recovery

The intention with the recovery experiment was to investigate the method bias in the proposed UPLC-MS/MS method. The method recovery was conducted by spiking known concentrations of the target analytes at three levels covering the low, medium, and high concentration range. An overview of spiked concentrations for each replicate is stated in **Table 6**. Due to the

availability of various serum matrices, recovery was evaluated in new-born calf serum (Lot 2) and real human serum with low (Lot 1) and high (Lot 3) endogenous values of T.

Table 6: Spike concentrations (nmol/L) in low, medium, and high level used to evaluate method recovery. The spiked concentrations at all levels remained the same independent of the serum lot used.

	Spike concentration (nmol/L)		
Compound	**Low**	**Medium**	**High**
Androgens			
TT	7.0	18	35
A4	4.9	12	25
11KT	6.4	16	32
11OHA4	7.2	18	36
Progestogens			
P	6.0	15	30
17OHP	5.4	14	27
Glucocorticoids			
F	81	203	406
E	16	39	78
B	8.4	21	42
11DF	2.5	6.2	13
DOC	2.1	5.3	11

The relative spike recovery was further calculated using *Equation 3* and given in percentage (%). It is expected that the method recovery, from the sample preparation until the UPLC-MS/MS, will be 100% for target analytes. Thus, positive bias is reflected by recovery greater than 100% and negative bias by recovery below 100%. Every replicate was treated equally and underwent the entire analysis procedure for the proposed in-house developed UPLC-MS/MS analysis.

$$\text{Recovery } (\%) = \frac{\bar{x}' - \bar{x}}{x_{spike}} * 100 \qquad \textit{Equation 3}$$

x_{spike}: Spiked concentration
$\bar{x}'$: Concentration in spiked sample
$\bar{x}$: Concentration in non-spiked sample with endogenous analyte concentrations

3.6.5 Carry-over

The measurement of carry-over was conducted to observe the potential analyte transfer from one serum sample to the next. Sufficiently low carry-over ensures that the samples with lower concentrations do not attain falsely elevated concentration levels of the target analyte when

analysed subsequently to samples with hormone concentrations in the upper range. Analyte carry-over was evaluated by analysing a blank PBS sample, free from endogenous concentrations of target analytes, following the highest calibration solution. Carry-over was measured by *Equation 4* with the measurand concentration of the blank sample divided by the measured concentration of the highest calibrator.

$$\text{Carry} - \text{over}\ (\%) = \frac{[\text{Blank PBS sample}]}{[\text{Highest calibrator}]} * 100 \qquad \textit{Equation 4}$$

3.6.6 Correlation and bias

Correlation between IA and LC-MS/MS were measured for TT, F, 17OHP, A4 and P. The remainder of the analyte in the steroid panel could not be measured due to the absence of available assays. The concentrations for F, P and TT were measured with the established chemiluminescent immunoassay (Abbott Alinity) at Stavanger University Hospital. Samples from 37 volunteers were measured and compared with the quantifications done with the proposed UPLC-MS/MS analysis. Additionally, a comparison of results was done for the analytes TT, F, 17OHP and A4, by analysis of 29 samples from an external quality assessment (EQA) scheme (Labquality, Helsinki, Finland). The average reported concentrations by LC-MS/MS analysis were compared with results obtained by our novel method. However, no further information about the LC-MS/MS methods utilized by the external laboratories was provided.

The described experiments above were also used to assess the bias of the in-house developed UPLC-MS/MS method for TT, F, 17OHP, A4 and P, in addition to the relative spike recovery experiments for the remaining analytes. Bias for the BT quantitation was evaluated comparing with the calculated bioavailable T (cBT) obtained by using the Vermeulen-algorithm with albumin and SHBG concentrations acquired through photometry and immunoassay, respectively. Moreover, the free fraction of T found in saliva (sFT) was also measured for bias evaluation of BT. The analysis of sFT was carried out by UPLC-MS/MS at the Stavanger University Hospital as previously presented by Nadarajah et al. (2017).

3.7 Data analysis

Drawing of the chromatograms and calibration curves, as well as quantification of concentrations, were done by using the software TargetLynx (Waters). Further calculations, data analysis and statistics were done by using Microsoft Excel v2208 and PAST (Hammer et al., 2001). Figures were composed with ChemDraw 21.0.0, Microsoft Excel v2208 and Microsoft PowerPoint v2208.

4. Results and Discussion

Due to the magnitude of the analytes presented in this study, sections will mostly present testosterone, TT and BT, to emphasize the method's usefulness in determination of hyperandrogenism and hypogonadism. However, tables will present the data for every analyte when available. Additional illustrations (i.e., chromatograms and calibration curves) for the remainder of the steroid panel will be given as supplemental data in *Section 7*.

4.1 Optimized sample preparation

4.1.1 Extraction of bioavailable testosterone

The sample preparation applied in this study was SALLE with 100% ACN as the organic solvent and AMS as the salting-out reagent. This technique allowed analyte extraction and removal of sample contaminants such as phospholipids and proteins from the serum sample matrix (Tang & Weng, 2013). However, sufficient extraction of BT requires an optimized SALLE procedure using AMS. According to Loric et.al., the saturation of AMS is crucial for obtaining a maximum amount of SHBG in the precipitate and to keep albumin in the supernatant (**Figure 24**). Thus, the 50% AMS saturation was achieved with 125 µL AMS and 125 µL serum in 1+1 ratio for the BT extraction (**Figure 22**).

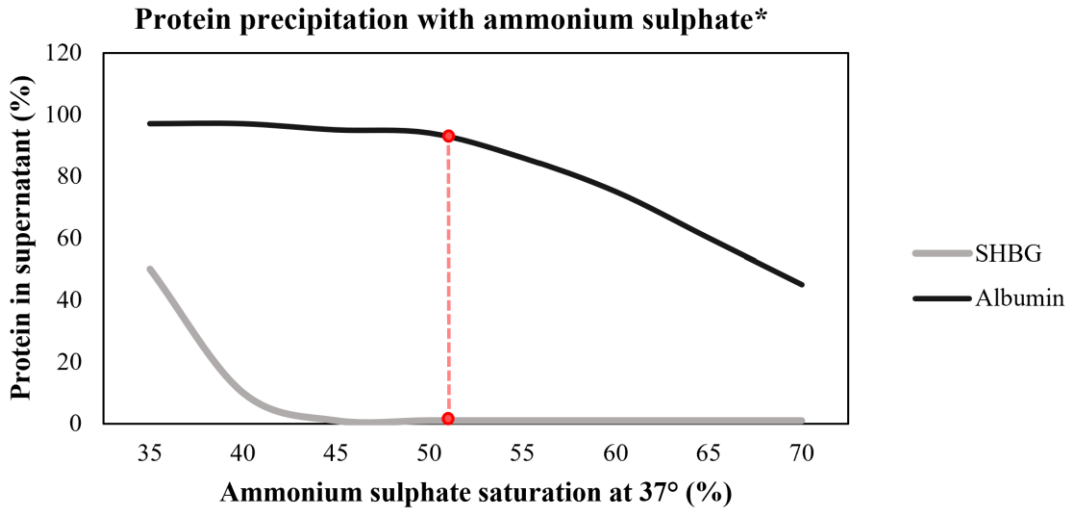


Figure 24: The effects of the salt saturation of ammonium sulphate (AMS) in the precipitation of SHBG and albumin with 37°C assay temperature as presented by Loric et. al. *Illustration adapted and modified from Loric et al. (1988).

The precipitation of SHBG occurred prior to the addition of the ISTD to avoid possible interactions of the transport proteins in the matrix and the deuterium-labelled testosterone. Thus, leaving the BT extraction non-corrected for analyte loss by ISTD. This solution was applied to enable constant ISTD amount throughout the whole analysis to prevent fluctuating and falsely elevated analyte signals. However, measures were taken to ensure high repeatability of the SHBG precipitation and the extraction of BT by examining the temperature and saturation of the AMS. **Figure 25** shows the values of BT in serum (n = 10) in low, mid- and high-level QCs using AMS solutions incubated at 21°C and 37°C. Temperature affects the saturation of AMS as higher temperatures lead to increased salt dissolution in aqueous solution. It was therefore of interest to investigate the difference of SHBG precipitation with higher temperatures of AMS.

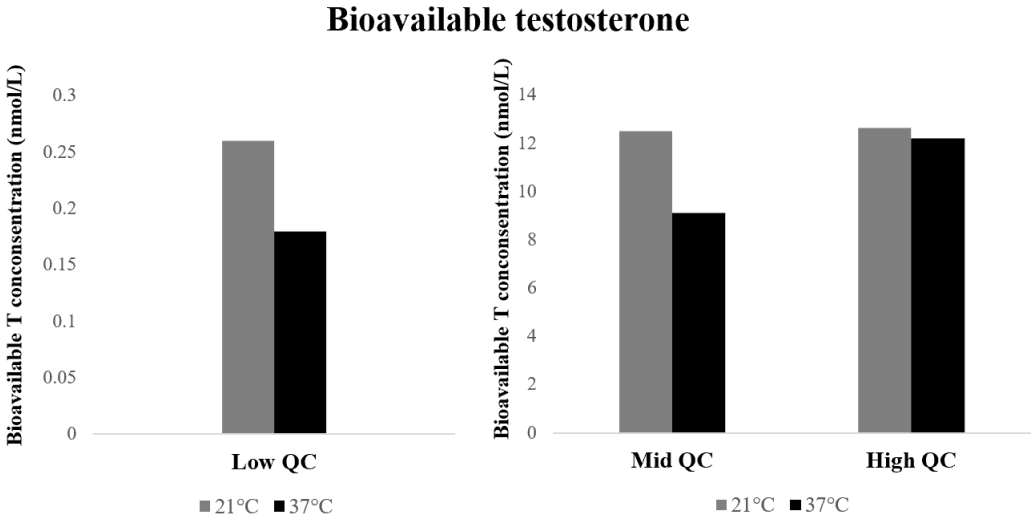


Figure 25: Bar plots illustrating the measured bioavailable T (BT) concentrations in the low, mid, and high-level QCs using saturated AMS stored at 21°C and 37°C. Due to the considerable difference in concentrations between the low and the mid/high-level QCs, the second axis with the concentrations in nmol/L are differentiated.

There was an observed decrease in BT concentrations in across all levels when using the AMS stored at 37°C **(Figure 25).** The mixture for extracting BT consists of 125 µL serum and 125 µL AMS corresponding to 50% salt saturation for maximum SHBG and minimal albumin precipitation (Loric et al., 1988). However, AMS with higher incubation temperature will result in more salt saturation, thus increasing the protein precipitation. The decreased BT concentrations indicates the increased precipitation of albumin due to the higher salt saturation of AMS upon reaching 37°C. The main finding of this experiment concurs with the data

provided by Loric et.al. where higher salt saturation > 50% will effectively start precipitating albumin from serum, thus precipitating more of the albumin-bound T in the bioavailable fraction of T.

The binding between SHBG and T is shown to be temperature dependent with less binding affinity to the transport protein at 37°C (Shanbhag & Södergård, 1986). It should be noted that it is solely the AMS solution that was incubated at 37°C whilst the rest of the analysis was carried out in room temperature with room-tempered samples. However, the temperature of the AMS solution is not necessarily 37°C by the time the AMS solution is mixed with the serum matrix for BT extraction when considering the time from when the AMS solution was taken from the incubator to the start of the fully-automized sample preparation. The possible temperature differences in the AMS solution between samples can therefore increase the extraction variabilities due to the changes in SHBG affinity, as well as the salt saturation and albumin precipitation. Moreover, the procedure of using AMS stored at 37° is time-dependent requiring rapid addition to the serum samples before the temperature decreases. Recognizing the potential extraction variation between samples and analyses deems that the AMS solution should be maintained at a constant temperature. The most practical solution to hinder saturation differences is to store the AMS solution at room temperature (~21°C) to ensure adequate saturation for BT extraction.

Interestingly, there was an observed sudden change in the SHBG precipitation between the analyses (data not shown) during the seasonal change from autumn to summer, even when the AMS flask was kept at a laboratory bench in room temperature during this period. The ambient temperature in the laboratory was assumed to have changed with colder temperatures at night resulting in varying AMS temperature and its saturation in the following day when the analysis was conducted. As a solution, the AMS solution was incubated at 25°C until further analysis. Vigorous shaking of the flask and letting it sit for 30 minutes prior to the analysis may also improve the homogeneity of the solution and further ensure the stable AMS saturation and BT extraction.

4.1.2 Protein precipitation by ammonium sulphate

The fully-automized sample preparation involves two separate procedures: one for the extraction of BT and the second for the extraction for the remaining analyte panel (Section

3.4.1; **Figure 22**). Separate 96-well microplates was required for each protocol, thus resulting in the analysis of two microplates. The first microplate is referred to as the Total Plate (TP) and the second as the Bioavailable Plate (BP). The difference between TP and BP lies in the additional step for the BT extraction that precipitates SHBG using AMS as the precipitation agent prior to SALLE. The analysed supernatant from BP is therefore expected to be free from SHBG, but the other carrier proteins (e.g., albumin and CBG) remains intact. This implies that the expected analyte concentrations, apart from T, would be equivalent in TP and BP. It should be noted that only BT assessment was planned for the BP. **Figure 26** illustrates the measured analyte concentrations in TP and BP.

The data concurs with the findings of Vermeulen et.al. that approximately 60% of T is bound to SHBG corresponding to the observed decrease (40%) of TT in BP (Vermeulen et al., 1971). Effective precipitation of SHBG was achieved using AMS as the precipitation agent. However, decrease in analyte concentrations was detected in BP indicating that AMS also precipitates a small amount of proteins other than SHBG. The significant decrease especially applies to the glucocorticoids and progestogens F (6%, $p = 0.002$), B (14%, $p = 0.0003$), 11DEO (18%, $p = 0.0006$), 17OHP (7%, $p = 0.006$), DOC (20%, $p = 0.01$) and P (16%, $p = 0.01$). The least decrease in analyte concentration was found for E (3%, $p = 0.03$), but still significant with Student's t-test at 95% confidence level. These steroid hormones bind to the carrier protein CBG further implying that AMS also precipitates CBG in a lesser extent compared to SHBG (Dunn et al., 1981).

The use of 1+1 ratio of AMS and serum (i.e., 50% salt saturation) has been presented by Loric et. al. to maximize the precipitation of SHBG and minimize the loss of albumin (Loric et al., 1988). Concentration non-significant decrease for A4 (6%, $p = 0.06$) and increase for 11OHA4 (2%, $p = 0.6$) was found in the BP. A4 has high binding affinity to albumin and binds greatly to this carrier protein (Nerusu et al., 2017). Thus, the observed modest loss of A4 in BP implies that the method precipitates non-significant amounts of albumin. This indicates that minimal amounts of albumin-bound T are precipitated and further solidifies the argument that 1+1 ratio of AMS and serum effectively minimizes albumin precipitation upon BT extraction. Interestingly, protein precipitation by AMS showed sparse effects on 11OHA4 suggesting binding to albumin, but not SHBG. This experiment also demonstrated a decrease in the 11-oxygenated androgen 11KT by 6% ($p = 0.34$), comparable to A4 and F, suggesting that SHBG may not be the primary carrier protein for this potent androgen in the same manner as T.

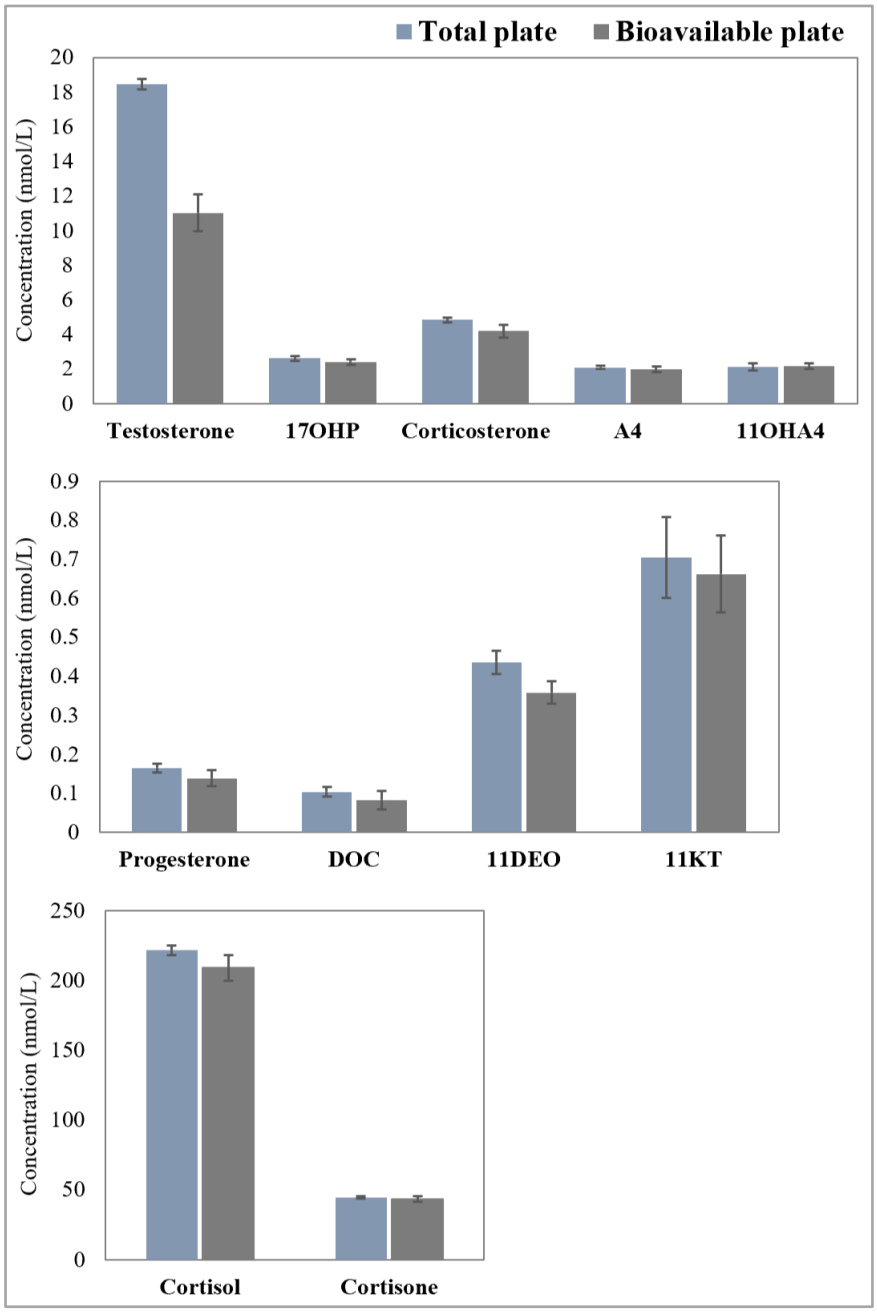


Figure 26: Analyte concentrations in the total plate (TP) and bioavailable plate (BP) in human serum (*n* = 10). SHBG removal was conducted on BP prior to SALLE. Three separate bar graphs were generated due to the large difference in hormone concentrations. Concentrations are given mean ± standard deviation in nmol/L. **17OHP**: 17α-hydroxyprogesterone, **A4**: androstenedione, **11OHA4**: 11β-hydroxyandrostenedione, **DOC**: deoxycorticosterone, **11DEO**: 11-deoxycortisol, **11KT**: 11-ketotestosterone.

4.1.3 Choice of extraction solvent

The polar, water-miscible solvent ACN is widely used in SALLE for the preparation of biological samples (Valente et al., 2013). The study conducted by Urge et. al. included post-

spiking experiments to demonstrate the efficient use of ACN for the extraction of F, E and T from human serum. Moreover, their work also showed that SALLE is superior for minimizing matrix effects compared to LLE and PPT (Urge et al., 2023). Sufficient phase separation was achieved with 100% ACN, but it was feasible to examine whether solvent mixtures of ACN may result in enhanced analyte extraction and decreased ion suppression. The applicable solvent additives were MeOH and Ace which are water-miscible solvents with chemical properties to change the solvent polarity. The salting-out effect seen in SALLE is dependent on the solvent polarity and solvent additives to extract analytes with greater polar properties (e.g., cortisol) (Grover & Ryall, 2005). However, the method optimalization revolved around achieving the highest extraction rates for testosterone. The data shown in **Figure 27** shows the ion suppression and analyte extraction for mixture solvents with 0-30% (v/v) MeOH or Ace in ACN for testosterone.

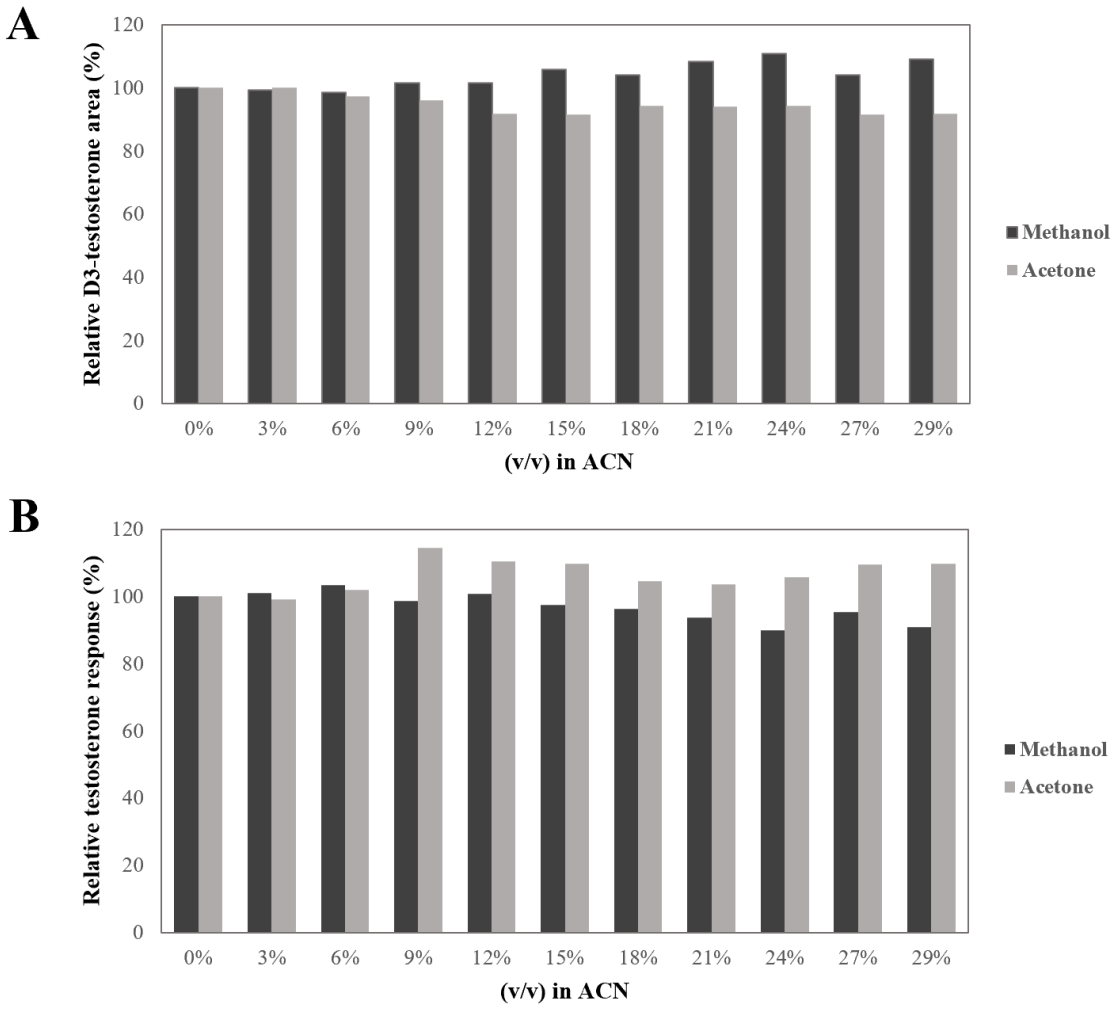


Figure 27: Bar plots illustrating the effects of solvent mixtures with methanol and acetone in acetonitrile (ACN) (% v/v). (**A**) Relative ISTD peak area for D3-testosterone describing ion suppression (**B**) Relative analyte response for testosterone. Measured in human serum (n = 3) with relative ISTD peak area and analyte response in percent (%) compared with 100% ACN solvent.

The data presented from this experiment shows slightly decreased ion suppression with higher concentrations of MeOH in the solvent mixture, further indicating that MeOH as solvent additive results in ion enhancement. Moreover, it demonstrates Ace to be causing minor ion suppression above 3% Ace in the solvent (**Figure 27A**). Despite the low ion suppression caused by MeOH in the solvent, the analyte response is remarkably lower compared to Ace. (**Figure 27B**). The observed ion enhancement with MeOH indicates that the decreased analyte response cannot be accounted for the presence of phospholipids, although MeOH more effectively dissolves phospholipids than ACN (Alzweiri et al., 2008). However, the extraction solvent with added MeOH could have altered the solvent polarity resulting in lowered solubility of T in the organic phase. Alternatively, MeOH could affect the salting-out effect of SALLE and hinders T from partitioning to the solvent phase. As a result, the use of pure ACN in the extraction solvent will minimize the amount of phospholipids in the sample extract subjected to direct injection to the UPLC-system (Lahaie et al., 2010). Considering the contribution of the solvent additives on ion suppression and analyte response, as well as the possibility of enhanced contribution of phospholipids to the matrix effects, it was deemed that pure ACN is suitable for the SALLE procedure in this study. The use of pure ACN further minimizes errors related to pipetting and handling of solvent mixtures which is advantageous regarding the method's utility as a clinical routine analysis.

Analyte extraction for T in various solvent mixtures is shown in **Figure 28** as the relative analyte peak area compared to neat ACN. The solvent mixtures with MeOH as the solvent additives demonstrate slight increases in analyte extraction when compared to the neat ACN solvent. On the contrary, the addition of Ace shows decreases in analyte extraction, except for the 9% Ace/ACN (v/v) sample. The latter sample may have been affected by random errors resulting in deviation from the overall trend as ISTD was added prior to the extraction. The solvent mixture of 3-6% MeOH/ACN resulted in higher analyte extraction and minimal ion suppression indicating that modest increase in solvent polarity is beneficial. However, the SALLE extract will be injected directly to the UPLC-MS/MS system. It is therefore an advantage to utilize a solvent with high analyte extraction without the dissolving phospholipids into the extract to avoid potential column clogging (Jemal et al., 2010). Considering ion suppression, analyte extraction and practicability, it was decided to use 100% ACN for a neat SALLE supernatant for direct injection.

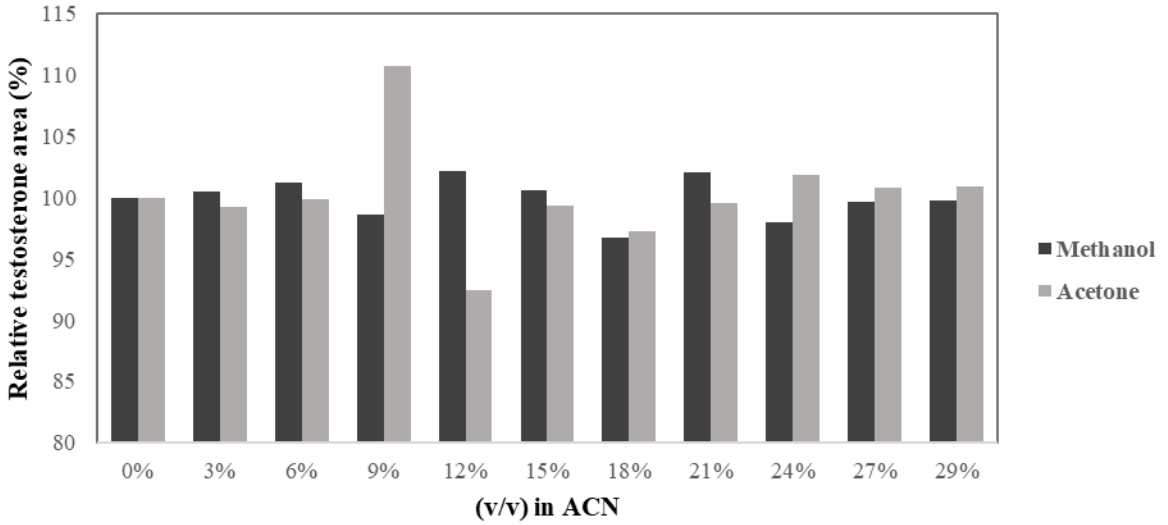


Figure 28: Bar plots illustrating the effects of solvent mixtures with methanol and acetone in acetonitrile (% v/v) on the relative testosterone peak area (%).

4.2 Optimized chromatographic separation with UPLC-MS/MS

4.2.1 Mobile phase composition

The use of UPLC-MS/MS ensures sensitive and selective analyte detection, but such analyses require appropriate instrument adjustments to acquire satisfactory separation and retention of target analytes (Vogeser & Parhofer, 2007). The analytical column used was C18 stationary phase using Waters™ BEH technology enabling high operational pH without dissolving the silica solid support (Wyndham et al., 2003). The proposed method in this study was designed as a routine analysis, and one of the criteria is the use of common mobile phases and columns. There was no need to test other reversed-phase stationary phases as the column utilized is already in use in routine analyses at the Stavanger University Hospital. However, further research remains in exploring alternative stationary phases such as phenyl biphenyl and pentafluorylphenyl (PFP) for better separation of isobaric steroid hormones (Keevil, 2016). Nonetheless, four mobile phase compositions were evaluated for their effects on peak shape, peak sensitivity, and retention. **Figure 29** shows the chromatograms for testosterone with the mobile compositions ACN/FA, MeOH/FA, ACN/NH_4OH and MeOH/NH_4OH.

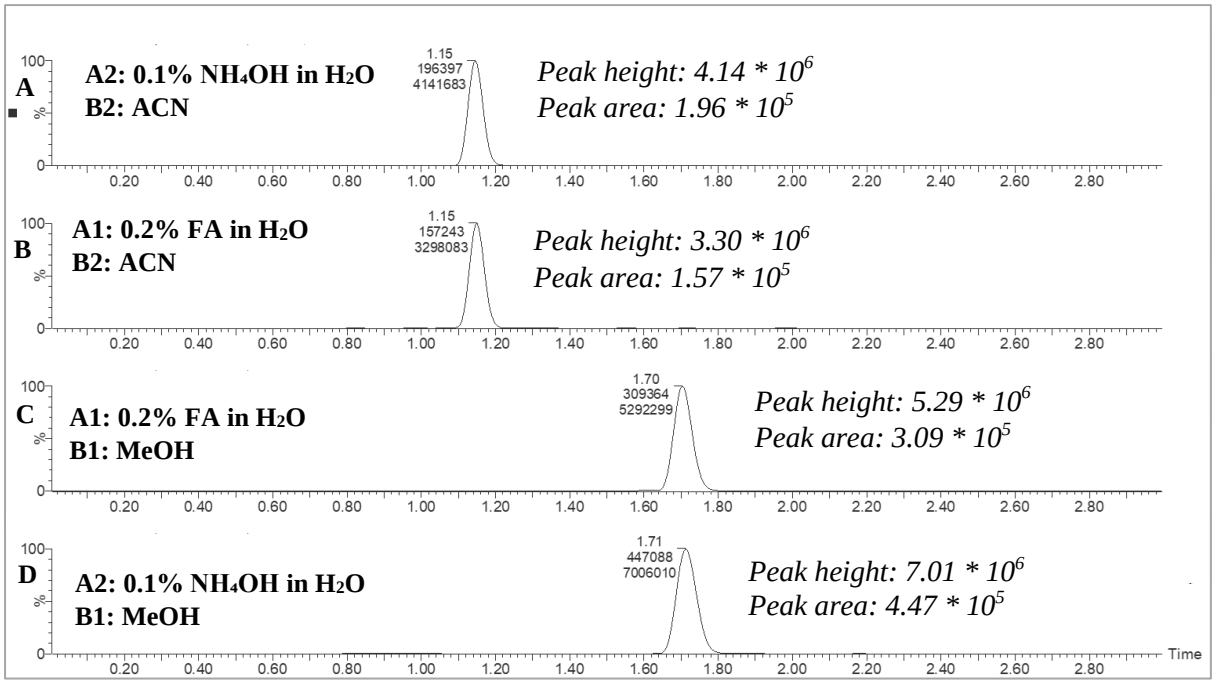


Figure 29: Chromatograms for D3-testosterone with fragment ion m/z 97.1 for the choice of mobile phase composition. The employed composition of mobile phases is indicated on the figure with their respective chromatograms. Additional information of the retention time and peak sensitivity are given.

The analyte signal for T was relatively high using 0.1% NH_4OH independent of using MeOH or ACN (**Figure 29A and D**) indicating that NH_4OH in the MP enhances the ionization and gives high analyte signals when coupled with ESI+ as it functions as the proton-donor despite the elevated pH (i.e., absence of H_3O^+) (Mess et al., 2009). None of the target analytes are pH-dependent with ionizable functional groups and therefore enables the use of NH_4OH in the mobile phase, unlike the thyroid hormones requiring low pH (Wang & Stapleton, 2010).

Higher analyte peak height can be achieved with MeOH/NH_4OH (**Figure 29D**) compared to ACN/NH_4OH (**Figure 29A**). However, the use of ACN/NH_4OH gives more symmetrical and narrower peaks, in addition to faster analysis time although lower peak heights (i.e., sensitivity) was achieved. The retention time for D3-testosterone decreased by half minute (0.55 min; 33 s) by using ACN. ACN has lower viscosity than MeOH and can often be applied to higher volumetric flowrates than MeOH when coupled with high operating pressure of UPLC systems withstanding up to 20 000 psi (Plumb et al., 2007). With sub-2 µm particle size of the UPLC columns, the theoretical plate height will not increase much even at mobile phase flow rates well above what is considered optimum flow rates of columns with larger particle sizes in addition to increasing the peak capacity (Swartz, 2005). Furthermore, the decreased particle size of UPLC packing particles contributes to higher linear velocity and decreased band

broadening because of the reduced contribution of the C-term in the Van Deemter Equation seen in *Equation 5* (van Deemter et al., 1956). The resistance of mass transfer in the SP (C_S) and MP (C_M) will therefore lessen due to the reduced particle size of the SP packing and enables higher linear velocity with lower MP viscosity using ACN. Considering the advantages of higher linear velocity and sensitive analyte detection, the mobile phase composition further used in the finalized method was ACN as the organic solvent with 0.1% NH_4OH in H_2O with flowrate 0.800 mL/min to achieve clean analyte separation and fast analysis without sacrificing the chromatographic efficiency.

$$H = A + \frac{B}{u} + C_s u + C_M u \qquad \textit{Equation 5}$$

Abbreviations: H: theoretical plate height, A: Eddy diffusion, B: Longitudinal diffusion, C: Mass transfer in stationary phase (S) and mobile phase (M), u: linear velocity.

4.2.2 Gradient elution and chromatographic separation

The gradient elution was adjusted to obtain satisfactory separation of all analytes, especially of the isobaric compounds with shared MRM-transitions. Gradient elution in liquid chromatography enables manipulation of analyte elution time and becomes particularly important in multiplex methods with separation of multiple analytes. The increase of the organic modifier in MP must also be considered due to the consequence of increasing eluting strength. It is crucial that the increase in the organic mobile phase B (i.e., ACN) is gradual to elute the analytes with retention factors for sufficient separation when utilizing a relatively high flowrate at 0.800 mL/min. The gradient elution can therefore be divided into four sections A, B, C and D as illustrated in **Figure 30**.

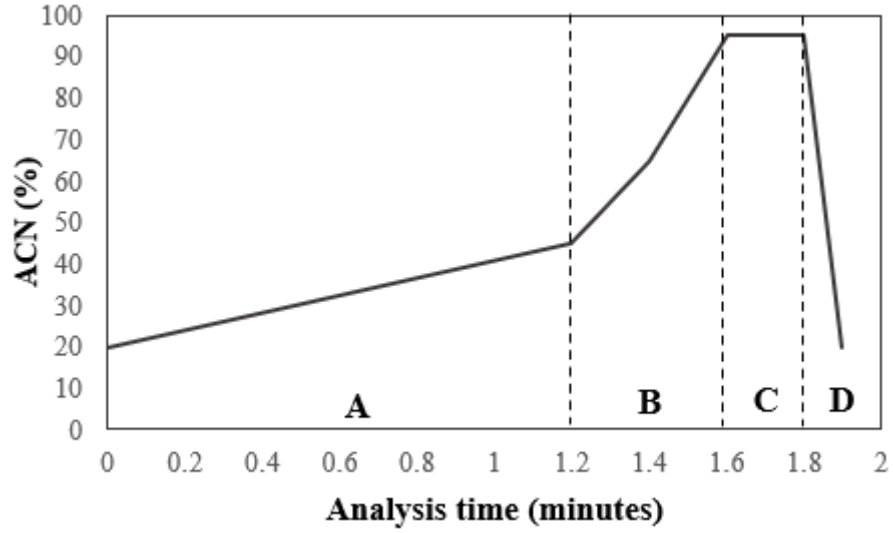


Figure 30: Schematic illustration of the gradient elution following the volume (v/v %) of the organic mobile phase ACN. Section A: elution of polar to mid-polar analytes, Section B: elution of non-polar analytes, Section C: column wash, Section D: mobile phase reconstitution and column re-equilibration.

The gradient elution was initiated by the gradual increase of the ACN from 20-45% in 1.2 minutes to elute the polar compounds with satisfactory separation, as seen in Section A. Section B is characterized by the sudden increase of ACN going from 45-65-95% in 0.4 minutes which promoted the elution of the more non-polar analytes as the mobile phase's elution strength increases. The gradient elution then plateaued with high ACN amount at 95% for 0.2 minutes in Section C to ensure complete analyte elution and column wash for any remaining matrix. Finally, the mobile phase was reconstituted by decreasing ACN to the initial conditions in Section D to re-equilibrate the column.

As mentioned, separation of isobaric steroid hormones poses certain challenges in achieving complete separation of analytes (Keevil, 2013). Firstly, ionization of isobaric compounds may result in equal fragment ions with the same m/z ratio despite column separation. The lack of difference in the m/z ratio hinders the quadrupoles in the tandem mass spectrometry to separate the analytes. Secondly, isobaric analytes with similar chemical properties can co-elute as modest interactions with the SP and MP may not be sufficient for adequate column separation. Such challenges can be solved by analyte derivatization to alter the analytes' chemical properties and interactions with the stationary phase, thus their retention and elution time (Higashi & Ogawa, 2016). Likewise, separation improvements can be accomplished by alternative reversed-phase SP to C18 columns such as phenyl hexyl (Szarka et al., 2013), improved mass resolution by using time-of-flight mass spectrometers (Flores-Valverde & Hill,

2008) and adjusting the mobile phase elution strength to alter the retention of the isobaric analytes (Vanhaecke et al., 2011).

The isobaric hormones with shared MRM-transitions in this study are 11DF/B (**Figure 31**) and DOC/17OHP (**Figure 32**). Exploring alternative stationary phases to C18 and mass analyser was not applicable for this study as the proposed method is designed as a routine analysis using the existing C18 column already in use. Nevertheless, two viable solutions were explored to avoid false quantification of the mentioned compounds due to the significant contribution of their respective isobaric interferences. The first option was to adjust the gradient elution by increasing the elution strength of the mobile phase to alter the retention factors for one or both analytes. **Figure 31A** shows the merged analyte peaks for 11DF and B with the gradient elution 10-70% B from 0.0 – 1.6 min compared to the separated analyte peaks shown in **Figure 31B** when the elution was changed to 20-45% B for 0-1.2 min, 45-65% B for 1.20-1.40 min, 65-95% B for 1.40-1.60 min. Both gradient elutions were conducted with ACN with 0.1% NH_4OH in H_2O at flowrate 0.800 mL/min.

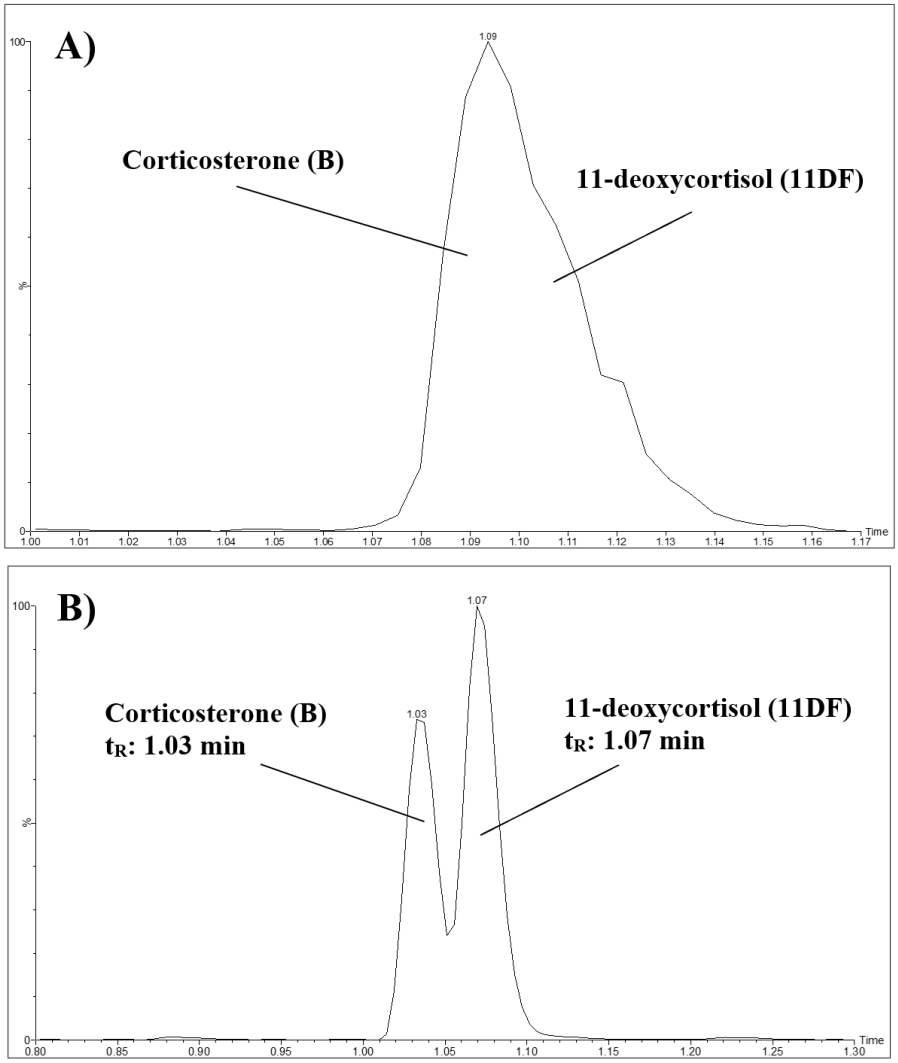


Figure 31: Chromatogram of the isobaric compounds corticosterone (B) and 11-deoxycortisol (11DF) with the shared MRM-transition m/z 347.2 > 97.1. (**A**) Merged analyte peaks were observed with 10-70% B from 0.0 – 1.6 min; A: 0.1% NH_4OH in H2O and B: ACN at 0.800 mL/min flowrate. (**B**) Separated analyte peaks were observed with 20-45% B for 0-1.2 min, 45-65% B for 1.20-1.40 min, 65-95% B for 1.40-1.60 min 10-70% B; A: 0.1% NH_4OH in H2O and B: ACN at 0.800 mL/min flowrate. Retention times (t_R) was 1.03 min for B and 1.07 min for 11DF.

Luckily, corticosterone utilizes MRM-transition (m/z 347.1 > 121.1) as the quantitative ion ensuring specific quantification separate from 11DF. However, the shared MRM-transition m/z 347.1 > 97.1 was used for the quantification of 11DF. Proper peak integration for 11DF was achieved by integrating from peak end of B perpendicular to the baseline to the peak end in baseline. The alteration the gradient elution by increasing the organic modifier in the mobile phase in a shorter timeframe worked well for the analyte separation of 11DF and B. Moreover, the partial separation of the isobaric compounds with differing chemical properties may be further enhanced by having longer analysis time to aid increased interactions with the stationary phase resulting in higher retention time.

To compare, the isobaric compounds 17OHP and DOC are fully separated prior in reaching the ion source and resulted in separated analyte peaks even with a shared MRM-transition as their quantifying ion (**Figure 32**). The most important parameter in correct analyte identification is retention time (t_R), and it is therefore important that the retention times of isobaric compounds with shared MRM-transition is distinguishable from each other. Following the sufficient chromatographic separation of DOC and 17OHP, the MRM-transition m/z 331.2 > 97.1 were used as the quantifying fragment ion as it gave the highest sensitivity for both analytes.

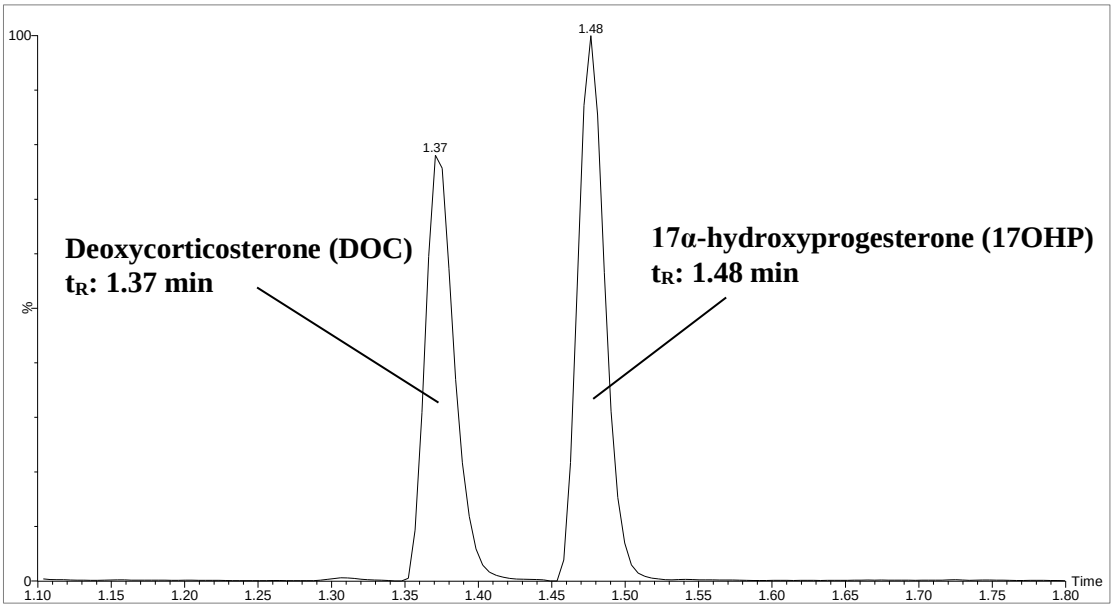


Figure 32: Chromatogram of deoxycorticosterone (DOC) and 17α-hydroxyprogesterone (17OHP) depicting full analyte separation despite using the same MRM-transition m/z 331.2 > 97.1. The difference in the retention time (t_R) between DOC and 17OHP is 0.11 min.

To summarize, the finalized gradient elution resulted in sharp and symmetrical peaks where all analytes are separated from interferences, in addition to high peak height indicating the method's high sensitivity. **Figure 33** to **Figure 35** gives an overview of the chromatograms for all analytes and their retention times in the proposed method. The analytes measured in this study lacked ionizable groups and was unaffected by the high MP pH by using 0.1% NH_4OH.

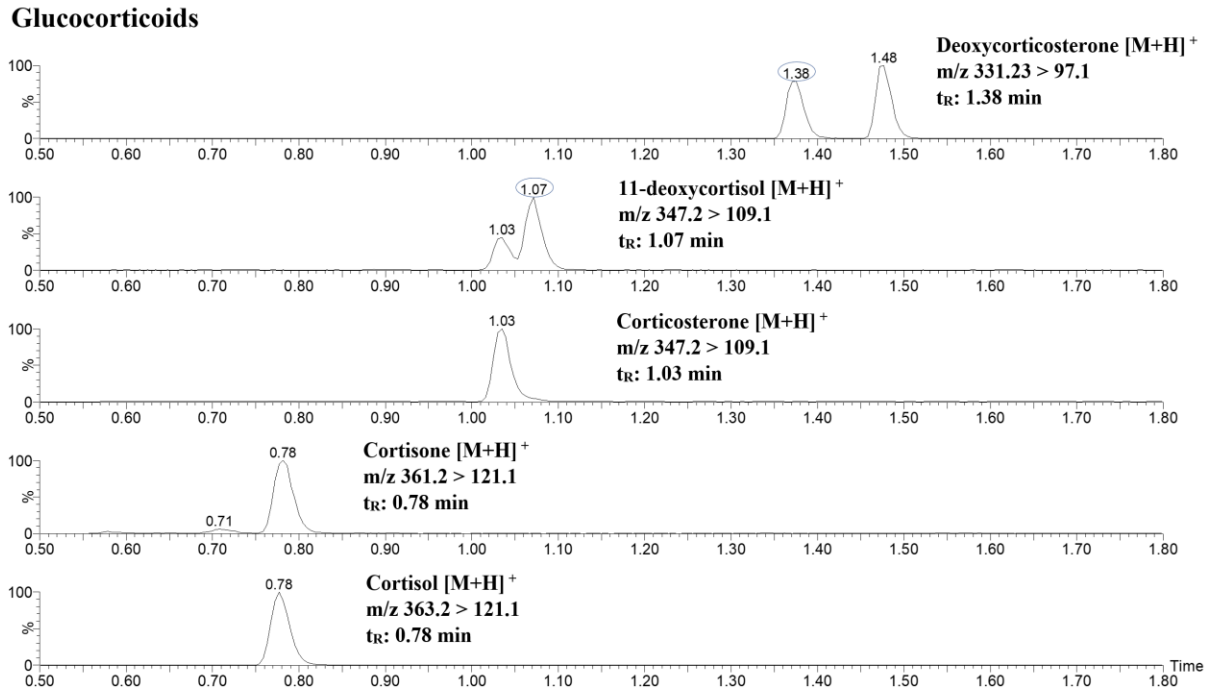


Figure 33: Chromatograms of glucocorticoids with their MRM-transition and retention time. Analytes with multiple peaks from isobaric interferences in their chromatograms will have the retention time of correct analyte peak circled for clarification.

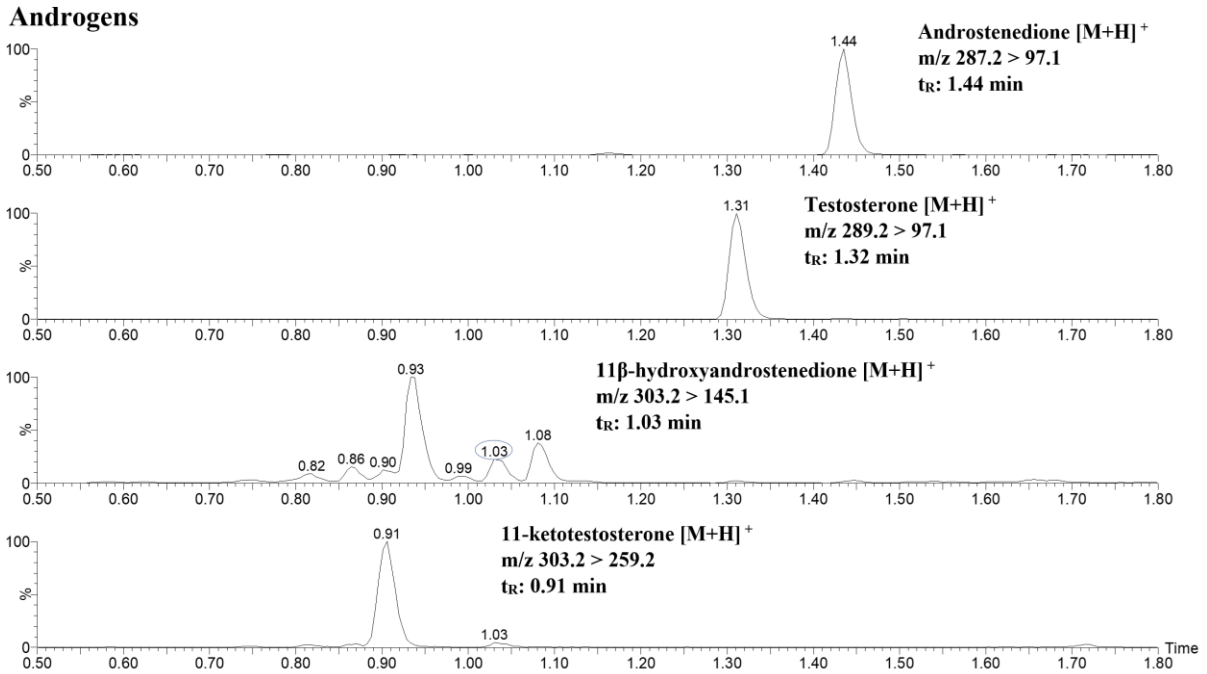


Figure 34: Chromatograms of androgens with their MRM-transition and retention time. Analytes with multiple peaks from isobaric interferences in their chromatograms will have the retention time of correct analyte peak circled for clarification.

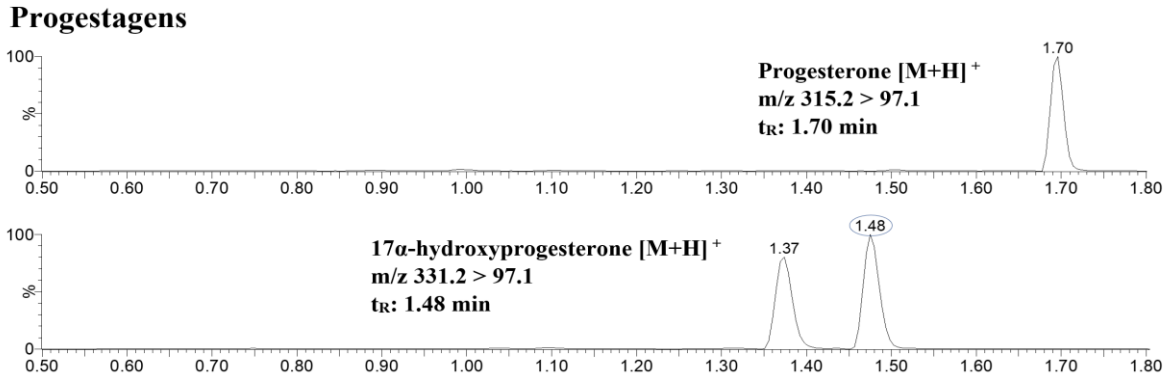


Figure 35: Chromatograms of progestogens with their MRM-transition and retention time. Analytes with multiple peaks from isobaric interferences in their chromatograms will have the retention time of correct analyte peak circled for clarification.

4.3 Method validation

4.3.1 Repeatability and intermediate precision

One of the most important validation parameters is the measurement of uncertainty expressing the method's reliability in correctly quantifying both within analysis and between analyses. The precision of a method is expressed as the intra-assay variation (i.e., repeatability) and the inter-assay variation (i.e., intermediate precision or reproducibility) (Magnusson & Ornemark, 2014). The measured imprecisions for all analytes in the proposed method is given in **Table 7** as the mean ± standard deviation and the relative imprecision in percent in multiple concentration levels when possible.

The proposed method for steroid hormone analysis shows promising repeatability with inter-assay variation < 20% for every analyte over the entire concentration range. The highest variation seen belongs to the bioavailable T at 0.19 nmol/L with 17% variation. The intra-assay variation describing the method's repeatability also has < 20% variation for most analytes in the mid-level and high-level concentration range. Imprecision above 20% are seen for the low-level range of bioavailable T, 17OHP, A4 and DOC with 38%, 22%, 27% and 22% coefficient of variation, respectively.

Remarkably, between-run and within-run variation for most analytes in the mid-level and high-level concentration are well within 10% imprecision. The low imprecision demonstrates acceptable repeatability and reproducibility of the method and further proves the reliability of the quantification conducted with the proposed method using SALLE and UPLC-MS/MS.

Most importantly, diagnoses such as hypogonadism in men and hyperandrogenism in women has androgen concentrations in the mid- to high-level concentrations where the method displays satisfactory imprecision for this concentration range. Thus, implying the fitness of the novel method as a diagnostic tool.

The bioavailable T shows the highest imprecision across all concentration ranges with variance ranging from <20% — 40% in the high and low level, respectively. This illustrates the difficulty of reproducible precipitation of SHBG and implication of an inconstant precipitation of SHBG within samples and between analyses. Furthermore, the reasons behind the variability should be examined to acquire acceptable repeatability and reproducibility. The variable and incomplete SHBG precipitation may be due to variations in the AMS saturation and competitive binding from other hormones such as oestrogens found in women and DHT in men (Shanbhag & Södergård, 1986). Additionally, the step involving BT extraction in the sample preparation was not corrected by ISTD resulting in higher imprecision due to the lack of correction for possible analyte loss and extraction variations.

Greater concentration fluctuations are observed in the lower concentration ranges, and the total uncertainty of the method has greater impact in low concentrations than in high concentrations. The effects of random and systematic errors will be more pronounced in the lower concentration level resulting in higher measurement variability. The low-level sample presented in **Table 7** represents real human serum from healthy volunteers of both genders. The naturally low concentrations of T in women will ultimately result in high imprecision (Davison & Davis, 2003), but the method demonstrated low imprecision below 10% for TT in this concentration range enabling reproducible and repeatable detection of TT in females. Therapeutic ranges of relevant analytes for hyperandrogenism, CAH and hypogonadism are well within the mid-level to high-level concentrations (O'Reilly et al., 2014; Tonetto-Fernandes et al., 2006; Ueland et al., 2022; Winters, 2020).

Table 7: Inter- and intra-assay variation for all measured analytes. Mean and standard deviation given in nmol/L and relative imprecision in percentage. All values were measured in morning serum samples. Low (n = 15), medium (n = 16), two high (High 1: n = 11, High 2: n = 16) concentration levels are given if available. **T**: testosterone, **F**: cortisol, **E**: cortisone, **P**: progesterone, **17OHP**: 17α-hydroxyprogesterone, **A4**: androstenedione, **B**: corticosterone, **DOC**: deoxycorticosterone, **11DF**: 11-deoxycortisol, **11KT**: 11-ketotestosterone and **11OHA4**: 11β-hydroxyandrostenedione.

		Inter-assay variation		**Intra-assay variation**	
Analyte		Mean ± standard deviation, nmol/L	Imprecision, %	Mean ± standard deviation, nmol/L	Imprecision, %
Total T	Low	0.64 ± 0.04	6.7	0.65 ± 0.05	8.2
	Mid	7.04 ± 0.16	2.3	6.93 ± 0.37	5.4
	High 1	18.9 ± 0.31	1.6	18.7 ± 0.98	5.2
	High 2	22.9 ± 0.59	2.6	24.6 ± 1.6	6.3
Bioavailable T	Low	0.19 ± 0.03	17	0.27 ± 0.1	38
	Mid	3.34 ± 0.39	11	3.57 ± 0.4	11
	High 1	8.58 ± 0.64	7.5	11.1 ± 2.1	19
	High 2	9.81 ± 0.87	8.8	13.9 ± 3.0	21
F	Low	231 ± 12	5.0	225 ± 15	6.6
	Mid	355 ± 16	4.4	345 ± 37	11
	High	705 ± 47	6.7	717 ± 75	11
E	Low	32.6 ± 1.1	3.5	30.5 ± 1.2	4.0
	Mid	45.6 ± 1.8	4.0	45.3 ± 1.8	3.9
	High	78.2 ± 2.9	3.7	78.3 ± 3.1	4.0
P	Low	1.31 ± 0.06	4.9	1.32 ± 0.10	7.9
	High	2.87 ± 0.22	7.6	2.73 ± 0.14	5.3
17OHP	Low	0.40 ± 0.05	12	0.40 ± 0.09	22
	Mid	1.35 ± 0.11	8.1	1.40 ± 0.07	4.8
	High	3.27 ± 0.27	8.2	3.09 ± 0.21	6.8
A4	Low	1.17 ± 0.20	17	0.99 ± 0.27	27
	Mid	2.68 ± 0.10	3.5	3.29 ± 0.57	17
	High	4.29 ± 0.12	2.8	4.05 ± 0.44	11
B	Low	5.52 ± 0.42	7.6	5.88 ± 0.86	15
	Mid	8.10 ± 0.30	3.8	8.40 ± 0.60	7.1
	High	12.8 ± 0.36	2.8	13.4 ± 0.82	6.1
DOC	Low	0.10 ± 0.01	11	0.10 ± 0.02	23
11DF	Low	0.87 ± 0.09	10	0.84 ± 0.13	16
11KT	Low	0.70 ± 0.1	15	0.60 ± 0.1	17
11OHA4	Low	2.12 ± 0.20	9.6	4.23 ± 0.47	11

4.3.2 Limit of detection and limit of quantification

The limit of detection (LOD) and quantification (LOQ) illustrates the sensitivity of the proposed instrumental analysis for steroid hormones with UPLC-MS/MS. **Table 8** summarizes important method validation parameters as linearity, working range, LOD, LOQ and carry-over for the analytes included in the method. Across all analytes the LODs ranged from 0.01 to 0.12 nmol/L and the LOQs from 0.03 to 0.29 nmol/L. The method shows sensitive detection, especially for testosterone, enabling the measurement of T concentrations in women. **Figure 36** shows the chromatograms of the lowest T and BT concentration measured in real human serum with 0.36 nmol/L and 0.18 nmol/L, respectively. The analyte peak for T shows to have little to no interference indicating satisfactory sample clean-up and separation, in addition to high analyte signal with clear distinguishment from the baseline.

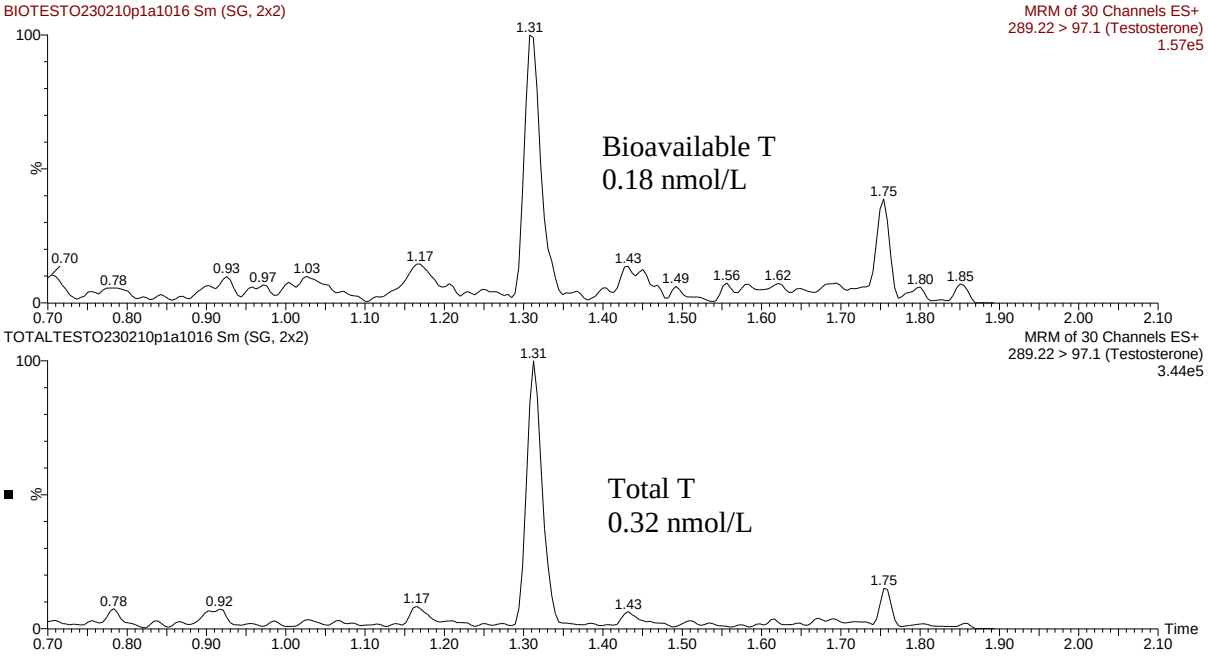


Figure 36: Chromatograms of bioavailable T (upper) and total T (lower) with smoothing factor 2x Savitsky-Golay for the lowest observed concentrations from a real human serum sample with 0.18 nmol/L and 0.32 nmol/L, respectively. Chromatograms are derived from Waters MassLynx with additional information on retention time, MRM-transition (quantifying fragment ion), concentration, and peak sensitivity for testosterone.

The proposed method using UPLC-MS/MS shows superiority in quantifying T concentrations in the low level compared to conventional methods such as IAs. The latter method exhibits poor sensitivity for the measurements of TT in low concentrations due to analyte cross-

reactivity and unsatisfactory limits of quantifications (Herold & Fitzgerald, 2003; Yang et al., 2019). The sensitivity demonstrated by the in-house developed method is advantageous for providing correct diagnosis and treatment plan of endocrinological disorders regardless of endogenous concentrations of T. The observed analyte LOQs are below 0.3 nmol/L, thus enabling the confident quantifications of steroid hormones as their concentrations related to endocrinological conditions are greater than the reported LOQ. Biochemical assays are one of the Rotterdam criteria for diagnosing PCOS (Teede et al., 2018) and sensitive assays using UPLC-MS/MS can provide more accurate and representative hormone profiles for the diagnosis of PCOS (Taylor et al., 2015).

4.3.3 Linearity and working range

The linearity is dependent on the ability of the instrument to detect and correctly translate the measured concentration in the calibrators into a linear relationship between the analyte concentration and analyte response. The linear calibration was not weighted. Satisfactory linearity was found for all analytes was observed with $R^2 > 0.996$ using an ISTD corrected calibration and standard addition calibration for analytes F, E and B due to high endogenous concentrations in the sample matrix.

As defined by the *Eurachem Guide*, the working range begins from the analyte LOQ until the highest concentration with observed linearity (Magnusson & Ornemark, 2014). Linearity was observed for all analytes up to the highest calibration curve. Thus, the analyte working range beginning from the LOQ to highest calibrator concentration is applicable for the analytes measured in this study. The measured linear and working range of the analyte panel is summarized in **Table 8** and calibration curves in *Section 7: Supplemental data*.

4.3.4 Analyte carry-over

The analyte carry-over must be considered when developing an analysis method for patient samples, most especially when analysing analytes with great concentration difference between gender and patient condition. To state examples, testosterone levels in women are 20-fold lower than those found in men, and 17OHP levels are 100-fold higher in individuals with CAH (Ueland et al., 2022). It is therefore essential that analyte carry-over is as little as possible to ensure that samples with low concentrations of naturally occurring hormones are not falsely

elevated when subsequently analysed after those in the upper concentration range. Analyte carry-over was evaluated to be < 1% and found tolerable for all analytes (**Table 8**).

Table 8: Overview of the validation parameters including linearity given with Pearson's R, working range, limit of detection (LOD) and limit of quantification (LOQ) given in nmol/L, in addition to method recovery and carry-over in percentage. LODs and LOQs were measured in PBS sample matrix (n = 10), recovery in 96% human albumin/PBS and the rest of parameters in human serum. **TT**: total testosterone, **BT**: bioavailable testosterone, **F**: cortisol, **E**: cortisone, **P**: progesterone, **17OHP**: 17α-hydroxyprogesterone, **A4**: androstenedione, **B**: corticosterone, **DOC**: deoxycorticosterone, **11DF**: 11-deoxycortisol, **11KT**: 11-ketotestosterone and **11OHA4**: 11β-hydroxyandrostenedione.

	Compound	Linearity (R^2)	Working range (nmol/L)	LOD (nmol/L)	LOQ (nmol/L)	Carry-over (%)
Androgens	TT	0.999	0.04 – 87.7	0.01	0.04	0.28
	BT	0.999	0.04 – 87.7	0.01	0.04	0.31
	A4	0.999	0.05 – 61.4	0.02	0.05	0.30
	11KT	0.999	0.24 – 80.2	0.09	0.24	0.03
	11OHA4	0.998	0.29 – 89.7	0.12	0.29	0.29
Progestogens	P	0.999	0.10 – 74.7	0.04	0.10	0.79
	17OHP	0.998	0.03 – 67.7	0.02	0.03	0.25
Glucocorticoids	F	0.999	0.1 – 1092	0.04	0.10	0.07
	E	0.999	0.08 – 206	0.03	0.08	0.11
	B	0.999	0.16 – 109	0.05	0.16	0.09
	11DF	0.999	0.11 – 31.1	0.04	0.11	0.21
	DOC	0.999	0.03 – 26.4	0.01	0.03	0.35

4.3.5 Method recovery

Experiments to evaluate the recovery can uncover systematic errors embedded in the method analysis, further affecting the precision and accuracy of the conducted measurements. It was therefore important to assess method recovery in a wide range of concentrations (i.e., low, medium and high) levels for every analyte. According to the *Eurachem Guide*, evaluation of bias can be conducted through recovery experiments (Magnusson & Ornemark, 2014). In this study, recovery was examined in NBCS serum and real human serum with varying levels of endogenous T concentrations to determine recovery disparity between origins of serum matrix and presence of interferences. Values obtained for method recovery is given in **Table 9**.

Table 9: Results for method recovery in three concentration levels: low, medium, and high (n = 1) for each level. Lot 1: Human serum with low endogenous T concentration, Lot 2: New-born calf serum with low endogenous concentration and Lot 3: Human serum with high endogenous T concentration. **TT**: total testosterone, **BT**: bioavailable testosterone, **F**: cortisol, **E**: cortisone, **P**: progesterone, **17OHP**: 17α-hydroxyprogesterone, **A4**: androstenedione, **B**: corticosterone, **DOC**: deoxycorticosterone, **11DF**: 11-deoxycortisol, **11KT**: 11-ketotestosterone and **11OHA4**: 11β-hydroxyandrostenedione.

		Recovery (%)		
Compound	**Level**	**Lot 1**	**Lot 2**	**Lot 3**
Androgens				
TT	Low	104.3	103.5	111.4
	Medium	101.6	105.4	104.8
	High	105.5	103.0	104.1
BT	Low	40.6	64.3	156.3
	Medium	46.8	57.2	74.5
	High	45.8	73.8	83.6
A4	Low	90.3	98.4	109.1
	Medium	93.0	99.3	99.6
	High	96.5	99.1	106.7
11KT	Low	103.3	126.3	126.6
	Medium	108.3	124.7	123.3
	High	104.7	110.1	115.8
11OHA4	Low	92.8	111.5	108.0
	Medium	113.3	108.8	117.6
	High	104.1	108.1	109.4
Progestogens				
P	Low	105.1	99.5	105.7
	Medium	102.9	100.7	99.8
	High	112.3	111.6	88.5
17OHP	Low	103.1	109.7	107.8
	Medium	98.8	114.7	96.7
	High	103.1	112.2	99.0
Glucocorticoids				
F	Low	51.6	118.6	108.2
	Medium	84.5	106.4	99.8
	High	87.4	103.8	102.2
E	Low	105.1	108.7	108.2
	Medium	91.2	113.4	99.6
	High	97.4	107.6	103.9
B	Low	130.7	117.1	110.5
	Medium	110.1	111.8	109.2
	High	112.0	116.6	110.2
11DF	Low	113.6	87.5	104.6
	Medium	100.0	103.7	109.9
	High	106.2	100.1	104.4
DOC	Low	116.7	117.5	122.8
	Medium	112.4	119.6	105.1
	High	112.2	124.4	107.8

The method recovery found for all analytes, excluding BT, shows adequate recovery ranging from 90% to 130%. There was no observed difference in recovery between NBCS and human serum across the whole concentration range. However, there was variability in recovery using serum lot 1, obtained from a female volunteer, with lower relative recovery compared to the other serum lots for the analytes A4, 11OHA4, B, and F in the lower concentration range. The effects of interferences originating from the serum matrix is expected to become greater in the lower concentration range. Further experiments on matrix effects by post-spiking experiments should be explored in future studies. Overall, the recoveries were satisfactory for the analytes extracted by the procedure for total hormone extraction.

For the bioavailable T, there was an apparent difference in recovery depending on the origin of the sample matrix. The recovery was measured to be less than 50% when using real human serum obtained from a female volunteer (serum lot 1) compared to NBCS (serum lot 2) or human serum from a male volunteer (serum lot 3) with higher recoveries. For further information, the female volunteer from whom the serum lot 1 was obtained from were taking oral contraceptives. It is thought that serum lot 1 will therefore have increased SHBG levels as oral contraceptives promotes SHBG production from the liver (De Leo et al., 2010). Furthermore, the presence of high oestradiol levels may compete with T for SHBG binding despite having lower binding affinity to SHBG (Dunn et al., 1981). Moreover, women will naturally have increased levels of oestrogens compared to men, thus giving comparatively increased SHBG levels even with low T concentrations regardless of age (Aribas et al., 2021; Elmlinger et al., 2005). The increased oestrogens and SHBG in serum lot 1 may have led to higher precipitation of SHBG-bound spiked T upon BT extraction possibly explaining the lower recoveries across the entire concentration range using female serum. Moreover, the data for serum lot 1 demonstrates a stable recovery rate below 50% for all concentration levels suggesting SHBG saturation for T. However, serum lot 2 and 3 demonstrates fluctuations in recovery even though they are higher compared to serum lot 1. This may arise from the method's general variability for the analyte BT. As previously mentioned, experiments on matrix effects should be investigated further.

4.3.6 Bias and method trueness

Three experiments were conducted to assess the method trueness and bias, in addition to the recovery experiments presented in Section 4.3.4. First, real human samples (n = 37) from healthy volunteers obtained through the BRIS-project were analysed with the novel UPLC-MS/MS method and the current established immunological methods utilized at the Stavanger University Hospital. **Figure 37** shows bias and correlation for the total T concentration in females and males when comparing the two methods of analysis.

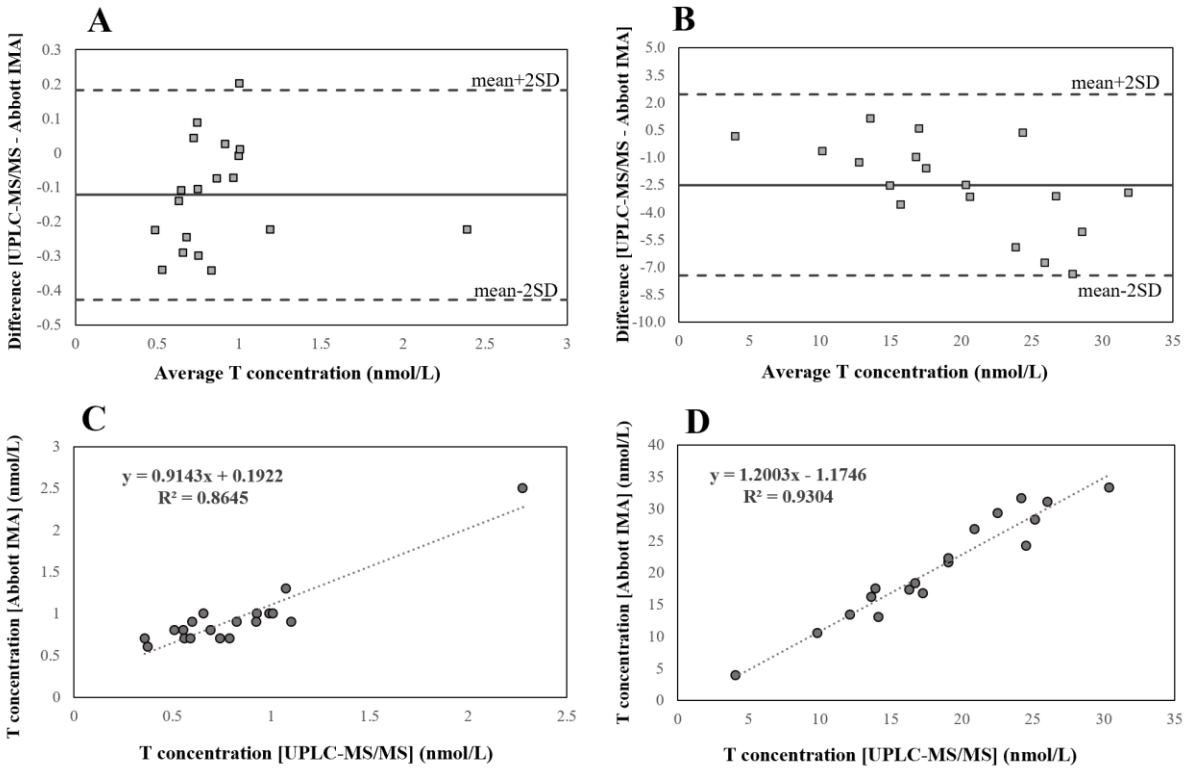


Figure 37: Results of the method comparison between the in-house developed UPLC-MS/MS method and the established immunoassay (Abbott IMA) for TT at Stavanger University Hospital. Real human serum samples obtained through the BRIS-project from 19 female and 18 male healthy volunteers. (**A**) Bias in TT levels in the lower concentration range (female samples). (**B**) Bias in TT levels in the upper concentration range (male samples) (**C**) Correlation in TT conc. in the lower concentration range (female samples). (**D**) correlation in TT conc. in the upper concentration range (male samples). Concentration given in nmol/L, linear relationship as y = ax + b and the correlation coefficient as Pearson R^2.

The analysis of 19 female samples demonstrates no systematic bias found for total T concentrations in the lower concentration range, and may imply varied quantifications conducted by the utilized IA (**Figure 37A**). However, the trend of bias becomes more apparent with higher total T concentrations found in 18 male samples (**Figure 37B**). Data shows that the

in-house developed UPLC-MS/MS has a positive bias for lower total T concentrations from 5-20 nmol/L, and negative bias with higher concentrations beyond 20 nmol/L. Cross-reactivity is known to occur with IAs and it becomes apparent with higher total T concentrations as metabolites with similar chemical structure as T increases accordingly (Krasowski et al., 2014). Moreover, the correlation is observed to have $R^2 = 0.86$ and $R^2 = 0.93$ for the low and high concentration levels, respectively, between the in-house developed UPLC-MS/MS method and the established CMIA. The satisfactory correlation between the methods indicates the trueness of the in-house developed method and its ability in correctly quantifying the TT concentrations in serum.

One of the greatest advantages of an UPLC-MS/MS assay is the possibility for a multiplex method capable of measuring multiple analytes in a single analysis run (Keevil, 2016). Immunological methods are based on the interaction between the target analyte and their corresponding antibody, and the IAs used in this study can only detect one analyte per assay (Gosling, 1990). The proposed UPLC-MS/MS method can therefore replace multiple assays and being cost- and time-efficient in the long-run despite the higher instrumentation cost and maintenance. Alongside the possibility of multiple analyte detection, UPLC-MS/MS quantifies with higher sensitivity and specificity than IAs due to the lack of analyte cross-reactivity (Krasowski et al., 2014). Moreover, analytes with similar chemical structure as the target analyte can bind and interact with the antibodies resulting in falsely elevated analyte concentrations. Steroid analysis using UPLC-MS/MS separates the target hormone from hormones with similar chemical structure by chromatography and ionization ensuring specific detection free from cross-reactivity (Taylor et al., 2015).

Secondly, quality controls used in EQA scheme by Labquality were measured with the proposed method. The aim of this experiment was to observe the correlation and bias when compared to external laboratories using existing LC-MS/MS method for TT, as shown in **Figure 38A** and **38B**, respectively.

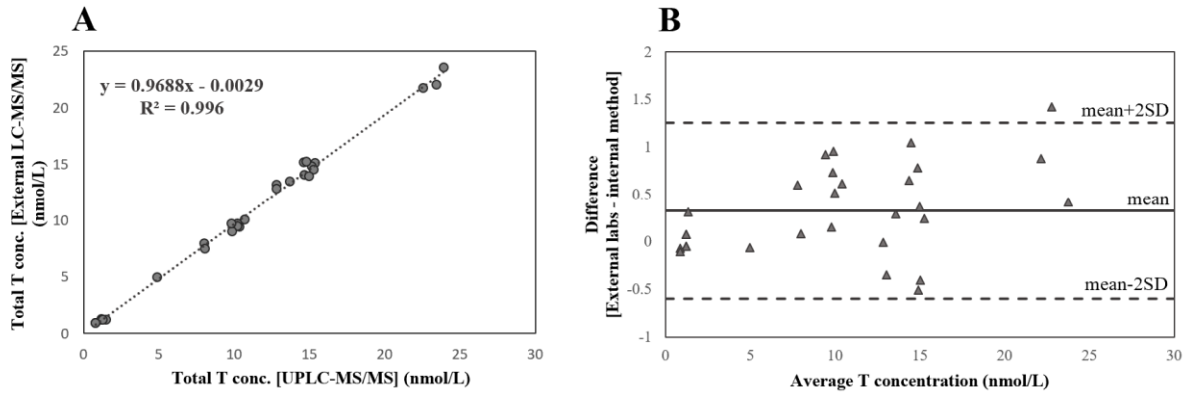


Figure 38: Measurements of total T in samples received by an external quality assessment (EQA) scheme (Labquality). Results from the new method were compared with average results from laboratories using LC-MS/MS. without specifications on the methods used. (**A**) Correlation of total T concentrations measured (nmol/L) and (**B**) Bias of measurements.

This experiment demonstrates the inter-laboratory comparability of the proposed method, and data shows exceptional correlation with $R^2 = 0.996$ (**Figure 38A)** between the proposed method and other LC-MS/MS methods. No information was provided about the LC-MS/MS methods used by the external laboratories, but it can be assumed that the sample preparation used can either be PP, SPE or LLE as the mentioned methods are most used for steroid hormone analysis (Keevil, 2016). Currently, the use of SALLE as sample preparation in steroid hormone analysis has been proposed in few studies (Li et al., 2019; Urge et al., 2023; Wang et al., 2012). The data in this experiment demonstrates that extraction of testosterone and sample preparation using SALLE is equivalently capable as the conventional methods used for sample clean-up and analyte extraction. Additionally, the high correlation demonstrates excellent trueness in the measurements for TT with the in-house developed UPLC-MS/MS method.

The proposed method was calculated to have a satisfactory negative bias with 3% (data not shown) in average in the inter-laboratory comparison. **Figure 38B** illustrates the positive bias in the lower concentrations below 10 nmol/L and negative bias in the upper concentration range above 15 nmol/L compared to the external laboratories. The same trend was observed when the proposed UPLC-MS/MS method was compared with the established CMIA (**Figure 37B**). Overall, the in-house measured TT concentration correlates well with the measurements conducted in external laboratories further demonstrating the low bias and high trueness of the method.

The third experiment conducted to evaluate the trueness for the quantification of BT is to compare the proposed UPLC-MS/MS method for BT, the cBT and cFT with the Vermeulen-algorithm (Vermeulen et al., 1999), and sFT obtained from the established UPLC-MS/MS method at the Stavanger University Hospital (Nadarajah et al., 2017) (**Figure 39**). For this experiment, only the male samples obtained through the BRIS-project is used. The FT concentrations found in females were near or below the LOD for the sFT analysis and were therefore not included.

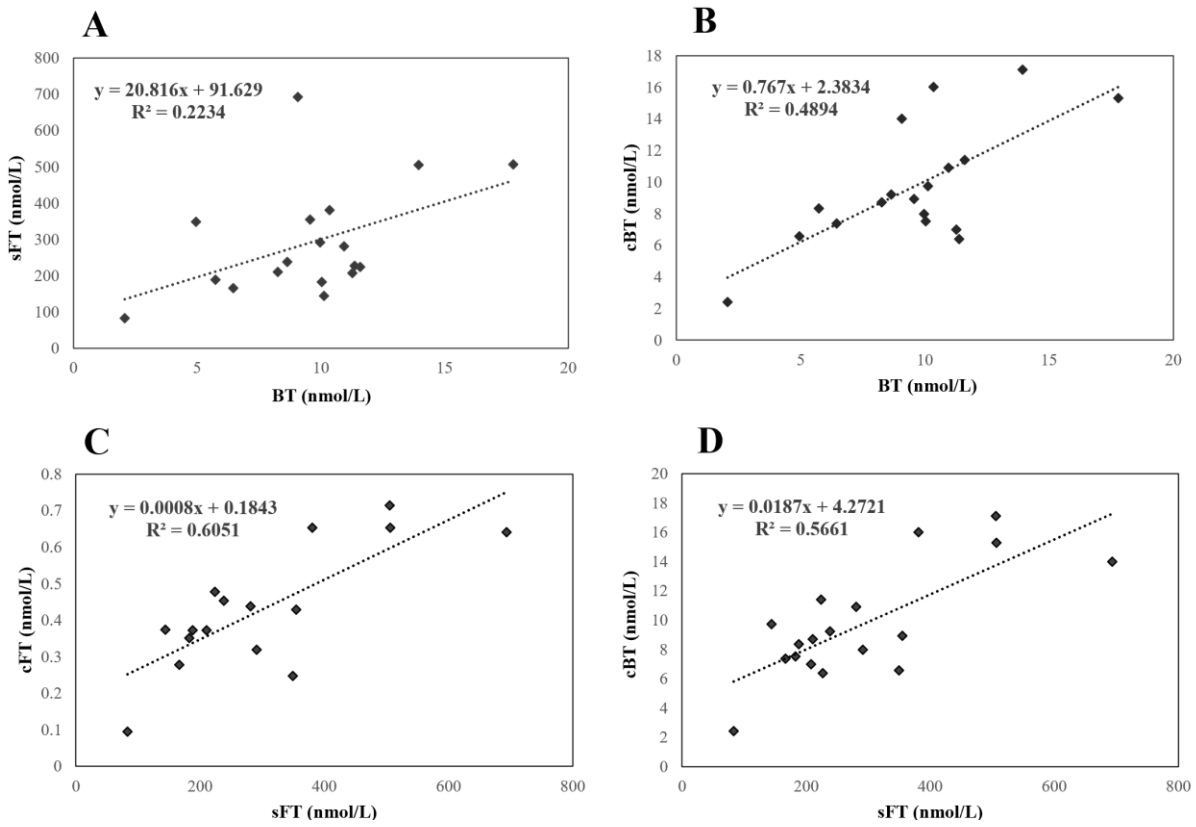


Figure 39: Results for the correlation analysis of bioavailable T (BT) in the upper concentration range. Samples obtained from 18 healthy male volunteers from the BRIS-project. (**A**) Correlation between BT and sFT (free T in saliva) measured by UPLC-MS/MS. (**B**) Correlation between calculated BT (cBT) and BT. (**C**) correlation between calculated free T (cFT) and sFT. (**D**) correlation between cBT and sFT. Concentrations given in nmol/L.

Poor correlation with R^2 = 0.22 was found between the BT measured with the in-house developed method and sFT as seen in **Figure 39A**. It is assumed that the bioavailable fraction of T would be proportional to the sFT as the BT consists of both the free fraction and the albumin-bound T (Zakharov et al., 2015). However, the high variability in recovery and reproducibility of BT results in inadequate correlation, even in the higher concentration range.

Furthermore, the BT shows better correlation with $R^2 = 0.49$ with the cBT obtained by using the Vermeulen-algorithm (**Figure 39B**). Nevertheless, the data shows that the proposed UPLC-MS/MS method has positive bias compared to cBT where the linear relationship shows a slope value of 0.77. Obtaining the cBT by the Vermeulen-algorithm utilizes values of TT, SHBG and albumin measured by immunological methods. The satisfactory correlation with the measurements for TT between the proposed method and IA (**Figure 39D**), in addition to the reliable SHBG and albumin measurements, indicates that the difference in analysis method could not have been the cause of the poor correlation found for BT. The correlations found for BT can be seen as substandard and reveals that further work on the reproducibility and reliability of the SHBG-precipitation for BT extraction using SALLE must be done.

The question arises regarding the practicality of quantifying the BT using SALLE when one can use mathematical equations (e.g., Vermeulen-algorithm) for the cBT. However, the concentrations of SHBG, TT and albumin must also be quantified to obtain cBT and cFT (Ho et al., 2006). In the current study, three different assays were utilized to acquire the parameters needed to calculate cBT and cFT. In contrast, the proposed method could extract the BT alongside 11 other steroid hormones from a single sample clean-up and analysis procedure using the more sensitive and specific assay UPLC-MS/MS.

The free T, thus the bioactive T, can also be quantified from saliva rather than serum with non-invasive sample collection by ejecting saliva onto a collection tube (Turpeinen et al., 2012). sFT demonstrates sufficient correlation with the cFT with $R^2 = 0.61$ and with the cBT with $R^2 = 0.57$, seen in **Figure 39C** and **39D**, respectively. The method for the quantification of sFT can serve as an alternative to the BT measurement in the novel method to acquire the advantages of using a specific and sensitive UPLC-MS/MS assay (Keevil et al., 2014). However, the method proposed by Nadarajah et. al. for the determination of steroid hormones in saliva requires derivatization resulting in a more time-consuming sample preparation than SALLE (Nadarajah et al., 2017). The BT extraction using SALLE shows potential, but the challenges with complete analyte recovery and reproducible quantifications must be further examined to achieve a reliable assay with SALLE and UPLC-MS/MS for the determination of BT.

4.3.7 Clinical significance of the novel method

The proposed method was designed and optimized to become a routine analysis. It fulfils the criteria for routine analyses such as having fast analysis for substantial number of samples and high efficiency in cost- and time-management (Shackleton, 2010). It is therefore feasible to discuss the method's clinical significance and application. The BRIS-project provided a population cohort with 38 samples divided between 20 female and 18 male samples, and the multiplex analysis resulted in 38 hormone profiles of assumed healthy individuals. However, one sample was excluded from the data analysis due to pregnancy and heighted hormonal concentrations. Three samples were suspected to have abnormal androgen levels upon examining the hormone profiles. The samples, labelled Sample A, B and C, were determined to have deviating androgen concentrations from the reference ranges established by Stavanger University Hospital, Oslo University Hospital and Haukeland University Hospital (**Table 10**).

Table 10: Reference ranges for relevant androgens for the determination of hypogonadism and hyperandrogenism measured with LC-MS/MS. **TT**: total testosterone, **FT**: free testosterone, **BT**: bioavailable testosterone, **A4**: androstenedione, **11KT**: 11-ketotestosterone, **11OHA4**: 11β-hydroxyandrostenedione, **17OHP**: 17α-hydroxyprogesterone, **FAI**: free androgen index, **SHBG**: Sex-hormone Binding Globulin, **N/A**: not available.

Analytes	Reference ranges
TT	Male: [b]18 - 40 years old: 7.2 – 24 nmol/L [b]41-120 years old: 4.6 -24 nmol/L [c] >18 years old: 6.7 – 31.9 nmol/L Female: [b]18 – 49 years old: >1.9 [b] 50 - 120 years old: > 1.1 [c]18 – 50 years old: 0.3 – 1.9 nmol/L [c] > 51 years old: 0.2 – 1.1 nmol/L
FT	Male: [a] 92-344 pmol/L Female: [a] 6-69 pmol/L
BT	N/A
A4	Male: [b,c]18 – 40 years old: 1.2 – 47 nmol/L [b,c]41 – 120 years old: 0.8 – 3.1 nmol/L Female: [b,c]18 – 49 years old: 0.9 – 7.5 nmol/L [b,c]50 – 120 years old: 0.5 – 2.9 nmol/L
11KT	N/A
11OHA4	N/A
17OHP	Male: [b]12 – 120 years old: < 6.7 nmol/L [c] >18 years old: < 5.0 nmol/L e Female: [b] Follicular phase: > 2.4 nmol/L [c] Follicular phase: < 2.5 nmol/L [b] Luteal phase: > 8.6 nmol/L [b] Post-menopausal: > 1.5 nmol/L
FAI (10TT/SBHG)	Female: [d] > 0.6

[a] *Stavanger University Hospital*
[b] *Hormone Laboratory at Oslo University Hospital*
[c] *Hormone Laboratory at Haukeland University Hospital*
[d] *PCOS International Guideline (Teede et al., 2018)*

The hormone profiles of relevant androgens regarding hyperandrogenism and hypogonadism for Sample A, B and C are given in **Table 11**. The analytes most used as biomarkers for hyperandrogenism and hypogonadism are testosterone (TT, BT and FT) and A4 (Fanelli et al., 2013). According to the PCOS International Guidelines, FAI is a useful marker for the assessment for women with hyperandrogenism when quantification of the bioavailable or free T is absent. However, FAI is deemed unreliable in the presence of low SHBG levels which is found in women with hyperandrogenism and obesity (Keevil et al., 2018). Furthermore, 17OHP is featured in the hormone profiles to exclude CAH in cases with hyperandrogenism (Ueland et al., 2022). The 11-oxyandrogens 11KT and 11OHA4 may also be used to determine whether androgen excess originates from the HPG-axis (i.e., the gonads) or the HPA-axis (i.e., the adrenal glands (Turcu et al., 2020). All samples had morning cortisol levels within the normal reference ranges, and hyperandrogenism due to Cushing's syndrome can be disregarded (Fegan et al., 2007). Due to the relevance of gender and age of hormonal levels, further information on this is given to compare the measured concentrations with the established reference ranges.

Table 11: Hormone profile of sample A, B and C with concentrations (nmol/L) of relevant analytes for determination of hyperandrogenism and hypogonadism. The mean values for women (n = 19) and men (n = 18) with the age spectrum are also shown. Serum samples were obtained through the BRIS-project at the Stavanger University Hospital from assumed healthy individuals with three samples with suspected hypogonadism and hyperandrogenism. Values higher or lower than the established reference ranges are highlighted in **bold**. Age and gender are provided for comparison to the reference ranges. Free T in saliva (sFT) was conducted as previously reported by Nadarajah et al. (2017). **TT**: total testosterone, **FT**: free testosterone, **BT**: bioavailable testosterone, **A4**: androstenedione, **11KT**: 11-ketotestosterone, **11OHA4**: 11β-hydroxyandrostenedione, **17OHP**: 17α-hydroxyprogesterone, **FAI**: free androgen index, **SHBG**: Sex-hormone Binding Globulin, **y/o**: years old, **N/A**: not available.

Analytes	**Sample A (F, 54 y/o)**	**Sample B (F, 23 y/o)**	**Sample C (M, 30 y/o)**	**Mean (F, 23-65 y/o)**	**Mean (M, 24-60 y/o)**
	nmol/L	**nmol/L**	**nmol/L**	**nmol/L**	**nmol/L**
TT	**1.1**	**2.3**	**4.1**	0.93	18.3
BT	0.6	1.5	2.1	0.45	9.6
A4	**3.8**	**15.2**	8.2	4.3	3.1
11KT	1.6	2.7	1.6	0.85	0.80
11OHA4	10.3	5.8	7.7	3.9	4.3
17OHP	1.8	2.7	3.1	2.4	2.7
FAI	0.29	2.8	N/A	0.28	N/A
	pmol/L	pmol/L	pmol/L		
sFT	15	**80**	**83**	25	291

Sample A (female, 54 years old) and sample B (female, 23 years old) have androgen levels complementary to women with hyperandrogenism (Yoshida et al., 2018). Sample B has concentrations of TT, sFT, A4 , and level of FAI beyond the normal ranges clearly illustrating elevated androgen levels and consistent with hormone levels found in PCOS. However, the hormone profile of the previous sample differs as Sample A only has slight elevation of A4 and TT concentration in the upper range with normal sFT and FAI levels. Interestingly, the concentration of 11-oxyandrogens for sample A and 11KT for sample B are increased when compared to the normal population of the 19 female samples from BRIS (**Figure 40**).

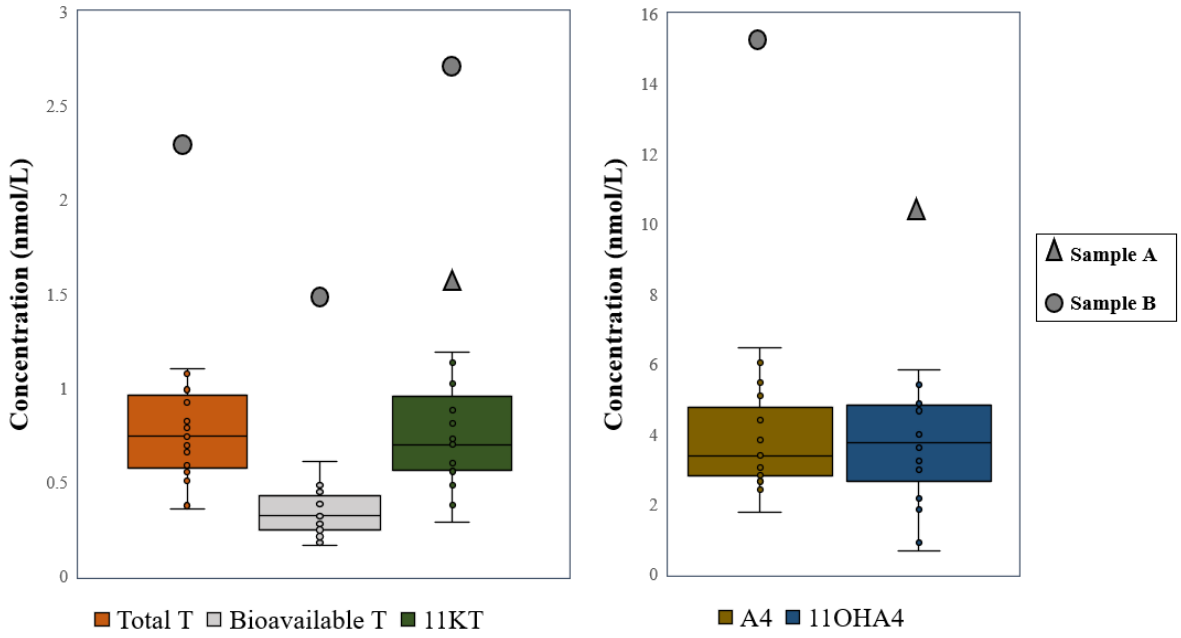


Figure 40: Boxplots of generated normal ranges for female (n = 19) participants in the BRIS-project for relevant androgens. Mean shown as horizontal line with upper and lower limits within 95% confidence. Outliers labelled as Sample A and Sample B for clarity. **T**: testosterone, **11KT**: 11-ketotestosterone, **A4**: androstenedione, **11OHA4**: 11β-hydroxyandrostenedione.

The considerable elevation of 11-oxydrogens of sample A indicates androgen excess with adrenal origin as the elevation of TT and A4 are only slightly increased implying the limited contribution of the gonads in androgen excess. The similar findings have been found by Yoshida et. al. where women with hyperandrogenic symptoms has increased concentrations of 11-oxygenated androgens, but normal levels of TT and A4 (Yoshida et al., 2018). Moreover, sample A has an FAI and FT value within the normal range further demonstrating androgen excess from the adrenals (O'Reilly et al., 2016). According to the PCOS International Guideline, the levels of FAI must be above 0.6 and T levels two-times higher than the set reference ranges (Teede et al., 2018). Additionally, two out of three Rotterdam-criteria must

be met to categorize PCOS resulting in multiple phenotypes (Guastella et al., 2010). Sample A exhibits the PCOS phenotype without androgen excess of the classical androgens (i.e., TT). Thus, sample A would not have been assessed for PCOS with hyperandrogenism as the classical androgens were within the normal concentration ranges. As previously mentioned, biochemical assays most often solely measure TT, A4 and FAI to identify hyperandrogenism in women with suspected PCOS (Keevil, 2019; Teede et al., 2018). With the in-house developed UPLC-MS/MS hormone analysis, sample A was revealed to have elevated levels of 11KT and 11OHA4 indicating androgen excess with adrenal origin. Moreover, sample B was demonstrated to have hypersecretion of androgens from both the gonads and adrenals due to the elevated concentrations of TT and 11KT with equivalent androgenic potency (Pretorius et al., 2017).

Another sample from the BRIS-project showed TT levels consistent with hypogonadism, namely sample C. Hypogonadism is characterized by the diminished testosterone production resulting in androgen deficiency (Basaria, 2014). The hormone profile for sample C, as shown in **Table 11**, displays concentrations of TT and sFT below the established reference ranges. The measured BT concentration can be considered relatively low compared to the mean concentration found for the population cohort. The 11OHA4 and 11KT concentration for sample C is elevated compared to the male population of 18 men from the BRIS-project. However, 11KT in sample C is comparable to levels found in eugenic men (Caron et al., 2021; Turcu et al., 2021), as well as 11OHA4 when compared to the data provided in some studies (Schiffer et al., 2023; Turcu et al., 2021). There are discrepancies on measured 11OHA4 concentrations amongst eugenic men as the studies conducted by Caron et al. and Davio et al. shows approximately halved 11OHA4 concentrations than what is found in this current study (Caron et al., 2021; Davio et al., 2020). Interestingly, there are asymptomatic men diagnosed with hypogonadism where the decrease of their primary androgen (i.e., testosterone) do not display any clinical manifestations (Lackner et al., 2007). Is it possible that the decrease of gonadal androgen production is compensated by an increased production of adrenal androgens with similar potency as T?

The 11-oxygenated androgens are resurfacing as utile biomarkers for hyperandrogenism, especially for androgen excess from the adrenal glands as seen in CAH (Turcu et al., 2016) and from the ovaries seen in PCOS (O'Reilly et al., 2016). They may represent the intracrine relationship between the steroidogenesis of adrenal glands and gonads in various

endocrinological diseases (Turcu et al., 2020). The potential of 11-oxyandrogens as alternative biomarkers must be further evaluated by reliable assays (i.e., LC-MS/MS) and larger population cohorts in pursuance of reference range establishment. Uncovering their functional role can further enhance our understanding of the steroidal pathways, and the possibility of conducting multiplex analysis can provide hormonal patterns more representative for the endocrinological conditions.

5. Conclusion

5.1 Summary

The current study explored a novel procedure of extracting steroid hormones from serum using salting-out assisted liquid-liquid extraction (SALLE). The utilization of acetonitrile as extraction solvent and ammonium sulphate as the salting-out reagent revealed itself to work brilliantly at extracting multiple steroid hormones consisting of glucocorticoids, progestogens and androgens (including bioavailable T) from human serum. The proposed sample preparation showed to be cost- and time-efficient, in addition to being fully automated minimizing the labour required from the operator.

The proposed method with UPLC-MS/MS has proven to quantify androgens (i.e., total T, BT, A4), 11-oxyandrogens (i.e., 11KT, 11OHA4), progestogens (i.e., P, 17OHP) and glucocorticoids (F, E, B, 11DF and DOC). The LODs and LOQs ranged from 0.01 – 0.12 nmol/L and 0.03 – 0.29 nmol/L, respectively. Carry-over was satisfactory with < 1% for all analytes. All hormones showed acceptable linearity with R^2 > 0.996 in clinically relevant working ranges. The intermediate precision and repeatability were found to be < 30% for all analytes from the lower to the upper concentration range. Exclusion from the latter statement is bioavailable T with coefficient of variation ranging from 19-38% for the high to low concentration range, respectively.

The in-house developed multiplex UPLC-MS/MS analysis is high-throughput with 2.67-minute cycle time per injection capable of analysing large sample quantities within a short timeframe. Multiple endocrinological conditions such as PCOS in women, hypogonadism in men, CAH and Cushing's syndrome can be diagnosed, in addition to the exclusion of other differential diagnoses such as hormone-secreting tumours through hormone profiling. Not only is the proposed UPLC-MS/MS method capable as a diagnostic tool, but it is also suitable for therapeutic drug monitoring for patients undergoing hormonal therapy. To conclude, the novel method can potentially replace multiple IAs and provide an assay for analytes lacking available IAs by simultaneously detecting 11 steroid hormones in human serum with satisfactory sensitivity and selectivity.

5.2 Further research

5.2.1 Sample preparation

SALLE for sample clean-up and analyte extraction of steroid hormones in serum was shown to yield high, reproducible, and fast extractions with the possibility of direct sample introduction to the UPLC-MS/MS system. However, this extraction method is somehow limited by the lack of analyte enrichment as seen in the conventional LLE. One of the possibilities to achieve higher analyte enrichment with SALLE is to minimize dilution in the final solvent extract. This can be accomplished by having narrower wells in the microplate **(Figure 41)** where optimal phase separation can be generated with small volumes of ACN whilst avoiding injection of the precipitate into the UPLC-MS/MS instrument.

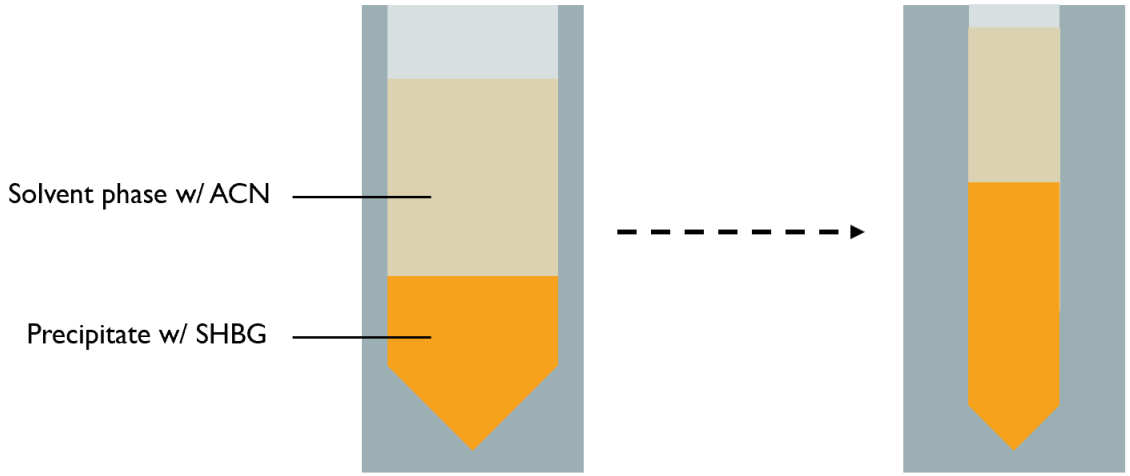


Figure 41: Proposed solution for minimizing dilution in SALLE with less solvent volume by using narrower wells in 96-formats

Further research is needed to minimize the variations in the BT extraction and SHBG-precipitation from serum as values found for high and low levels are unsatisfactory. Moreover, the spiked recovery experiment displayed varying interactions to SHBG suggesting that experiments must be conducted considering SHBG levels. This study has shown the tremendous potential of using AMS and SALLE as a direct extraction method of BT from human serum. However, the large variability observed for BT extraction, especially in the lower concentration ranges, illustrates the importance of understanding the root causes to produce a robust and reproducible analysis.

5.2.2 UPLC-MS/MS analysis

The UPLC-MS/MS method presented in this study was developed with the intent to become a routine analysis. However, columns with phenyl biphenyl or pentafluorylphenyl phases may achieve better separation of isobaric compounds. Additionally, longer analysis enables increased analyte interactions with the stationary phase, thus increasing its retention to avoid co-elution and improve separation. The gradient elution can be further tuned to attain the optimal elution strength of the mobile phase to control the retention of isobaric compounds ensuring adequate separation.

Multiplex analyses enable the generation of hormone profiles, which are beneficial for diagnosing, therapeutic drug monitoring and differentiation of endocrinological conditions. The proposed method can determine 11 analytes, including the bioavailable testosterone. The list of analytes can be extended to include other steroid hormones relevant for various endocrinological cases. For example, 21-deoxycortisol for CAH and oestrogens for women's health and sex-cord stromal tumours. The sample preparation SALLE shows to effectively extract steroid hormones from serum, and the UPLC-MS/MS method can be altered to cater the separation requirements of the additional analytes. Such alterations include switching the electrospray ionization mode from positive to negative and adjusting the mobile phase pH to promote analyte ionization when needed. For instance, detection of oestradiol may be possible upon the use of ESI- due to the high MP pH in the current method that favours deprotonation of the phenol group, thus aiding the generation of negatively charged ions. The method proposed in this study, using SALLE with UPLC-MS/MS for serum steroid hormone analysis, shows incredible potential in expansion of the analyte panel and application for various endocrinological conditions.

6. Reference list

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7. Supplemental data

7.1 Calibration curves

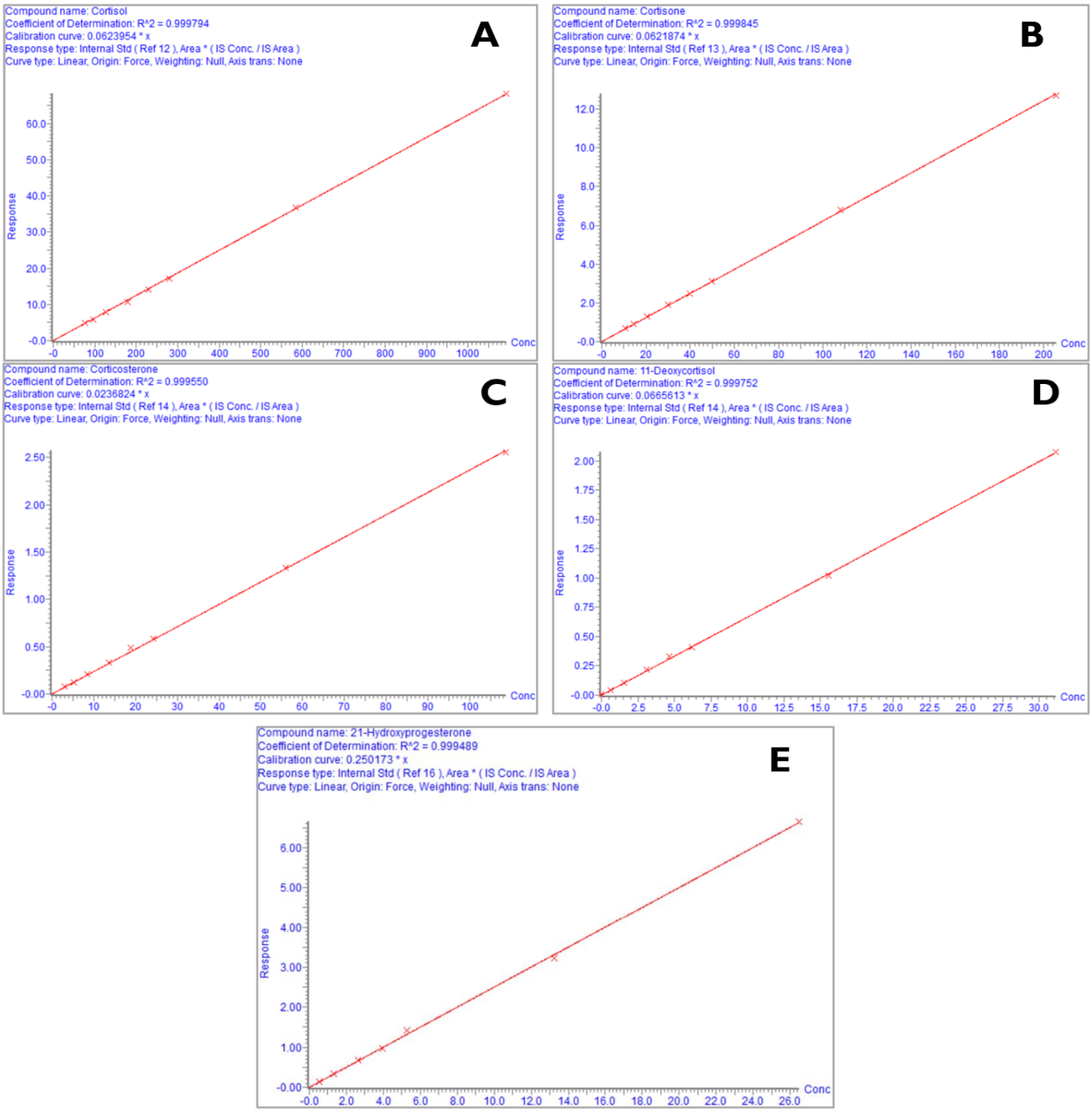

Figure S1: Calibration curves for glucocorticoids: (**A**) Cortisol, (**B**) Cortisone, (**C**) Corticosterone, (**D**) 11-deoxycortisol and (**E**) Deoxycorticosterone, for progestogens: (**F**) progesterone and (**G**) 17 hydroxyprogesterone and for androgens: (**H**) Testosterone, (**I**) Androstenedione, (**J**) 11-ketotestosterone, (**K**) 11-hydroxyandrostenedione

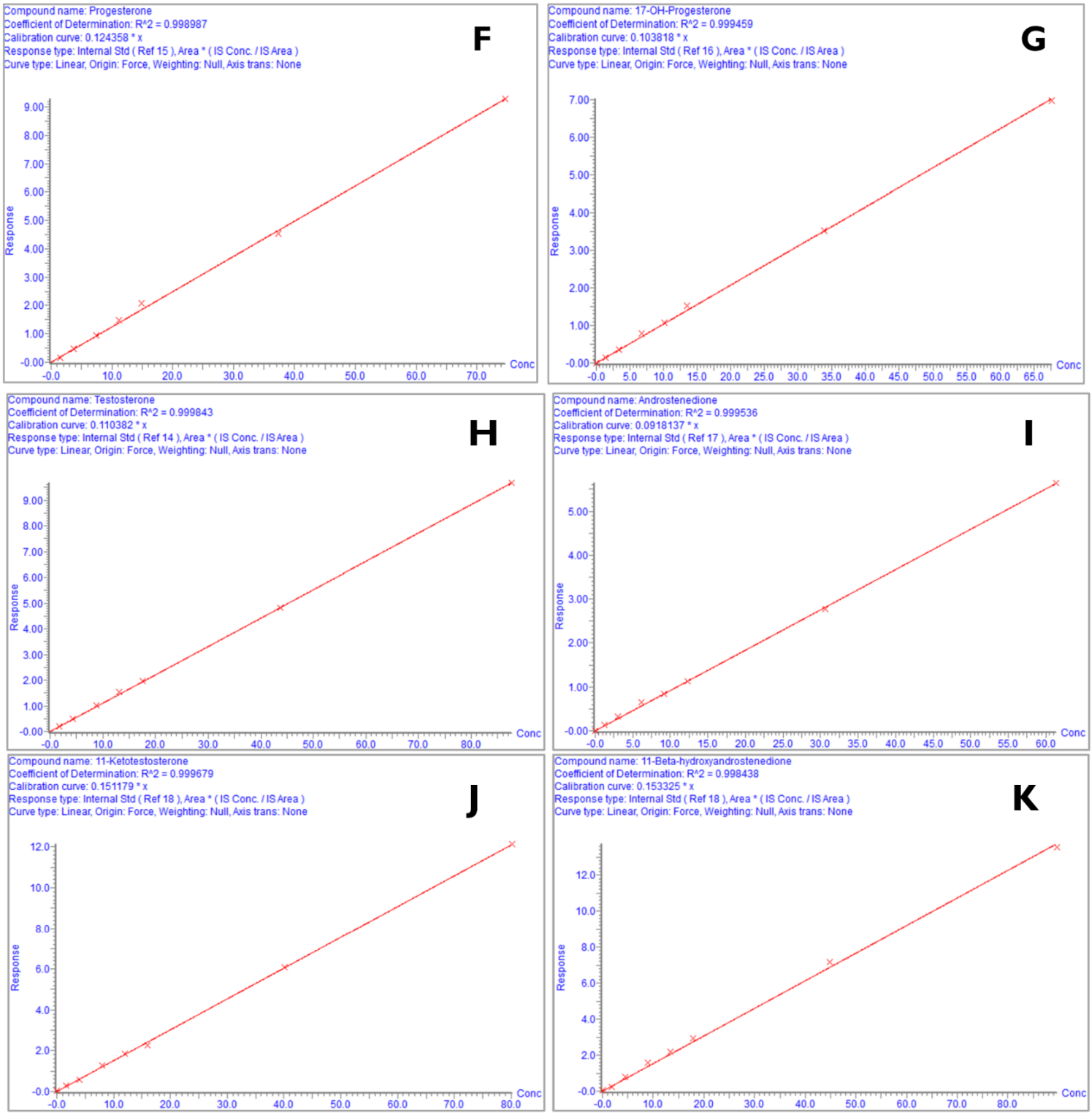

Figure S1: Continued.

7.2 Comparison between UPLC-MS/MS and immunoassay

The in-house developed UPLC-MS/MS method was subjected to method comparison, and the linear relationship and bias was observed for progesterone, 17α-hydroxyprogesterone, testosterone, androstenedione and cortisol (**Figure S3**).

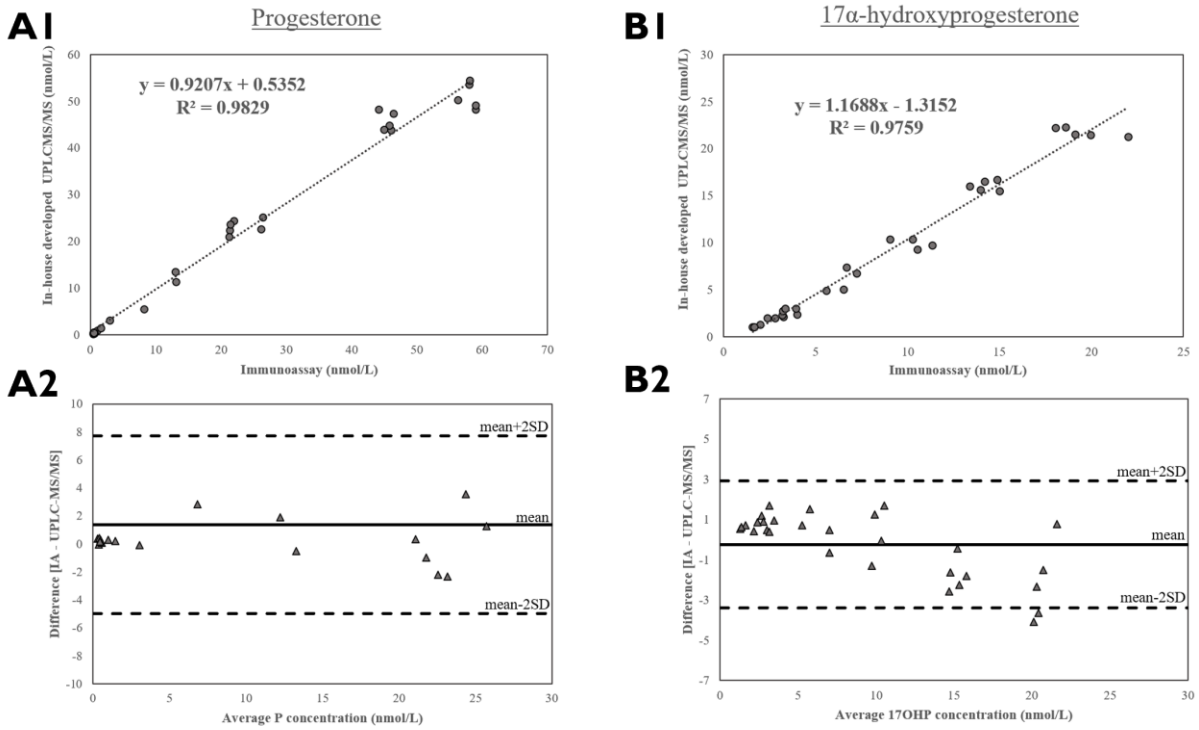

Figure S2: Results of method comparison between immunoassays and the in-house developed method with UPLC-MS/MS. Measurements were conducted on the Labquality external quality controls (n = 29) for the analytes (**A**) progesterone, (**B**) 17α-hydroxyprogesterone, (**C**) testosterone, (**D**) androstenedione, (**E**) cortisol. Additional comparison was conducted for (**F**) cortisol with the CMIA method used in the Stavanger University Hospital. The linear relationships between the methods are shown in A1, B1, C1, D1, E1 and F1. Bias was assessed with Bland-Altmann plots given in A2, B2, C2, D2, E2 and F2.

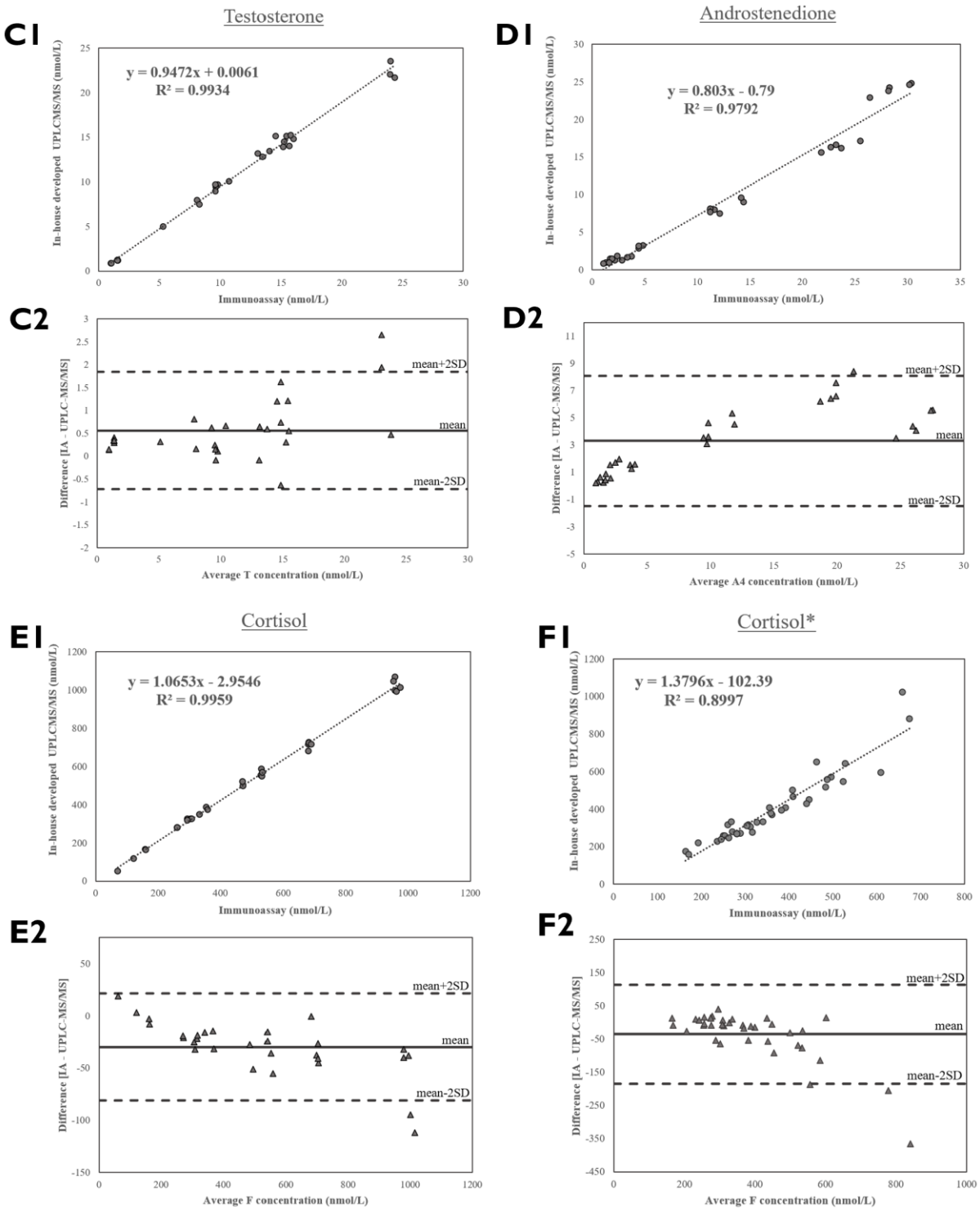

Figure S2: Continued.

7.3 Interlaboratory comparison with LC-MS/MS

Interlaboratory assessment was conducted to evaluate the linear relationship and bias of the in-house developed UPLC-MS/MS method with Labquality external quality controls (n = 29) for the analytes androstenedione (**Figure S3**), cortisol (**Figure S4**) and 17α-hydroxyprogesterone (**Figure S5**).

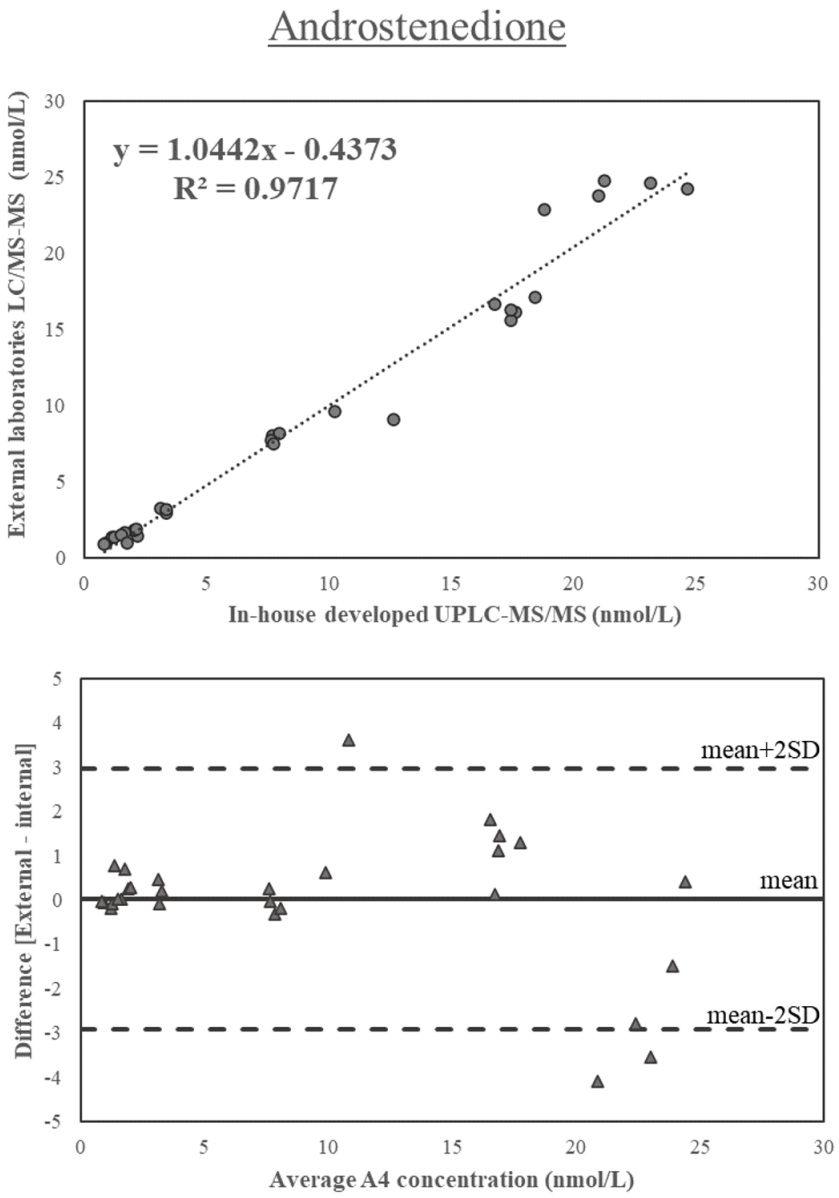

Figure S3: The linear correlation (upper) and bias assessment (lower) of androstenedione (A4) in an interlaboratory analysis using Labquality external quality controls (n = 29)

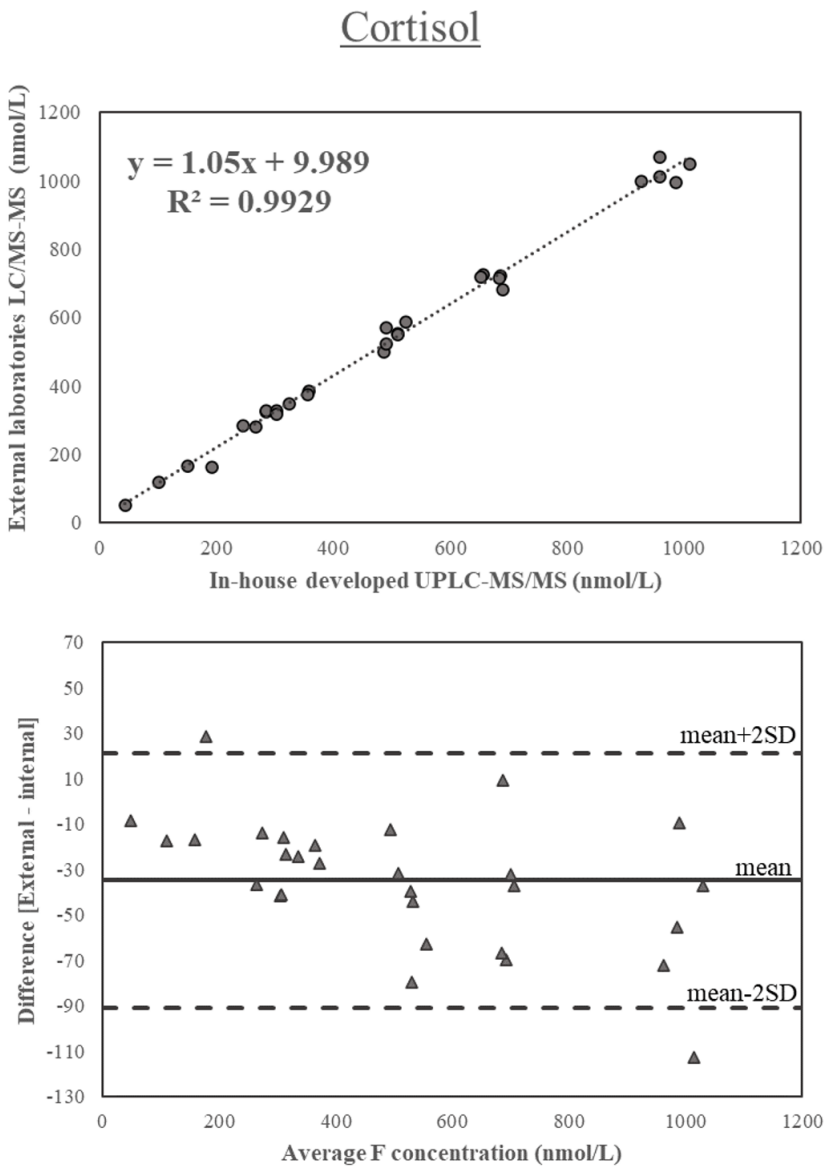

Figure S4: The linear correlation (upper) and bias assessment (lower) of cortisol (F) in an interlaboratory analysis using Labquality external quality controls (n = 29)

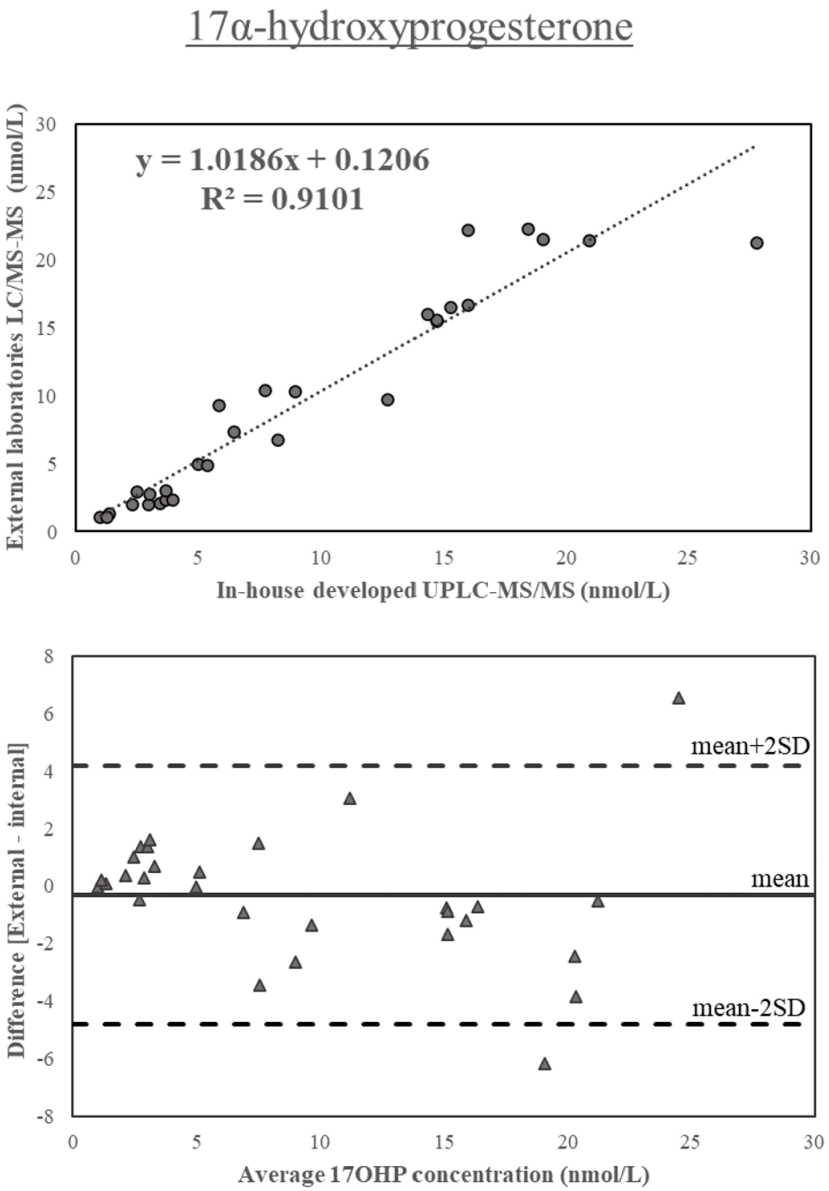

Figure S5: The linear correlation (upper) and bias assessment (lower) of 17α-hydroxyprogesterone (17OHP) in an interlaboratory analysis using Labquality external quality controls (n = 29)

7.4 Blood Reference in Stavanger (BRIS)

The reference ranges generated from 34 healthy individuals through the BRIS biobank (**Table S1**). Pregnant women (*n* = 1) and individuals with suspected endocrinological conditions (*n* = 3) are excluded from the total 38 obtained samples. Serum samples were acquired between 07.00 and 09.00 am to ensure minimal circadian variations in the hormonal levels. The reference ranges presented are solely based on gender, however, future improvements should involve separate concentration ranges based on age and BMI (i.e., SHBG-levels). The volunteers answered a questionnaire (**Figure S6**) and consent form (**Figure S7**) prior to sample taking.

Table S1: Measured reference ranges from healthy volunteers with 17 females and 17 males for all analytes with mean value and standard deviation in nmol/L. **TT**: total testosterone, **BT**: bioavailable testosterone, **F**: cortisol, **E**: cortisone, **P**: progesterone, **17OHP**: 17α-hydroxyprogesterone, **A4**: androstenedione, **B**: corticosterone, **DOC**: deoxycorticosterone, **11DF**: 11-deoxycortisol, **11KT**: 11-ketotestosterone and **11OHA4**: 11β-hydroxyandrostenedione.

		Female (23-65 y/o)	**Male (24-60 y/o)**
Compound		Mean ± standard deviation, nmol/L	Mean ± standard deviation, nmol/L
Androgens	TT	0.72 ± 0.22	19.18 ± 5.68
	BT	0.32 ± 0.11	10.0 ± 3.01
	A4	3.67 ± 1.36	2.81 ± 1.00
	11KT	0.70 ± 0.26	0.75 ± 0.34
	11OHA4	3.41 ± 1.46	4.14 ± 1.88
Progestogens	P*	9.35 ± 12.89	0.23 ± 0.13
	17OHP*	1.96 ± 1.80	2.7 ± 1.27
Glucocorticoids	F†	393.72 ± 190.47	358.26 ± 111.47
	E	55.20 ± 12.85	51.40 ± 7.59
	B†	12.78 ± 11.38	16.17 ± 12.84
	11DF	0.65 ± 0.53	0.91 ± 0.64
	DOC	0.11 ± 0.06	0.10 ± 0.06

*P and 17OHP concentrations are known to vary during the menstrual cycle (McNatty et al., 1975). The data do not regard the menstrual cycle of the female volunteers and the measured variability does not necessarily reflect the method performance.

†F, E and B concentrations are highly dependent of the circadian rhythm (Dickmeis, 2009). High variability may arise due from differences in wake-up time between individuals.

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Questionnaire

Blood Reference In Stavanger – BRIS

First name:		Last name:	
Norwegian personal number (11 digits):			
Sex (M/F):	Age (years):	Height (cm):	Weight (kg):
Vegetarian/vegan? (Yes/No):		Blood donor? (Yes/No):	
Allergies? (Yes/No):	High blood pressure? (Yes/No):		Diabetes? (Yes/No):
If you drink coffee or tea, state the number of cups per week (0 = do not drink coffee/tea):			
If you smoke, state the number of cigarettes per week (0 = non-smoker):			
If you exercise, state the number of intervals per week (0 = do not exercise regularly)			
If you drink alcohol, state the number of units per week (0 = do not drink/seldom drinks): 1 unit = 1 glass of wine/ 1 can of beer or a drink			
Diagnosis/diseases:			
Do you use medicine? (Yes/No):	Name of the medicine:		
Do you take vitamins, minerals, tran or any other supplements? (Yes/No):			
Name of the supplement:			
On daily average, how much fruits and vegetables do you eat?			

Information regarding the sample(s) taken:

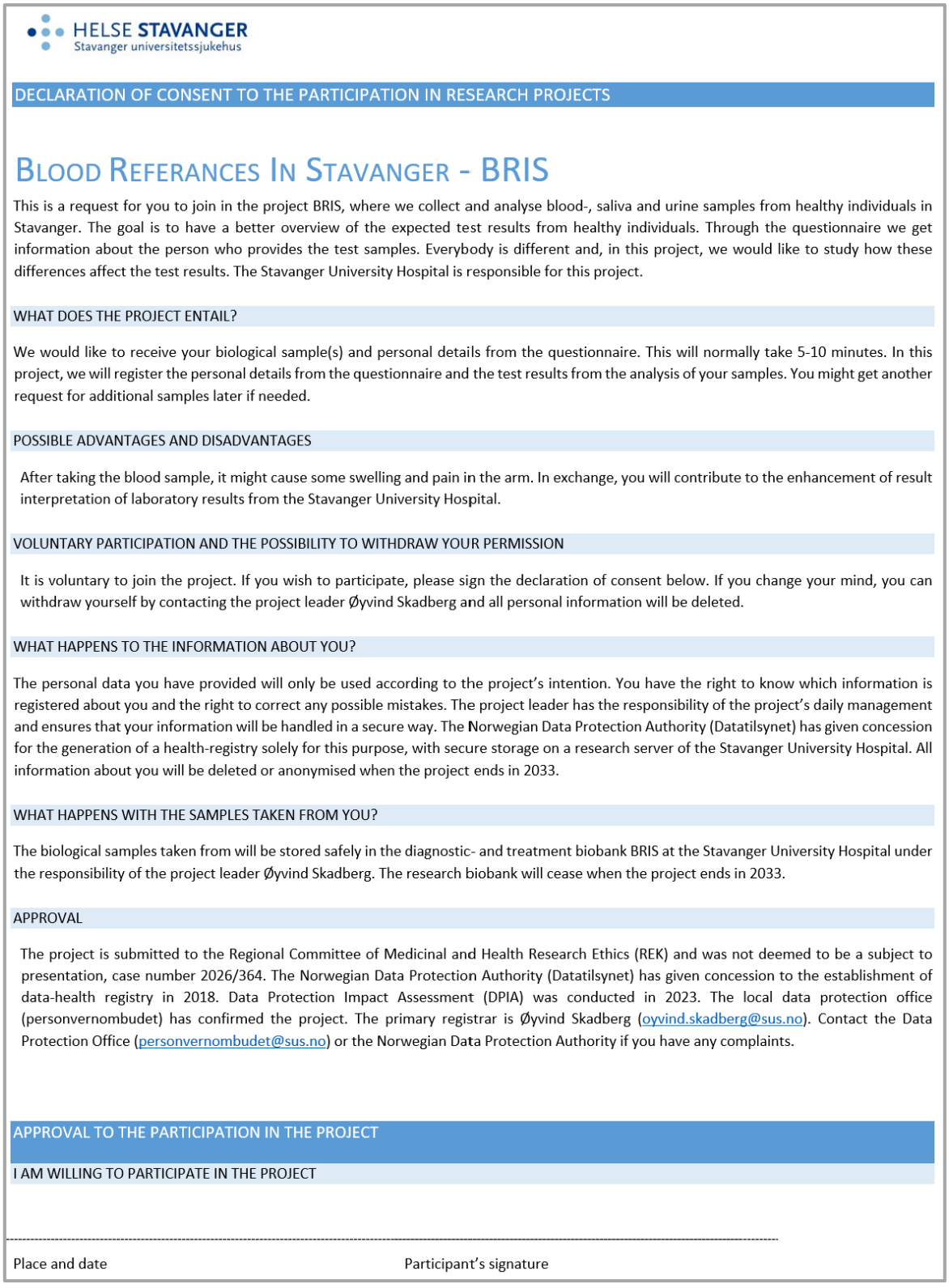
Circle the appropriate answer: Blood sample Urine sample Saliva sample

Sample nr. (From the requisition form)	Date (dd:mm:yyyy)	Sample taken at (hh:mm)	Woke up at (hh:mm)	Number of hours since last meal, snacks or drinks (besides water)

Following questions are directed to female subjects:

Pregnant? (Yes/No):	Pregnancy duration (months):	Breastfeeding? (Yes/No):
Use of any contraceptives (p-pills etc.)? (Yes/No):	About menstruation cycles, time since the start of last menstruation? (days)	

Figure S6: BRIS questionnaire



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DECLARATION OF CONSENT TO THE PARTICIPATION IN RESEARCH PROJECTS

BLOOD REFERANCES IN STAVANGER - BRIS

This is a request for you to join in the project BRIS, where we collect and analyse blood-, saliva and urine samples from healthy individuals in Stavanger. The goal is to have a better overview of the expected test results from healthy individuals. Through the questionnaire we get information about the person who provides the test samples. Everybody is different and, in this project, we would like to study how these differences affect the test results. The Stavanger University Hospital is responsible for this project.

WHAT DOES THE PROJECT ENTAIL?

We would like to receive your biological sample(s) and personal details from the questionnaire. This will normally take 5-10 minutes. In this project, we will register the personal details from the questionnaire and the test results from the analysis of your samples. You might get another request for additional samples later if needed.

POSSIBLE ADVANTAGES AND DISADVANTAGES

After taking the blood sample, it might cause some swelling and pain in the arm. In exchange, you will contribute to the enhancement of result interpretation of laboratory results from the Stavanger University Hospital.

VOLUNTARY PARTICIPATION AND THE POSSIBILITY TO WITHDRAW YOUR PERMISSION

It is voluntary to join the project. If you wish to participate, please sign the declaration of consent below. If you change your mind, you can withdraw yourself by contacting the project leader Øyvind Skadberg and all personal information will be deleted.

WHAT HAPPENS TO THE INFORMATION ABOUT YOU?

The personal data you have provided will only be used according to the project's intention. You have the right to know which information is registered about you and the right to correct any possible mistakes. The project leader has the responsibility of the project's daily management and ensures that your information will be handled in a secure way. The Norwegian Data Protection Authority (Datatilsynet) has given concession for the generation of a health-registry solely for this purpose, with secure storage on a research server of the Stavanger University Hospital. All information about you will be deleted or anonymised when the project ends in 2033.

WHAT HAPPENS WITH THE SAMPLES TAKEN FROM YOU?

The biological samples taken from will be stored safely in the diagnostic- and treatment biobank BRIS at the Stavanger University Hospital under the responsibility of the project leader Øyvind Skadberg. The research biobank will cease when the project ends in 2033.

APPROVAL

The project is submitted to the Regional Committee of Medicinal and Health Research Ethics (REK) and was not deemed to be a subject to presentation, case number 2026/364. The Norwegian Data Protection Authority (Datatilsynet) has given concession to the establishment of data-health registry in 2018. Data Protection Impact Assessment (DPIA) was conducted in 2023. The local data protection office (personvernombudet) has confirmed the project. The primary registrar is Øyvind Skadberg (oyvind.skadberg@sus.no). Contact the Data Protection Office (personvernombudet@sus.no) or the Norwegian Data Protection Authority if you have any complaints.

APPROVAL TO THE PARTICIPATION IN THE PROJECT

I AM WILLING TO PARTICIPATE IN THE PROJECT

Place and date Participant's signature

Figure S7: BRIS consent form