THE UNIVERSITY OF
SYDNEY

Liquid Chromatography Tandem Mass Spectrometry Measurements of Steroids in Biological Samples for Clinical Research Studies: Methodology and Application

Gurmeet Kaur Surindar Singh

The University of Sydney

PhD

March 2015

Declaration

This thesis is submitted to the University of Sydney in fulfilment of the requirement for the Degree of Doctor of Philosophy.

I declare that this thesis is my own work and effort and that has not been submitted for any other award. Where other sources of information have been used, they have been duly acknowledged.

Ethical approval from Human Research Ethics Committee, University of Sydney (HREC 13094) and Sydney Local Health District Human Ethics Committee within National Health and Medical Research Council Guidelines for Human Experimentation (NHMRC) were granted for the studies presented herein.

Signature:…….

Date: 27/03/2015

Dedication

I would like to dedicate my thesis to my dear father. Daddy, you are always in my thoughts. I still remember the last hug you gave me. I wish you were here to see my success.

Acknowledgements

Firstly, I express my greatest gratitude to both my supervisors, Professor David Handelsman and Reena Desai for their continuous support, expertise and scientific guidance throughout the last three and half years of my doctoral work. Thank you for believing in my capabilities and pushing me to realize my potential that made this dissertation possible. I wish to thank Reena for all her guidance on the mass spectrometry work. I would also like to thank Dr. Ron Newman for providing indispensable advice during the initial part of my doctoral work. Your continuous support and encouragement has driven me to pursuit my goals.

I owe my deepest appreciation to our collaborators Professor Katharine Steinbeck and Ben Balzer for their expertise and contribution for the puberty hormonal study. I would like to thank Leo Turner for his help and contribution for the subcutaneous steroid study.

A special thanks to Annet Doss and Reena Desai for their friendship and unconditional love. I cherish all the good time we have spent together and making my stay in Australia, a memorable one. I am fortunate to have known the two of you and made lifelong friends.

I am grateful to Mark Jimenez for his advice and contribution to this work. My sincere thanks go to my friends Rasmani Hazra, Trupti Trivedi, Marie Merheb and Mashani Mohamad for their friendship and support. I would also like to thank everyone from the Andrology Laboratory and Anzac Research Institute. I would like to acknowledge Universiti Teknologi MARA and Ministry of Higher Education, Malaysia that provided the necessary financial support throughout my doctoral study.

I would like to express my deepest appreciation to my dear mother. I admire her strength, dedication and positive spirit towards life. I also wish to thank my sisters, Daljeet and Surenjeet and brother, Kishen for their unconditional love and support. A special thanks to my soulmate, Gopal Subramaniam for being there for me when I needed you the most, uplifting my spirit and keeping me sane. Thank you for all your love, patience and support.

Finally, I thank *Waheguru Ji/Raam Ji*, for the blessings that He bestowed upon me.

Publications

Peer-reviewed journals

Singh GKS, Turner L, Desai R, Jimenez M, Handelsman DJ 2014 Pharmacokinetic-pharmacodynamic study of subcutaneous injection of depot nandrolone decanoate using dried blood spots sampling coupled with ultrapressure liquid chromatography tandem mass spectrometry assays. J. Clin. Endocrinol. Metab. 99:2592-2598

Singh GKS, Jimenez M, Newman R, Handelsman DJ 2014 Immunoreactive LH in long-term frozen human urine samples. Drug Test. Anal. 6: 336-341

Singh GKS, Balzer, BWS, Desai R, Jimenez M, Steinbeck KS, Handelsman DJ Requirement for specific gravity and creatinine adjustments for urinary steroids and luteinizing hormone concentrations in adolescents. Ann Clin Biochem (first published online on 16 March 2015).

Other peer-reviewed journals

Handelsman DJ, Jimenez M, Singh GKS, Spaliviero J, Desai R, Walters KA 2015 Measurement of testosterone by immunoassays and mass spectrometry in mouse serum, testicular and ovarian extracts. Endocrinology 156: 400-405

Abstract presented at conferences

Singh GKS, Newman R, Desai R, Jimenez M, He JX, Simanainen U, Steinbeck K, Handelsman DJ Validation and application of an ultra-sensitive liquid chromatography (LC)-tandem mass spectrometry (MS) assay to measure androgens and estrogen in human urine. The annual Scientific Meeting of Endocrine Society of Australia and the Society for Reproductive Biology (ESA/SRB), Gold Coast, Queensland, Australia 2012.

Singh GKS, Desai R, Jimenez M, Handelsman DJ Quantitation of testosterone and nandrolone using dried blood spots (DBS) for steroid pharmacological study. 61st Conference on Mass Spectrometry and Allied Topics, American Society of Mass Spectrometry (ASMS), Minneapolis, Minnesota, United States 2013.

Desai R, Singh GKS, Turner L, Jimenez M, Handelsman DJ. Dried Blood Spots (DBS) Blood Sampling coupled with Liquid Chromatography, Tandem Mass Spectrometry (LC-MS/MS) assay to investigate pharmacokinetics and pharmacodynamics of subcutaneous

injection of steroid esters in an oil vehicle. Indian Society of Mass Spectrometry (ISMAS-WS) Parwanoo, Himachal Pradesh, India 2014

Singh GKS, Turner L, Desai R, Jimenez M, Handelsman DJ. Pharmacokinetic-pharmacodynamic study of subcutaneous injection of nandrolone decanoate in an oil vehicle using dried blood spots (DBS) blood sampling coupled with liquid chromatography, tandem mass spectrometry (LC-MS) assays. 16th International Congress of Endocrinology and the 96th Endocrine Society Annual Meeting (ICE/ENDO), Chicago, Illinois 2014

Balzer BWR, Singh GKS, Kelly PJ, Paxton K, Hawke CI, Handelsman DJ, Steinbeck KS. Urinary sex steroids, luteinizing hormone and anthropometric markers of puberty: Preliminary results from an intensive longitudinal cohort study. 16th International Congress of Endocrinology and the 96th Endocrine Society Annual Meeting (ICE/ENDO), Chicago, Illinois 2014

Singh GKS, Turner L, Desai R, Jimenez M, Handelsman DJ. Pharmacokinetic-pharmacodynamic study of subcutaneous injection of nandrolone decanoate using dried blood spots (DBS) blood sampling coupled with LC-MS/MS. 20th International Mass Spectrometry Conference (IMSC), Geneva, Switzerland 2014

Turner L, Singh GKS, Desai R, Jimenez M, Handelsman DJ. Pharmacokinetic-pharmacodynamic study of subcutaneous injection of nandrolone decanoate in an oil vehicle using dried blood spots (DBS) blood sampling coupled with liquid chromatography, tandem mass spectrometry (LC-MS) assays. The Annual Scientific Meeting of Endocrine Society of Australia and the Society for Reproductive Biology (ESA/SRB), Melbourne, Victoria, Australia 2014

Abbreviations

ACAT	acyl-coenzyme A:cholesterol acyltransferase
ACTH	adrenocorticotropin
AKR	aldo-keto reductase
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
APPI	atmospheric pressure photo ionization
APS	adenosine 5'-phosphosulfate
AR	androgen receptor
Asn	asparagine
BMI	body mass index
CBG	corticosteroid binding globulin
CDC	Centres for Disease Control
CTP	carboxyl terminal peptide
CV	coefficient of variation
CYP	cytochrome P450
DBS	dried blood spot
DELFIA	dissociation-enhanced lanthanide fluorescence immunoassay
DHEA	dehydroepiandrosterone
DHEAG	dehydroepiandrosterone glucuronide
DHEAS	dehydroepiandrosterone sulphate
DHT	dihydrotestostorone
DHTG	dihydrotestosterone glucuronide
E_2	estradiol
E_2G	estradiol glucuronide
ECD	electron-capture detector
EMA	European Medicines Agency
ER	estrogen receptor
ESI	electrospray ionization
FDA	Food and Drug Administration
FID	flame ionization detector
FSH	follicle stimulating hormone
FT ICR	Fourier transform ion cyclotron resonance
GAB	granulosa cell aromatase bioassay

GABA	γ-aminobutyric acid type A
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GH	growth hormone
GlcNAc	N-acetylglucosamine
GnRH	gonadotropin-releasing hormone
hCG	human chorionic hormone
HPG	hypothalamic-pituitary-gonadal
HSD	hydroxysteroid dehydrogenase
hv	photons energy
ICL	immunochemiluminometric
IE	ionization energy
IF	immunofluorometric
IGF-1	insulin-like growth factor l
im	intramuscular
IRMA	immunoradiometric assays
LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography tandem mass spectrometry
LDL	low density lipoprotein
LH	luteinizing hormone
LLE	liquid-liquid extraction
LLOQ	lower limit of quantification
m/z	mass-to-charge ratio
MICT	mouse interstitial cell testosterone assay
MKRN	makorin ring finger 3
MO-TMS	methyloxime-trimethylsilyl ether
MRM	multiple-reaction-monitoring
MS	mass spectrometer
MTBE	methyl tert-butyl ether
N	nandrolone
NADPH	nicotinamide adenine dinucleotide phosphate
ND	nandrolone decanoate
P450scc	cytochrome P450 side-chain cleavage enzyme
PAPS	3’ phosphoadenosine-5’-phosphosulfate
PP	protein precipitation

Q	quadrupole
QC	quality control
r	correlation coefficient
R^2	correlation of determination
rhCG	recombinant human chorionic hormone
RIA	radioimmunoassay
RICT	rat interstitial cell testosterone assay
S/N	signal to noise ratio
sc	subcutaneous
SD	standard deviation
SDR	short-chain dehydrogenase/reductase
SEM	standard error of mean
SG	specific gravity
SHBG	sex hormone binding globulin
SIM	selected-ion-monitoring
SPE	solid phase extraction
SRM	selected-reaction-monitoring
StAR	steroidogenic acute regulatory protein
SULT	sulphotransferases
T	testosterone
TG	testosterone glucuronide
TOF	time-of-flight
TSH	thyroid stimulating hormone
UDP	uridine diphosphate
UDPGA	uridine diphosphate-glucuronic acid
UGT	uridine diphosphate glucuronosyltransferases
UV	ultraviolet
WHO	World Health Organization
17-OHP	17-hydroxyprogesterone
3α-diol	5α androstan-3α-17β-diol
3β-diol	5α androstan-3β-17β-diol

Table of Contents

Declaration i

Dedication ii

Acknowledgements iii

Publications iv

Abbreviations vi

List of Figures xii

List of Tables xv

ABSTRACT 1

Chapter 1 Literature Review 5

1.1 Steroid hormones 5

1.1.1 Structure and classification 5

1.1.2 Physiological action 8

1.2 Steroid metabolism 10

1.2.1 Steroidogenesis (Biosynthesis of steroids) 10

1.2.2 Phase I metabolism 14

1.2.3 Phase II metabolism (conjugate formation) 17

1.3 Gonadotropin structure and regulation 23

1.4 Steroid hormone production and action 29

1.4.1 Androgens 29

1.4.2 Estrogens 32

1.5 Puberty 35

1.5.1 Hormonal changes in puberty 35

1.5.2 Activation of hypothalamus-pituitary-gonadal axis during development 39

1.5.3 Growth and skeletal maturation during puberty 43

1.5.4 Body composition during puberty 46

1.6 Analytical measurements of LH and FSH 48

1.7 Analytical techniques of steroid measurement 52

1.7.1 Immunoassay 52

1.7.2 Gas chromatography- mass spectrometry 54

1.7.3 Liquid chromatography-mass spectrometry 56

1.8 Mass spectrometry 64

1.8.1 Triple quadrupole mass spectrometer 64

1.8.2 Ionization interfaces 67

1.8.3 Matrix effect....73
1.8.4 Sample preparation....77
1.9 Aims of study....80
Chapter 2 Materials and Methods....82
2.1 LC-MS/MS assay....82
2.1.1 Chemicals....82
2.1.2 Instrumentation....82
2.1.3 LC-MS/MS method for DBS steroids analysis....82
2.1.4 LC-MS/MS method for urinary steroids analysis....93
2.1.5 LC-MS/MS method for serum steroids analysis....98
2.2 LH immunoassay....101
2.2.1 Immulite....101
2.2.2 DELFIA....101
2.3 Urine creatinine measurement....102
2.4 Urine specific gravity measurement....102
Chapter 3 Pharmacokinetic-Pharmacodynamic Study of Subcutaneous Injection of Depot Nandrolone Decanoate Using Dried Blood Spots Sampling Coupled With LC-MS/MS Assays....104
3.1 Introduction....104
3.2 Study Design....106
3.3 Data analysis....107
3.4 Results....108
3.5 Discussion....114
Chapter 4 Immunoreactive LH in long-term frozen human urine samples....119
4.1 Introduction....119
4.2 Study Design....120
4.3 Data analysis....121
4.4 Results....121
4.5 Discussion....127
Chapter 5 Requirement for Specific Gravity and Creatinine Adjustments for Urinary Steroids and Luteinizing Hormone Concentrations in Adolescents....131
5.1 Introduction....131
5.2 Study Design....133
5.3 Data Analysis....134

5.4 Results 134
5.5 Discussion 145
Chapter 6 Urinary Sex Steroids, Luteinizing Hormone and Anthropometric Markers of Puberty 150
6.1 Introduction 150
6.2 Study design 151
6.3 Data analysis 153
6.4 Results 153
6.4.1 Cohort characteristics 153
6.4.2 Hormone measurements 155
6.4.3 Association between Serum and Urine Hormone Measurements and Anthropometry 160
6.5 Discussion 161
Chapter 7 Conclusions 163
7.1 Blood spot sampling after sc steroid injection 163
7.2 Urinary LH immunoassays in stored samples 164
7.3 SG and creatinine adjustments for urinary hormones 165
7.4 Urinary puberty hormones 165
References 167
Appendix 207

List of Figures

Figure 1.1 Cholesterol structure.....6
Figure 1.2 Classification of steroids according to basic backbone structure (androstane, estrane and pregnane) and example of steroid classes: sex steroids (androgen, estradiol and progesterone), corticosteroids and mineralocorticoid.....7
Figure 1.3 Genomic (A) and non-genomic (B) pathways of steroid hormones.....9
Figure 1.4 Classic and backdoor pathways of DHT synthesis.....13
Figure 1.5 Biosynthetic pathway and steroidogenic enzymes involved in the pathway. 15
Figure 1.6 Androgens (5α-DHT and testosterone), glucocorticoid (cortisol), estradiol (estrogen) and progestin (progesterone) phase I metabolism by hydroxysteroid dehydrogenase enzyme (aldo-keto reductase; AKRs and short-chain dehydrogenase; SDRs). 16
Figure 1.7 Phase II metabolism of testosterone. 22
Figure 1.8 Location of glycosylation sites in the human glycoprotein hormone α and β-subunits. 24
Figure 1.9 Crystal structure of human chorionic gonadotropin (hCG; left) and human follicle-stimulating hormone (hFSH; right).....26
Figure 1.10 Chemical structure of four main androgenic steroids: testosterone, dihydrotestosterone, dehydroepiandrosterone and androstenedione. 31
Figure 1.11 Structures of estrone, estradiol and estriol. 34
Figure 1.12 Pubertal rating according to Tanner stages. 38
Figure 1.13 A proposed model for the control of the timing of puberty in primates.....42
Figure 1.14 Ionization capabilities of ESI, APPI and APCI according to the molecular weight and polarity of the analytes. 58
Figure 1.15 Representation of precursor ion and product ion selection of MS1 and MS2, respectively in the tandem mass spectrometer.....67
Figure 1.16 Schematic diagram of ESI source.....69
Figure 1.17 Schematic diagram of APCI source 70
Figure 1.18 Schematic diagram of APPI source.....71
Figure 1.19 Schematic diagram of APPI ionization mechanism. 71
Figure 1.20 Matrix effects evaluation (a) post-column infusion and (b) post-extraction spike. 76

Figure 2.1 Relationship between amount of blood and weight of filter card (n=5).....86
Figure 2.2 Bland-Altman plots for comparison of testosterone (T) concentration obtained from whole DBS versus plasma (left panel) and 6 mm punch disc versus plasma (right panel). 87
Figure 2.3 Dried blood spot method analytes chromatogram.....90
Figure 2.4 Urinary steroids method chromatogram.....97
Figure 2.5 Serum steroids method chromatogram.....100
Figure 3.1 Structures of nandrolone and nandrolone decanoate.....106
Figure 3.2 Plot of correlation (Passing-Bablok-upper panel) and deviance (modified Bland-Altman-lower panel) of individual capillary and serum testosterone in 32 samples obtained from 8 participants before and weekly for 3 weeks after sc injections of ND.....109
Figure 3.3 Plot of correlation (Passing-Bablok-upper panel) and deviance (modified Bland-Altman-lower panel) of individual capillary and serum nandrolone in 32 samples obtained from 8 participants before and weekly for 3 weeks after sc injections of ND.....110
Figure 3.4 Plot of mean and SEM of capillary nandrolone, testosterone and change in testosterone from pre-injection baseline in 8 participants before and daily for 21 days after subcutaneous injections of 100 mg nandrolone decanoate in 2 mL arachis oil.....111
Figure 3.5 Plots of non-linear curve fitting of T (left panel), N (right panel) and T suppression (lower panel) of capillary blood collection from 8 participants before and daily according to exact individual collection time points after SC injection of ND.....112
Figure 3.6 Plot of mean and SEM of capillary, serum and venous nandrolone (left panel), testosterone (right panel) and serum LH and FSH (lower panel) in 8 participants before and weekly for 3 weeks after subcutaneous injections of 100 mg ND.....113
Figure 4.1 Comparison of urinary LH concentrations obtained using IF (left panels) and ICL (right panels) measured before and after four years of storage at -20 °C.....123
Figure 4.2 Comparison of urinary LH concentrations measured by IF and ICL in 2008 (left panels) and 2012 (right panels).....124
Figure 4.3 Plot of urine LH assayed with Delfia (left panels) and Immulite (right panels) for year 2008 (upper panels) and 2012 (lower panels).....125
Figure 5.1 Plot of urinary creatinine measurements of adolescents groups according to age (upper panel) and Tanner stage (lower panel). 136
Figure 5.2 Comparison of urinary LH, E2, T, DHT and DHEA concentrations adjusted by SG and creatinine.....137

Figure 6.1 Cross-sectional plots of serum LH (top), E2 (centre) and T (bottom) at baseline and 12-month follow-up, by self-rated Tanner stage. 156
Figure 6.2 Plots of mean and SEM of individual changes from baseline (0) in urine LH, E2 and T concentrations in female (left panels) and male (right panels) at 3, 6, 9 and 12 months. 158

List of Tables

Table 1.1 List of steroid substrates metabolized by UGT enzymes. 19
Table 1.2 List of steroid substrates metabolized by sulfate isoforms. 21
Table 1.3 Examples of endogenous steroids analysis by GC/LC-MS. 59
Table 1.4 Examples of immunoassay and GC/LC-MS comparative studies for quantitation of testosterone (T) and estradiol (E2). 63
Table 1.5 Basic principles of different types of mass analyzers 66
Table 1.6 Summary of ionization techniques (ESI, APCI and APPI) commonly used for steroid LC-MS/MS analysis 72
Table 2.1 List of chemicals for steroid LC-MS/MS assay. 83
Table 2.2 List of reagents for steroid LC-MS/MS assay. 83
Table 2.3 Mass spectrometry parameters for nandrolone, testosterone and d3-testosterone measurements. 88
Table 2.4 LC-MS/MS DBS method validation data. 91
Table 2.5 Stability data of testosterone and nandrolone on DBS at three different storage conditions for short (42 days) and long term storage (742 days). 92
Table 2.6 Mass spectrometry parameters for estradiol, testosterone, dihydrotestosterone, dehydroepiandrosterone and its corresponding isotopically labeled internal standards. 94
Table 2.7 List of possible interfering compounds for LC-MS/MS urinary and serum methods. 96
Table 2.8 LC-MS/MS urinary steroids method validation data. 97
Table 2.9 LC-MS/MS serum steroids method validation data. 100
Table 4.1 Comparison of urine LH determined using two immunoassays. 126
Table 4.2 Comparison of urine LH determined using Immulite and Delfia (2008 and 2012) versus serum LH determined using Delfia (2008). 126
Table 5.1 Comparison of unadjusted against SG and CR adjusted urinary hormone measurements. 138
Table 5.2 Comparison of mean urinary hormone measurements (unadjusted, SG adjusted and CR adjusted) and F- ratios according to Tanner stage in female. 139
Table 5.3 Comparison of mean urinary hormone measurements (unadjusted, SG adjusted and CR adjusted) and F- ratios according to Tanner stage in male. 140
Table 5.4 Comparison of mean urinary hormone measurements (unadjusted, SG adjusted and creatinine adjusted) and F- ratios according to age groups in female. 141

Table 5.5 Comparison of mean urinary hormone measurements (unadjusted, SG adjusted and creatinine adjusted) and F- ratios according to age groups in male. 142
Table 5.6 Comparison of mean serum hormone measurements and F- ratios according to Tanner stage in female and male. 143
Table 5.7 Comparison of mean serum hormone measurements and F- ratios according to age groups in female and male. 144
Table 5.8 Pearson's correlation coefficient and confidence intervals (in parentheses) of paired urinary and serum LH, E2, T, DHT and DHEA (n=343). 145
Table 6.1 Baseline and 12-month follow-up anthropometry measurements. 154
Table 6.2 Baseline and 12 month follow-up WHO and CDC z-scores (for age). 154
Table 6.3 Self-Rated Tanner staging at baseline and 12 month follow-up. 155
Table 6.4 Baseline and 12-month follow-up serum LH, E_2 and T. 155
Table 6.5 Mean urine LH, E2 and T. 159
Table 6.6 Mean urine hormone measurements for girls by menarcheal status. 159
Table 6.7 Regression results for serum (left) and urine (right) hormones and anthropometric markers of puberty. 160

ABSTRACT

Steroids are endogenous compounds derived from cholesterol which can be divided into five families comprising of estrogens, androgens, progestins, mineralocorticoids and glucucorticoids. Most circulating steroids are conjugated to a hydrophilic moiety (e.g. glucuronide and sulfate) or bound to carrier proteins (e.g. sex hormone binding globulin and albumin) with a small proportion in a non-protein bound form. The production of steroids is regulated by the pituitary gonadotropins i.e. luteinizing hormone (LH) and follicle stimulating hormone (FSH) that mediate their bioactivity by binding to cognate G protein-coupled receptors expressed on the surface of specific target cells in the gonads – Leydig cells for LH and Sertoli cells for FSH. Steroids exert biological activities by binding to their specific intracellular receptors in both reproductive and non-reproductive target tissues. Accurate measurement of reproductive hormones is vital to evaluate neonatal disorders of sexual differentiation, pubertal status, gonadal function, and hormonal doping in sports. Steroids from biological fluids, notably serum and urine, can be measured using immunoassays and gas chromatography mass spectrometry (GC-MS); however, these traditional assays have significant limitations. The advent of liquid chromatography tandem mass spectrometry (LC-MS/MS) coupled with soft ionization (e.g. atmospheric pressure photo ionization: APPI) method has expanded the scope for measurement of small molecules from biological matrices with higher accuracy, specificity, sensitivity and requires less sample preparation. The overall aim of this study is to develop and validate LC-MS/MS methods to measure steroids from biological samples for clinical research studies.

The first part of this thesis was to develop methodologies and analysis of androgens i.e. testosterone (T) and nandrolone (N) from dried blood spot (DBS) samples using LC-MS/MS. Conventionally T esters are used as replacement therapy for T deficient men and is usually

administered by intramuscular (im) injections to produce long-acting depots which is more convenient in sustaining T release for weeks to months and thereby assists in maintaining long-term adherence to treatment. However, administration of androgen esters via deep im injections into the gluteal muscle requires medical personnel (to give the injection at the right site) and yet the injectate is often deposited into subcutaneous (sc) fat due to the thick sc fat layer in most men. IM injections are also associated with local pain and discomfort, injection site infection or bleeding and may not be suitable for patients with bleeding disorders or using anti-coagulants. Thus, the aim of this clinical study was to investigate the feasibility and pharmacology of sc injection (at a single abdominal site) of nandrolone decanoate (ND), as a typical androgen ester in an oil vehicle, in healthy men and to collect whole blood samples from finger pricks onto filter cards at home for 21 days to investigate the pharmacokinetics of single sc injection of ND and its pharmacodynamic effects on endogenous T. A LC-MS/MS method was developed to enable measurement of serum T and N from the whole blood spot. This bypassed using a subsample to avoid non-homogenous distribution of blood and hematocrit effect on the filter paper. The assay had lower limits of quantitation of 50 (T) and 156 (N) pg/mL, respectively using 50 μL of blood. In the clinical study, daily serum N peaked 2.50 ± 0.25 (SEM) ng/mL at a median (range) of 6 (4-13) days causing a reduction in serum T from 3.50 ± 0.57 ng/mL at baseline to a nadir of 0.38 ± 0.13 (SEM) ng/mL (89 ± 3% suppression) at a median (range) of 8 (5-16) days. This study demonstrates that (a) DBS sampling with LC-MS/MS steroid assays achieves frequent time sampling in the community without requiring clinic visits, venesection or frozen serum storage and (b) an androgen ester in oil vehicle can be delivered effectively by sc injection avoiding the need for medically supervised deep im injections.

The second part of this thesis evaluated the measurement of urinary LH immunoreactivity using immunofluorometric (IF) and immunochemiluminometric (ICL) LH assays after

prolonged frozen storage. These commercially available LH immunoassays are developed and validated for human blood samples but not urine thus LH assays intended for use with urine samples needs thorough validation. LH was measured in serial urine samples following administration of a single injection of one of two doses of recombinant human chorionic hormone (rhCG) with assays run at the end of study (2008) and again after 4 years of frozen (-20 °C) storage where samples were stored without adding preservatives. The ICL assay showed quantitatively reproducible LH measurements after prolonged -20 °C storage. However, the IF immunoassay gave consistently lower LH levels relative to ICL (2008) with a further proportionate reduction after 4 years of sample storage (2012). Yet, both the assays displayed similar patterns of the time-course of urine LH measurement both before and after 4 years of frozen storage. We found that both immunoassays are suitable for urinary LH measurements with ICL assay being more robust for quantitative urinary LH measurement such as for anti-doping purpose whereas the IF could be applicable for research studies where urine LH levels are compared within-study but not in absolute terms.

Urinary hormone concentrations are often adjusted to correct for hydration status. The third part of this thesis investigated whether first morning void urine hormones in growing adolescents require adjustments for urine dilution/concentration and, if so, whether urinary creatinine or specific gravity (SG) are better adjustments. The study population was adolescents aged 10.1 to 14.3 years initially who provided fasting morning blood samples at 0 and 12 months (n=343) and first morning urine every three months (n=644). LC-MS/MS method was developed and validated to measure unconjugated T, estradiol (E_2), dihydrotestostorone (DHT) and dehydroepiandrosterone (DHEA) in human urine. In addition, urine LH was measured using the previously validated ICL assay. Unadjusted, creatinine and SG-adjusted hormonal concentrations were compared by Deming regression and Bland-Altman analysis and grouped according to self-rated Tanner stage or chronological

age. Correlations of paired serum and urinary hormonal concentration of unadjusted and creatinine and SG adjusted were also compared. Fasting first morning void hormone concentrations correlated well and were unbiased between unadjusted or adjusted by either creatinine or SG. Urine creatinine concentration increases with Tanner stages, age and male gender whereas, urine SG was not influenced by Tanner stage, age or gender. Adjustment by creatinine or SG of urinary LH, E_2, T, DHT and DHEA concentrations did not improve correlation with paired serum concentrations. The study demonstrates that urine steroid and LH concentrations in first morning void samples of adolescents are not significantly influenced by hydration status and may not require adjustments; however, if desired, both creatinine and SG adjustments are equally suitable.

The final part of this thesis aimed to describe the longitudinal relationships of within-individual hormone and anthropometric changes during puberty. Temporal changes in urine and serum hormones over 12 months to standard measures of pubertal development were assessed. A community sample of 104 adolescents (57 female) was studied over 12 months with annual anthropometric assessment, blood sampling and self-rated Tanner staging and urine collected every 3 months. Serum and urine sex steroids (T, E_2) were measured by the developed and validated LC-MS/MS method and LH by ICL assay. A high proportion (92%) of scheduled samples were obtained with low attrition rate of 6.7% over the 12 months. The study demonstrated that the urine hormone measurements correlated cross-sectionally and longitudinally with age, anthropometry and Tanner stage. This study successfully developed a feasible and valid sampling methodology and measurements for puberty hormones in urine, which allows a sufficiently intensive sampling frequency to monitor individual pubertal progression in adolescents.

Chapter 1 Literature Review

1.1 Steroid hormones

1.1.1 Structure and classification

Steroids can be classified into five families depending on its structural and biological basis. These comprise estrogens, androgens, progestins, mineralocorticoids and glucocorticoids. Vitamin D, bile acids and thyroid hormones also have close structural resemblance to the steroid family of molecules. Steroids are lipophilic compounds derived from cholesterol, a sterol which is made up of three hexagonal carbon rings (A, B and C) and a pentagonal carbon ring (D) to which a side chain is attached (Figure 1.1). This four-membered hydrocarbon core has two important methyl groups are attached at position 18 (attached to C13) and 19 (attached to C10). The carbon rings fused in a *trans* orientation to form a planar structure. The orientation of the substituent groups on the steroid skeleton is either above (β) or below (α) the plane. According to the chemical structure, steroids are divided into cholane, cholestane, androstane, estrane and pregnane. Cholanes (bile acids) and cholestanes (vitamin D and cholesterol) are known as sterols with long and branched hydrocarbon side chains attached to D ring. Partial cleavage of the cholestane, C27 sterol side chain (ring-D) produces pregnane C21 series steroids (progestins and corticosteroids), whereas the total cleavage of the side chain yields androstane C19 series steroids (androgens). Finally, removal of the methyl group between the A- and B-rings results in production of estrane C18 series steroids (estrogens). The structures of the three classes of steroids are shown in Figure 1.2. Various functional groups such as hydroxy, keto and methyl-groups are located at different positions of the carbon backbone in all series depending on the compound.

Each steroid has common alike structure and stereochemistry. However, each class of steroids displays distinct physiologic activities depending on the family of nuclear transcription factors the molecules activate via its cognate (steroid hormone receptor)

(Strauss 2014). Progestagens, androgens and estrogens are classified as sex steroids whereas corticosteroids and mineralocorticoids are adrenal steroids (Figure 1.2). The ovary, testis, adrenal cortex and placenta are the endocrine organs that specialize in steroid hormone production. The ovaries secrete estrogens and progestagens, the testis produces mainly androgens and the adrenal produces both corticosteroids (mineralo- and glucucorticoids) and sex steroid precursors. During pregnancy, the placenta produces progestagens and estrogens.

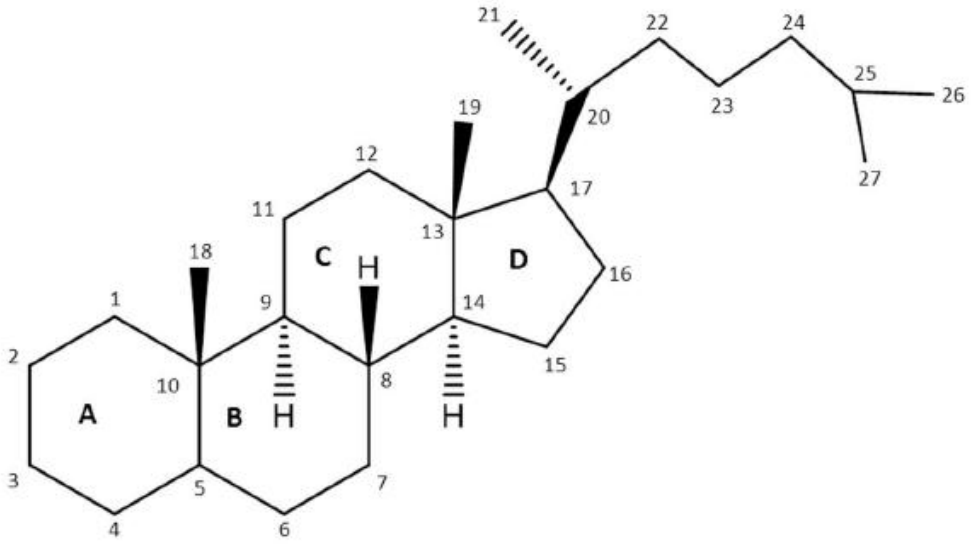


Figure 1.1 Cholesterol structure

The rings are identified with capital letters and carbon atoms are numbered. The bold and cross-hatched wedges represents the bonds extending above and below the plane, respectively.

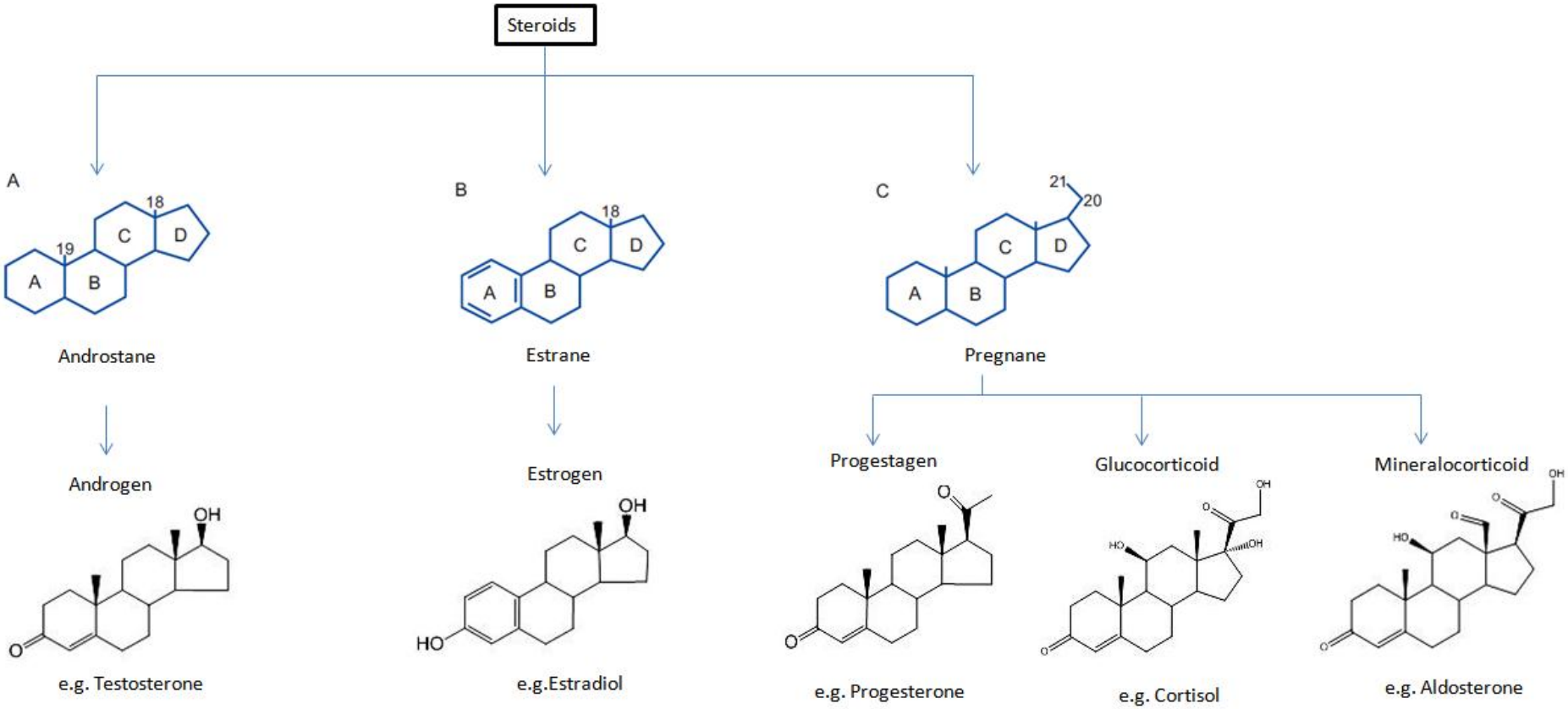


Figure 1.2 Classification of steroids according to basic backbone structure (androstane, estrane and pregnane) and example of steroid classes: sex steroids (androgen, estradiol and progesterone), corticosteroids and mineralocorticoid.

1.1.2 Physiological action

The production of steroids occurs in the mitochondria and smooth endoplasmic reticulum of cells in the adrenal cortex, the gonads or the placenta. Steroids are hydrophobic molecules that can penetrate biological membrane of lipophilic cells and are not stored in intracellular vesicles like peptide hormones. They are then transported rapidly in the bloodstream to reach targeted cells where the steroids bind to its specific receptor, which acts as transcriptional activators of steroid-responsive genes. Besides genomic response which may take between a few hours to days to manifest, steroids also regulate non-genomic responses (or 'rapid actions') that occurs within seconds to an hour (Norman et al. 2004). The pathway of steroid hormone biological responses is shown in the schematic diagram below (Figure 1.3).

In biological fluid, small proportion of steroids circulate in unbound form (2-3%) and the remaining are in conjugated form with a hydrophilic moiety such as glucuronide or sulfate or they are bound to 50-60 kd carrier protein glycoproteins, such as sex hormone binding globulin (SHBG), corticosteroid binding globulin (CBG) as well as albumin (Dunn et al. 1981). Albeit the steroid binding proteins circulate at low concentration compared to albumin, they have higher binding affinity to certain steroids. SHBG and CBG have high binding affinity towards sex steroids (testosterone and estradiol) and corticosteroids, respectively (Kicman 2010). The binding of steroids to the circulating binding proteins serves as a reservoir of the hormone, protects the steroids from rapid inactivation or urinary/biliary excretion and ensures ubiquitous distribution of the steroids (Kronenberg et al. 2011). According to the free hormone hypothesis, the free steroids are physiologically available to bind to their target cell, whereas the steroids bound to the carrier proteins are believed to have different and limited physiological activity (Mendel 1989). Contrary to this latter concept, recent studies have found that the cell membrane endocytic receptors, also known as the megalin receptor, expressed in the reproductive tissues mediates cellular uptake of circulating

protein-bound sex steroids**.** Once transported into the cells, lysosomes degrades the carrier protein bound and releases the steroids allowing it to bind to its receptor that further induces steroid responsive-genes (Hammes et al. 2005). SHBG bound androgens and estrogens are involved in the development and maturation of reproductive organs (Hammes et al. 2005, Kahn et al. 2008).

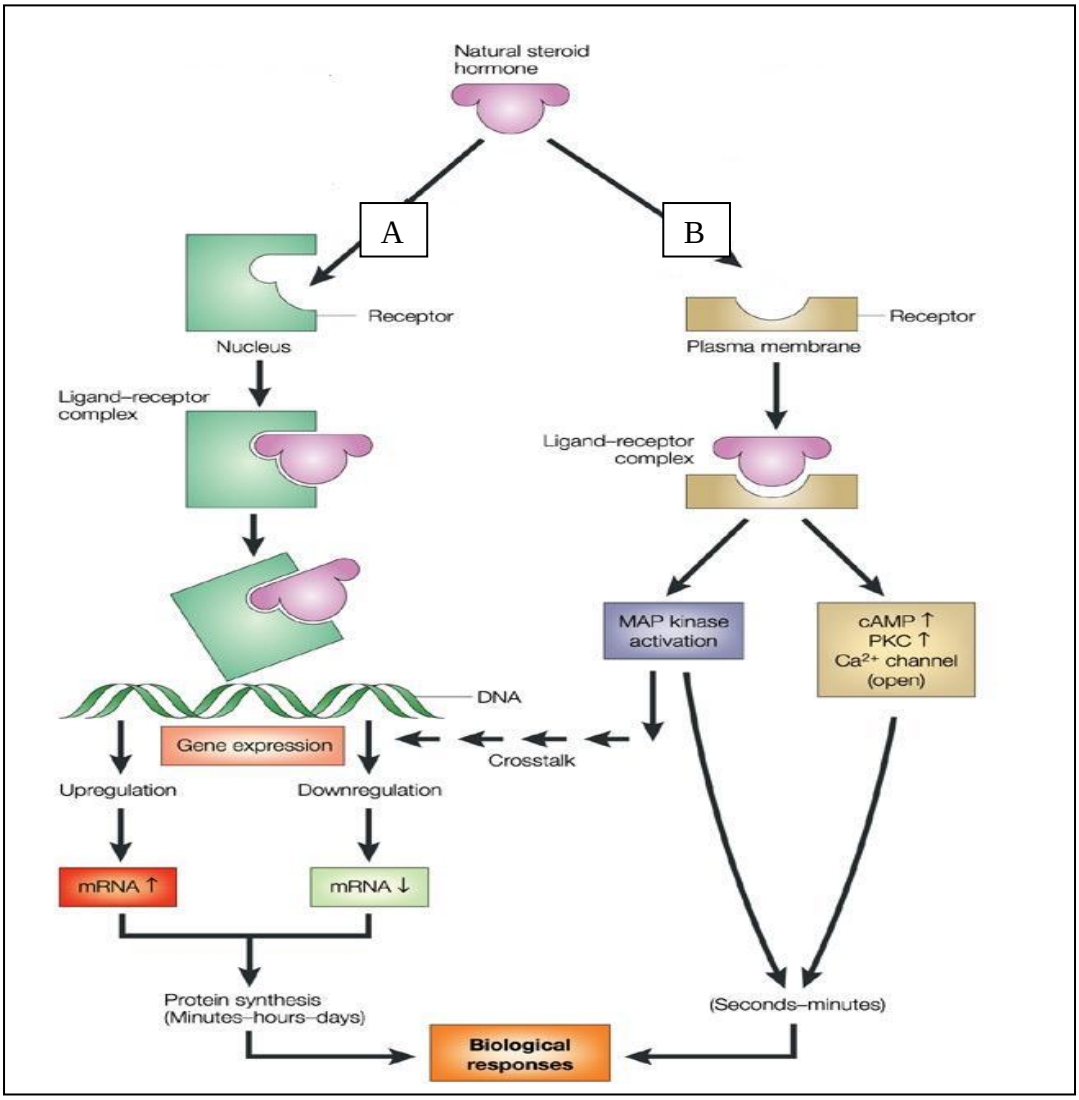


Figure 1.3 Genomic (A) and non-genomic (B) pathways of steroid hormones

A. In the genomic pathway, binding of steroids to the nuclear receptors (present either in the nucleus, in the cytoplasm complexed to chaperones or in between the cytoplasm and nucleus) leads to up- or down regulation of gene transcriptional responses regulating protein synthesis within minutes to days. **B.** Non-genomic biological responses of steroid involves occupancy of steroids to the plasma membrane of the cell, which in turn activates direct biological responses by intracellular signaling molecules such as mitogen activated protein (MAP) kinase, protein kinase A (PKA), cyclic adenosine monophophate (cAMP), and opening of calcium channels or indirect biological responses through genomic pathway [adapted from (Norman et al. 2004, Vogeser and Parhofer 2007)].

1.2 Steroid metabolism

1.2.1 Steroidogenesis (Biosynthesis of steroids)

The synthesis of steroids specifically takes place in the adrenal cortex (zona fasciculata, reticularis and glomerulosa), testicular Leydig cells, ovarian granulosa and theca cells and the placental syntiotrophoblast cells (Miller and Bose 2011). Cholesterol is a starting point for biosynthesis of all steroids hormone, bile acids and vitamin D. The three sources of cholesterol are from dietary low density lipoprotein (LDL)-uptake, stored cholesterol and *de novo* synthesis. LDL is taken up by the steroidogenic cell via specific receptor-mediated endocytosis. Within the cell, stored lipoprotein cholesterol esters are then converted by the lysosomal acid lipase into free cholesterol which is the substrate for steroidogenesis (Brown et al. 1978, Gwynne and Strauss 1982). Excessive cholesterol uptake by the cell is either transferred out as an external lipoprotein or stored within the cell cytoplasm as lipid droplets upon cholesterol esterification catalyzed by the acyl-coenzyme A:cholesterol acyltransferase (ACAT). As required, the stored lipid droplets are hydrolized by acid lipase to release free cholesterol (Miller and Bose 2011). Cholesterol is also synthesized *de novo* by the smooth endoplasmic reticulum of the steroidogenic cells catalyzed by at least 17 enzymes. The cellular cholesterol synthesis and lipoprotein uptake by the steroidogenic cells is stimulated by the tropic peptide hormones (Miller and Bose 2011).

The conversion of cholesterol to steroids is regulated by a series of enzyme reactions. The two main classes of enzymes involved in the steroids conversion pathway are the cytochrome P450 (CYPs) heme containing proteins and hydroxysteroid dehydrogenases (HSDs) (Miller and Auchus 2011, Payne and Hales 2004). The CYP enzymes includes type I or type II which is located in the mitochondria and endoplasmic reticulum, respectively. Whereas the HSD enzymes can be classified into aldo-keto reductase (AKR) and short-chain dehydrogenase/reductase (SDR) families (Miller and Auchus 2011). CYP enzymes mediate

hydroxylation, aromatization and carbon-carbon bond cleavage using molecular oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor which irreversibly oxidize steroids (Miller 2005). *In vitro*, the HSD reactions are reversible directing reactions to either oxidation of hydroxysteroids or reduction of ketosteroids depending on conditions, for example, pH, cofactor and substrate availability. However *in vivo*, HSD enzymes catalyzes unidirectional reactions and are classified as dehydrogenases or reductases where pyridine nucleotide acts as cofactors (Agarwal and Auchus 2005).

Steroidogenesis is first initiated from the conversion cholesterol (C27) to the first steroid pregnenolone (C21) via the action of CYP11A (cytochrome P450 side-chain cleavage enzyme: P450scc) (removes six carbon units) (Waterman and Simpson 1985) and steroidogenic acute regulatory protein (StAR) (Lin et al. 1995). CYP11A is expressed in all steroidogenic cells as a rate-limiting step at the start of steroidogenesis (Ishimura and Fujita 1997, Oonk et al. 1990, Payne and Youngblood 1995, Pelletier et al. 2001, Strauss et al. 1996). The StAR protein is responsible for transporting the hydrophobic cholesterol substrate from the outer to the inner mitochondrial membrane where this reaction takes place (Miller and Auchus 2011, Miller and Bose 2011). The CYP11A enzyme reaction introduces hydroxyl groups at C20 and C22 and cleaves the cholesterol side chain between these carbons (Payne and Hales 2004). After this rate limiting step, pregnenolone is transported out of the mitochondria into the endoplasmic reticulum where the remainder of steroidogenesis takes place. Pregnenolone further undergoes 17α-hydroxylation to produce 17α-hydroxypregnenolone in a reaction catalyzed by CYP17 (P450c17). In the adrenal zona reticularis, ovarian theca cells and Leydig cells, the CYP17 enzyme also catalyzes cleavage of C17 and C20 (lyase) bond to produce dehydroepiandrosterone (DHEA, C19 steroid) (Payne and Hales 2004). The 3β-HSD type 2 converts 5-ene-3β-hydoxylated steroids (Δ^5) to 4-ene-3-oxo steroids (Δ^4), resulting in conversion of DHEA to androstenedione in the gonad

and adrenal (Payne and Hales 2004). The 17β-HSD type 3 which is expressed exclusively in the testicular Leydig cells converts androstenedione to testosterone (Geissler et al. 1994). Whereas, the 17β-HSD type 5 which is the only form of 17β-HSD found the ovarian theca cells catalyzes the production of testosterone from androstenedione (Pelletier et al. 1999). Testosterone is converted to dihydrotestosterone (DHT) by the irreversible 5α-reductase type 2 enzyme reaction in the prostate and external genitalia (Thigpen et al. 1993). Additionally, DHT can also be generated through the "backdoor pathway" bypassing DHEA, androstenedione and testosterone, as intermediates. This pathway involves the conversion of 17-hydroxyprogesterone (17-OHP) to produce androstanediol which is an immediate precursor of DHT. The conversion involves 3α and 5α-reduction activity followed by sequential CYP17 and 17β-HSD type 3 enzyme activity. Finally, androstanediol can be back-converted to DHT by 3α-oxidation reaction (Auchus 2004). Figure 1.4 outlines the classic and backdoor pathways involved in DHT biosynthesis. In humans, DHT generated by both pathways plays indispensible role in male fetal sexual development but role of the backdoor pathway in mature individuals remains unclear (Flück et al. 2011, Greaves et al. 2014).

In the ovary, the androstenedione produced by the theca cells is later diffused into the granulosa cells, where the aromatase enzymes, CYP19 (P450arom) catalyses hydroxylations of androstenedione to produce estrone (C18 steroid with phenolic A ring). Estrone is later converted to estradiol by the 17β-HSD type 1 (Miller and Auchus 2011). The aromatase enzyme also converts testosterone to estradiol in brain, bone and adipose tissues but not liver (Perel and Killinger 1979, Sasano et al. 1997). In the adrenal zona fasciculata, the enzyme 3β-HSD converts 17-hydroxypregnenolone to 17-OHP (Payne and Hales 2004). Consequently, CYP11B1 (P450c11β1) and CYP21 (P450c21) enzymes converts 17-OHP to 11-deoxycortisol and cortisol. The biosynthesis pathway of androgens, estrogens,

corticosteroids (minerolocorticoid and glucocorticoid) and progestogen metabolites are outlined in Figure 1.5.

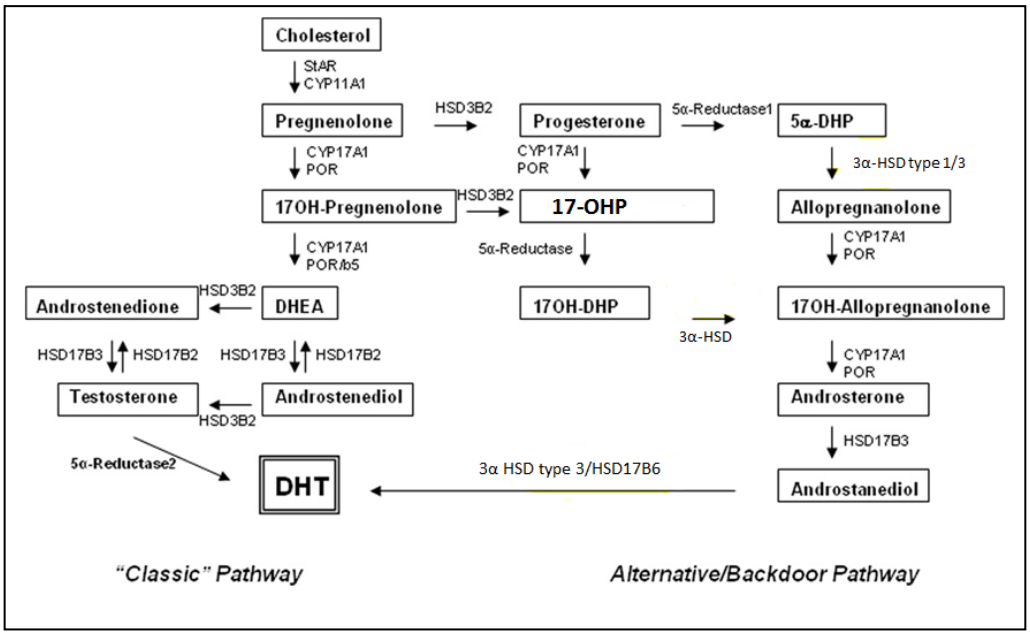


Figure 1.4 Classic and backdoor pathways of DHT synthesis.

The classic and backdoor pathways involved in DHT biosynthesis are shown on the left and right, respectively. Adapted from (Flück et al. 2011, Holčapek et al. 2010).

The classic pathway requires the following protein and enzymes: StAR (steroidogenic acute regulatory protein), CYP11A (cytochrome P450 side-chain cleavage enzyme, P450scc), CYP17A1 (17α-hydroxylase/17,20-lyase, P450c17), HSD3B2 (3β-hydroxysteroid dehydrogenase, type 2), HSD3B2 (3β-hydroxysteroid dehydrogenase, type 2), HSD17B3 (17β-hydroxysteroid dehydrogenase, type 3) and 5α reductase, type 2.
Additional enzymes involved in the backdoor pathway includes: 5a-reductase, type 1 (5a-reductase 1, 3a-reductase, type 3 and possibly 3a-reductase, type 1 and HSD17B6 (17β-hydroxysteroid dehydrogenase, type 6).

Steroid abbreviations: 17-OHP: 17-hydroxyprogesterone; 17OH-DHP: 17-hydroxy-dihydroprogesterone (or 5a-pregnane-17a-ol-3,20-dione); 17OH-allo: 17-hydroxy-allopregnanolone (or 5a-pregnan-3a,17a-diol-20-one); 5a-DHP: 5a-dihydroprogesterone (or 5a-pregnane-3,20-dione), and 3a-OH-DHP: allopregnanolone (or 3a-hydroxy-dihydroprogesterone or 5a-pregnane-3a-ol-20-one).

1.2.2 Phase I metabolism

Phase I metabolism involves converting active steroids into inactive metabolites that are substrates for phase II conjugation for eventual excretion in the urine and bile. The inactivation primarily occurs in the liver although there are also metabolic enzymes present in the steroid target tissues (Strauss 2014). The enzymes that are involved in the phase I steroid metabolism include 3α/3β-HSDs, 5β-reductase, 11β-HSDs, 17β-HSDs and 20α-HSDs that acts on C3, C5, C11, C17 and C20 positions of steroid, respectively (Rižner and Penning 2014). However, most of the target tissues also express HSD isoforms that are capable of converting the inactive steroids metabolites back into active form (Bélanger et al. 2003). Thus, these enzymes acts as 'switches' that tightly regulate the receptor occupancy by the steroids and balance the synthesis and metabolism of the steroids (Penning 2011). In the liver, 5α and 5β reductase converts circulating testosterone to form 5α-DHT and 5β-DHT, respectively. In the prostate and androgen target tissues, DHT is reduced by 3α-HSD, 3β-HSD and 17β-HSD forming androsterone, 5α androstan-3α-17β-diol (3α-diol), 5α androstan-3β-17β-diol (3β-diol), androstanedione and epiandrosterone (Bélanger et al. 2003, Dufort et al. 2001). Basically steroids with 3-keto- $\Delta^{4,5}$ structure may first be 5α-reduced and subsequently 3α-reduced to form stereoisomeric tetrahydrosteroids which are later conjugated in phase II metabolism and finally excreted in the urine (Jin and Penning 2001). However, the 17β-HSD type 6 can catalyze oxidation of 3α-diol back to form 5α-DHT in the prostate (Penning 2011). In the kidney, the 11β-HSD type 2 catalyzes the oxidation of potent glucucorticoid, cortisol into its inactive counterpart cortisone to enable the less abundant aldosterone to occupy the mineralocorticoid receptors (Strauss 2014). Conversely in the liver, adipose tissue and skeletal muscle, the inactive cortisone is reduced back to the inactive moiety cortisol by 11β-HSD type 1 (Strauss 2014). In breast tissues, 17β-HSD type 1 converts estrone to estradiol whereas 17β-HSD type 2 and type 4 inactivates estradiol by

catalyzing the reverse reaction. Progesterone is converted by 20α(3α-)-HSD to a weaker progestin metabolite, 20α-hydroxyprogesterone whereas 17β-HSD type 2 directs the reverse reaction (Rižner and Penning 2014). Figure 1.6 illustrates the HSD enzymes involved in the activation and inactivation of androgens, glucucorticoid, estrogens and progestins.

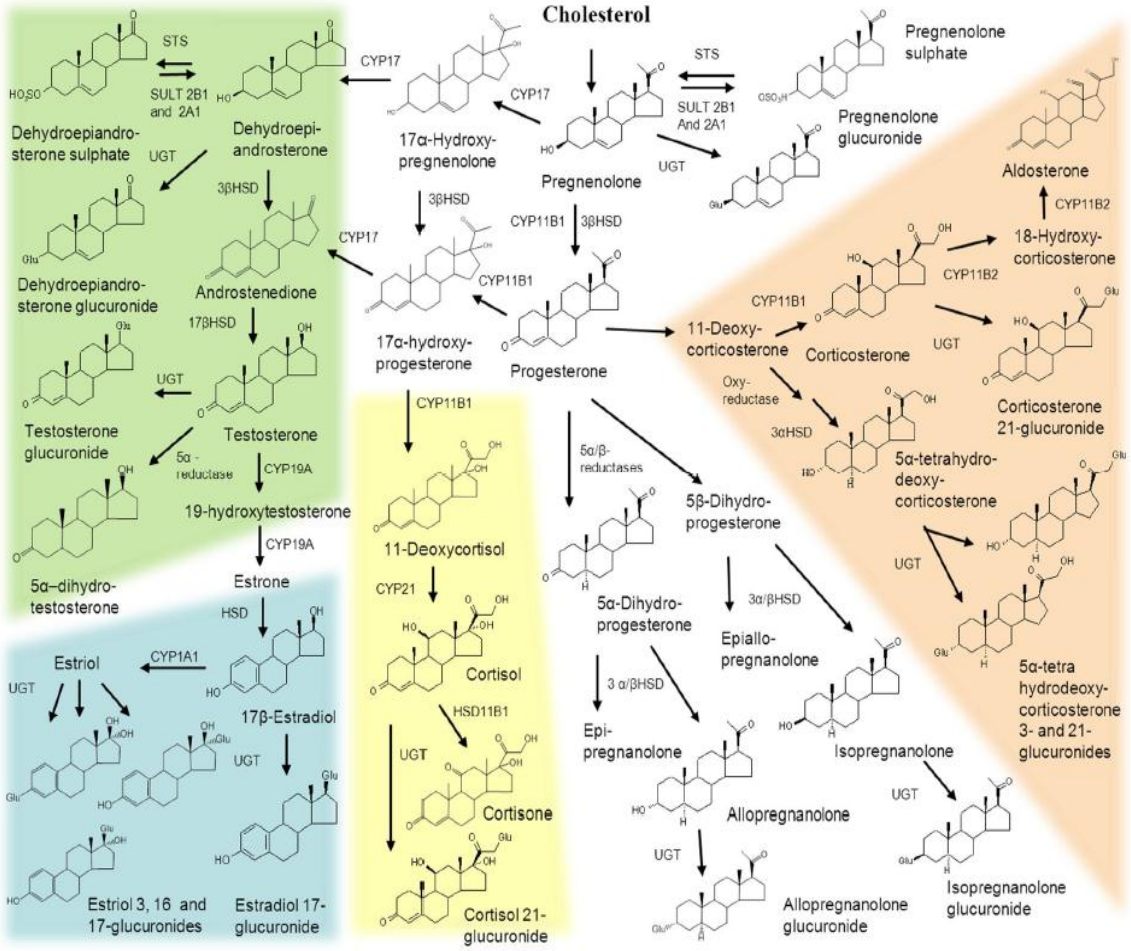


Figure 1.5 Biosynthetic pathway and steroidogenic enzymes involved in the pathway.

The pathway is divided into colour zones to depict the different classes of steroids; androgen (green), estrogen (blue), glucocorticoid (yellow), mineralocorticoid (orange) and progestogens metabolites (white) [adapted from (Jäntti et al. 2010)].

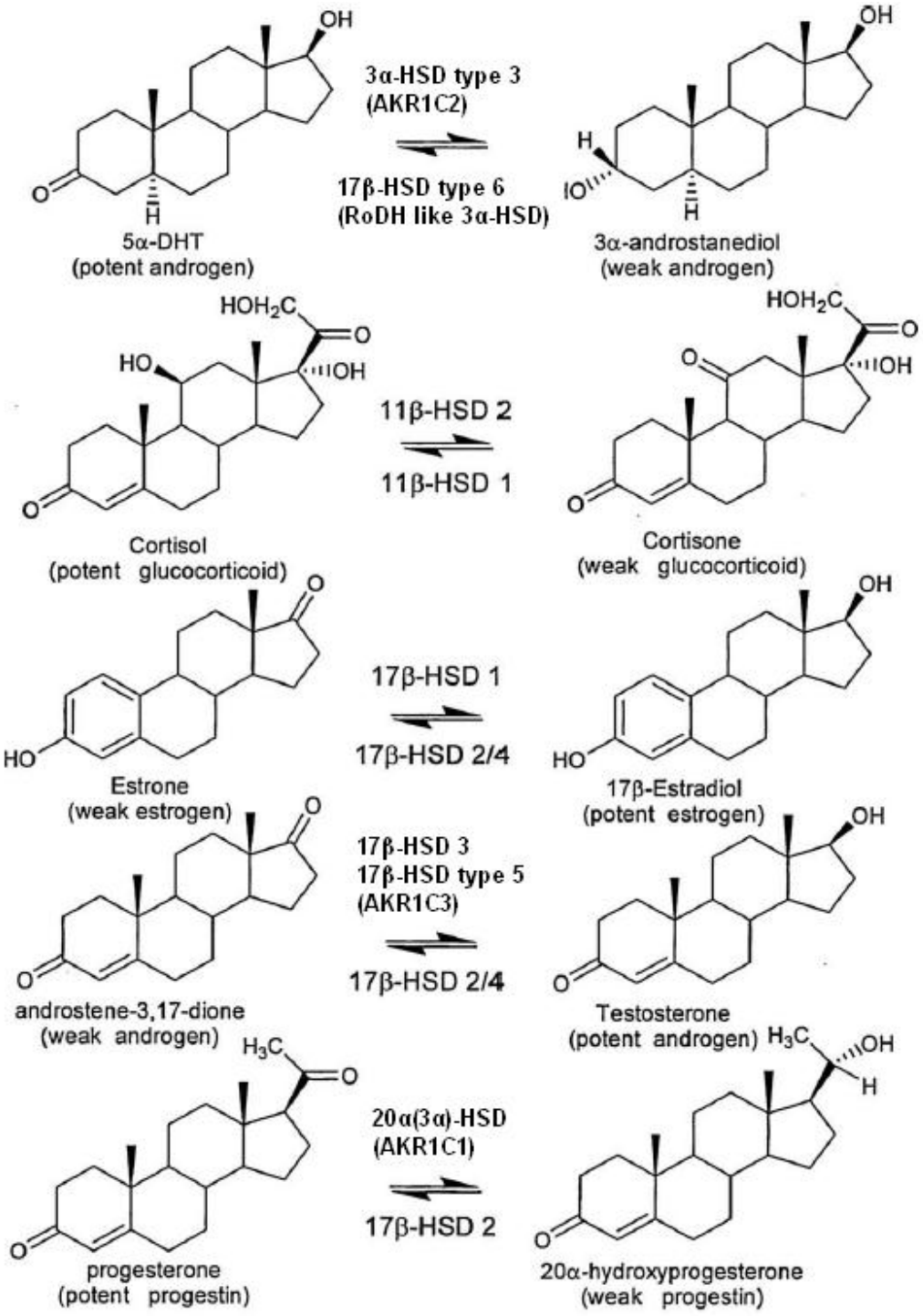


Figure 1.6 Androgens (5α-DHT and testosterone), glucocorticoid (cortisol), estradiol (estrogen) and progestin (progesterone) phase I metabolism by hydroxysteroid dehydrogenase enzyme (aldo-keto reductase; AKRs and short-chain dehydrogenase; SDRs).

The enzymes act in pairs to regulate the steroid occupancy towards the specific receptor at target tissues. The steroids are activated and deactivated by the specific enzymes to form potent steroids weak steroids, respectively which the latter is readily available for phase II metabolism. Adapted from (Penning 2011).

1.2.3 Phase II metabolism (conjugate formation)

Steroid hydroxyl metabolites from phase I metabolism further undergo conjugation reactions to form hydrophilic conjugates with lower affinity for cognate receptor and plasma proteins compared to their parent steroid (Andrew 2001, Tukey and Strassburg 2000). This metabolic pathway is catalyzed by enzymes which inactivate steroidal biological activity and increases the polarity of the steroids to aid their excretion (urine and bile). Phase II metabolism involves conjugation of steroids with glucuronides or sulfates which are the most abundant conjugated derivatives. Other cholesterol metabolite conjugates involved in phase II metabolism includes N-acetylglucosamine (GlcNAc), amino acids, glucose and galactose (Goto et al. 2005, Marschall et al. 1989, Vessey 1978, Wietholtz et al. 1991). Only small amount of steroids are excreted in unconjugated form in urine (e.g. less than 3% of urinary androgens secreted in free form) (Dehennin and Matsumoto 1993). Generally steroid conjugates are regarded inactive, however there are conjugated steroids that remain in circulation (e.g. DHEA sulfate) or stored locally in tissues (e.g. estrone sulfate) serving as a reservoir for back conversion to its active hormones or as steroid hormone precursor, reactions catalyzed by sulfatase enzyme (Andrew 2001, Hobkirk 1985, Reed et al. 1996, Zhu and Conney 1998).

Glucuronidation: This is a major route of steroid elimination. This reaction involves covalent linkage (conjugation) of glucuronic acid moiety (glycosyl group) derived from uridine diphosphate-glucuronic acid (UDPGA) co-substrate to lipophilic substrates that commonly contains hydroxyl, carboxyl or nitrogen group. This mechanism is catalyzed by an endoplasmic reticulum membrane enzyme, uridine diphosphate (UDP)-glucuronosyltransferases (UGTs) (Figure 1.7) (Court 2014, King et al. 2000, Tukey and Strassburg 2000). The co-substrate UDPGA is formed in the cytosol from the oxidation of UDP-glucose catalyzed by the UDP-glucose dehydrogenase (Zamek-Gliszczynski et al.

2006). The human genome encodes 19 UGT enzymes which are most abundant in the liver, although some are also expressed in other tissues such as the intestine, kidney, mammary gland and prostate (Gaganis et al. 2007, Mackenzie et al. 2005, Ohno and Nakajin 2009). The distribution of the enzyme varies in tissues according to age, gender, hormonal status, genetic factors and environmental exposure (Ritter 2000). Based on the homology of primary structure, UGTs can be divided into four families: UGT1, UGT2, UGT3 and UGT8 (Mackenzie et al. 1997). UGT1 and UGT2 are the two main enzyme families involved in steroid glucuronidation comprising of three subfamilies: UGT1A, UGT2A and UGT2B (Barbier and Bélanger 2003, Hum et al. 1999, Mackenzie et al. 1992). The UGT1 isoforms have specificity for bilirubin, amines and phenolic compounds as substrate, whereas the UGT2 targets the elimination of steroids, bile acids and opioids (You 2004). Metabolism of most estrogens and androgens are catalysed by subfamily UGT1A and UGT2B, respectively. All of the UGT1A isoforms members specificity (with exception to UGT1A6) are mainly directed towards C18 steroid substrates such as estradiol, estrone (UGT1A1; UGT1A10), 2-hydroxyestrone (UGT1A3) and 4-hydroxyestrone (UGT1A9) (Albert et al. 1999, Basu et al. 2004, Cheng et al. 1998, Mojarrabi et al. 1996, Strassburg et al. 1998). In humans, there are seven UGT2B isoforms isolated namely UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17 and UGT2B28 with broad overlapping substrate specificities (Girard et al. 2003, Riedy et al. 2000). All of the UGT2B isoforms are involved in the glucuronidation of steroids with exception to UGT2B10 and UGT2B11 (Turgeon et al. 2001). The UGTB4 catalyzes the conjugation of 3α-diol, estriol and hydroxyestrone (Lévesque et al. 1999). Whereas, UGT2B7 is involved in conjugation of different classes of steroids which includes the 5α-reduced metabolites of cortisol, progestins and androgens (Girard et al. 2003). UGT2B7, UGT2B15 and UGT2B17 enzymes catalyzes glucuronidation of major circulating C19 steroid metabolites at 17β-hydroxy position (e.g. DHT, testosterone and 3α-

diol). The UGT2B7 and UGT2B17 enzymes (but not UGT2B15) also catalyzes the C19 steroid metabolites at 3α hydroxy position (e.g. androsterone) (Beaulieu et al. 1996, Chen et al. 1993, Turgeon et al. 2001). The UGT2B28 type 1 catalyzes the conjugation of 3α-diol, estradiol, androstane and testosterone (Lévesque et al. 2001). Androsterone glucuronide circulates 20 times higher compared to 3α-diol glucuronide concentration in the circulation and both the glucuronide forms are indicators of androgen metabolism in peripheral tissues (Bélanger et al. 1986, Horton and Lobo 1986). Table 1.1 summarizes glucuronidation of various steroids by UGT conjugating enzymes.

Table 1.1 List of steroid substrates metabolized by UGT enzymes.

UGT isoform	Steroid substrate
1A1	Estradiol, estrone
1A3	2-hydroxyestrone, estrone
1A4	16α-OHP
1A7	Estriol, 2-hydroxyestradiol
1A8	2-methoxyestradiol, 4-hydroxyestrone, 16,17-epiestriol
1A9	4-hydroxyestrone, 11α-OHP
1A10	Estradiol, estrone, estriol, 2-hydroxyestradiol, 4-hydroxyestrone, androsterone
2B4	3α-diol, estriol, hydroxyestrone
2B7	Cortisol, progestins, DHT, testosterone, androsterone, 3α-diol, estradiol, estriol, 11α-OHP
2B15	DHT, testosterone, 3α-diol, 16α-OHP
2B17	DHT, testosterone, androsterone, 3α-diol, 16α-OHP
2B28	Estradiol, androsterone, testosterone, 3α-diol

Abbreviations: OHP: hydroxyprogesterone; 3α-diol: 5α androstan-3α-17β-diol; DHT: dihydrotestosterone
References are given in text.

Sulfation: Sulfate conjugation (also called sulphonation) is another important conjugation reaction that modulate the pharmacological activity of steroids. This reaction is catalysed by cytosolic sulphotransferases (SULTs) that attaches a sulfonate group (SO_3^-) from an enzyme cofactor, 3' phosphoadenosine-5'-phosphosulfate (PAPS) to an acceptor group (O-, N- or S-) (Figure 1.7) (Mulder et al. 1990). The formation of PAPS involves two reactions: first the conjugation of the cytosolic inorganic sulphate with adenosine monophosphate catalyzed by ATP sulfurylase to form adenosine 5'-phosphosulfate (APS) followed by the phosphorylation of APS by the APS kinase to form PAPS (Zamek-Gliszczynski et al. 2006). The sulfation and glucuronidation reaction have overlapping substrates, with the sulfation reaction occuring at low substrate concentration due to its high affinity, whereas at high substrate concentration, the activation of the glucuronidation reaction occurs as the SULT enzymes saturates or its cofactor PAPS are exhausted (Pang et al. 1994, Zamek-Gliszczynski et al. 2006). The SULT enzymes display unique tissue distribution with varying degree of activity. SULT enzymes for steroid metabolism are located in the cytosol, mainly in cells of the liver and gastrointestinal tract, although they are also found in the brain, kidney, lung, prostate and placenta (Coughtrie 2002). Four families of SULTs namely, SULT1, SULT2, SULT4 and SULT6 have been identified in human with at least 13 distinct members (Gamage et al. 2006, Li et al. 2008). The SULT1 members (or known as phenol SULT), are involved in sulfation of various estrogens such as estrone and 17β-estradiol (SULT1A1 and SULT1E1) (Falany 1997, Falany et al. 1994, Pasqualini 2009). Whereas SULT2 members (or known as hydroxysteroid SULT) are divided into SULT2A1, SULT2B1a and SULT2B1b. SULT2A1 also referred as DHEA sulfotransferase catalyses a broad range of steroids including the 3α, 3β, and 17β-hydroxyl groups as well as 3-phenolic hydroxyl estrogens (Falany 1997, Falany et al. 1994). SULT2B1a and SULT2B1b sulfonates pregnenolone, DHT and DHEA. However, SULT2B1b has higher cholesterol sulfation activity compared to

pregnenolone (Meloche and Falany 2001, Strauss 2014). Table 1.2 listed the sulfate isoforms involved in steroid metabolism.

Table 1.2 List of steroid substrates metabolized by sulfate isoforms.

SULT isoform	Steroid substrate
1A1	Estradiol, estrone, 2-hydroxyestradiol, 4-hydroxyestradiol, 4-hydroxyestrone
1E1	Estradiol, estrone, DHEA, pregnenolone and androstenediol
2A1	DHEA, testosterone, androsterone, epiandrosterone, androstenediol, pregnenolone, etiocholanolone, estrone and estradiol
2B1a	Pregnenolone, DHT, epiandrosterone, DHEA, androstenediol
2B1b	Pregnenolone, DHT and DHEA

Abbreviations: DHT: dihydrotestosterone; DHEA: dehydroepiandrosterone
References are given in text.

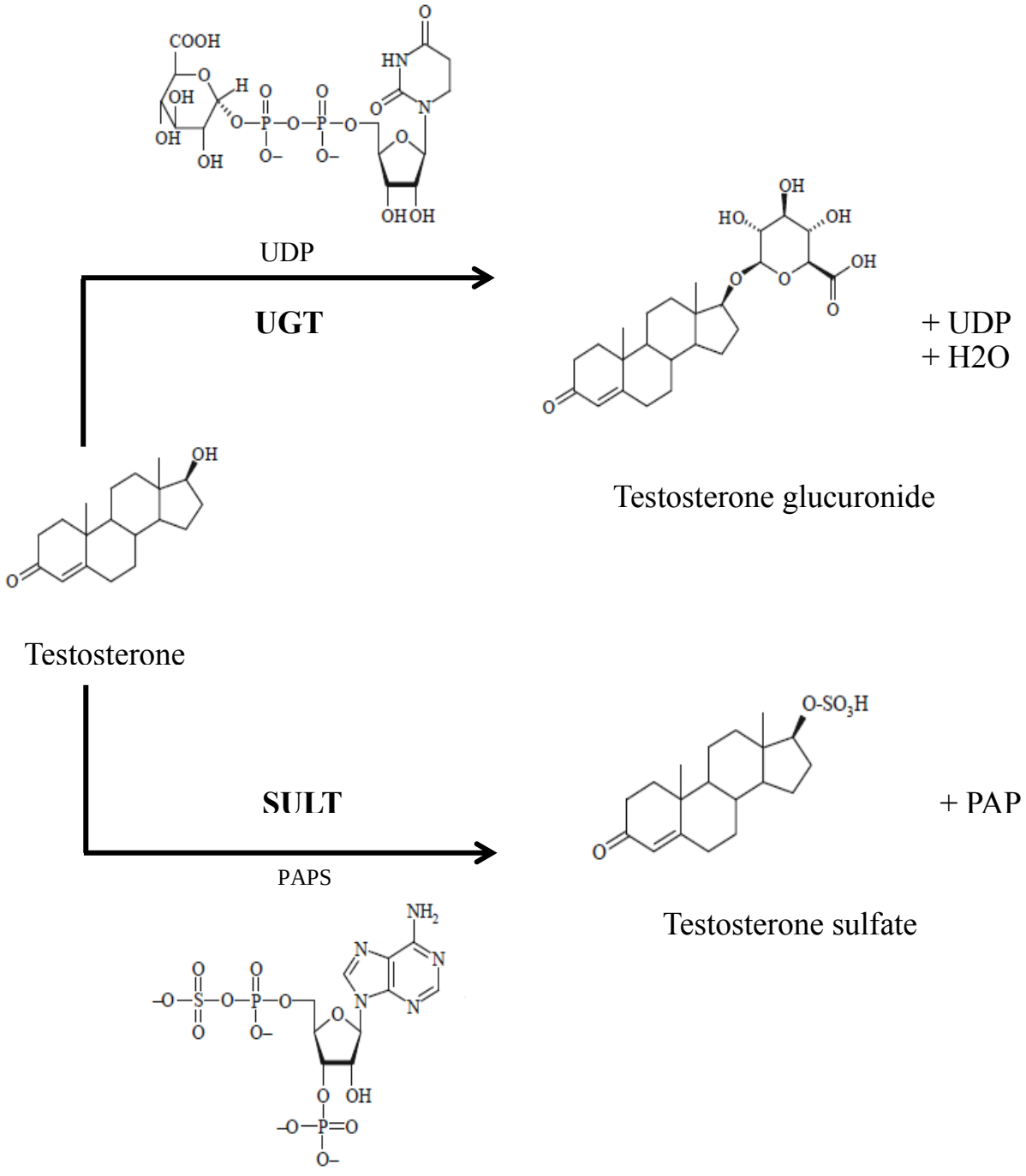


Figure 1.7 Phase II metabolism of testosterone.

Glucuronidation and sulfation reactions catalyzed by UDP-glucuronosyltransferases (UGTs) and sulphotransferases (SULTs).

1.3 Gonadotropin structure and regulation

The pituitary luteinizing hormone (LH), follicle-stimulating hormone (FSH) and placental human chorionic hormone (hCG) are gonadotropins that, together with thyroid stimulating hormone (TSH), comprise the glycoprotein hormone family (Pierce and Parsons 1981). The glycoprotein hormones are large proteins with a molecular mass of 30 – 40 kDa. They are heterodimers featuring a common α-subunit with a specific β-subunit that confers biological specificity by binding to their specific G protein-coupled receptors. Although the subunits (mainly α) exist in "free" form in the pituitary or circulation, they are biologically inactive. Thus, both subunits must be combined as α/β heterodimers and form a strong and specific noncovalent interaction complex that enables hormonal activation (Pierce and Parsons 1981). The α-subunit contains 92 amino acid residues whereas the LHβ, FSHβ and hCGβ have 121, 110 and 145 amino acids residues respectively (Stenman et al. 1997, Themmen and Huhtaniemi 2000). Each glycoprotein has specific oligosaccharide structure bound on the β-subunit that determines its physiological specificity and differentiates the members of the glycoprotein hormones (Robinson et al. 2007, Ulloa-Aguirre et al. 2001). However, more than 85% of the first 114 amino acids sequence of LH and hCG β-subunit are significantly similar which is potentially responsible for the similar biologic activity of their respective dimers (Garcia-Campayo et al. 1997). Figure 1.8 illustrates the specific location of the oligosaccharide glycosylation of the four glycoprotein hormones. The α-subunit consists of two N-linked oligosaccharides, positioned at asparagine (Asn)-52 and Asn-78. Study on the mutagenesis of hCGα oligosaccharides revealed that they have distinct functions; the Asn-52 is involved in the formation of intact glycoprotein dimer whereas the Asn-78 is important for the protein stability (Fares 2006, Matzuk and Boime 1988). Studies have also reported disruption in the glycoprotein secretion with the absence of the β-subunit N-linked oligosaccharides (Lash et al. 1992, Matzuk and Boime 1988). The carbohydrate portion is

also involved in the folding, assembly and clearance of the gonadotropin (Thotakura and Blithe 1995).

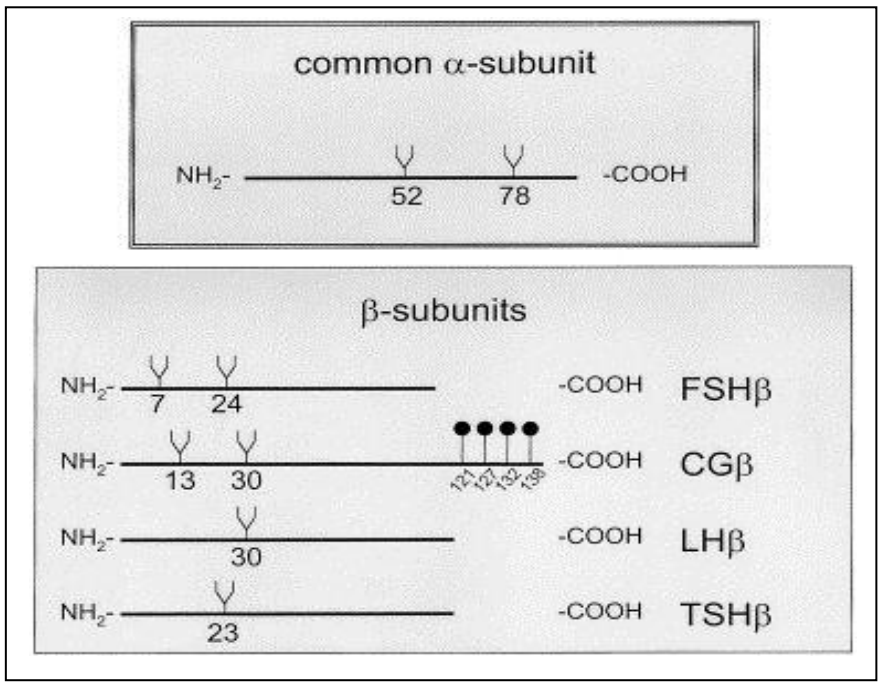


Figure 1.8 Location of glycosylation sites in the human glycoprotein hormone α and β-subunits.

The horizontal bar and the branched-like structures represent the amino acids and the N-linked oligosaccharides, respectively. The subunits contains one (LHβ and TSHβ) or two (the common α-subunit, FSHβ and hCGβ) N-glycosidically linked oligosaccharides attached to the amino acids residues (locations of the oligosaccharides attachment to the amino acids are indicated with the numbers). Additionally, the hCGβ-subunit consists of four O-glycosidically linked oligosaccharides in the carboxyl terminal extension (adapted from (Ulloa-Aguirre et al. 2001).

The hCG is distinct from the other glycoprotein due to the O-linked glycosylation to a carboxyl terminal peptide (CTP) extension of the hCGβ subunit (Figure 1.8) which prolong the half-life and enhance the *in vivo* bioactivity of the hormone (Fares 2006, Kalyan and Bahl 1983, Matzuk et al. 1990). Studies have shown insertion of CTP domain of the hCGβ subunit into other glycoproteins β-subunit (e.g. FSH and TSH) resulting in increase of the CTP-

modified hormones' half-life which proved to be beneficial for therapeutic purposes (Bouloux et al. 2001, Fares et al. 1992, Joshi et al. 1995). For *in vitro* fertilization, CTP-modified FSH is used to induce the development of ovarian follicle maturation with only a single dose due to its long half-life in contrast to multiple doses using recombinant human FSH analog (Balen et al. 2004).

The oligosaccharide contains side chains that terminate with sialic acid, galactose, fucose as well as sulfated N-acetylgalactosamine (only in LH). These complex carbohydrate side chains influences the glycoprotein hormones' bioactivity and clearance rates by the liver and kidney (Ulloa-Aguirre et al. 2001). The glycoprotein hormones with highly sialylated or more acidic oligosaccharides (e.g. FSH and hCG) have longer plasma half-life but reduced in potency and lower receptor binding activity *in vitro* compared to the galactose and sulfated or more basic oligosaccharides (e.g. LH) which are cleared rapidly from the plasma by specific hepatic receptors (Fiete and Baenziger 1997, Fiete et al. 1991, Ulloa-Aguirre and Timossi 2000). The glycosylation patterns of the glycoprotein hormones are variable depending on various physiological changes that occur according to age and sex (Ulloa-Aguirre et al. 2001). Studies have shown that the secretion of gonadotropin glycosylated variants with low sialic acid or sulfate content during the preovulatory phase of the menstrual cycle compared to the early, mid-follicular and luteal phases which are believed to be influenced by high estrogen secretion (Birken et al. 2007, Padmanabhan et al. 1988, Wide and Bakos 1993). In males and postmenopausal women, the gonadotropins circulate in more acidic/sialylated isoforms (Veldhuis et al. 1989, Wide 1985, Wide 1989). Other examples include the release of the less sialyted FSH isoforms which are more biologically active during the mid-puberty in boys compared to other stages of puberty (Campo et al. 2007). Due to the heterogeneity of the gonadotropin carbohydrate side-chains (microheterogeniety), many assays fail to either detect or differentiate between these isoforms. For example, throughout pregnancy the hCG

isoforms changes and a common hCG immunoassay employing monoclonal antibodies was unable to detect certain hCG isoforms produced during the early stages of pregnancy (Birken et al. 2001, Kovalevskaya et al. 1999).

The cross-linked disulfide bond existing between the subunits stabilizes the glycoprotein structure and is responsible in maintaining the three-dimensional structure to the subunits which is important in maintaining the biological activity of the dimeric structure (Ulloa-Aguirre and Timossi 2000). Figure 1.9 illustrates the known crystal structures of hCG and human FSH.

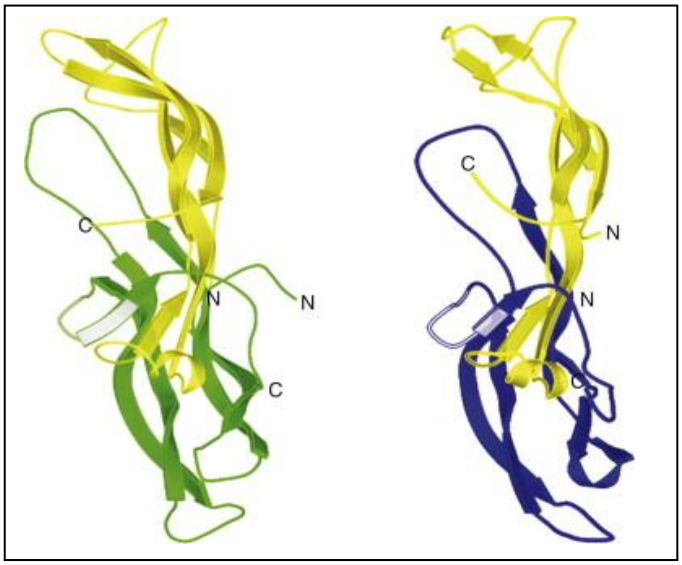


Figure 1.9 Crystal structure of human chorionic gonadotropin (hCG; left) and human follicle-stimulating hormone (hFSH; right).

The α-subunit (yellow) and the β-subunit (hCG in green; hFSH in blue) have similar folding forming highly elongated molecules. Both the subunits intertwined with a 20 amino acid residue region of the β-subunit known as seatbelt (white region) wraps around and latched a portion of the α-subunit. The two gonadotropins exhibit differences in the conformation at the C-terminal portions of the seatbelt. As for the LH and TSH, no structures are yet available. However, it is believed that the conformation of LH and TSH are closely related to the known structures. Adapted from (Ascoli and Narayan 2014)

The gonadotropin-releasing hormone (GnRH) (previously known as luteinizing releasing hormone), regulates the release of LH and FSH. GnRH is a decapeptide hormone synthesized by the cell body of the hypothalamic neurons that projects axon to the median

eminence of the brain. Regardless of the developmental stage, the secretion of GnRH from their terminals into the pituitary-portal blood supply is in a pulsatile or episodic manner. This pattern of release is absolutely critical as continuous release of GnRH causes desensitization of the GnRH receptors which further supresses the release of LH and FSH by the gonadotrope cells (Belchetz et al. 1978). Additionally the ratios of the LH and FSH released are also infuenced by the GnRH pulse frequency. High GnRH frequencies leads to higher LH secretion but lower FSH secretion, thus the LH/FSH ratio is high. However, the ratio decreases with the lowering of GnRH frequencies, due to increase in FSH and lower LH secretion (Wildt et al. 1981). Thus the GnRH pulse frequency and amplitude plays a pivotal role in the regulation of episodic release of LH and FSH into the general circulatory system. These gonadotropins binds to the glycoprotein hormone receptors to regulate hormonal and reproductive function of the gonads. The gonadotropin receptors are characterized by the large N-terminal extracellular domain that determines the recognition and binding affinity of their appropriate hormones (Braun et al. 1991). Both LH and hCG mediates their bioactivity through a single LH/CG receptor which is expressed in Leydig, theca, granulosa and luteal cells whereas FSH binds to its specific follicle stimulating hormone receptors expressed in the granulosa and Sertoli cells (Caltabiano et al. 2008, Pierce and Parsons 1981).

In males, the placental hCG plays an important role to stimulate fetal steroidogenesis and Leydig cell growth and differentiation after the initial gonadotropin-independent phase (Huhtaniemi et al. 1977). hCG also circulates at very low concentrations in men (produced by testes) and nonpregnant women which increases around menopause (produced by the ovaries) (Handelsman 2006, Stenman et al. 1997). Meanwhile, the circulating concentration of LH stimulates the activity of steroidogenic enzymes including CYP11A and CYP17α-hydroxylase in the testicular Leydig cells for the production of testosterone (Wu et al. 2007). LH is also responsible in maintaining high intratesticular testosterone levels required for

spermatogenesis. Testosterone binds to the androgen receptors (ARs) expressed in the somatic cell in the testes (Sertoli, Leydig and peritubular) but not germ cells to initiate spermatogenesis (Verhoeven et al. 2010). The FSH is also involved in the initiation and maintenance of spermatogenesis but is not essential to spermatogenesis (Singh et al. 1995). Studies on transgenic FSH mice revealed that both the FSH and testosterone binds to their specific receptors expressed in Sertoli cells in order to initiate and maintain spermatogenesis (Allan and Handelsman 2005).

In females, LH regulates ovarian steroid synthesis. LH stimulates the thecal cells and the luteinized granulosa cells of the corpus luteum to produce androgen and precursors as well as progesterone, respectively. The androgen precursors then move to the neighboring granulosa cells where they are aromatized into corresponding estrogens under the control of FSH (Themmen and Huhtaniemi 2000). LH is also involved in regulating cholesterol availability for steroidogenesis and stimulates the activity of CYP11A to catalyze formation of pregnenolone from cholesterol. LH stimulates the gene expression and production of enzymes involved in steroidogenesis (Kaiser 2011). Mid-cycle peak of LH terminates preovulatory follicle growth and triggers ovulation and stimulates the corpus luteum to synthesize progesterone in the second half of the ovulatory cycle (Themmen and Huhtaniemi 2000). In the ovary, granulosa cells express FSH receptors and are the only target cells of the FSH action. FSH is required for granulosa cell differentiation, modulates LH receptor expression in granulosa cells and regulates the estrogen production (Richards and Pangas 2010). FSH is also important for follicular recruitment, selection and growth (Zeleznik and Pohl 2006).

Taken together, the regulation of gonadotropins secretion is under positive control of the hypothalamic GnRH. The gonadotropins in turn regulate the gonadal secretion of androgen, estrogen and progesterone that exert their effects by binding to their specific

receptors. These are expressed in both reproductive and nonreproductive target tissues including the reproductive tract and genitalia, breast, fat, bone, muscle, liver and kidney. The sex steroid hormone receptors are also expressed in the central nervous system and pituitary gland to enable steroid feedback regulatory mechanisms by the hypothalamic-pituitary-gonadal (HPG) axis. The gonadal steroids and peptide (inhibin) exert negative and stimulatory (positive) effects on the gonadotropin regulation and release, either directly at the level of pituitary gonadotrophs by the modulating the genes encoding LHβ and FSHβ or indirectly at the hypothalamus level to modulate patterned GnRH secretion.

1.4 Steroid hormone production and action

1.4.1 Androgens

Androgens are produced by the gonads and androgen precursors by the adrenal glands in response to LH and adrenocorticotropin (ACTH), respectively, in both sexes. Testosterone and DHT are the two major and most potent androgens, both of which have a 17β-hydroxyl group and 3-oxo group (Figure 1.10). DHEA, dehydroepiandrosterone sulphate (DHEAS) and androstenedione are steroids with minimal androgenic activities but are pro-androgens (Mo et al. 2006). Testosterone is the most abundant androgen in mammalian males whereas the other weaker androgen precursors circulate at the same concentration in male and female. Testosterone is primarily produced by the testicular Leydig cells in men (approximately 95%), the thecal and stroma cells of the ovaries in women (25% through direct secretion and 25% through peripheral conversion of androgen precursors) and at a lesser amount in the adrenal zona reticularis and peripheral tissues (liver, skin and adipose) from conversion of androstenedione (Kicman 2008, Palacios 2007). Daily production of testosterone in eugonadal men is approximately 3-7 mg per day whereas only 0.1-0.3 mg is produced in women (Burger 2002, Kicman 2010). Approximately half of the circulating testosterone in women is produced peripherally by androstenedione conversion (Bardin and Lipsett 1967).

As described previously, circulating testosterone also acts as a pro-hormone at the target tissues when converted into DHT by 5α-reductase and into estradiol by aromatase (Figure 1.5) (Kicman 2008). Approximately 50% of the DHT production in men originates from this peripheral conversion and the remaining is secreted by the testes (Hammond et al. 1977, Saez et al. 1972). Although testosterone circulates at 10-fold higher concentration than DHT, the potency of DHT is approximately 3-10 fold greater than testosterone (Barbier and Bélanger 2008, Wright et al. 1996). Testosterone and DHT binds to the same AR under physiological conditions. However, DHT binds with a higher affinity than testosterone resulting in activation of target genes even at lower concentrations compared to testosterone (Kicman 2008, Wright et al. 1996). Despite binding to the same AR, testosterone and DHT have different physiological roles in males. During sexual differentiation, testosterone stimulates the urogenital sinus and Wolfian duct development that leads to formation of internal male genitalia (epididymis, seminal vesicle and vas deferens) (Themmen and Huhtaniemi 2000). Postnatally, testosterone maintains spermatogenesis, maturation of sexual characteristics, and plays a role in masculization and libido. During embryogenesis, DHT is responsible for development of urogenital sinus (the male external genitalia, urethra and prostate) (Marchetti and Barth 2013). During puberty, DHT is involved in the development of phallus and prostate, and the appearance of virilizing features such as body and facial hair. In adults, DHT is also associated with modulation of prostatic function, and is believed together with other androgens to be involved in the homeostasis between cell proliferation and cell apoptosis (Carson III and Rittmaster 2003, Isaacs 1984).

Androstenedione and DHEA are major androgen precursors. The adrenal glands and gonads produces large amount of androstenedione. DHEA and its sulfated analog (DHEAS) are primarily produced by the adrenal cortex and lesser amount by the gonadal tissues (Labrie et al. 1995). DHEA and DHEAS are also known as neurosteroids, whereby

the production of these steroids occurs locally by the neurons and glia of the brain (Lazaridis et al. 2011). These steroids are postulated to regulate brain function. For example, the DHEA acts on γ-aminobutyric acid type A (GABA) receptors as an agonist whereas DHEAS acts on the same receptor as an antagonist (Park-Chung et al. 1999). DHEAS circulates in the bloodstream at a higher concentration (250-500 times) compared to its unconjugated form and is the most abundant androgen in human circulating 20 times higher than other steroid hormone (Ebeling and Koivisto 1994, Kroboth et al. 1999, Labrie et al. 1997). The enzyme steroid sulfatase acts on DHEAS to produce DHEA and further converted into other androgenic and estrogenic compounds in reproductive (endometrium, ovary, prostate, testis) and non-reproductive tissues (bone, breast, skin and brain) (Reed et al. 2005). During gestation, the sulfatase enzyme is highly expressed in the placental syncytiotrophoblast regulating the production of estrogen in the placenta from the sulfated DHEA produced by the fetal adrenal (Salido et al. 1990).

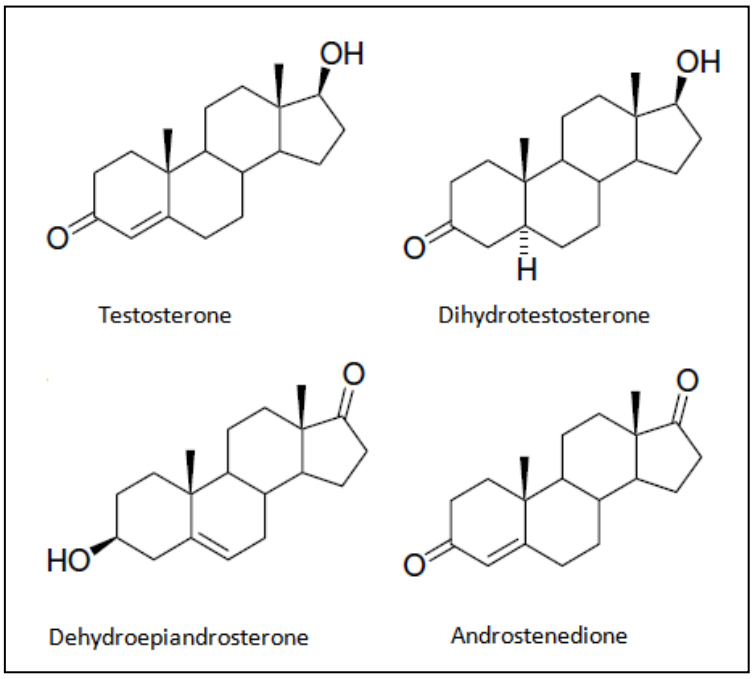


Figure 1.10 Chemical structure of four main androgenic steroids: testosterone, dihydrotestosterone, dehydroepiandrosterone and androstenedione.

1.4.2 Estrogens

The only naturally occurring estrogen is estradiol (or 17β estradiol) with one pro-estrogen (estrone) and phase I metabolite estriol (Figure 1.11). Typically estradiol and estrone circulate at higher concentration compared to estriol (Wiren 2007). Aromatase is expressed primarily in the ovarian granulosa cells (premenopausal women) and placental syncytiotrophoblast (pregnant women) to catalyze the synthesis of estrogen from androgens. Testosterone is aromatized into the potent estrogen, estradiol whereas the adrenal precursor androstenedione and DHEA are converted into the weaker pro-estrogen, estrone (Nawata et al. 1995). The aromatase enzyme is also expressed in the peripheral tissues (adipose and skin) which are the primary site for estrogen formation from C19 steroids precursor in both men and postmenopausal women (Simpson and Davis 2001, Simpson et al. 1994). The aromatase enzyme is also expressed in the muscle, brain, testicular Sertoli and Leydig cells and the osteoblast (Frieden et al. 1968, Longcope et al. 1978, Roselli et al. 1987, Saez et al. 1972, Sasano et al. 1997). Approximately 80% of the daily estradiol production in eugonadal men is produced peripherally from testosterone and the remainder is secreted directly by the testes (Baird et al. 1965). Estradiol has high affinity for estrogen receptors (ER) compared to the other pro-estrogens, hence known as the only potent bioactive estrogen (Jones 1992). In premenopausal women, the circulating level of estradiol is between 68 and 107 pg/mL across the menstrual cycle with higher levels during ovulation and is reduced in postmenopausal women to less than 5 pg/mL (Rothman et al. 2011). Although the levels of estradiol decrease dramatically in postmenopausal women, its metabolite estrone produced from the conversion of androstenedione in the adipose tissue remains unchanged (Ruggiero and Likis 2002). In men, the mean concentration of estradiol is about 20-30 pg/mL and its daily blood production rate is approximately 30-40 µg (Vermeulen et al. 2002).

Although throughout adulthood, men have plasma estradiol levels equivalent to postmenopausal women, estrogen action plays a pivotal role in the regulation of LH feedback. Recent studies have shown that testosterone and estradiol act independently on the LH regulation presumably via AR and ERα (Pitteloud et al. 2008). Studies have reported that estrogens act in the regulation of LH through the aromatization of testosterone at the pituitary level but testosterone does not require aromatization at the hypothalamic level to regulate LH secretion (Hayes et al. 2000, Pitteloud et al. 2008, Rochira et al. 2005). The role of estradiol on negative feedback regulation was demonstrated through studies conducted in normal or GnRH-deficient men where administration of aromatase inhibitor resulted in elevated of LH (Finkelstein et al. 1991, Marynick et al. 1979). In female, during the menstrual cycle, the concentration of estradiol regulates the negative or positive feedback action on gonadotropins. The serum estradiol levels gradually increase with the progression of follicular phase which resulted in inhibition of the gonadotropins secretion via the negative feedback. As the serum estradiol begins to rise rapidly during the late follicular phase, the positive feedback action leads to a preovulatory surge of LH and FSH. Thus estrogens play an important role in the gonadotropin regulation for ovulation (Ulloa-Aguirre and Timossi 2000). Estrogens are also involved in the bone maturation, mineralization, prevention of osteoporosis and maintenance of cardiovascular health in both men and women (De Ronde et al. 2003, Grumbach and Auchus 1999). During puberty, estrogens are responsible in the development of secondary sex characteristics in females which includes breast development by stimulating alveolar growth, subcutaneous adipose tissue distribution, typical female body proportion and estrogen dependent changes of the genital tract (external genitalia, uterus, vagina, fallopian tubes and ovaries) (Dösch et al. 2001).

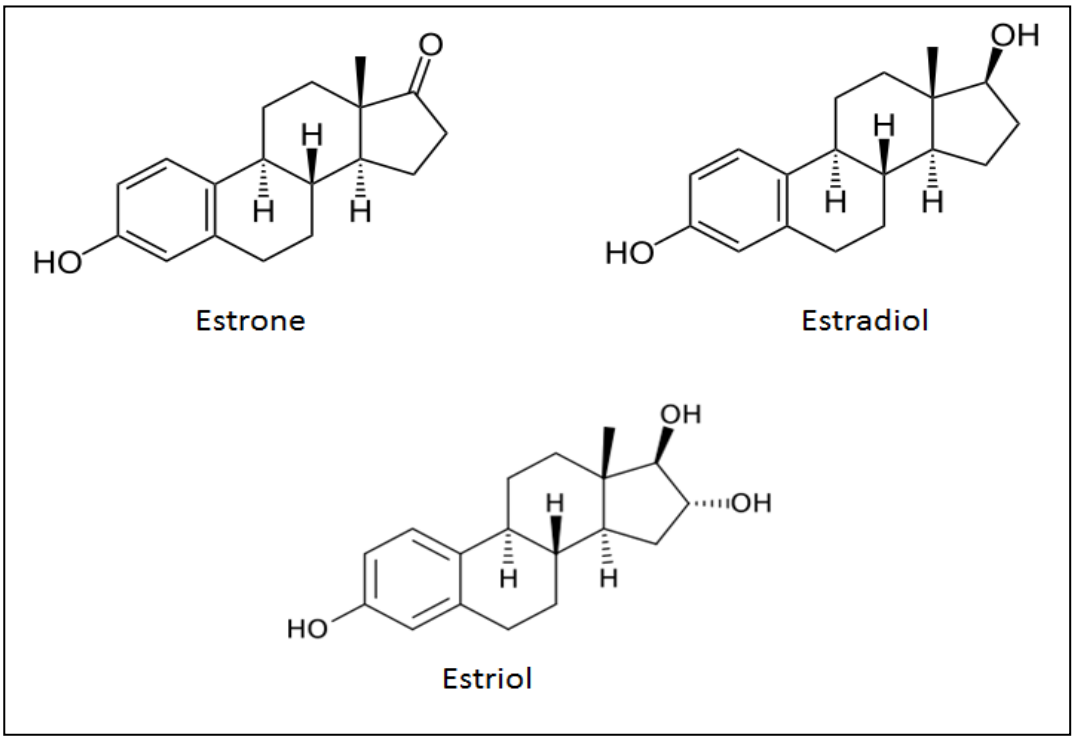


Figure 1.11 Structures of estrone, estradiol and estriol.

Estradiol is the most potent estradiol in non-pregnant women predominately produced by the granulosa cells from androgens. Estrone is produced via conversion of androstenedione in the adipose tissue. Estriol, a phase 1 metabolite of estradiol is produced peripherally and by the placenta during pregnancy.

1.5 Puberty

1.5.1 Hormonal changes in puberty

Puberty is a biological event that results in physical and reproductive maturity. This process is coordinated by a complex cascade of neuroendocrine changes which initiates secondary sexual characteristics, maturation of the genitalia, increase in growth velocity and ends with the acquisition of reproductive capability and attainment of adult body habitus and height (Plant and Barker-Gibb 2004, Styne and Grumbach 2011). The pubertal transition involves two independent but temporarily overlapping physiological processes namely, adrenarche and gonadarche.

Adrenarche refers to substantial increase of adrenal androgens, firstly DHEAS followed by DHEA and androstenedione, in response to ACTH (Styne and Grumbach 2011). The stimulus for this adrenal androgen steroidogenesis is unclear. The event of adrenarche only occurs in human and higher primates (chimpanzee, gorilla). Thus studies on its development in lower animals model are regarded as uninformative (Dorn and Biro 2011). The maturation of the adrenal cortex occurs from the age of about 6 to 7 years onwards in girls and 7 to 8 years onwards in boys (Ducharme et al. 1976, Korth-Schutz et al. 1976). The increase of adrenal androgens production occurs between 1 to 2 years before the changes of other pubertal hormones (Rogol et al. 2002) with higher levels reported in girls than in boys (Apter et al. 1979, Courant et al. 2010). The rise of adrenal androgens continues during gonadarche, with higher DHEAS levels in males compared to females after the age of 15 (de Peretti and Forest 1978). Adrenal androgen continues to increase on until the third decade of life and thereafter DHEAS levels gradually decline in both sexes (Ibáñez et al. 2000). Adrenarche is associated with the appearance of sexual hair (pubarche), axillary hair, adult apocrine odor and appearance of acne in some individuals (Rogol et al. 2002).

Gonadarche refer to the reactivation of the HPG axis at the end of the prepubertal phase resulting in dramatic surge in gonadal steroid production and initiation of folliculogenesis and ovulation in female and spermatogenesis in male (Witchel and Plant 2014). Gonadarche also leads to the final maturation of primary sex organs (ovaries and testis) and external signs of puberty. The stages of sexual development have been well defined by Tanner and Marshall as a predictable and ordered sequence of event (Marshall and Tanner 1969, Marshall and Tanner 1970). This clinical assessment of pubertal maturation known as Tanner staging remains the primary system used as the descriptive standards for assessing pubertal development. Other measures of puberty status includes Petersen Pubertal Development Scale (PDS) which is a self-report that focuses on physical changes in growth and development (Petersen et al. 1988). Morris and Udry (Morris and Udry 1980) also introduced self-report method along with line drawings of external physical changes. Other groups combined method of physical examinations for pubertal staging and pubertal hormone concentrations to define pubertal status. Tanner stages are divided into five anatomically defined stages which include pubic hair growth, development of breast in females and the development of phallus in male (Carel and Leger 2008). Figure 1.12 illustrates the Tanner stages of both girls and boys. Overall, girls usually enter and complete each stages of puberty before boys. The timing (onset) and tempo (rate) is affected by many factors including genetic factors although it even varies among individual from the same ethnicity and gender (Clark and Rogol 1996, Euling et al. 2008).

In girls, the appearance of breast buds (thelarche) underneath the areola is the initial sign of gonadarche (Tanner stage 2) which occurs between the age of 8 and 13 years indicating increased ovarian estrogen production (Jenner et al. 1972). As puberty progresses, the areola size, erectility and colour changes. Estrogen action is also demonstrated by the production of vaginal secretion and the enlargement of labia minora and majora. The adrenal

and ovarian androgen secretion leads to the development of pubic hair. Approximately 2.5 years after the appearance of breast buds, the first menstrual cycle (menarche) takes place (Marshall and Tanner 1969). The duration of pubertal development in girls and boys normally takes between 3-3.5 years to complete or in some cases occur earlier (within 2 years) or later (5 years) (Dorn et al. 2006).

In boys, the first sign of gonadarche is associated with the enlargement of the testes and a thinning and reddening of scrotum which occurs between the age of 9 and 13 years (Rogol 2002). The volume of testes increases from 1-2 mL (prepubertal) to 4 mL at the onset of puberty (Tanner stage 2) coincides with the increase of testicular androgen production (August et al. 1972). The testicular volume dramatically increases to about 10-fold by the end of pubertal development with the initiation of spermatogenesis and proliferation and differentiation of the Sertoli cells (Marshall 1975). Adrenal and testicular androgen secretion reflects the manifestation of pubic and axillary hair and development of acne (Greiner and Kerrigan 2006). As stated above, the two main hormones that mediate the biological manifestations of puberty are estradiol and testosterone, in girls and boys, respectively. However, most studies in the past have utilized assay methodologies that are considered inaccurate to measure low levels of these hormones in early and mid-puberty (Ankarberg-Lindgren and Norjavaara 2008, Moal et al. 2007, Taieb et al. 2003).

Adrenarche and gonadarche are two separate maturational events (Sklar et al. 1980). This is evident as the increase in adrenal androgens occurs when HPG axis is still inactive before the initiation of gonadarche. However, absence of adrenarche does not prevent gonadarche and vice versa. Girls with Turner's syndrome, despite the absence of functioning ovaries, have normal adrenarche (Saenger 1996) but does not result in reproductive maturation. This was also noted in patients with hypogonadotropic hypogonadism (Counts et

al. 1987). Additionally normal onset of gonadarche was noted in boys with primary adrenal insufficiency condition (Urban et al. 1980).

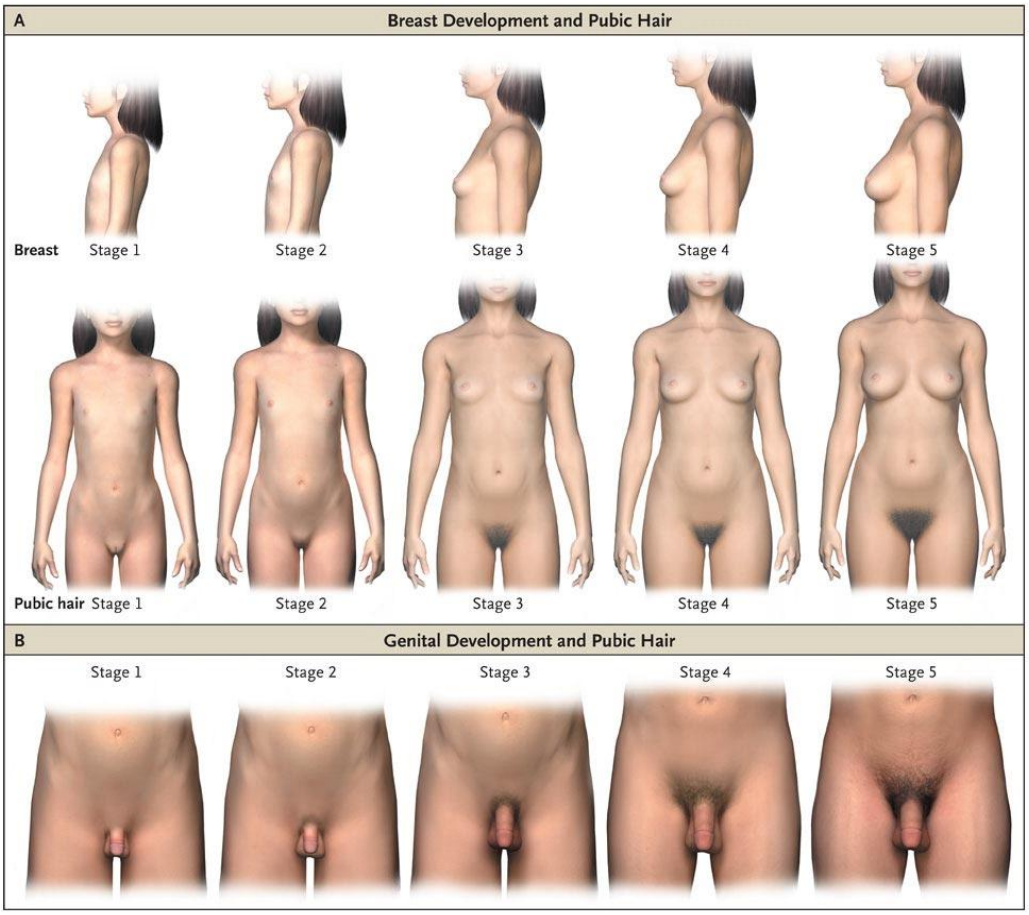


Figure 1.12 Pubertal rating according to Tanner stages.

Panel A demonstrates the development of breast and pubic hair in girls rated from stage 1 (preadolescent) to 5 (mature). The development of breast buds (stage 2) marks the onset of gonadarche. Adrenarche begins at stage 2.

Panel B illustrates the genital development and pubic hair development in boys rating from stage 1 (preadolescent) to 5 (adult). Stage 2 genital development marks the onset of gonadarche, which is characterized by enlargement of the testis and scrotum accompanied by changes in scrotal skin texture and color (reddening). The onset of pubic hair growth begins in stage 2.

Here the pubic hair and genital/breast development is illustrated simultaneously; however it should be scored separately as they do not necessarily take place at the same time. Adapted from (Carel and Leger 2008).

1.5.2 Activation of hypothalamus-pituitary-gonadal axis during development

In humans, the activation of the HPG axis first occurs *in utero*. The diffusely distributed network of hypothalamic neurons expressing gonadotropin releasing hormone-I (GnRH-I) gene known as GnRH pulse generator activates the release of fetal pituitary LH and FSH. The fetal pituitary contains detectable levels of gonadotropins at the 10^{th} week of gestation and the gonadotropins are detectable in fetal circulation by $12\text{-}14^{th}$ week of gestation in response to the decreasing placental estrogen (Seminara et al. 1998, Styne and Grumbach 2011, Witchel and Plant 2014). The fetal testes expresses LH/hCG receptors that activates the Leydig cell to synthesize testosterone important for male gonadal maturation *in utero* (Biro and Dorn 2005) whereas at this stage the ovary development of female fetuses remains quiescent (Witchel and Plant 2014). The fetal ovary apparently does not express gonadotropin receptors and appears to be independent of gonadotropins and gonadal function (Huhtaniemi et al. 1987, Themmen and Huhtaniemi 2000). The circulating gonadotropins concentrations are much higher in female compared to male fetus. It is postulated that the male fetus have early development of negative feedback by testosterone (Grumbach and Gluckman 1994). At the end of pregnancy, the fetal LH and FSH levels decline progressively and the GnRH pulses are inhibited with the increase of maternal and fetal estrogen levels and the development of negative feedback mechanism (Grumbach 2004, Nagata et al. 2006).

In male neonates, within a couple of minutes after birth, LH secretion surges approximately 10 times greater than the cord blood concentration resulting in secretion of testosterone which lasts for about 12 hours (Corbier et al. 1990, De Zegher et al. 1992). However, this LH surge is absent in female neonates (Grumbach 2005). After the first few days of birth, as the placental sex steroids inhibition is removed, the HPG axis activity commences. The circulating levels of LH and FSH rise intermittently to adult values or even occasionally higher at 2-3 months age and then declines to prepubertal levels between 6 to 9

months of age in boys and 2 to 3 years of age in girls (Andersson et al. 1998, Kuiri-Hänninen et al. 2011, Wright et al. 1996). This period is known as the neonatal surge or minipuberty. After this period of postnatal gonadotropin surge, the GnRH pulse generator becomes quiescent until its reactivation before onset of puberty. However, studies utilizing sensitive assays have shown that gonadotropins are released at low levels in pulsatile pattern primarily at night in prepubertal children with greater amplitude of FSH than LH pulses (Albertsson-Wikland et al. 1997, Apter et al. 1989, Wu et al. 1991). Low levels of gonadal steroids circulate during this childhood period (Rogol 2010).

Puberty represents the final step in the maturational process of the HPG axis. The onset of puberty is referred as (re)activation of the previously quiescent neuroendocrine reproductive axis which is reflected by increase in GnRH secretion (Ojeda and Skinner 2006, Plant and Skinner 2006). During this period, the reactivation of GnRH secretion transforms from low-level irregular pattern to a regular and pulsatile pattern which is detectable even prior to the exhibition of pubertal external signs (Harris and Levine 2003, Watanabe and Terasawa 1989). Marked increase in the amplitude and frequency of GnRH secretion enhanced the release of gonadotropin. At early progression of puberty, the pulses occur predominantly at night resulting in augmented LH secretion which in turn stimulates early morning release of gonadal steroids. Once again the gonadal steroid is able to inhibit GnRH secretion that cause decline in the gonadotropin and gonadal steroid levels throughout the day. As puberty progresses the gonadotropin pulses occurs both during the night and day as the negative feedback sensitivity of the hypothalamus decreases resulting in a more stable elevation of gonadal steroids hormones (Grumbach 2004, Grumbach 2005). At each pubertal stage, overnight LH values are approximately the same in both the genders but girls have higher FSH values (Manasco et al. 1997).

The specific neural and molecular mechanism that regulates the activation of the GnRH neuron during the initiation of puberty is not fully understood. However, previous studies have associated neurotransmitter and neuropeptide such as glutamate (Plant et al. 1989), γ-amino butyric acid (GABA) (Keen et al. 1999), neurokinin B (Topaloglu et al. 2008), leptin (Cheung et al. 2001), neuropeptide Y (El Majdoubi et al. 2000) and glial cell regulatory system (Grumbach 2004) with activation of GnRH neurons at puberty. In the last decade, neuropeptide kisspeptin has emerged as an important regulator of GnRH neurons in a number of mammalian species which led to the perception that kisspeptin signaling controls the timing of puberty (Terasawa et al. 2013). The involvement of kisspeptin in regulating reproductive axis was first reported by two independent groups in 2003. Their studies have shown that mutation or deletion of kisspeptin receptor, Gpr54 (G-protein receptor 54) recently renamed as Kiss1 receptor (Kiss1R) in humans and mice resulted in striking deficits in reproductive function, including failure to initiate puberty and infertility (de Roux et al. 2003, Seminara et al. 2003). In the following years, many studies have emerged unraveling the role of kisspeptin in regulation of puberty and fertility. Exogenous treatment of kisspeptin accelerates the age of puberty in prepubertal rats by early activation of gonadotropic axis (Matsui et al. 2004). Studies also demonstrated increase in LH and FSH levels in adult rodents and primates (including human) with administration of kisspeptin (Dhillo et al. 2005, Kinoshita et al. 2005). This suggests that the secretion of kisspeptin is necessary not only for puberty to occur, but required for the maintenance of reproductive function. The increase in kisspeptin expression in the hypothalamic region immediately before the onset of puberty further elucidates the role of this neuropeptide in the maturation of the reproductive axis (Navarro et al. 2004, Silveira et al. 2010). Kisspeptin plays a critical role in the control of reproductive axis at different stages of life including postnatal, pubertal development and

adulthood. Figure 1.13 illustrates a model demonstrating the role of kisspeptin signaling in controlling the timing of puberty in primates.

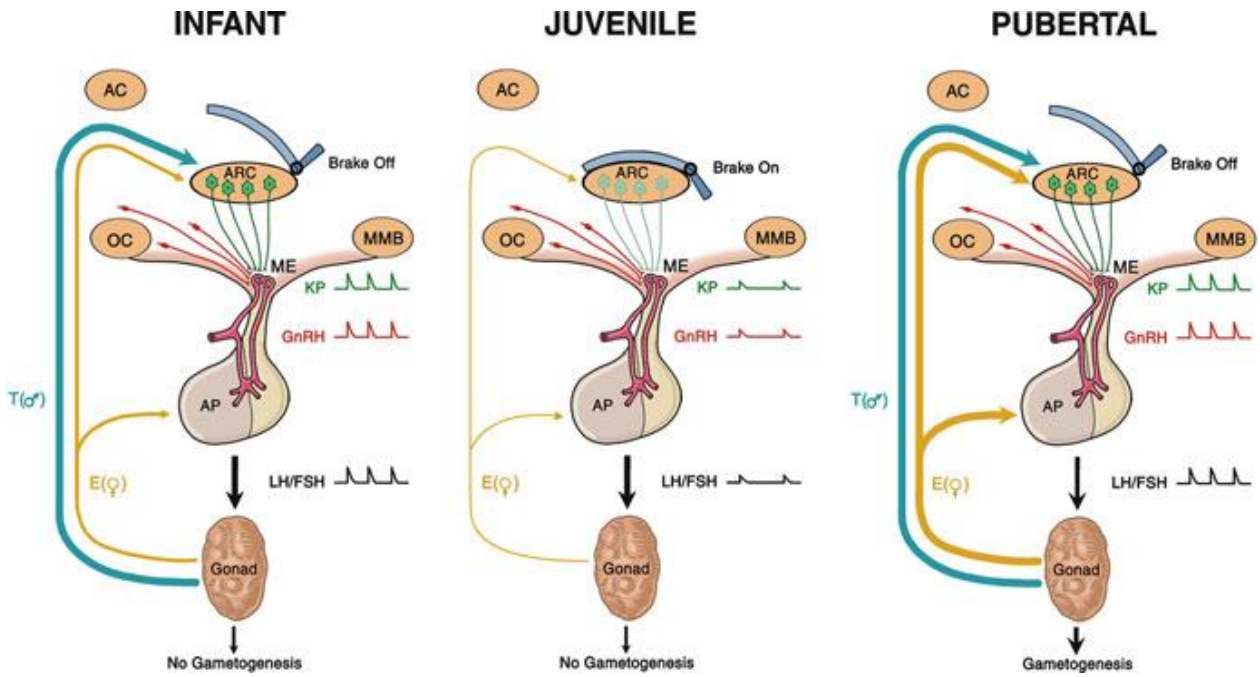


Figure 1.13 A proposed model for the control of the timing of puberty in primates.

During infancy (left panel), the robust activity of GnRH pulse generator leads to the intermittent release of kisspeptin in the ME, that causes corresponding pattern of GnRH release in the portal circulation. This further leads to the secretion of LH and FSH by the AP.

During the developmental progression from infancy to juvenile stage, a neurobiological brake results in inhibition of pulse generation by GnRH (reduce production of GnRH) and reduces the pulsatile release of kisspeptin into the ME (middle panel).

Puberty is initiated when this neurological brake is release which then triggers reactivation of GnRH pulse generator and release of kisspeptin in the ME (right panel). However, the nature of the neurobiological brake during infancy and its release at the end of juvenile developmental phase is not well understood.

The thickness of the arrows marked in blue (T, male) and gold (E_2, female) indicates the degree of negative feedback by the gonadal steroids produced by the gonads (testis and ovaries) on LH secretion at the different developmental stages. Adapted from (Hu et al. 2005, Terasawa et al. 2013)

Abbreviations: T: testosterone; E_2: estradiol: AC: anterior commissure; AP: anterior pituitary gland; ARC: arcuate nucleus; OC: optic chiasm; ME: median eminence; MMB: mamillary body.

More recent genetic studies of familial central precocious puberty have linked another possible gene known as makorin ring finger 3 (MKRN3) with initiation of puberty (Abreu et al. 2013, Settas et al. 2014). MKRN3 is a maternally imprinted gene that contains no introns and is located on chromosome 15q11.2 in Prader-Willi syndrome critical region. Mutations in MKRN3 gene has been associated with acceleration of puberty initiation leading to development of pubertal signs in both sexes which supports MKRN 3 role as a physiological inhibitor of hypothalamic activity during childhood (Abreu et al. 2013, Macedo et al. 2014, Schreiner et al. 2014). Studies have suggested that the MKRN 3 might function as a silencer of downstream genes that controls the activation of puberty such as kisspeptin (Hagen et al. 2015, Ojeda and Lomniczi 2014).

1.5.3 Growth and skeletal maturation during puberty

Puberty is accompanied by rapid sexually dimorphic changes in body composition, size and shape. Growth spurt is a major physical change that occurs during puberty which encompass 15-20% of the final adult height (Juul 2001). During childhood, growth is relatively stable primarily depending on the dietary intake, hormonal balance (growth and thyroid hormone), general health and adequate psychosocial environment (Rogol 2010). Generally, in the first year of life, a child grows 25 cm and 12 to 13 cm the following year. After this age, the child growth is stable about 5 to 6 cm until puberty (Rogol et al. 2002). The stable growth rate slows to a nadir (preadolescent dip) just before the onset of puberty and thereafter accelerates during mid-puberty. Although pubertal development in both genders takes about 4.5 years to complete, there is a marked difference in the timing of pubertal growth spurt between males and female. In girls, growth spurt occurs earlier compared to boys. However the growth spurt in girls does not reach the same magnitude of that in boys (Clark and Rogol 1996).

In girls, the growth spurt is commonly detected with the first sign of puberty (breast development and pubic hair) (Pinyerd and Zipf 2005). Peak height velocity occurs between

Tanner stage 2 and 3 (breast stage 2 [B2] and breast stage 3 [B3]) at about 11 to 12 years of age that averages about 9 cm annually with about 25 cm of total height gained (Marshall and Tanner 1969). However, peak height velocity varies between individual around this median pattern; 40% occurring at B2, 30% at B3, 20% at B4 and 10% at B1 (Coste et al. 2002). As the peak height velocity is about a year before menarche, the growth slows down to about 2.5 cm in height until completion of growth although it varies among individuals (Styne and Grumbach 2011). In males, peak growth velocity is observed at the Tanner genital stage 3 to 4 between the age of 13 and 14 years with an average of 10.3 cm per year, gaining about 28 cm of height during the pubertal growth period (Marshall and Tanner 1970). In boys, peak height velocity also varies between individuals with 60% occurring at genital stage 3 (G3), 28% at G4 and less than 8% at G2 and G5 (Coste et al. 2002). 95% of boys complete height velocity by Tanner genital stage 5 (Styne and Grumbach 2011). The pubertal growth rate decreases rapidly after the gender-specific peak in height velocity with increase of 1 cm per year or less in height after the age of 14.5 and 17 years in girls and boys, respectively (Veldhuis et al. 2005). In boys, the combination of higher peak velocity and for a longer duration of growth compared to girls results in an average height difference of 13 cm between men and women (Greiner and Kerrigan 2006). Peak height velocity is also greater in youths who mature earlier (Veldhuis et al. 2005).

Pubertal growth spurt is marked by the significant increase of growth hormone (GH), insulin-like growth factor l (IGF-1), estrogens and androgens. During childhood, the GH hormone secretion rates are stable and secretion pattern is similar in both genders with striking day-night rhythm (Martha et al. 1989). The secretion of GH is highest at early hours of sleep followed by small episodic bursts throughout the night/day (Finkelstein et al. 1972). The GH pulses are also released during the day, however at low levels (Miller et al. 1982).

During puberty, the activation of GH/IGF-1 axis and gonadal sex steroids not only demonstrates independent effects on growth, but the synergistic effect between them alters linear growth and body composition. Normal growth is attained with the presence of both GH and gonadal sex steroids. The growth spurt is impaired in conditions when either hormone is absent (Rogol et al. 2002). The increase in GH/IGF-1 axis during puberty is influenced by sex steroids. Elevated concentration of sex steroids especially estrogens stimulate 24-hours GH levels in a maximal pulse amplitude and increase in mass of GH released per burst which subsequently stimulate the hepatic IGF-1 production (Mauras 2001). Pulsatile secretion of GH increases between 1.5 and 3 fold which is accompanied by more than 3 fold serum IGF-1 concentration increase during puberty (Juul et al. 1994). The secretion of GH differs between genders that are parallel with the pattern of height velocity. In girls, the GH/IGF-1 levels rise significantly at Tanner B2 and peaks at Tanner B3-B4. Meanwhile in boys the increase of GH/IGF-1 occurs later during the pubertal development, peaking at Tanner G4 (Albertsson-Wikland et al. 1994). At the end of pubertal stage and adulthood, the levels of GH and IGF-1 reduces significantly even though the gonadal steroids levels are high (Martha et al. 1989).

The growth plate of bones undergoes functional and structural changes during growth. The growth plate contains chondrocytes and is divided into three zones; resting (containing immature cells close to the epiphyseal bone), proliferative (containing replicating and mature chondrocytes) and hypertropic (containing large chondrocytes). In both boys and girls, estrogens play an important role to modulate the secretion of GH and act directly on the growth plate maturation and fusion that leads to termination of longitudinal growth rates (Nilsson et al. 2005). During early puberty, low circulating levels of estradiol increases GH secretion followed by IGF-1 synthesis that stimulate chondrocyte growth in the proliferation zone of the growth plate and initiates pubertal growth spurt. As puberty progresses, augmentation of estradiol leads to apoptosis of hypertropic chondrocytes and stimulates

invasion of the growth plate by osteoblast. The completion of growth depends on estrogen action via ERα that induces exhaustion of chondrocytes proliferation that leads to fusion of the epiphyseal plate (Lazar and Phillip 2012, Weise et al. 2001). In conditions such as precocious puberty when premature exposure of estrogen occurs, skeletal growth accelerates leading to early epiphyseal fusion and decreased in final height (Carel et al. 2004). On the contrary, conditions featuring lack of estrogens (e.g. hypogonadism) causes delay in epiphyseal fusion and tall stature (Sedlmeyer and Palmert 2002).

Testosterone requires aromatization to estradiol in order to influence the GH/IGF-1 axis. This is evident in patients with aromatase deficiency where lack of estradiol lead to reduced epiphyseal closure, lack of pubertal growth spurt and the linear growth continues even after reaching adulthood (Morishima et al. 1995). Other studies have also shown administration of a non-aromatized androgen (DHT) in pre- and peripubertal boys does not increase GH secretion (Eakman et al. 1996, Veldhuis et al. 1997). Testosterone exerts its effect indirectly by enhancing the abundance and responsive of IGF-1 receptors in the growth plate chondrocytes (Lazar and Phillip 2012). Although non-aromatized androgens do not alter the GH/IGF-1 axis, they may have direct effects on bone growth independent of estrogens as AR expression is present within the growth plate cartilage (Nilsson et al. 2003).

1.5.4 Body composition during puberty

Dramatic changes in body composition occur *in utero*, across infancy, childhood and puberty. Endocrine factors play a key role in these dimorphic changes regulating the increase in bone density, fat free mass (lean mass) and the distribution and amount of adipose tissue. In early development, infant boys are heavier than girls due to greater lean mass whereas the fat mass are unchanged between the sexes (De Bruin et al. 1996). Thus proportionally newborn female have more subcutaneous fat than males with slightly higher mean skin-fold thickness (Rodriguez et al. 2005, Rodríguez et al. 2004). During prepubertal years, the differences in

body composition in both sexes are modest compared to postpubertal. Boys and girls weigh approximately 23 kg and 22 kg respectively by the age of 7 years (Veldhuis et al. 2005).

As puberty progresses, the body composition markedly changes. In girls, the total body fat increases steadily with the mean of approximately 5.5 kg at the age of 8 years to about 15 kg at 16 years. The increase beyond this stage is considerably slow. In boys, the total body fat increases from 5.5 kg to 11 kg between the age of 8 and 14 years and falls beyond this stage to approximately 9 kg at the age of 16 years and reaches a plateau (Siervogel et al. 2000). The fat free mass increases in girls by the age of 6 years and attain stability by the age of 15 to 16 years. Boys acquire fat free mass more quickly and for longer duration than girls during puberty with a steady increase between the age of 8 and 18 years (Siervogel et al. 2000, Veldhuis et al. 2005). The pattern of higher fat distribution and lower fat free mass in girls is not only hormonally driven but is also related to lower energy expenditure during puberty (Goran et al. 1998). Body mass index (BMI) calculated as weight (kg) divided with the square of stature (m^2) provides useful information to define over-weight and obesity (Siervogel et al. 2000).

1.6 Analytical measurements of LH and FSH.

The importance of gonadotropin measurement as biochemical indicator of endocrinological status and for therapy monitoring has led to development of various specific and sensitive assays. The measurement of LH and FSH can be divided into two assay systems. The first type of measurement is the immunoassays that quantitate the mass of immunoreactive hormone molecules of the sample. This assay includes the radioimmunoassay (RIA), immunofluorometric assay (IF) and immunochemiluminometric assay (ICL). The second type of assay measures the biological activity of the gonadotropin such as receptor binding assay and bioassays that quantitate functional aspects of gonadotropins. The bioactive to immunoactive ratio provides a useful index to assess qualitative changes in the gonadotropin for clinical diagnostics (Jaakkola et al. 1990).

RIA: The first immunoassay was developed by Yalow and Benson for peptide hormones measurements in the late 1950s, for which their work was awarded a Nobel Prize in Medicine to Yalow in 1972 (Yalow and Berson 1959). The development of RIA proved to be beneficial in studying gonadotropin regulation in health and disease conditions (Jaffe and Midgley Jr 1969). The RIA technique is based on competitive binding of unlabeled gonadotropin in biologic fluid and radiolabelled gonadotropin to a limited number of binding sites on a specific antibody. As the amount of unlabeled hormone increases, the binding of radioactive labeled hormone binding to the antibody decreases. Immunoradiometric assays (IRMA) on the other hand are a variation of RIA in which the antibody is labeled instead of antigen. For LH RIA, antisera from hCG were initially used based on the resemblance in structure and immunologic properties (Wide et al. 1961). However, the hCG antisera were replaced with the availability of highly purified human LH and its antibodies (Odell et al. 1967, Odell et al. 1966). Although this assay for gonadotropin measurement is known to be robust and is extensively employed for endocrine research, there are a number of limitations associated

with it. This includes considerable cross-reactivity of the free α-subunit of LH in some RIAs system (Dahl and Sarkissian 1993). RIA is also limited in sensitivity to measure low levels of gonadotropins. This hampers the assay application for measurement of low or suppressed gonadotropin levels for physiological or clinical assessment. Some IRMA and RIA were unable to measure LH pulses in prepubertal children (Clark et al. 1997). Studies have also shown discrepancy in the gonadotropin values measured by polyclonal antibodies in RIA compared to monoclonal antibodies immunoassays (enzyme linked immunoassay) possibly due to the differences in the antibody specificity (Olivares et al. 2000). Other disadvantages such as radiation hazard associated with, short half-life of the iodine tracer and costly gamma counter gradually lead to the replacement of RIA methods with new non-radioactive immunoassays such as IF and ICL (Munro et al. 1991).

IF and ICL assays: These immunometric assays make use of two antibodies forming a sandwich. The first antibody bound to solid phase is used to capture the antigen. Whereas the second antibody labeled to signal transducer, commonly fluorescence (IF assay) or luminescence (ICL assay) is used to measure the concentration of the analyte. No radioactivity is involved in these assays. The gonadotropin molecules present in the sample will be enclosed ("sandwiched") between the antibodies. The development of commercially available two-site-directed assays has overcome the limitation of gonadotropin RIAs associated with sensitivity and cross-reactivity of the α-subunit. IF assays are able to measure low serum LH and FSH during early puberty development that was difficult to obtain with the RIA assays (Apter et al. 1989).

The dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) is a commercially available IF assay that utilizes europium as a label and time-resolved fluorescents as the detection method. This solid-phase assay is based on two site sandwich technique with high affinity monoclonal antibody. The monoclonal capture antibody is

immobilized on the plate which is directed against the specific β subunit of the glycoprotein hormone. The europium labeled detector monoclonal antibody is directed against the common α subunit (Lövgren et al. 1984). The long fluorescent decay time of the europium label enhances the sensitivity of the assay (Hemmilä et al. 1984).

Immulite is another widely used automated immunoassay technology which utilizes the enzyme-amplified chemiluminescent technology. The solid phase of the system consists of a polystyrene bead sealed in the test unit and coated with a capture monoclonal antibody against LH or FSH. The gonadotropin standard or sample binds to the monoclonal antibody coating the bead. A detector polyclonal antibody which is conjugated to alkaline phosphatase enzyme binds to the gonadotropin bound to the monoclonal antibody forming a sandwich. Finally, the chemiluminescent substrate added to the complex undergoes hydrolysis through the action of the alkaline phosphatase enzyme creating unstable anion that emits sustained light which is then measured using a luminometer (Reimers et al. 1996).

FSH Bioassay: The Steelman-Pohley was the earlier *in vivo* assay to assess FSH. For this assay, immature female rats are pretreated with hCG to increase sensitivity to exogenous FSH. This is detected by measuring its response to exogenous FSH which results in a linear dose-response increase in mean ovarian weight (Steelman and Pohley 1953). The problems associated with this ovarian augmentation assay includes that it is too susceptible to serum interference, lacks sensitivity and is cumbersome for routine clinical studies (Wang 1988).

The granulosa cell aromatase bioassay (GAB) and Sertoli cell aromatase assay are *in vitro* FSH bioassays that provide sufficient sensitivity for measurement of serum FSH. This non-species-specific assay is based on stimulation of estrogens production by the rat ovarian granulosa cell primary culture to measure biologically active FSH. Serum samples are pretreated with polyethylene glycol to remove inhibitory substances prior to assay (Dahl et al.

1988, Jia and Hsueh 1986). Meanwhile, the Sertoli cell bioassay is based on measurement of estradiol production from exogenous testosterone in the presence of FSH (Van Damme et al. 1979).

LH/hCG Bioassays: The mouse interstitial cell testosterone assay (MICT) or the rat interstitial cell testosterone assay (RICT) are *in vitro* bioassays commonly used by laboratories for LH and hCG bioactivity measurements. These assays are based on the stimulation of testosterone from dispersed Leydig cells and the assays measure all LH/hCG like bioactivity but do not cross-react with the biologically inactive free subunits of the gonadotropins (Dufau et al. 1976).

Receptor-binding assays: These assays are competitive protein binding assays similar to RIA but using a binding protein other than antibody, typically a receptor that utilizes radioligand gonadotropin (^{125}I-hCG/^{125}I-LH or ^{125}I-FSH) as a ligand and crude rat homogenate from rat testes or corpora lutea which is rich in LH/CG and FSH receptors, respectively (Catt et al. 1972, Leidenberger and Reichert Jr 1972, Sanzo and Reichert 1982).

1.7 Analytical techniques of steroid measurement

Steroid hormone analysis plays an indispensable role for the diagnosis of endocrinological disorder or gonadal function in clinical laboratories. Steroid hormones and their metabolites can be measured in various biological fluids such as plasma, serum, urine, amniotic fluid, saliva and tissue extracts (Table 1.3). Generally, binding assays or chromatographic methods are used for steroid quantitation. Quantitation of steroids using gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are superior to immunoassays with regards to specificity and sensitivity (Krone et al. 2010). Rapid growth in the mass spectrometry (MS) technology has enabled routine analyses of steroids in clinical and research laboratories (Stanczyk and Clarke 2010). Table 1.4 shows comparison studies between immunoassays and GC/LC-MS for testosterone and estradiol measurements.

1.7.1 Immunoassay

RIA to measure steroid was first developed in 1969 for estradiol analysis in serum/plasma (Abraham 1969). The 'indirect' RIA method involves extraction of steroid with organic solvent (to remove conjugated steroids and denature the SHBG or CBG protein bound to the steroids) and Celite or Sephadex column chromatography separation (to remove possible interfering structurally related precursor or metabolites) prior to RIA quantitation that increases the specificity of the assay (Abraham et al. 1972, Giton et al. 2009). Since steroid molecules are too small to be antigenic, they are covalently attached to carrier protein such as bovine serum albumin forming hapten (Abraham 1974). The specificity of the assay antibody increases when the binding of protein to the steroid is distant from the functional groups (Honour 2010).

The antibodies used for RIAs are available commercially for many but not all steroids. Tritiated steroid were used in earlier RIA methods which was then replaced with

iodinated derivatives that increased the assay sensitivity (Stanczyk 2004). The assay involves displacement of radioactively labelled steroid from the antibody binding site by steroid in the assay sample. Then the antibody-bound and the unbound steroid are separated either by activated charcoal where the unbound steroids is adsorbed (Wu and Lundy 1971) or by precipitation of the antibody-bound steroid complex forming a pellet upon centrifugation (Honour 2010). The liquid scintillant fluid is then added to the radioactive sample (only for the tritiated label) that produces light which is then measured using a photomultiplier (beta counter).

The RIA has high throughput of samples. Pre-assay purification method removes interfering metabolites so as to provide accurate and reliable steroid measurements. However, RIAs are cumbersome, requires prolonged incubation and time-consuming purification steps. Large sample volume is also required especially when quantifying samples with low steroid concentrations (Stanczyk and Clarke 2010). Due to all these disadvantages, direct (non-extracted) immunoassay such as IF, ICL or enzymatic assays were developed. These assays do not require radioactive ligands and steroid purification prior to steroid analysis. Additionally the direct immunoassays are simple, rapid and more affordable. However, steroid immunoassays generally lack in specificity because the antibodies cross-react with steroids having or sharing structural similarities, especially when present at higher concentration than the steroid of interest. For example in women and children, high DHEAS concentration may cross-react with testosterone measurements and in such condition there will be an overestimation of testosterone steroid concentrations (Heald et al. 2006, Moal et al. 2007). Cross-reactivity also occurs due to conjugated metabolites of the steroid of interest resulting in erroneous steroid measurement (Yarrow et al. 2013). Other inherent problem of using immunoassays includes inaccuracy and limited sensitivity to measure steroids at low concentrations. These immunoassays are unsuitable for steroids quantitation below the assay

validated range for example quantitation of estradiol in children, postmenopausal women and men (Stanczyk et al. 2010, Taieb et al. 2002) and testosterone measurement in children and women (Moal et al. 2007, Rothman et al. 2011, Taieb et al. 2003). Assay comparison studies demonstrated large inter-assay variability for testosterone and estradiol measurements (Table 1.4) (Handelsman et al. 2014, Sikaris et al. 2005).

The presence of heterophilic and auto-antibodies that form complexes with the reagent antibodies may also interfere with the immunoassays giving false negative or positive measurements (Boscato and Stuart 1988, Boscato and Stuart 1986). Additionally, direct immunoassays utilizing samples without extraction, chromatographic separation or authentic tracers (H^3, C^{14}) are susceptible to matrix and SHBG interference (Handelsman et al. 2014, Sikaris et al. 2005, Taieb et al. 2003). Removing the pre-immunoassay purification step was reported to falsely underestimate or overestimate analyte measurements (Marks 2002). Immunoassay performance was improved with extraction method prior to assay for samples with low estradiol in children (Ankarberg-Lindgren and Norjavaara 2008). However, studies have also reported positive bias in assays involving extraction and chromatographic separation methodologies compared to reference methods (Cawood et al. 2005, Wang et al. 2004). Furthermore, the presence of endogenous compounds such hydrophobic and amphiphatic lipids in serum samples has been reported to interfere with the RIA assays by interacting with the steroids or the antigen-antibody reaction (Rash et al. 1980).

1.7.2 Gas chromatography- mass spectrometry

The first gas chromatography (GC) to measure steroids was developed in 1960 which preceded the use of immunoassay in endocrine studies (Sweeley and Horning 1960, Vanden Heuvel et al. 1960). The coupling of GC and MS to measure sterols was first carried out in 1964 (Eneroth et al. 1964) followed by the GC-MS steroids analysis in the late 1960s in biological matrices (Sjövall and Vihko 1968).

GC-MS is a robust and widely used method for detection and analysis of major steroids and their metabolites in complex matrices such as urine (Weykamp et al. 1989). The GC consists of flexible silica-based column (approximately 30 m in length) that utilizes large gas volumes (normally helium) with high pressure as mobile phase and for chromatographic separation. Samples injected into the column are vaporized upon reaching the analyte boiling point which is about 300°C for steroids. The volatile analytes then move into the vacuum detector such as flame ionization detector (FID), electron-capture detector (ECD) or the mass spectrometer (MS). The MS detector is preferred for steroid analysis as it provides highly sensitive and selective detection.

The GC-MS is capable of providing baseline separation of steroids with structural similarities or α/β stereoisomers (Krone et al. 2010). However, prior to GC-MS analysis, non-volatile and thermolabile analytes have to be chemically derivatized, for example, using methyloxime-trimethylsilyl ether (MO-TMS) to enhance volatility and to withstand the GC high temperature during vaporization. Derivatization with suitable reagents increases the ionization efficiency by changing the steroid physical and chemical property by forming covalent linkage to the specific functional group of the analyte. Common concerns with derivatization includes incomplete derivatization, nonspecific derivatization and production of multiple products (Liberato et al. 1987). Steroids in conjugated form have to be enzymatically or chemically hydrolyzed before analysis as steroid conjugates have low volatility and degrade at high temperature (Caulfield et al. 2002). There are two types of enzyme solutions normally used for steroid deconjugation which includes *Escherichia coli* and *Helix pomatia*. β-glucuronidase extracted from *E.coli* deconjugates the glucuronide moiety from steroids whereas the β-glucuronidase/arylsulfatase extracted from *H. pomatia* hydrolyses both the glucuronide and sulfate conjugates. However, the use of *H. pomatia* yields unwanted steroid by-product (e.g. conversion of DHEA to androst-4-ene-3,17-dione)

and incomplete deconjugation (e.g. androsterone-3-sulfate and testosterone-17-sulfate) (Messeri et al. 1984, Vanluchene et al. 1982, Venturelli et al. 1995). This is not a problem with the enzymatic hydrolysis using the enzyme from *E.coli*. The GC-MS run time for multiple steroid profile analysis is also commonly very long thereby limiting the sample throughput. Although only small volume of sample is required for GC-MS analysis (1-5 μL), it requires extensive sample cleanup before analysis.

1.7.3 Liquid chromatography-mass spectrometry

In the 1980s, the development of liquid chromatography (LC) technology coupled with MS provided a much simpler and faster analysis of steroid compared to the GCMS (Liberato et al. 1987). To date, LC-MS/MS is now considered the most accurate method to measure small molecules from variety of biological matrices (Xu et al. 2007). The LC consists of pumps that delivers mobile phase under high pressure to maintain a constant flow rate thereby ensuring reproducible chromatography. Mobile phase for steroids separation consist of a mixture of organic solvents (e.g. methanol and acetonitrile) and water with modifiers (e.g. formic acid, ammonium acetate, ammonium formate). Samples injected into the column (stationary phase) separates the steroids and the effluent is then transferred into the MS. The LC separation is dependent on the physical or chemical characteristics of the analytes such as molecular size and presence of functional groups.

LC-MS analysis is highly sensitive and specific for steroid quantitations. A robust LC-MS method to quantitate steroids requires high quality chromatography to separate the steroid of interest from isobaric compounds (i.e. identical nominal mass to charge ratios) (Keevil 2013). Examples of isobaric compound pairs include cortisone/prednisolone, testosterone/DHEA and 11-deoxycortisol/corticosterone. However, separating these isobaric or interfering compounds may increase the assay runtime and requires more mobile phase. Multiple analytes can be measured with lesser sample preparation compared to GC-MS.

Additionally LC-MS steroid profile provides more useful data than single steroid measurement such as from using immunoassay. With the LC-MS system, the analysis operates at low temperature which is beneficial for measurement of unstable analytes such as conjugated steroids and thermolabile steroids (Borts and Bowers 2000, Bowers 1996). Derivatization step is added to increase the sensitivity of LC-MS methods for certain steroids analysis especially for analytes that are not effectively ionized such as estradiol and DHEA (Kushnir et al. 2008) (Table 1.3). Derivatization reagent reacts with a functional group (eg hydroxyl) to improve column retention and ionization efficiency (Xu et al. 2011). Dansyl chloride is commonly used for hydroxyl group to esterify estrogens (Nelson et al. 2004, Xu et al. 2005). However, derivatization for LC-MS adds complexity to the method, possibility of artifact formation and requires certain functional groups (Jäntti et al. 2010, Xu et al. 2010). Unlike GC-MS, the LC-MS has generally shorter run time and is not limited to measurement of volatile compounds and is able to measure heat-labile steroidal compounds.

The LC is combined with ionization source that ionizes compounds delivered by the LC in liquid droplet. The atmospheric pressure ionization (API) which was introduced in the 1980s facilitates ionization for MS analysis. Ionization techniques commonly used for steroids LC-MS/MS analysis includes electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI) (refer section 1.8.2 for details on ionization interface). The ESI, APCI and APPI are considered as “soft” ionization technique as they form protonated or de-protonated molecules without fragmentation (Souverain et al. 2004). This type of ionization is achieved by transferring minimum internal energy to the analytes during ionization (El-Aneed et al. 2009). However, conjugated steroids could be fragmented by the ion source (Field 2013).

The ionization technique selected for LC-MS method is dependent on the molecular weight and the polarity of the analytes (Figure 1.14). APCI and APPI are known to be less

susceptible to ion suppression or enhancement due to matrix effect as the ionization occurs in gas phase compared to ESI (liquid phase ionization) (Elviri et al. 2010, Holčapek et al. 2010). However, ionization with APCI is not completely free from ion suppression for certain compounds and the analyst should perform through investigation on ionization technique selection during method development (Sangster et al. 2004). Table 1.3 shows different ionization techniques used in previous studies to determine free and conjugated steroids measurement using MS.

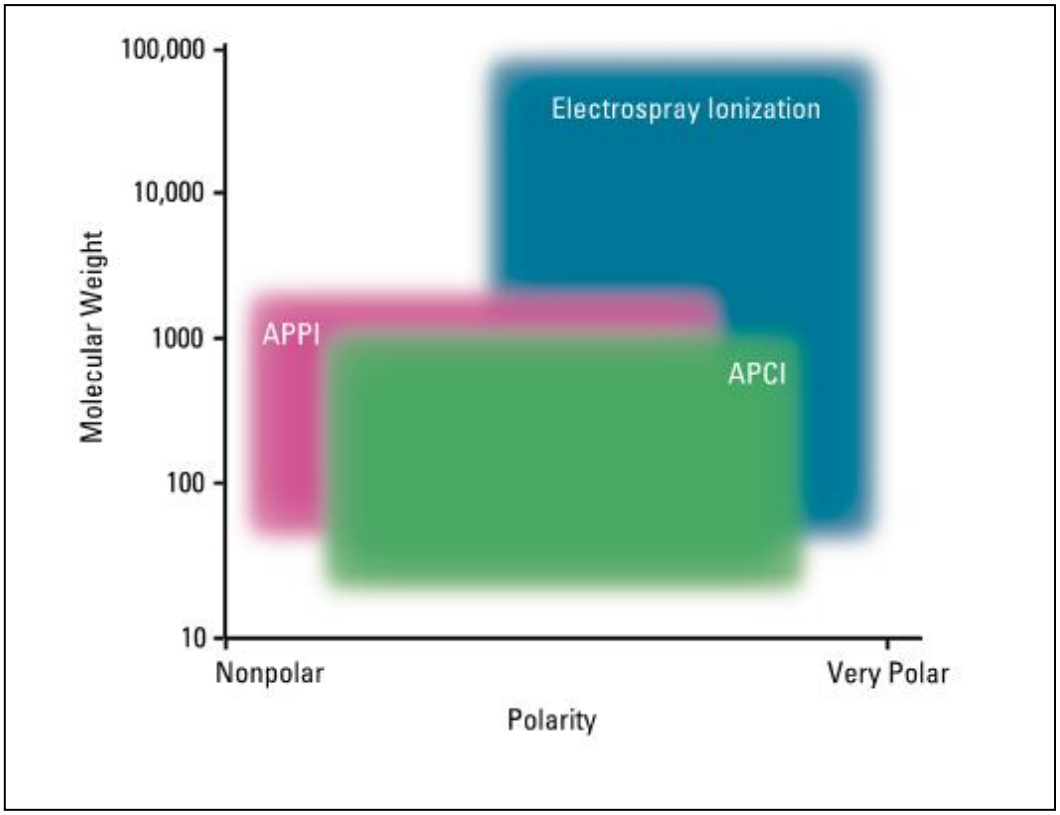


Figure 1.14 Ionization capabilities of ESI, APPI and APCI according to the molecular weight and polarity of the analytes (Holčapek et al. 2010).

Table 1.3 Examples of endogenous steroids analysis by GC/LC-MS.

Analyte	Analytical method	Biological Matrix	Sample preparation	Derivatization	Deconjugation	Assay validation	LOD/LOQ	Reference
T, ET, A, Etio, DHEA (Sulfates and glucuronides)	LC-ESI-MS/MS (- mode)	Urine (human)	SPE (Oasis WAX)	No	No	Yes	Not reported	(Strahm et al. 2008)
T, E, DHEA, DHT, 5α3α17β, 5β3α17β, 5A3β17α, 5A3β17β, EpiA, A, Etio	GC-MS	Urine (human)	SPE (C18)	ITMS/MSTFA	Chemical and Enzymatic (*E.coli*)	Partial	Not reported	(Dehennin et al. 1996)
ADT, CORT, E1, E2, E3, T, 5AD, ALD, 17OHPreg, DHEA, 5αDHT, 5βDHT, P5, THS, DOC, 5a-THB, HC, S, M, 2-OH E1; 16α-OH E1, 17-OHP, A4	LC-ESI-MS/MS (+ mode)	Urine (human)	SPE (Oasis HLB)	DMAB (all steroids accept for P5, 17-OHP, A4)	No	No	LOD: 5-500 pg/mL	(Dai et al. 2012)
15 estrogen and estrogen metabolites	LC-ESI-MS/MS (+ mode)	Urine (human)	LLE (dichloromethane)	Dansyl chloride	Enzymatic (*H. pomatia*)	Partial	LOQ: 0.04 ng/mL	(Xu et al. 2005)
23 estrogens, androgens and pregnanes (free/glucuronide conjugated)	LC-ESI-MS/MS (+ mode for all steroids except pregnandiol)	Urine (primate)	LLE (MTBE)	No	Chemical and Enzymatic (*E.coli*)	Yes	LOQ: 0.3-3 ng/mL	(Hauser et al. 2008)

E1, E2, E3, P4, P5	LC-ESI-MS/MS (+ mode for P4 and P5, - mode for estrogens)	Urine (human)	Online SPE (C18)	No	Enzymatic (*E.coli*)	Partial	6-61 pg on column	(Alvarez Sanchez et al. 2008)
T, ET, DHEA, A, Etio (free, sulfates and glucuronides)	LC-MS/MS (+ mode for free steroids and – mode for sulfates and glucuronide staeroids)	Urine (bovine and human)	SPE (Strata X)	No	No	No	LOD: 80-100 ng/mL	(Buiarelli et al. 2004)
TG, ETG, AG, EtioG	LC-ESI-MS/MS	Urine (human)	Filtration or LLE (ethyl acetate)	No	No	Yes	Filtration method: 0.25-1 ng/mL LLE method: 0.05-0.25 ng/mL	(Pozo et al. 2008)
AG, TG, E23G, E2-17G, E2-3G,17diG, DHEAS, TS, E1S, E2S, E2-3G17S, E2-3S17G, CRLG, CRLS, CRNS	LC-ESI-MS/MS (-mode)	Urine (human)	SPE	No	No	No	Not reported	(Antignac et al. 2005)
E1, E2	LC-APPI-MS/MS (- mode)	Tissues, serum (human)	Tissue homogenization (water), LLE (hexane:ethylacetate)	No	No	Yes	LOQ: 2.5-5.0 pg/mL	(Huhtinen et al. 2012)

T, DHT, 3α-diol, 3β-diol, E2, E1	LC-APPI-MS/MS (- mode: estrogens; + mode: androgens)	Tissues (Mouse testis, prostate, ovary, uterus	Tissue homogenization (EDTA in PBS), LLE (hexane:ethyl acetate)	No	No	Yes	LOQ: 0.3-40 pg	(McNamara et al. 2010)
E1, E2, E3, 17βT, 17αT, 5βDHT, 5αDHT, A, Etio	GC-MS/MS (- mode: estrogens; + mode: androgens)	Serum (human)	LLE (ether), SPE (ChromP), SiOH SPE column, HPLC	MSTFA-TNIS-DTT mixture	Enzymatic *(H.pomatia)*	Yes	LOD: 0.03-1.8 pmol/L LOQ: 0.07-3.7 pmol/L	(Courant et al. 2010)
DHEAS, A, F, CORT, S A4, E2, T, 17α-OHP, DHEA, P4	LC-APPI-MS/MS (- mode: A; + mode: all other steroids studied)	Serum (human)	PP (ACN)	No	No	Yes	LOD: 1.5-10 pg/mL	(Guo et al. 2006)
Seven C-21 adrenal steroids	LC-APCI-MS/MS (+ mode)	Serum (human)	PP ($ZnSO_4$/MeOH) Online extraction C18 silica monolithic pre-column	No	No	Yes	LOD: 0.10-2.75 nmol/L) LOQ: 0.30-12.40 nmol/L)	(Carvalho et al. 2008)
DHEAS, F, A4, E3, P4, DHEA, S, 17α-OHP, E2	LC-APPI-MS/MS (+ mode)	Serum (human)	PP (ACN)	No	No	Yes	Not reported	(Guo et al. 2004)
E1, E1S, E2, E13G, E2, 2MeOE1, 2MeOE2	TS-LC-MS/MS (- mode)	Serum (human)	LLE and SPE (Strata X)	No	No	Partial	Not reported	(Caron et al. 2009)

17OHP, T, A4	LC-APCI-MS/MS (+ mode)	Plasma/ Serum (human)	Online SPE (Oasis HLB)	No	No	Yes	LOD: 0.14-0.17nmol/L LOQ: 0.30-0.35 nmol/L	(Rauh et al. 2006)
A4, T, DHT	LC-ESI-MS/MS (+ mode)	Plasma	SPE (Oasis MAX)	No	No	Yes	LOQ: 0.29 ng/dL	(Kulle et al. 2010)
A4, T, DHT	LC-ESI-MS/MS (+ mode)	Cultured human cell lines	Online SPE (C4-alkyl-diol silica)	No	No	Yes	Not reported	(Chang et al. 2003)
T, DHEA	LC-ESI-MS/MS (+ mode)	Saliva (human)	SPE (Strata-X)	HMP	No	Yes	LOQ: 10 pg/mL	(Shibayama et al. 2009)
17OHPreg, 17OHP	LC-ESI-MS/MS (+ mode)	DBS (human)	SPE (Strata-X)	HP	No	Yes	LOQ: 0.5-1 ng/mL	(Higashi et al. 2008)
CORT, DOC, P4, 17α-OHP, F, DF, A4, T, DHT, F	LC-ESI-MS/MS (+ mode)	DBS (human)	LLE (acetone/ACN)	No	No	Yes	LOQ: 0.75-6.3 nmol/L	(Janzen et al. 2008)

Abbreviations: HMP: 2-hydazino-1-methylpyridine; HP: 2-hydrazinopyridine; DMAB: 4-Dimethlamino-benzoic acid; ACN: acetonitrile; MTBE: methyl tert-butyl ether; MSTFA-TNIS-DTT: N-methyl-N-(trimethylsilyl-trifluoroacetamide/trimethyliodosilane/dithiothreitol; T: testosterone; ET: epitestosterone; A: androsterone; M: mesterolone; Etio: etiocholanolone; DHEA: dehydroepiandrosterone; E1: estrone; E2: Estradiol; E3: estriol; 2-OH E1: 2-hydroxyestrone; 16α –OH E1; 16α-hydroxyestrone ; E1S: Estrone sulfate; E13G: Estrone glucorunide; 2MeOE1: 2-methoxy estrone; 2MeOE2: 2-methoxy estradiol: CORT: corticosterone; A4: Androstenedione; ADT: Androsterone; ALD: Aldosterone; P5: pregnenolone; 17α-OHP: 17α-hydroxyprogesterone; 17OHPreg: 17αhydroxypregnenolone; P4: progesterone; S: 11-Deoxycortisol; 21-Deoxycortisol: DF ; deoxycorticosterone: DOC; F:Cortisol; THS: tetrahydrodeoxycortisol; 5a-THB: 5a-tetrahydrocorticosterone; HC: Hydrocortisone; 17α-OHP: 17α-hydroxyprogesterone; E: epitestosterone; 5AD: 5-androstenediol; 5α3α17β: 5α -androstane-3 α,17β-diol; 5β3α17β: 5β-androstane-3 α,17β-diol ; 5A3β17α: 5androstane-3 β;17α –diol; 5A3β17β: 5androstane-3 β,17 β –diol; EpiA: epiandrosterone; AG: 5-androstane-17-glucuronide; TG: testosterone-17-glucuronide; E2-3G: 17-estradiol-3-glucuronide; E2-17G: estradiol-17-glucuronide; E23,17diG: estradiol-3,17-diglucuronide; DHEAS: dehydroepiandrosterone sulfate; TS: testosterone-17-sulfate; E2-3S: 17-estradiol-3-sulfate; E2-3G17S: estradiol-3-glucuronide,17-sulfate; E2-3S17G: estradiol-3-sulfate,17-glucuronide; CRLG: cortisol 21-glucuronide; CRLS: cortisol 21-sulfate; CRNS: cortisone 21-sulfate; TS: Turbo IonSpray; ESI: electrospray ionization; APCI: atmospheric pressure chemical ionization; APPI: atmospheric pressure photoionization; LC: liquid chromatography; GC: gas chromatography; MS: mass spectrometry; SPE: solid phase extraction; LLE: liquid-liquid extraction; PP: protein precipitation; LOD: lower limit of detection; LOQ: lower limit of quantitation: HPLC: High performance liquid chromatography; DBS: dried blood spot

Table 1.4 Examples of immunoassay and GC/LC-MS comparative studies for quantitation of testosterone (T) and estradiol (E2).

Analyte	Analysis technique comparisons	Samples (number)	Findings	Reference
T	GC-MS versus 10 immunoassay (8 direct non-isotopic immunoassays and 2 direct RIAs)	Serum from normal 50 men, 55 women and 11 children	• Men: Immunoassays underestimate T with 12% below GC-MS measurement. • Women: Degree of bias significantly higher in women with 46% above GC-MS concentration. • Children: Immunoassays measured higher T concentration for undetectable GC-MS levels (< 4.3 ng/dL).	(Taieb et al. 2003)
T	LC-API- MS/MS versus 6 immunoassays (4 automated and 2 RIAs)	Serum from 62 normal and 60 hypogonadal male.	• Deming regression demonstrated variability (underestimation or overestimation) of T measurements with most of the immunoassays studied compared to LC-MS/MS. • 90% of the samples measured with immunoassays demonstrated T concentrations of more than 20% from LC-MS/MS measurements.	(Wang et al. 2004)
T	LC-ESI- MS/MS versus 5 immunoassays (2 automated non-isotopic and 3 RIAs)	Serum from 28 children and 31 women	• Immunoassays overestimated T concentration and unreliable for T below 100 ng/dL.	(Moal et al. 2007)
T	LC-APCI- MS/MS versus one automated immunoassay	Serum/plasma from 107 children	• Immunoassay underestimated T for samples with concentrations >5 nmol/L. • High variability samples with T <5 nmol/L determined by immunoassay.	(Rauh et al. 2006)
T	GC-MS versus 7 automated immunoassays	Serum from 124 healthy men	• Deming and Passing-Bablok regression demonstrated variability in six immunoassays with significant difference in slope and intercept when compared to GC-MS measurements. • Reported wide variability in immunoassay performance against valid healthy young men reference population.	(Sikaris et al. 2005)
E_2	LC-APPI-MS/MS versus 5 direct non-isotopic immunoassays	Serum from 101 healthy older men	• Only three immunoassays were able to measure E2 concentrations in all the samples. • Upward bias ranging between 6 and 74% for E2 measured with immunoassays compared LC-MS/MS (method certified with 3 certified reference materials).	(Handelsman et al. 2014)
E_2	GC-MS/MS versus 7 immunoassays (3 indirect and 4 direct assays)	Serum from 40 postmenopausal women	• The three indirect assays that involve extraction prior to assays demonstrated better correlation (0.94, 0.91 and 0.88) with GC-MS/MS measurements compared to the 4 direct assays (without extraction).	(Lee et al. 2006)

1.8 Mass spectrometry

All MS comprise of four basic components - the inlet, ion source, mass analyzer and detector (Figure 1.15). Six basic types of mass analyzers are currently available. These includes quadrupole, ion trap, time-of-flight (TOF), Orbitrap, double focusing magnetic analyzer and Fourier transform ion cyclotron resonance (FT ICR) (Holčapek et al. 2010). Each has different mass accuracy, mass resolution parameters and capability to measure different of mass-to-charge (*m/z*) range (Holčapek et al. 2010). Recent advancement to the MS with higher mass resolution allowed separation of co-eluting isobaric compounds that have the same nominal masses but different exact masses (Xian et al. 2012). The mass analyzer measures gas phase ions according to their *m/z* ratio, where the charge is produced by addition or loss of proton(s), cation(s), anion(s) or electron(s). Utilizing electrical fields, these charged molecules are separated according to their *m/z* that further allows its mass measurements. Table 1.5 lists the basic principles of the six mass analyzers in use today. The most common mass analyzer for quantitative steroid analysis is the triple quadrupole instrumentation which is also known as tandem MS (Keevil 2013, Rauh 2010).

1.8.1 Triple quadrupole mass spectrometer

The triple quadrupole MS consists of two resolving quadrupoles (Q1 and Q3) and a collision cell positioned in between the quadrupole (Q2) (Kushnir et al. 2011). Sample steroids separated by the LC are first ionized by the ion source before further transfer into the mass spectrometer where the first quadrupole monitors the precursor ions. The analyte(s) of interest (precursor ion) is selected at Q1 according to the *m/z* ratio and all other compounds are filtered out. Then, the second quadrupole (collision cell) performs the collision between the collision gas (e.g. argon, helium and nitrogen) and the introduced steroids producing fragmented or product ions. Finally, the third quadrupole monitors the product ions of the

steroids according to the radiofrequency set and removes the unwanted fragment ion. The targeted precursor and product ion finally reaches the ion detector (Figure 1.15).

New LC-MS/MS method development requires infusion of the standard steroid reference and its isotope labeled internal standard separately using a syringe pump and a 'tee mixer' to introduce the analyte into the LC mobile phase and the ion source. Analyte quantitation can be carried out by two common techniques, selected-ion-monitoring (SIM) or multiple-reaction-monitoring (MRM), which are commonly used for a single or triple quadrupole mass spectrometer, respectively. MRM (also known as selected-reaction-monitoring; SRM) mode provides high selectivity and sensitivity compared to SIM. High background chemical noise in SIM mode caused by isobaric compounds and impurities leads to lower signal to noise ratio (S/N) for the target analyte (Bakhtiar and Majumdar 2007). The analyte of interest should be tested in positive or negative ion modes to determine the most suitable and efficient ionization conditions with highest S/N ratio. For MRM, the specific ion transition for each analyte is selected based on precursor ion and product ions pairs, which is then used for quantitation of steroids from the biological samples (Shibata et al. 2014). During the method development, other MS parameters such as ion source voltage and temperature, collision cell energy, declutering potential, entrance potential, collision cell exit potential and dwell time are optimized. Biological samples have to be pretreated prior to sample quantitation by MS and the method has to be validated for limit of quantitation (lower and upper), accuracy and precision, recovery, reproducibility, stability and matrix effect according to the appropriate guidelines such as US Food and Drug Administration guidance (FDA 2001) or European Medicines Agency (EMA 2011).

Table 1.5 Basic principles of different types of mass analyzers

(Barrow et al. 2005, El-Aneed et al. 2009, Glish and Vachet 2003, Ho et al. 2003, Hu et al. 2005, Moens and Jakubowski 1998, Nikolaev et al. 2014, Vogeser and Parhofer 2007).

Mass analyzer	Main principles
Quadrupole	• Consist of four metal rods installed in parallel orientation • RF and DC voltages applied to rods guide the ions with desired *m/z* ratios to pass through and move towards the detector while other molecules are guided out and neutralized • Mass range: <4000 Da; Resolution: <4000
Ion trap	• Consist of three hyperbolic electrodes: ring, entrance and exit end cap electrode • RF and DC potential applied to the ring electrode produces 3-dimensional quadrupolar potential field • The ions losses kinetic energy and are trapped forming an ion "cloud" • Ions are ejected according to their *m/z* ratios into the detector • Mass range: <4000 Da; Resolution: <4000
Time-of-flight (TOF)	• Ion generated by ion source is accelerated into flight-tube • Smaller ions travel faster compared to molecules with higher mass • Molecules with different *m/z* travel at different time intervals into the detector • Mass range: >1 MDa; Resolution: 4000
Orbitrap	• Consist of two electrodes: outer electrode (barrel shape) and inner electrode (spindle shape) • DC voltage applied • Ions are pulsed into the orbitrap, rotates around the central electrode and oscillate along the horizontal line • *m/z* of different ions are determined from different frequencies of the oscillation by Fourier transform • Mass range: <6000 Da; Resolution: 150,000
Double focusing magnetic analyzer	• Involves two sectors: electrostatic and magnetic • Ions with same kinetic energy are directed from electrostatic sector (act as a kinetic energy selector) to the magnetic sector before ion separation and detection • Mass range: 10,000 Da; Resolution: 60,000
Fourier transform ion cyclotron resonance (FT ICR)	• Consist of an analyzer cell (or known as Penning trap) with two excitation, detection and trapping plates each • Analyzer cell is located within strong magnetic field • Ions generated by ion source moves into the analyzer cell and RF applied to the excitation plates causes excitation of the ions • Ions starts to precess in center of magnetic field resulting it to orbit (known as cyclotron motion) • Ions with lower *m/z* have higher cyclotron frequencies compared to ions with higher *m/z* • Ion packets frequencies are detected by the detection plates and the frequency analysis is performed using Fourier transformation • Frequencies are converted to *m/z* to give a mass spectrum. • Mass range: 10,000 Da; Resolution: >500,000

Abbreviations: RF: radiofrequency; DC: direct current; *m/z*: mass/charge

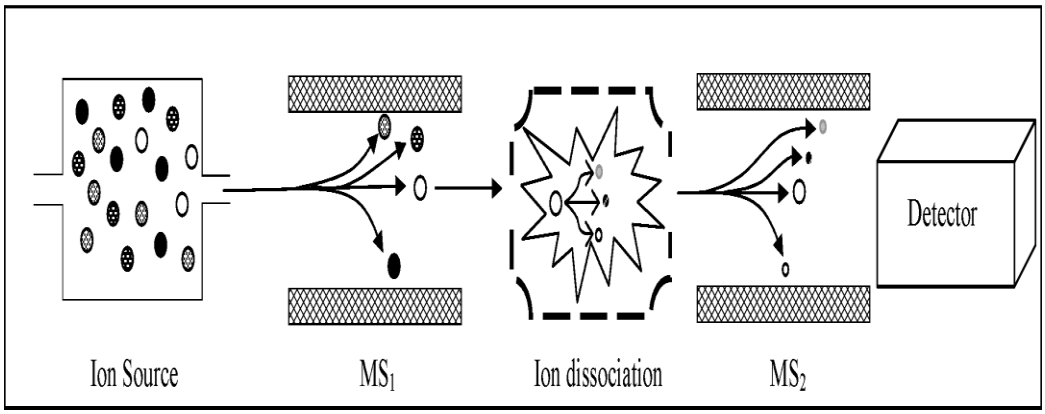


Figure 1.15 Representation of precursor ion and product ion selection of MS1 and MS2, respectively in the tandem mass spectrometer (El-Aneed et al. 2009).

1.8.2 Ionization interfaces

The eluent from the LC column must be effectively converted into gaseous phase before being measured by the MS. Since the introduction of electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI), LC-MS/MS has become widely used for steroid analysis with superior sensitivity and specificity (Table 1.3).

1.8.2.1 Electrospray ionization (ESI)

This ionization method, developed by Fenn and co-workers in 1988 (Fenn et al. 1989), led to award of a Nobel Prize in Chemistry to Fenn in 2002. ESI technique is based on the production of ions from liquid phase into gas phase by the dispersal of highly charged droplets at near atmospheric pressure followed by the formation of evaporated droplet. The LC mobile phase is sprayed from the tip of the metal capillary. The needle is applied with high voltage typically between 3-5 kV that leads to the formation of Taylor cone (Taylor 1964) (Figure 1.16). A fine spray of highly charged droplets is generated which then undergoes rapid evaporation due to heat and dry nitrogen (desolvation gas). The ion polarity is controlled by adjusting the capillary voltage that forms either positive or negative charged

ions. Evaporation of the solvent further reduces the droplet diameter and increases the droplet surface charge density. The parent droplets undergoes "Coulomb explosion" or "Coulomb fission" when the electrostatic forces overcome the surface tension of the droplets (Rayleigh limit is reached) forming smaller offspring droplets. This process continues until the charged analyte molecules escapes the droplets by field desorption and these analyte ions are guided into the mass analyzer by the electric field gradient (Banerjee and Mazumdar 2012, Gomez and Tang 1994) (Figure 1.16). To facilitate ionization, the presence of ionic solution is important. Mobile phase consisting of methanol/water or acetonitrile/water containing weak acids such as formic or acetic facilitate protonation (for positive mode ionization), whereas diluted ammonium hydroxide in aqueous solution or diluted volatile buffers (ammonium acetate or formate) facilitates deprotonation (for negative mode ionization) (Cech and Enke 2001). ESI is a soft ionization technique (i.e. non-disintegrating) suitable for polar compounds such as labile conjugates (glucuronides and sulfates) (Figure 1.14) (Kostiainen et al. 2003) or compound that forms charged adduct with ammonium or alkali cations (Himmelsbach 2012). The LC-ESI-MS/MS method is able to measure steroids in free and conjugated forms simultaneously (Moeller and Stanley 2012). However, steroids are commonly measured after hydrolysis, which is enzymatic or chemical deconjugation. Steroids with high proton affinity such as androgens, glucocorticoids and mineralocorticoids are easily ionized with ESI source due to the 3-oxo-4ene structure (Shibata et al. 2014). Derivatization is commonly carried out to improve ESI ionization efficiency and sensitivity for trace steroids or steroids with low ionization potential. Although derivatization may increase the sensitivity of steroids measurement for LC-ESI-MS methods (1-10,000 fold) (Dai et al. 2012) (Table 1.3), the derivatization step increases sample preparation time, causes loss or degradation of target steroids and increases "noise" (Higashi and Shimada 2004, Mao et al. 2004, Santa 2011).

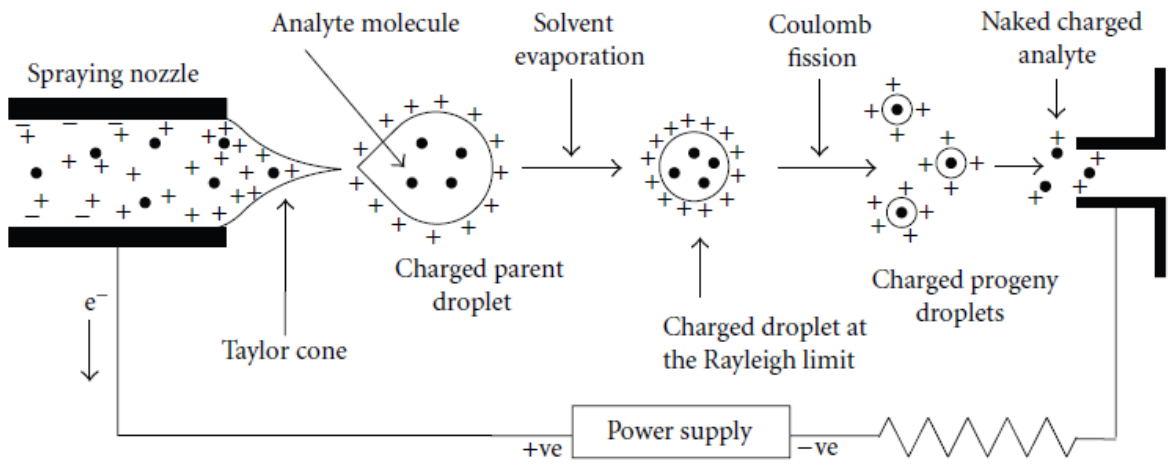


Figure 1.16 Schematic diagram of ESI source.

The highly charged capillary releases charged droplets. Size of the droplets decreases with the desolvation process and further droplet fission produces the gas phase analyte ions (Banerjee and Mazumdar 2012, Robb and Blades 2008).

1.8.2.2 Atmospheric pressure chemical ionization (APCI)

This is a chemical ionization process that ionizes analytes in gas phase. Mobile phase and analytes are first vapourized in the ion source with heat (from the nebulizer probe) and nebulizing gas flow. A sharp needle is positioned directly in the forming gas cloud and this needle generates electron from corona discharge which bombards the vapour. Solvent molecules become charged and the electrical charge is transferred to the analyte molecules (Figure 1.17). The LC mobile phase commonly used is a mixture of water and organic solvent (e.g. methanol and acetonitrile) which forms either protonated or deprotonated ions of these solvents that transfers their charge to ionizable analytes. APCI is preferred over ESI for steroid analysis as it is capable of ionizing non-polar or poorly ionized compounds (Kushnir et al. 2010) (Figure 1.14).

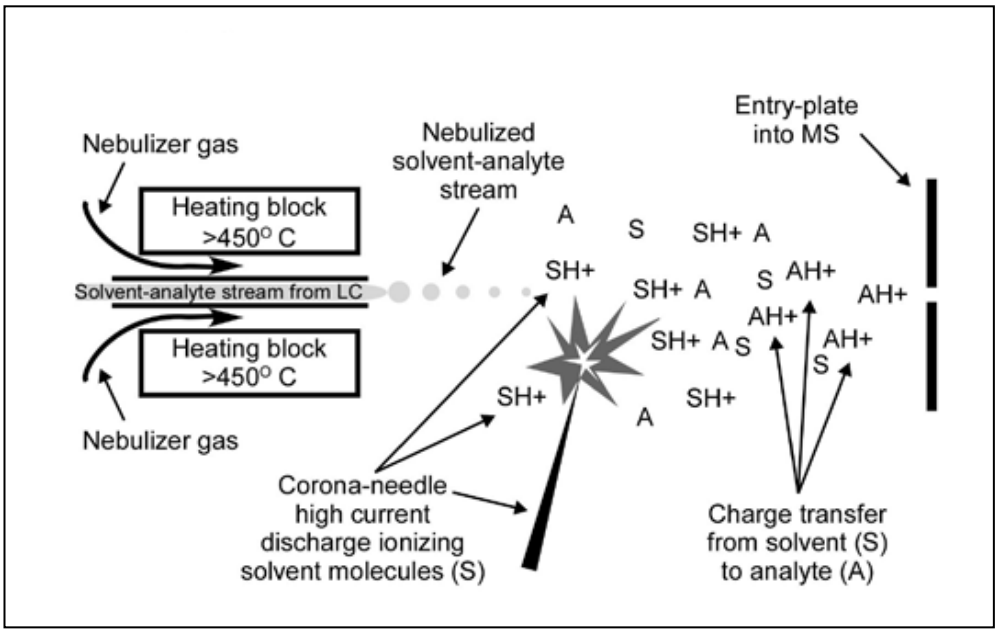


Figure 1.17 Schematic diagram of APCI source (Grebe and Singh 2011).

1.8.2.3 Atmospheric pressure photoionization (APPI)

This is a newer API source for LC-MS that also operates by chemical ionization in gas phase. Similarly to APCI, the solvent first undergo vaporization as it passes the heated nebulizer. Instead of the corona discharge needle used in APCI source, krypton lamp produces ultraviolet (UV) light that induce ionization via emission of photons in vacuum (Figure 1.18). The analyte ionization in this system is enhanced with the addition of dopant such as toluene and acetone that is involved in the chemical ionization (Robb and Blades 2006). Krypton lamp delivers photons energy (*hv*) of 10.0 and 10.6 eV and only ionizes ions with lower ionization energy (IE) including the dopant (e.g. toluene IE: 8.82 eV). Common LC mobile phase such as water (12.61 eV), methanol (10.85 eV) and acetonitrile (12.19 eV) with IE above the lamp photons *hv* are not ionized therefore do not aid analyte ionization (Robb and Blades 2008). APPI produces charged ions via two mechanisms: (1) direct photoionization of analyte by absorbing a photon of light from Krypton emission, (2) the dopant ionized by the UV lamp form free radicals followed by the reaction with the analyte by charge exchange (non-polar compounds) or proton transfer (polar compounds) (Robb and Blades 2006) (Figure 1.19). APPI ionization is more suitable for compounds with mid to low-polarity that

cannot be ionized by ESI and APCI (Figure 1.14). The sensitivity of APPI for steroid measurement in biological matrices is 3-5 fold higher than APCI (Alary 2001). Table 1.6 summarizes the properties of ESI, APCI and APPI.

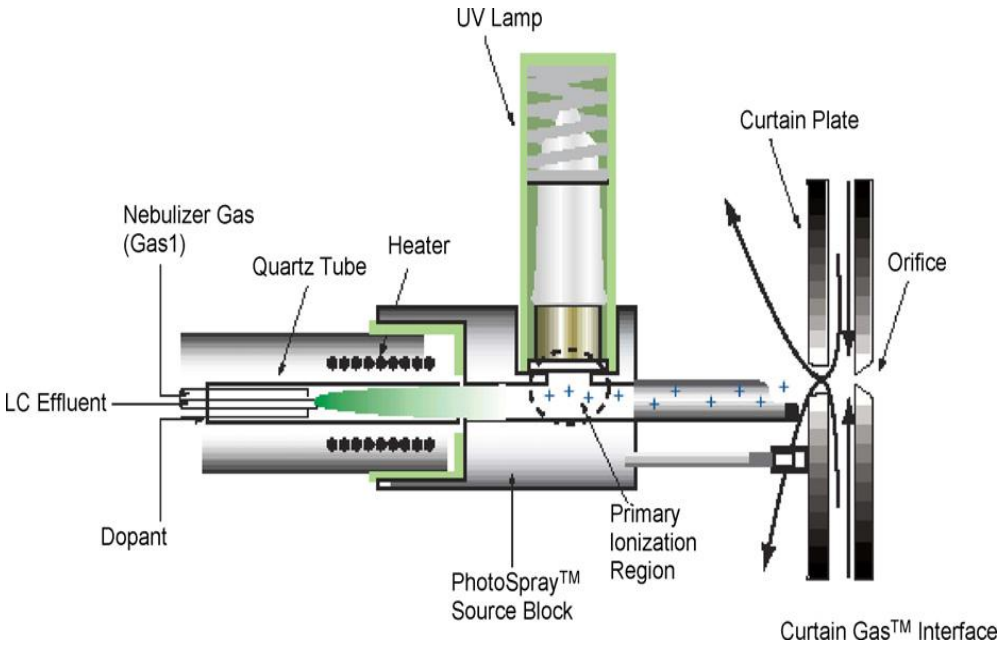


Figure 1.18 Schematic diagram of APPI source (Robb and Blades 2008).

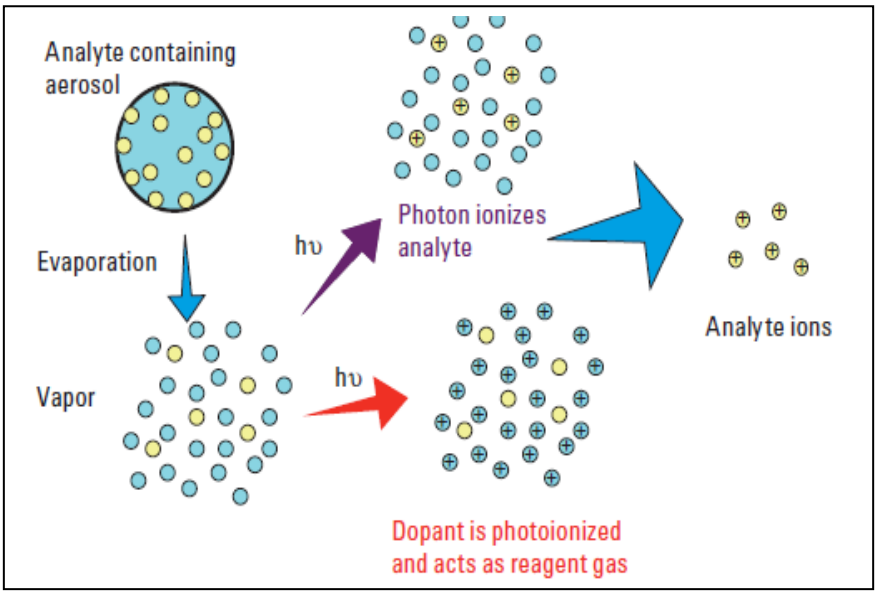


Figure 1.19 Schematic diagram of APPI ionization mechanism.

Adapted from http://www.chem.agilent.com/Library/technicaloverviews/Public/5990--7413EN.pdf

Table 1.6 Summary of ionization techniques (ESI, APCI and APPI) commonly used for steroid LC-MS/MS analysis
(Bakhtiar et al. 2002, Bos et al. 2006, Cech and Enke 2001, Kostiainen and Kauppila 2009).

	ESI	APCI	APPI
Ionization reaction phase	Liquid	Gas	Gas
Compound suitability (common mass range)	Polar and ionic compound (less than 100,000 Da)	Neutral or less polar (less than 1500 Da)	Mid to low polarity (less than 1500 Da)
Ideal compounds	non-volatile chargeable large molecules e.g. protein, peptide, polysaccharide, bile acids, steroid conjugates	Volatile, thermally stable and small molecules e,g. non-polar lipids, pesticides, drugs, steroids with conjugated double bond or keto group	Volatile, thermally stable and small molecules e.g. polycyclic aromatic hydrocarbons, drugs, non-polar lipids, pesticides, steroids with conjugated double bond and low polarity (pregnanes)
Matrix interference	More prevalent compared to APCI and APPI	Less prevalent compared to ESI	Less prevalent compared to ESI and APCI
Suitable flow rate	Few µL/min (operates at very low flow rate)	Up to 2 mL/min	Suitable for both low and high flow rates
Suitable solvents	Polar and medium polar	Polar and non-polar	Polar and non-polar

Abbreviations: ESI: electrospray ionization; APCI: atmospheric pressure chemical ionization and APPI: atmospheric pressure photoionization

1.8.3 Matrix effect

Urine, whole blood, serum, plasma and saliva are matrices commonly used for bioanalytical analysis. These matrices contain endogenous (e.g. phospholipids, salts, urea, amines and metabolites) and exogenous substances (e.g. buffers, anticoagulants, polymers from collection tubes, drugs and chemicals) that may co-elute with the compound of interest that may lead to ion suppression or enhancement (Antignac et al. 2005, Matuszewski et al. 1998, Mei et al. 2003). This phenomenon, known as matrix effect is defined as "The direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the samples" (Shah et al. 2000). It is important to identify and eliminate matrix effect using adequate validation procedure for bioanalytical method development as matrix effect dramatically affects the LC-MS method performance (especially for quantitative analysis) in terms of detection capabilities, reproducibility, linearity, selectivity, accuracy and limit of quantitation, leading to erroneous results (Gosetti et al. 2010, Trufelli et al. 2011).

For LC-MS analysis, matrix effects can be assessed either by post-column infusion (qualitative), post-extraction spike method (quantitative) or assessment of "relative" matrix effect (also known as standard line-slope method). The post-column infusion assessment involves simultaneous injection of sample extract and continuous infusion of analyte into the LC-MS/MS system. This assessment allows identification of the chromatographic region that could possibly be affected by matrix effects and appropriate changes should be made to the elution condition to shift the analyte of interest retention time away from the area affected in the chromatogram (Bonfiglio et al. 1999). However, this assessment does not provide the degree of matrix effect that is affecting the analyte quantitatively and is time consuming especially for optimizing a method with multiple analytes.

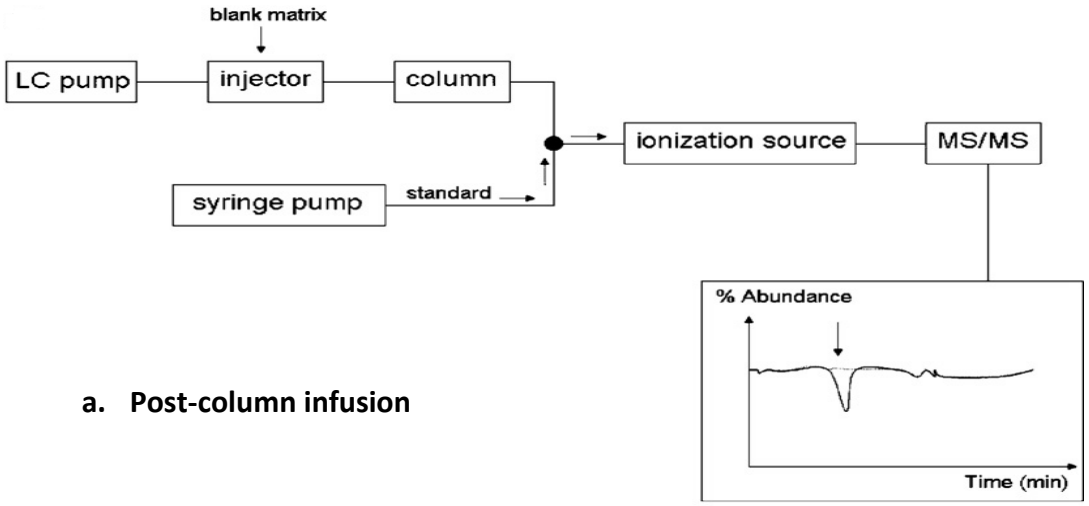
Post-extraction spike method is based on the response of analyte spiked after sample preparation against neat standard solution and is expressed as a percentage, with values above or below 100% indicates ion enhancement or suppression, respectively (Matuszewski et al. 2003). This method allows quantitative assessment for ion suppression or enhancement of multiple analytes simultaneously. The presence of matrix effect can also be determined by "relative" matrix effect assessment by directly comparing standard line slopes (made from five different lots of a biofluid) constructed using identical compound, IS, sample preparation and chromatographic conditions. The standard line slopes and precision of the slopes were calculated to determine the absence or presence of "relative" matrix effect on the quantification of the analytes assessed. For a method to be acceptable (free from matrix effect), the precision value of the standard line slopes should not exceed a cut-off value of 3-4% (Matuszewski 2006). Figure 1.20 illustrates the block diagram and MS chromatogram for the two most commonly used matrix effect assessments: post-column infusion and post-extraction spike.

The degree of interference caused by matrix effect in LC-MS analysis does not only depend on the sample matrix. Selection of sample preparation, mobile phase, reagents, chromatographic separation and ionization interface used for the LC-MS method developed also influences the extent of matrix effect (Annesley 2007, Chambers et al. 2007). There are steps that can be taken to overcome matrix effects such as appropriate modification to a cleaner sample preparation technique, sample dilution (limited by the method sensitivity and not suitable for monitoring trace compound), use of smaller sample volume (also limited by the method sensitivity) and utilization of stable labelled internal standard. Matrix effect can also be minimized by using lower LC flow rate, so that the ion source is exposed to lesser amount of contaminants. Other strategies include to separate the interfering compound from target analyte(s) by changing the chromatographic conditions and if possible, use other

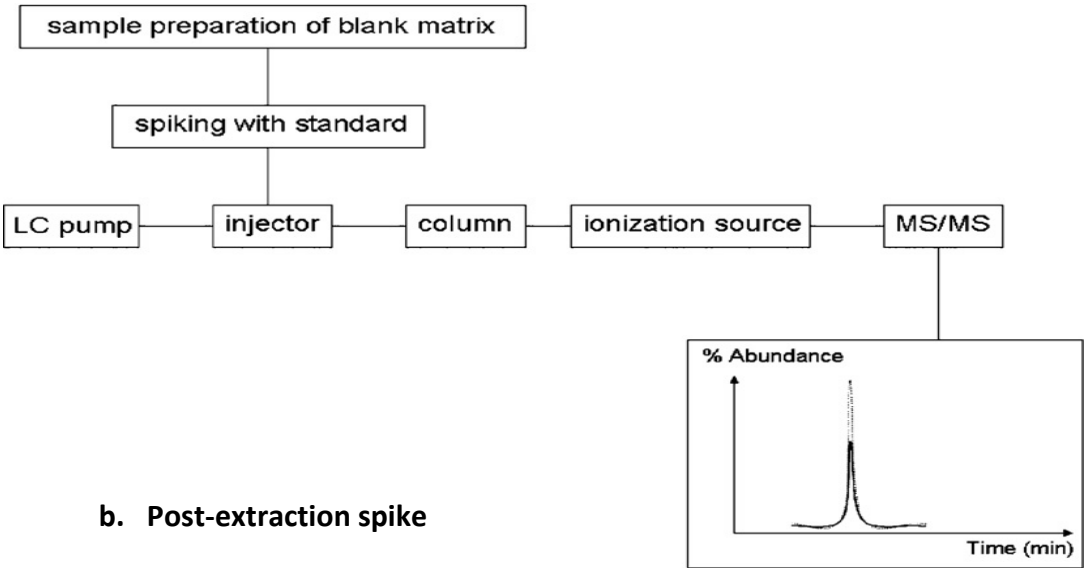
ionization technique that minimizes matrix effects (Chambers et al. 2007, Van Eeckhaut et al. 2009). Selection of appropriate MS ionization source (ESI, APPI, APCI) and mode (positive or negative) may also eliminate matrix effects (Hsieh et al. 2001, Janzen et al. 2008, Matuszewski et al. 1998, Mei et al. 2003).

Ion sources are prone to different degree of signal suppression or enhancement mainly because of difference in the ionization mechanisms (Himmelsbach 2012). A comparative study of LC-MS/MS method using three different ion sources, ESI, APCI and APPI for estradiol analysis in human serum and endometrial tissue demonstrated that ESI efficiently ionizes estradiol in negative polarity but estradiol is highly susceptible to ion suppression compared to the latter two ion sources (Keski-Rahkonen et al. 2013). APCI is also susceptible to signal suppression due to matrix effect but at a lower degree compared to ESI especially in the presence of hydrophobic interferences (Dams et al. 2003, Matuszewski 2006). For ESI, it is postulated that the signal suppression occurs when the analyte of interest competes for charge with co-eluting analyte. The co-analyte that has higher gas-phase proton affinity will be protonated first therefore reducing the intensity of the target analyte (Matuszewski et al. 2003). This is not the case for APPI, where its ionization mechanism is not based on charge affinity, therefore is less susceptible to ion suppression compared to ESI and APCI (Chen et al. 2009, Chu and Letcher 2008). The presence of high concentration non-volatile compounds (e.g. salts, ion-pairing agents, drugs) in the extracted sample matrix also lead to ion suppression by decreasing the ESI efficiency to form droplet and prevent evaporation (King et al. 2000). Furthermore, ESI is more susceptible to ion suppression in the presence of more polar analytes and the presence of molecules with higher mass that suppresses the signal of smaller molecules (Bonfiglio et al. 1999, Sterner et al. 2000). On the contrary, ion enhancement is caused by overlap of ions with specific *m/z* ratios, thus proper fragment ions selection with different combination of *m/z* may resolve the problem (Trufelli et al. 2011).

The degree of ion suppression differs between sample, compound and sample preparation (Bonfiglio et al. 1999).



a. Post-column infusion



b. Post-extraction spike

Figure 1.20 Matrix effects evaluation (a) post-column infusion and (b) post-extraction

a) For the post-column infusion, the analyte is infused into the LC stream using a 'tee-mixer' at a stable and constant flow to the MS ion source using an infusion pump. Sample extract (blank matrix) is injected via autosampler after extraction into the LC column. Elution of endogenous compound that interferes with the infused analyte will result in negative or positive peaks in the MS response due to ion suppression or enhancement, respectively. The arrow in the example above indicates ion suppression.

b) In the post-extraction spike method, comparison is made between the signal response of standard in sample extract (indicated as full peak in the chromatogram above) against the response obtained from standards in neat solution (dashed line). A reduction or increase in the response indicates ion suppression or enhancement, respectively. Adapted from (El-Aneed et al. 2009, Van Eeckhaut et al. 2009)

1.8.4 Sample preparation

Sample pretreatment prior to LC-MS is required to reduce the complexity of the sample. Sample preparation is necessary for the development of highly sensitive, accurate and specific steroid LC-MS/MS analysis (Shibata et al. 2014). Sample preparation involves extraction of the steroids typically with an organic solvent followed by purification step to remove unwanted matrix component from biological samples. Protein precipitation (PP), liquid-liquid extraction (LLE) and solid phase extraction (SPE) are common methods to separate analytes from sample matrix. However, it is important to evaluate whether the sample preparation technique selected: (1) provides a cleaner extract instead of magnifying (pre-concentrate) matrix effect and (2) is appropriate for the biofluids analyzed (Dams et al. 2003).

PP is achieved by adding solvent (e.g. acetonitrile, methanol and acetone) with low solubility and the precipitate of insoluble compounds is then separated from the soluble phase (e.g. centrifugation). The supernatant can be directly injected for analyte analysis or reconstituted in LC mobile phase before LC/MS analysis (Bakhtiar et al. 2002). PP is generally preferred for MS analysis for its simplicity but generally does not provide clean extract as many endogenous compounds such as fatty acids, lipids, phospholipids and endogenous peptides remains in the extract (Chambers et al. 2007). This is particularly a problem for ESI where the interfering compounds competes and co-elute with the target analyte by interfering with the droplet desolvation process (Bakhtiar and Majumdar 2007, Chambers et al. 2007). This co-eluting compound may also form adducts or react with the analyte of interest and interfere with the signal intensity (Gosetti et al. 2010). Studies have combined PP with other sample preparation to achieve cleaner samples (refer Table 1.3 for examples) (Carvalho et al. 2008). Some laboratories utilize a LC divert valve to elute early eluting compounds such as unwanted endogenous substances and salts to waste before the

extract is directed to the MS that may reduces matrix effect and MS contamination (Harwood and Handelsman 2009).

SPE is also widely used sample extraction method and provide cleaner extracts in comparison to PP (Chambers et al. 2007, Dams et al. 2003). This method involves separation of compounds between mobile and stationary phases which can be carried out either off-line (manual extraction) or on-line (automated extraction that is directly connected to the chromatographic system). On-line separation is less labour-intensive and time consuming compared to off-line SPE. The separation mechanism for SPE is similar to analytical chromatography where the analytes are retained in the stationary phase through the reverse-phase or ion exchanged SPE than removal of sample matrix followed by eluting the analyte with a strong organic solvent at the appropriate pH (Berrueta et al. 1995). Although SPE provides a cleaner extract compared to PP, studies have shown occurrence of matrix effect with this sample preparation method (Souverain et al. 2004). This is due to the pre-concentration step that increases unwanted interfering substance together with the target analyte(s). Possible alternative to remove the pre-concentration step (i.e. direct injection or dilution) but this will reduce the sensitivity (Dams et al. 2003).

LLE is widely used for extraction of small molecules such as drugs and hormones which gives clean extracts with high reproducibility (Janzen et al. 2008, Souverain et al. 2004, Xu et al. 2005). Besides being cost effective and highly selective, LLE sample extraction method is less susceptible to matrix effects compared to PP and SPE for common API ionization sources (Souverain et al. 2004). However, recovery of polar compounds has been reported to be low using LLE extraction depending on solvent pairs (Chambers et al. 2007). This extraction method involves separation of analytes from biological sample based on two different solubility properties. Non-polar analytes in aqueous sample (biological fluid) are extracted using an immiscible solvent (e.g. ethyl acetate, methy tert-butyl ether and

hexane), with phase separation leaving the salts and proteins in the aqueous phase. Samples are normally vortex-mixed with the organic solvents followed by centrifugation to separate the solvent from aqueous phase. The organic solvent is recovered by freezing the aqueous phase or pipette. The organic layer is then dried and the residue that consist the analyte is reconstituted in the LC mobile phase. However, in some cases, the hydrophobic extract is directly injected upon method optimization (Medvedovici et al. 2011). The drawbacks of using LLE include low recovery for polar analyte and ionic compounds, multiple extraction steps may be required, difficulty to automate the extraction method and formation of emulsion in samples containing high levels of phospholipids that may affect the accuracy and extraction recovery of the target analyte (Leung and Fong 2014, Van Eeckhaut et al. 2009). Neutralizing compound with ionizable functional groups by adjusting the pH prior to organic solvent extraction helps to achieve high extraction recovery (Hendriks et al. 2007). Examples of studies utilizing different sample purification methods for MS steroid analysis are listed in Table 1.3.

1.9 Aims of study

The overall aim of this study is to develop and validate sensitive LC-MS/MS assays for quantitative endogenous and exogenous steroids analysis from various biological matrices for specific clinical applications. Androgens (testosterone, DHT, nandrolone), pro-androgens (DHEA) and estrogen (17β-estradiol) were measured in various biological matrices including urine, serum and dried blood spots (DBS). LH was measured using validated ICL and IF assays for urine and serum. Urine is a preferred sample collection method as it provides convenience and is less intrusive especially for longitudinal field studies in children and adolescents. Urine sampling also avoids impracticality of multiple blood sampling for healthy volunteers and provides an integrated measurement especially for hormones secreted in pulsatile (LH) or diurnal (sex steroids in early puberty) manner. DBS is also a less invasive sampling method that requires only small volume of blood, simplified sample processing and is cost effective (i.e. shipping and sample storage without needing refrigeration). DBS sampling method is suitable for long-term pharmacological studies because the samples can be collected by the patients themselves with minimum training without the need of frequent visits to the clinic.

The specific aims of this thesis were:

I. To determine whether an androgen ester (nandrolone decanoate) marketed for intramuscular injection can be delivered effectively and safely as subcutaneous injection. To achieve this aim, DBS sampling method was optimized and LC-MS/MS method was developed and validated to measure testosterone and nandrolone from DBS.

II. To determine whether commercially available IF and ICL LH immunoassays optimized for blood samples can be used for urine samples kept after prolonged frozen storage.

III. To determine whether first morning void hormonal assessments in growing adolescents at various stages of pubertal progression requires adjustment to correct for hydration status and, if so, to determine whether creatinine or specific gravity are better adjustments.

IV. To validate the feasibility of frequent urine sampling regimen and urine assay methodology for LH and sex steroids measurements using ICL and LC-MS/MS, respectively. Samples were collected from growing adolescents for a longitudinal study to assess changes in urine LH, estradiol and testosterone over 12 months by reference to contemporaneous changes in anthropometry and self-reported Tanner stages.

Chapter 2 Materials and Methods

The chemicals, sample preparation and instrument settings used in this study are described in this section.

2.1 LC-MS/MS assay

2.1.1 Chemicals

The chemicals and reagents used are listed in Table 2.1 and Table 2.2. All reagents used were analytical and chromatographic grade.

2.1.2 Instrumentation

LC analysis was carried out on a Shimadzu Nexera UHPLC system (Shimadzu Scientific Instruments, Columbia, MD). MS/MS analysis was performed on an API-5000 triple-quadrupole mass spectrometer (Applied Biosystem/MDS SCIEX, Ontario, Canada) equipped with an APPI source. The MS/MS nitrogen gas was supplied by a nitrogen gas generator (Peak Scientific Instruments Ltd, Scotland).

2.1.3 LC-MS/MS method for DBS steroids analysis

2.1.3.1 Preparation of calibration standards and quality control

Calibration standards and quality controls (QCs) were made from artificial blood according to a modified method described previously (Higashi et al. 2008). Briefly, venous whole blood was collected from a volunteer in a lithium heparin tube (Vacuette®, Greiner Bio-one). The plasma separated from red blood cells by centrifugation at 1500 x g (at 4°C, 15 min) was discarded and the red blood cells were washed with 30 mL of saline (0.9% NaCl in distilled water) (to remove remaining plasma), centrifuged at 1500 x g for 15 min at 4 °C followed by discarding the supernatant; this step was repeated four times to remove any remaining plasma. Charcoal treated plasma was prepared by adding 50 mg of activated charcoal (BDH

Table 2.1 List of chemicals for steroid LC-MS/MS assay.

Chemical	Chemical name	Type	MW (g/mol)	Manufacturer
Testosterone	17β-hydroxyandrost-4-en-3-one	Standard	288.4	NMI, Sydney, Australia
Nandrolone	17β-hydroxy-19nor-4-andro-sten-3-one	Standard	274.4	Steraloids, Newport, RI, USA
17β-Estradiol	17β-estra-1,3,5(10)-estratriene	Standard	272.4	Steraloids, Newport, RI, USA
Dihydrotestosterone	17β-hydroxy-5α-androstan-3-one	Standard	290.4	NMI, Sydney, Australia
Dehydroepiandrosterone	5-androsten-3β-ol-17-one	Standard	288.4	NMI, Sydney, Australia
Testosterone glucuronide	17β-3-oxoandrost-4-en-17-yl b-D-glucopyranosiduronic acid	Standard	464.6	NMI, Sydney, Australia
17β-Estradiol glucuronide	1,3,5(10)-estratrien-3, 17β-diol 17-glucosiduronate	Standard	448.5	Steraloids, Newport, RI, USA
Dihydrotestosterone glucuronide	3-oxo-5α-androstan-17β-yl glucosiduronic acid	Standard	466.6	NMI, Sydney, Australia
Dehydroepiandrosterone glucuronide	5-androsten-3β-ol-17-one glucosiduronate	Standard	464.5	Steraloids, Newport, RI, USA
d_3-Testosterone	16,16,17-d_3-17β-hydroxyandrost-4-ene-3-one	Internal standard	291.4	NMI, Sydney, Australia
d_4-Estradiol	2,4,16,16-d_4-17β-estra-1,3,5(10)-estratriene	Internal standard	276.4	Cambridge Isotope Laboratory, USA
d_3-Dihydrotestosterone	5α-(16,16,17α-$^2H_3$)-5α-Androstan-17β-ol-3-one	Internal standard	293.5	NMI, Sydney, Australia
d_2-Dehydroepiandrosterone	16,16- d_2-5-androsten-3β-ol-17-one	Internal standard	290.4	Steraloids, Newport, RI, USA

Abbreviations: NMI: National Measurement Institute; MW: molecular weight; d: deuterated; AR: analytical reagent; HPLC: high performance liquid chromatography.

Table 2.2 List of reagents for steroid LC-MS/MS assay.

Reagent	Type	MW (g/mol)	Manufacturer
Methanol	Solvent (HPLC grade)	32.04	RCI LabScan, Bangkok, Thailand
Toluene	Solvent (HPLC grade)	92.14	RCI LabScan, Bangkok, Thailand
Methyl tert-butyl ether	Solvent (AR grade)	88.15	RCI LabScan, Bangkok, Thailand
Potassium carbonate (K_2CO_3)	Buffer	138.21	Sigma-Aldrich, St Louis, MO, USA
Disodium hydrogen phosphate ($NaHPO_4$ anhydrous)	Buffer	141.98	Sigma-Aldrich, St Louis, MO, USA
Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)	Buffer	156.01	UNIVAR Analytical Reagent, NSW, Australia
Sodium chloride (NaCl)	Salt	58.44	UNIVAR Analytical Reagent, NSW, Australia
Sodium azide (NaN_3)	Preservative	65.01	Ajax Chemical, Sydney, Australia

Chemicals, Victoria, Australia) to every milliliter of plasma. The mixture was stirred gently for an hour at room temperature followed by centrifugation at 1200 x g for 30 min. The supernatant was then filtered (filter paper #1, Advantec Co, Tokyo, Japan) to remove the remaining charcoal. The steroid-free plasma (tested with LC-MS/MS assay to ensure the plasma is free from endogenous testosterone) was added to the washed red blood cells to obtain a hematocrit of 0.45. The artificial blood prepared was then spiked with stock solution of testosterone (T) and nandrolone (N) to attain final concentrations of 0.05-32 ng/mL for T and 0.156-10 ng/mL for N. QCs were made up from a separate lot of artificial blood at low, medium and high concentrations (0.1, 1, and 16 ng/mL for T; 0.3, 1.25 and 5 for N). Fifty microliters of standards and QCs was spotted onto filter card (Whatman 903 protein saver cards, WHAT10534612; GE Healthcare, Westborough, MA), allowed to dry overnight at room temperature and stored in a sealed plastic bag at room temperature until analysis.

2.1.3.2 DBS sample preparation

Steroids were extracted from the whole DBS which were cut and transferred into 15 mL disposable borosilicate glass tubes (16×150 mm). One milliliter of methanol:water (1:1) was added followed by 10 µL of internal standard (25 ng/mL d_3-T). The mixture was shaken mechanically for 30 min and then again for another 20 min after addition of methyl tert-butyl ether (MTBE) (1.5 mL). The tubes were centrifuged at 300 x g for 5 min and followed by the transfer of the organic layer into 5 mL disposable borosilicate glass tubes tube (12 × 75 mm). The solvent was evaporated to dryness in heating block at 50 °C overnight in a fume hood. The dried residue was reconstituted in 70 µL of 50% methanol in water, transferred into 96-well polypropylene V-bottom microtiter plate (PerkinElmer, MA, USA) and 45 µL was injected into the LC-MS/MS system.

2.1.3.3 Blood volume determination

We aimed to use the whole capillary blood volume on the filter card rather than taking a subsample (e.g. by a punch) as that risked non-homogenous distribution of blood impregnation on the filter paper. To determine the capillary blood volume on the filter card, a linear equation was derived from a plot of weight of blood on filter card (weight of blood-impregnated filter paper minus weight of unused filter paper) versus volume of blood spotted on filter card (R^2= 0.997) (Figure 2.1). To generate this calibration, venous blood was collected from a volunteer (hematocrit, 0.45) and immediately a set of increasing volume of blood (10-250 µL) was spotted using a calibrated pipette onto filter cards with five replicates. The spots were dried overnight, cut out and weighed. To determine the accuracy of this equation, known volumes (40, 50, 60, 70 µL) of whole venous samples collected from 10 male volunteers (hematocrits 0.40-0.50) were spotted onto filter cards in triplicate and dried overnight. The estimated weight of the blood spot on the filter card (using a Mettler balance AE240) was then compared with the nominal volume of blood and accuracy (nominal volume x 100/actual volume) was 95-106% with coefficient of variation (CV) of 0.2-7.1% for all volumes tested.

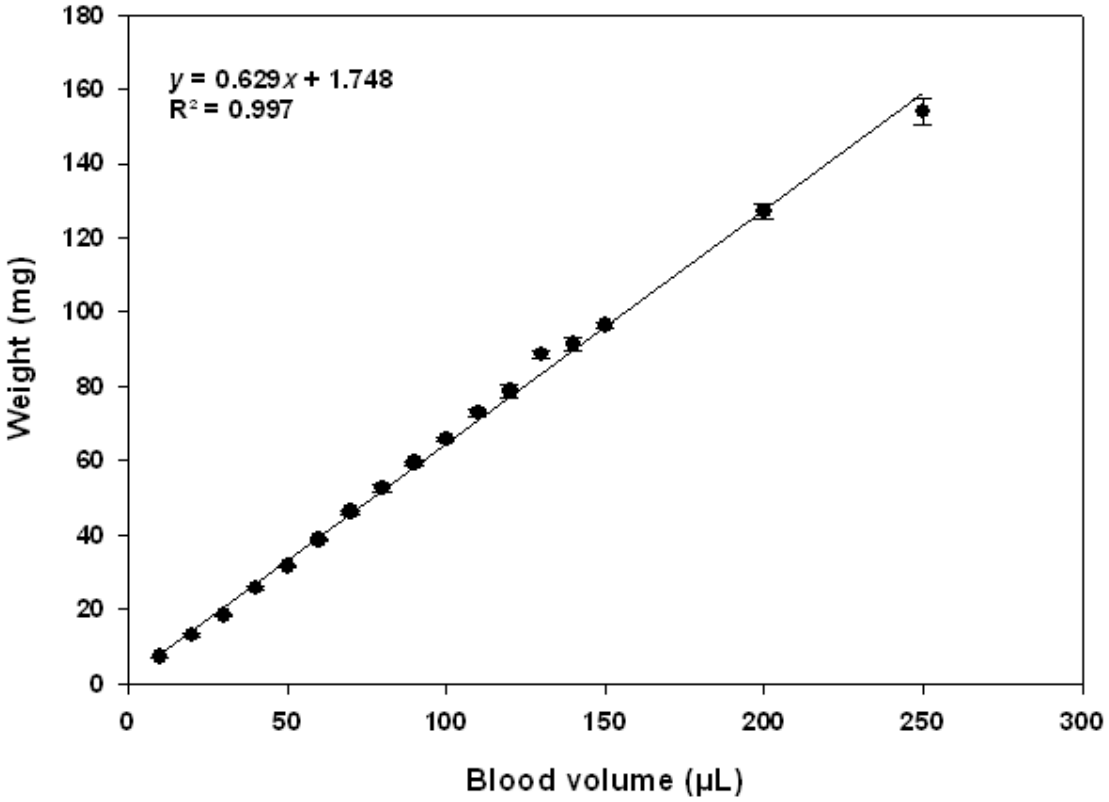


Figure 2.1 Relationship between amount of blood and weight of filter card (n=5).

To determine the capillary blood volume on the card, a linear equation derived from a plot; weight of blood on filter card versus volume of blood spotted on filter card (y= 0.629x + 1.748; R^2= 0.997) was used.

2.1.3.4 Comparison between whole DBS and fixed-sized disc

To determine the variation using whole DBS and fixed-sized paper disc, correlation between T venous DBS concentration obtained from two different sampling methods (1) whole blood spot and (2) 6 mm punch disc, were compared against plasma T concentration. For this purpose, venous blood was collected in lithium heparin tube from twenty male volunteers (hematocrit, 0.40-0.51). Fifty microliters of blood was spotted directly on the filter card using a calibrated pipette and the remaining blood was centrifuged at 1500 x g for 15 min at 4 °C to separate plasma. Plasma was extracted according to method described in Section 2.1.5.1. After overnight drying at room temperature, the DBS was either cut out entirely using a pair scissors or discs were punched out using a 6 mm puncher (n=3) from the same blood sample

followed by extraction and measured using LC-MS/MS assay. The plasma and DBS T concentration were correlated according to the formula [$plasma_T$ = DBS_T/(1-hematocrit)]. Venous whole DBS T was unbiased in relation to plasma T (standard deviation 6.3 ± 7.2 (SD) % in Bland-Altman analysis; Pearson correlation r=0.98) whereas the punch disc produced an upward bias (deviation 28 ± 9.2 %; r=0.97) (Figure 2.2). The finding indicates that the venous whole DBS T measurements correlate well with the corresponding plasma T levels. However, the punch disc overestimates the T concentration in comparison to plasma T levels. All further DBS measurements used whole blood spotted on the filter card and the volume of blood was determined by first cutting the whole DBS and weighing the blood spot followed by calculation using the equation from Figure 2.1 and adjusted for hematocrit.

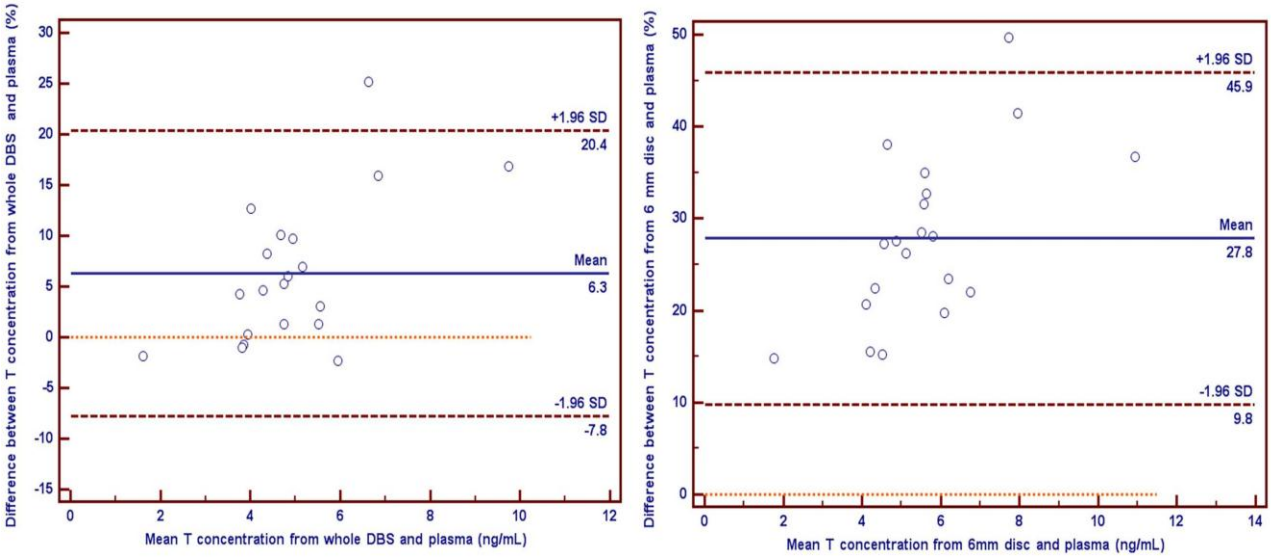


Figure 2.2 Bland-Altman plots for comparison of testosterone (T) concentration obtained from whole DBS versus plasma (left panel) and 6 mm punch disc versus plasma (right panel).

The Bland-Alman plots represent the difference between two sampling methods: 1) whole blood spot and 2) 6 mm punch disc against plasma T concentration. The solid and the dashed lines represents the observed average and 95% limit of confidence (± 1.96 SD), respectively.

2.1.3.5 LC-MS/MS parameters

The LC column was an Ascentis® Express Phenyl-Hexyl column (10 cm x 2.1 mm, 2.7 µm; Supelco, Sigma-Aldrich, PA; Cat. no. 53336-U) equipped with an Ascentis® Express Phenyl-Hexyl guard cartridge (0.5 cm x 2.1 mm, 2.7 µm; Supelco, Sigma-Aldrich, PA; Cat. no. 53524-U). The column temperature and autosampler were set at 35 and 4 °C, respectively. The elution solvents were water (A) (18MΩ quality; Millipore Milli-Q system, Bedford, MA, USA) and methanol (B). The gradient elution was performed at the flow rate of 0.75 mL/min with the organic solvent started with 10% B (0-0.10 min), 55-65% B (0.11-4.99 min), 100% B (5.00-5.70 min), 10% B (5.71-6.50 min). Toluene was used as the APPI dopant, delivered at the flow rate of 0.07 mL/min. T and N MS/MS analysis was carried out in positive ion mode. The MS conditions of N, T and d_3T are listed in Table 2.3. N, T and d_3T eluted at 3.62, 4.15 min and 4.19 min, respectively (Figure 2.3). The following MS parameters were used: ion source gas 1: 55 psi; ion source gas 2: 50 psi; curtain: 12 psi; collision gases: 6 psi; ion source temperature: 500 °C; ion spray voltage: 750V. Data were quantified using Analyst software (version 1.6, AB Sciex).

Table 2.3 Mass spectrometry parameters for nandrolone, testosterone and d3-testosterone measurements.

Steroid Ion Fragmented		MRM transition Q1→Q3	Dwell time (msec)	DP	EP	CE	CXP
N	$[M+H]^+$	275.2→109.1	500	190	10	40	12
T	$[M+H]^+$	289.3→109.1	500	190	10	35	12
d_3T	$[M+H]^+$	292.3→109.1	250	190	10	35	12

Abbreviations: N: nandrolone; T: testosterone; d_3T: deuterated testosterone; MRM: multiple-reaction-monitoring; DP: declustering potential; EP: entrance potential; CE: collision energy; CXP: collision cell exit potential.

2.1.3.6 Method validation

Linearity, lower limit of quantification (LLOQ), recovery, process efficiency, within and between day precision and accuracy were evaluated during the validation of the analytical method according to FDA guidance (FDA 2001). The linearity was evaluated on the basis of three calibration curves. Response of peak area ratio of T and N to internal standard (d_3T) was plotted against known steroid concentrations. The assay acceptance criterion for each back-calculated standard concentration was 15% CV from the nominal value. The LLOQ was evaluated by five replicates of the lowest concentrations of the calibration curve with the accepted deviation values of 20%. The calibration curve was quadratic for both T and N with 1/*x* weighing with correlation coefficient (r) greater than 0.999. The LLOQ was 50 pg/mL for T and 156 pg/mL for N using a 50 µL blood sample.

For within and between-day assay validation, QC samples were prepared in artificial blood as described in Section 2.1.3.1 with the T and N at three concentration levels of low, medium and high (0.1, 1, and 16 ng/mL for T; 0.3, 1.25 and 5 ng/mL for N) in five separate runs. The within-day precision (% CV) was 2.5-4.4% with accuracies of 95-108% and the between-day precision was 4.9-13.5% with accuracies of 100-106% at all QC levels (Table 2.4).

The recovery, matrix effect and process efficiency of the method was carried out according to the method described previously (Matuszewski et al. 2003) at low, medium and high concentrations of each compound. All extraction recoveries and process efficiencies were between 96-107% with no ion suppression or enhancement for either analyte (matrix effect recoveries between 96-102%). Refer Table 2.4 for the detailed validation data.

Specificity of T against isomers with same molecular weight, DHEA and epitestosterone (16 ng/mL) were evaluated. The retention time of these compounds did not co-elute with the retention time of T.

Stability of T and N on DBS was evaluated at 4°C, room temperature and 37°C for short term (42 days) and long term (742 days) at low, medium and high concentrations (0.1, 1, and 16 ng/mL for T; 0.3, 1.25 and 5 ng/mL for N) measured against fresh calibration spotted onto filter card and extracted immediately after overnight drying.

The stability of T on DBS was well maintained at 3 temperatures and all QCs tested with accuracies of 79.2-113.7% (CV 0.4 -11.8%) (Table 2.4). N was also stable on DBS at all 3 conditions and all QCs tested up to 42 days (accuracies 82.5-110.7%; CV 0.4-13%). Long term storage of DBS showed a decline in N stability at room temperature and 37°C in the medium and high QCs (accuracies 65.6-75.7%; CV 0.9-9.6%). The N stability was well maintained at 4°C for all QCs up to 742 days with accuracies of 99.9-116.6% (CV 4.2-7.7%) (Table 2.5).

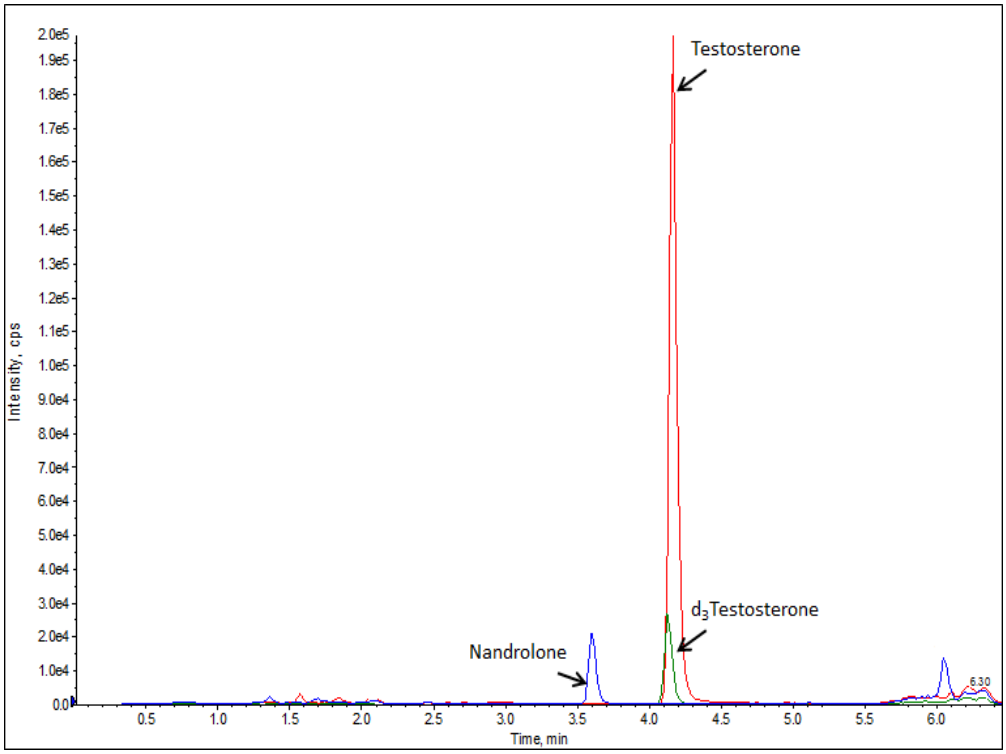


Figure 2.3 Dried blood spot method analytes chromatogram.

Table 2.4 LC-MS/MS DBS method validation data.

Analyte	LLOQ (pg/ml)	QC	Within-day (%)		Between-day (%)		ER (%)	ME (%)	PE (%)
			Accuracy	CV	Accuracy	CV			
T	50	Low	99.8	4.4	100.3	13.5	104.2	99.8	104.0
		Medium	98.4	3.0	103.1	5.2	106.4	95.6	101.7
		High	107.5	2.5	105.9	4.9	106.3	96.3	102.4
N	156	Low	95.0	3.0	101.5	10.6	106.5	95.8	102.0
		Medium	105.8	4.1	102.6	8.4	96.4	100.9	97.3
		High	105.2	3.8	103.8	5.7	99.5	101.7	101.2

Abbreviations: LLOQ: lower limit of quantitation; QC: quality control; ER: extraction recovery; ME: matrix effect; PE: process efficiency; CV: coefficient of variation

Table 2.5 Stability data of testosterone and nandrolone on DBS at three different storage conditions for short (42 days) and long term storage (742 days).

Time (days)	Storage condition	Testosterone						Nandrolone					
		Low		Medium		High		Low		Medium		High	
		Accuracy	CV	Accuracy	CV	Accuracy	CV	Accuracy	CV	Accuracy	CV	Accuracy	CV
3	RT	90.9	7.4	106.7	3.0	105.6	5.3	105.3	5.5	105.7	4.2	106.3	6.2
	4C	97.6	3.1	101.1	3.4	107.3	5.5	110.7	2.8	93.8	9.2	105.5	7.0
	37C	94.4	3.5	96.7	1.6	108.7	3.7	98.6	6.2	107.0	2.4	107.7	3.9
7	RT	91.8	9.0	98.7	4.0	103.9	4.0	99.4	6.8	94.6	5.2	96.3	2.2
	4C	101.3	5.5	104.7	3.9	105.3	0.6	108.3	0.9	103.3	4.3	100.7	1.2
	37C	94.0	11.6	99.5	3.5	108.3	0.9	90.4	4.5	103.2	3.6	101.7	1.4
14	RT	96.5	7.1	94.8	4.4	98.2	1.9	103.8	6.9	87.6	1.1	88.7	0.9
	4C	104.6	5.7	99.9	4.5	98.7	3.5	91.4	5.2	98.1	6.0	98.1	1.0
	37C	93.2	6.0	97.5	5.8	93.7	5.0	88.3	2.9	85.7	5.2	85.5	3.2
28	RT	99.0	8.0	97.0	1.5	101.5	4.7	85.7	2.1	86.6	12.2	91.5	3.8
	4C	88.1	8.1	103.0	8.3	106.0	2.4	94.2	9.5	94.8	2.6	97.0	4.6
	37C	87.0	11.8	95.0	4.4	95.6	4.4	86.4	13.0	82.5	8.1	88.4	6.4
42	RT	105.0	11.0	89.9	3.1	91.1	4.2	87.0	3.0	85.7	2.3	91.2	6.4
	4C	101.4	6.8	99.5	10.1	98.3	0.4	99.9	7.5	92.8	11.0	98.1	0.4
	37C	93.8	2.3	86.3	2.1	87.9	5.2	86.3	3.6	83.0	7.3	85.0	7.0
742	RT	107.0	8.1	92.9	1.4	94.4	0.7	101.4	4.3	75.7	9.6	74.0	4.0
	4C	109.3	6.7	104.3	2.4	107.7	2.9	111.6	7.7	109.63	7.4	99.9	4.2
	37C	113.7	5.2	89.2	4.3	79.2	3.9	106.7	3.0	74.1	6.6	65.6	0.9

Abbreviations: RT: room temperature; CV: coefficient of variation

2.1.4 LC-MS/MS method for urinary steroids analysis

2.1.4.1 Urine sample preparation

Urine samples were extracted by LLE. Urine (500 µL) was transferred into 15 mL disposable borosilicate glass tubes (16×150 mm), spiked with 10 µL of internal standards (25 ng/mL d_3T, 75 ng/mL d_4E_2, 200 ng/mL d_2DHEA and 250 ng/mL d_3DHT) and had 500 µL of 0.1M phosphate buffer (pH 6.2) and 10 µL β-glucuronidase from *Escherichia coli* K12 (Roche Diagnostic, Mannheim, Germany; Cat. no.: 03707598001) added. After overnight incubation at room temperature, 100 µL of potassium carbonate (20%: w/v) was added followed by 1.5 mL of MTBE. The mixture was shaken mechanically for 15 min, centrifuged at 2700 x g for 15 min followed by transfer of the organic layer into clean 5 mL disposable borosilicate glass tubes tube (12×75 mm) and evaporated to dryness in a heating block at 50 C overnight in a fume hood. The dried residue was reconstituted in 75 µL of 20% methanol in water, transferred into 96-well polypropylene V-bottom microtiter plate and 50 µL was injected into the LC-MS/MS system.

2.1.4.2 LC-MS/MS parameters

The LC elution solvents were water (A) and methanol (B). For urine extracts, separation was achieved on a Ascentis® Express Phenyl-Hexyl column (10 cm x 2.1 mm, 2.7 µm) equipped with a Ascentis® Express Phenyl-Hexyl guard cartridge (0.5 cm x 2.1 mm, 2.7 µm). The gradient elution was performed at the flow rate of 0.6 mL/min with the organic solvent starting with 10% B (0-0.10 min), 55% B (0.11-4.99 min), 65% B (5.00-6.50 min), 65-100% B (6.50-7.00 min), 100% B (7.01-8.00 min), 10% B (8.01- 9.50 min). The column temperature and autosampler were set at 40 and 4 C, respectively. Toluene was used as the APPI dopant, delivered at the flow rate of 0.06 mL/min.

Following LC separation, samples were subjected to MS/MS analysis. E_2 was detected in negative ion mode whereas the androgens (T, DHT and DHEA) were detected in positive ion mode. The optimization of MRM settings and LC-MS/MS system conditions for each analyte have been listed in Table 2.6. The following MS parameters were used: ion source gas 1: 55 psi; ion source gas 2: 50 psi; curtain: 12 psi; collision gases: 6 psi; ion source temperature: 500 °C; ion spray voltage: 750V (for positive mode) and -750V (for negative mode).

Table 2.6 Mass spectrometry parameters for estradiol, testosterone, dihydrotestosterone, dehydroepiandrosterone and its corresponding isotopically labeled internal standards.

Steroid	Ion Fragmented	MRM transition Q1→Q3	Dwell time (msec)	DP	EP	CE	CXP
E_2	$[M-H]^-$	271.1→145.0	100	-100	-10	-57	-15
d_4E_2	$[M-H]^-$	275.1→147.0	75	-100	-10	-57	-15
T	$[M+H]^+$	289.3→109.1	100	80	10	35	15
d_3T	$[M+H]^+$	292.3→109.1	75	80	10	35	15
DHT	$[M-H_2O+H]^+$	273.3→123.3	100	80	10	31	15
d_3DHT	$[M-H_2O+H]^+$	276.3→123.3	75	80	10	31	15
DHEA	$[M-2H_2O+H]^+$	253.1→197.1	100	80	10	30	15
d_2DHEA	$[M-2H_2O+H]^+$	255.3→197.2	75	80	10	30	15

Abbreviations: E_2: estradiol; d_4E_2: deuterated estradiol T: testosterone; d_3T: deuterated testosterone; DHT: dihydrotestosterone; d_3DHT: deuterated dihydrotestosterone; DHEA: dehydroepiandrosterone; d_2DHEA: deuterated dehydroepiandrosterone; MRM: multiple-reaction-monitoring; DP: declustering potential; EP: entrance potential; CE: collision energy; CXP: collision cell exit potential; Q1: first quadrupole; Q3: third quadrupole.

2.1.4.3 Method validation

The LC-MS/MS method for urinary steroid quantitation was validated for linearity, LLOQ, recovery, matrix effects, process efficiency, within and between day precision and accuracy according to FDA guidance for bioanalytical method validation (FDA 2001). For the validation and calibration, blank urine comprised a pool of urine from two children aged 3-4 years of age was used. The linearity was evaluated on the basis of three calibration curves. Response of peak area ratio of the steroids to internal standard was plotted against known steroid concentrations using quadratic regression with $1/x$ weighing. The assay acceptance criterion for each back-calculated standard concentration was 15% CV from the nominal value. The LLOQ was evaluated by five replicates of the lowest concentrations of the calibration curve with the accepted deviation values of 20% using 500 µL of urine.

The calibration curves fitted quadratic functions ranging from; 0.05-32 ng/mL E_2, 0.025-32 ng/mL T, 0.1-32 ng/mL DHT and 0.2-128 ng/mL DHEA, with correlation coefficient (r) greater than 0.999. The precision was 2.6-7.2% (within-day) and 4.8-9.7% (between-day) with accuracies of 97-110% (within-day) and 95-108% (between-day) for all QC levels (steroids spiked as glucuronides at 0.2, 4 and 32 ng/mL for T; 0.8, 4 and 32 ng/mL for E_2, DHT and DHEA) (Table 2.8).

The recovery, matrix effects and process efficiency were quantified as described previously (Matuszewski et al. 2003) at low (0.4 ng/mL), medium (1.6 ng/mL) and high (16 ng/mL) concentrations of each compound. The extraction recovery and process efficiency were between 91-118%, whereas the matrix effect recoveries were between 92-119% for all the analytes spiked at low, medium and high concentrations (Table 2.8).

The β-glucuronidase enzyme was evaluated for hydrolysis efficiency at room temperature (overnight). Five replicates were spiked with testosterone glucuronide (TG), estradiol

glucuronide (E_2G), dihydrotestosterone glucuronide (DHTG) and dehydroepiandrosterone glucuronide (DHEAG) at 40 ng/mL and another five replicates were spiked with the unconjugated steroid at the equivalent amount (25 ng/mL of T, DHT and DHEA; 24 ng/mL of E_2). The samples were then extracted according to the method described in section 2.1.4.1. The area ratios of the analyte to internal standards were compared and the enzyme deconjugation efficiency was between 88-102% after overnight incubation at room temperature for all the analytes.

For specificity, structurally related compounds that potentially may interfere with the method were also evaluated (Table 2.7). All the steroids listed did not interfere with the retention time of E_2, T, DHT and DHEA.

Table 2.7 List of possible interfering compounds for LC-MS/MS urinary and serum methods.

Analyte	Possible interfering compound(s)	Manufacturer
E_2	Estrone (1,3,5[10]-estratrien-3-ol-17-one)	Steraloids, Newport, RI, USA
T	Epitestosterone (17α-hydroxyandrost-4-ene-3-one) Dehydroepiandrosterone (5-androsten-3β-ol-17-one)	National Measurement Institute, Sydney Australia
DHT	Androsterone (17β-hydroxy-5α-androstan-3-one), 3α-diol (5α-androstane-3α,17β-diol) 3β-diol (5α-androstane-3β,17β-diol)	National Measurement Institute, Sydney Australia
	Etiocholanolone (5β-Androstan-3α-ol-17-one), Epietiocholanolone (5β-Androstan-3β-ol-17-one), 5-androstenediol (5-androsten-3β,17β-diol), Epiandrosterone (5α-androstan-3β-ol-17-one),	Steraloids, Newport, RI, USA

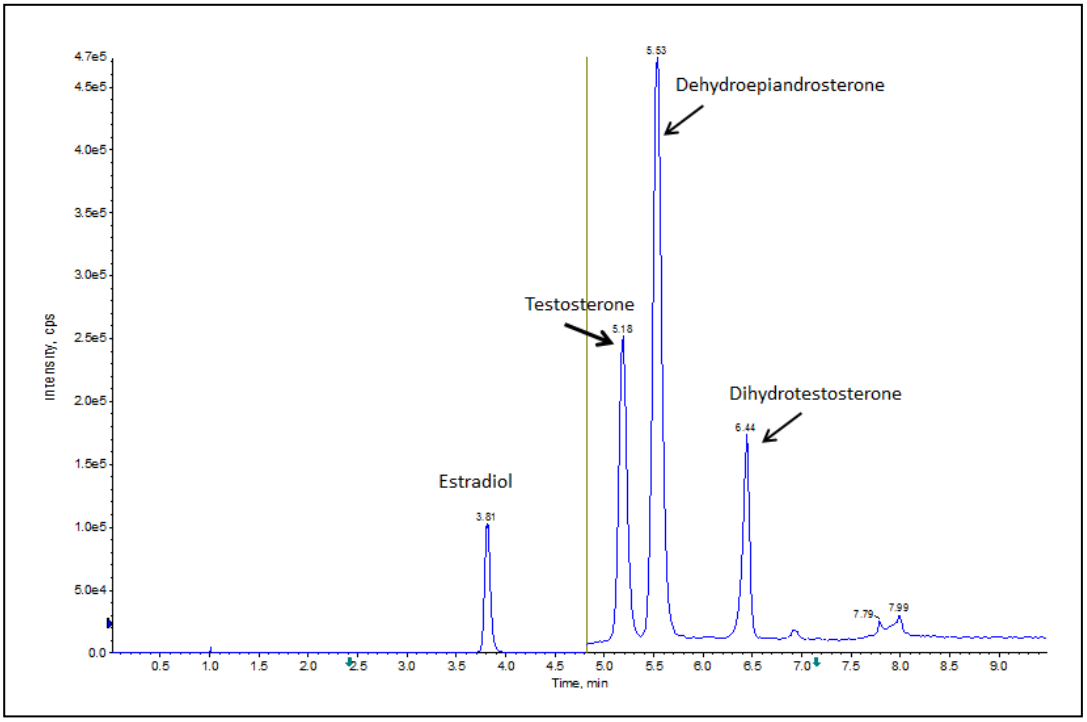


Figure 2.4 Urinary steroids method chromatogram.

Table 2.8 LC-MS/MS urinary steroids method validation data.

Analyte	LLOQ (pg/ml)	QC	Within-day (%)		Between-day (%)		ER (%)	ME (%)	PE (%)
			Accuracy	CV	Accuracy	CV			
E_2	50	Low	99.9	4.5	95.4	6.3	97.0	106.4	103.2
		Medium	104.4	3.4	100.1	5.6	96.0	101.6	97.5
		High	105.0	2.6	104.2	9.7	95.1	106.3	101.1
T	25	Low	97.2	3.6	103.2	7.2	97.3	103.9	101.1
		Medium	100.7	4.4	104.3	7.8	94.4	106.0	100.0
		High	102.4	2.8	95.6	6.7	92.7	107.9	100.0
DHT	100	Low	103.4	7.2	99.1	6.6	100.5	105.0	105.6
		Medium	102.6	3.7	99.7	5.8	103.3	91.7	94.7
		High	102.6	4.4	95.6	7.7	90.6	107.7	97.6
DHEA	200	Low	110.2	5.2	97.3	4.8	97.5	118.6	115.6
		Medium	101.1	5.5	101.4	7.8	101.7	117.3	118.1
		High	99.3	5.6	107.9	6.4	98.5	111.5	109.8

Abbreviations: LLOQ: lower limit of quantitation; QC: quality control; ER: extraction recovery; ME: matrix effect; PE: process efficiency; CV: coefficient of variation.

2.1.5 LC-MS/MS method for serum steroids analysis

2.1.5.1 Serum sample preparation

Serum samples were extracted by LLE. Serum aliquots (200 µL) were transferred into 5mL disposable borosilicate glass tubes (12×75 mm), spiked with 50 µL of internal standard (5 ng/mL d_3T and d_4E_2; 6.25 ng/mL d_3DHT; 7.5 ng/mL d_2DHEA) and had 1 mL of MTBE added. The tubes were vortex mixed for 1 min, allowed to phase separate at 4 °C for 1 h before being placed in a −80 °C freezer for 30 min to freeze the lower aqueous layer. The upper organic layer was decanted into clean glass tubes and the solvent evaporated overnight at 50 °C in a fume hood. The residue was then reconstituted in 75 µL of 20% methanol in water, transferred into 96-well polypropylene V-bottom microtiter plate and 50 µL was injected into the LC-MS/MS system.

2.1.5.2 LC-MS/MS parameters

LC separation was achieved on a Phenomenex Kinetex XB-C18 column (5 cm x 2.1 mm, 1.7 µm; Cat. no. OOB-4498-AW) protected by Phenomenex C18 guard cartridge (Cat. no. AJO-8782). The LC column temperature and autosampler were set at 40 and 4 °C, respectively. The elution solvents were water (A) and methanol (B). A gradient elution was performed at a flow rate of 0.5 mL/min with 25% B (0-0.10 min), 52-62% B (0.11-4.30 min), 100% B (4.31-5.45 min), 25% B (5.46-7.00 min). Toluene was used as the APPI dopant, delivered at the flow rate of 0.05 mL/min. The optimization of LC-MS/MS MRM settings and system conditions for serum assay was identical with the urine assay. Please refer section 2.1.4.2 for the MS/MS parameters.

2.1.5.3 Method validation

The LC-MS/MS method for urinary steroid quantitation was validated for linearity, LLOQ, recovery, matrix effects, process efficiency, within and between day precision and accuracy according to FDA guidance for bioanalytical method validation (FDA 2001). For the validation and calibration, 4% bovine serum albumin in phosphate buffer was used. The linearity was evaluated on the basis of three calibration curves. Response of peak area ratio of

the steroids to internal standard was plotted against known steroid concentrations using quadratic/linear regression (according to analyte) with 1/*x* weighing. The assay acceptance criterion for each back-calculated standard concentration was 15% CV from the nominal value. The LLOQ was evaluated by five replicates of the lower concentrations of the calibration curve with the accepted deviation values of 20% using 200 µL of serum.

The calibration curves were quadratic for T and DHEA (ranging between 0.01-16 ng/mL and 0.02-32 ng/mL, respectively) and linear for E_2 and DHT (ranging between 5-800 pg/mL and 0.01-16 ng/mL, respectively) with the r greater than 0.999. The precision was 5.3-13.5% (within-day) and 8.1-15.0% (between-day) with accuracies of 90-113% (within-day) and 95-111% (between-day) for all QC levels (0.01, 0.04 and 0.4 ng/mL for E_2; 0.025, 0.8, and 8 ng/mL for T; 0.2, 0.8 and 8 ng/mL for DHT; 0.05, 1.6, and 16 ng/mL for DHEA) (Table 2.9).

The recovery, matrix effects and process efficiency were quantified as described previously (Matuszewski et al. 2003) at low, medium and high concentrations of each compound (0.005, 0.08 and 0.4 ng/mL for E_2; 0.05, 0.4, and 8 ng/mL for T and DHT; 0.02, 0.8, and 16 ng/mL for DHEA). All the extraction recovery and process efficiency were within the range of 79-113%, whereas, the matrix effect were between the range of 81-107% for all the analytes spiked at low, medium and high concentrations (Table 2.9).

For specificity, structurally related compounds that potentially may interfere with the method were also evaluated (Refer Table 2.7 for compound list). All the steroids listed did not interfere with the retention time of E_2, T, DHT and DHEA.

Table 2.9 LC-MS/MS serum steroids method validation data.

Analyte	LLOQ (pg/ml)	QC	Within-day (%)		Between-day (%)		ER (%)	ME (%)	PE (%)
			Accuracy	CV	Accuracy	CV			
E_2	5	Low	96	13.0	101	14.5	79	101	80
		Medium	90	11.2	104	11.0	103	93	96
		High	103	7.7	104	9.9	111	81	89
T	25	Low	104	13.4	103	15.0	92	87	91
		Medium	97	5.3	103	9.0	92	83	83
		High	113	8.4	105	11.0	100	103	105
DHT	100	Low	101	13.5	111	12.9	89	88	91
		Medium	102	8.9	95	10.4	112	107	113
		High	107	8.9	100	11.3	87	102	89
DHEA	50	Low	99	12.5	106	14.6	98	97	100
		Medium	110	7.1	106	8.1	99	104	95
		High	107	5.6	107	9.7	91	91	100

Abbreviations: LLOQ: lower limit of quantitation; QC: quality control; ER: extraction recovery; ME: matrix effect; PE: process efficiency; CV: coefficient of variation.

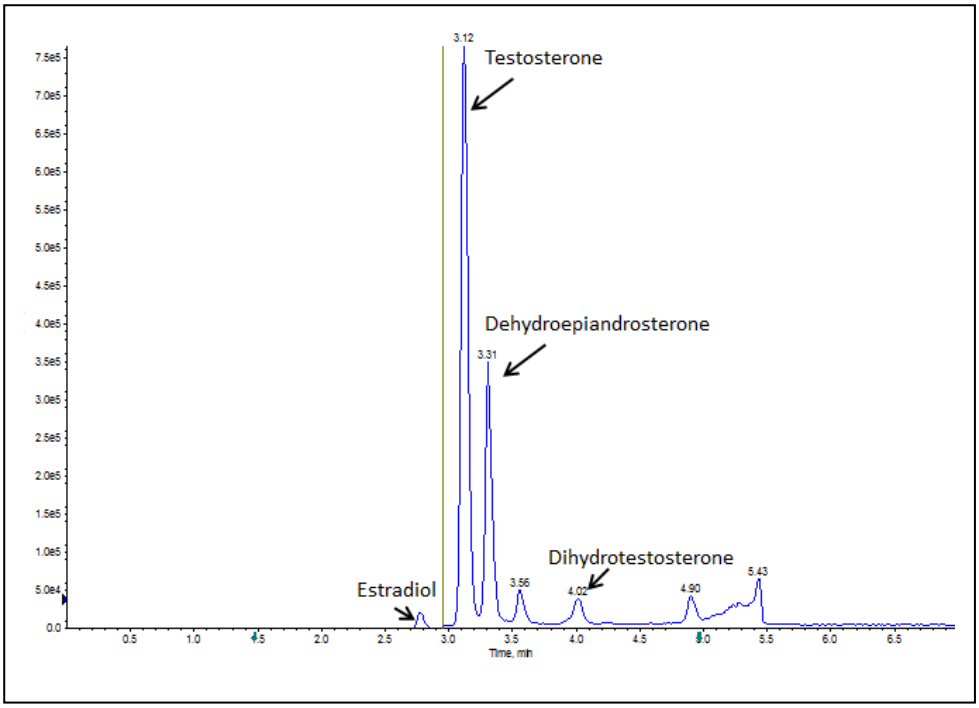


Figure 2.5 Serum steroids method chromatogram.

2.2 LH immunoassay

Urine and serum LH were measured by commercially available immunoassays an ICL immunoassay system: IMMULITE® 1000 LH, Siemens and IF assay: Delfia hLH assay, Perkin Elmer, Rowville, Melbourne, Victoria. The within-assay CV were <10%. Urine FSH assays (Immulite, DELFIA) did not pass validity tests (dilutional linearity, quantitative spike recovery) and were not used in this study.

2.2.1 Immulite

The ICL is a solid-phase two-site chemiluminescent immunometric assay that detects intact LH and LHβ subunit. The solid phase of the system consists of a polystyrene bead sealed in the test unit and coated with a capture monoclonal antibody against LH. The standards or samples (approximately 100 µL) were first aliquot in the disposable sample cups. Each sample cup was loaded on the loading rack followed by a test unit containing the bead. The sample (50 µL) and alkaline phosphatase (conjugated to polyclonal goat anti-LH) were automatically pipetted into the test unit by the programmed Immulite system. Following incubation (with intermittent agitation), the reaction fluid was removed by high speed mixing followed by series of washing to remove the unbound material. Chemiluminescent substrate added to the complex undergoes hydrolysis through the action of the alkaline phosphatase enzyme creating unstable anion that emits sustained light emission measured by the photon counter.

2.2.2 DELFIA

The IF assay utilizes the immobilized monoclonal human LHβ subunit as capture antibody and europium-labeled human LHβ subunit as detector antibody. The assay was carried out according to the manufacturer instructions. Briefly, 25 µL of standards and samples were aliquot in microtitration strip coated with mouse monoclonal anti-hLH followed by 200 µL of

assay buffer. The strips were incubated for an hour on a plate shaker (Wallac 1296-001 Plateshaker) at room temperature. The strips were then aspirated and each strip was washed with a plate washer (Wallac 1296-026 Platewasher). Two hundred microliter of tracer solution (europium labeled anti-β human LH IgG) was added followed by 15 min incubation on a shaker at room temperature. The strips were aspirated and washed before adding 200 µL of enhancement solution. The fluorescence was read using a time-resolved fluorometer (Perkin Elmer Multimode Plate Reader Enspire).

2.3 Urine creatinine measurement

Creatinine concentrations were determined by the colorimetric alkaline-picrate (Jaffé) method (CREJ2, Roche Diagnostics, Cat. No. 04810716 190) on a Cobas C501 analyzer (Roche Diagnostics GmbH, Indianapolis, IN). Calibrators (Roche Diagnostics, Cat. No. 10759350 190) were used for this automated system to generate a linear curve ranging between 375 and 55000 µmol/L and the limit of detection of 375 µmol/L. The assay requires 13 µL of potassium hydroxide (900 mmol/L, phosphate 135 mmol/L, pH > 13.5) and 17 µL of picric acid (38 mmol/L, pH 6.5) diluted in 77 µL and 30 µL of distilled water, respectively for sample volume of 10 µL. All reagents were from ready to use cassettes and were handled automatically by the analyzer.

2.4 Urine specific gravity measurement

Urine specific gravity (SG) was measured by immersing a reagent strip (ChoiceLine 10, Roche Diagnostics) in freshly voided urine sample. Dipstick color changes were compared visually with the color chart to estimate the SG (range between 1.000 and 1.030; 0.005 unit difference between consecutive colour).

Chapter 3

Pharmacokinetic-Pharmacodynamic Study of Subcutaneous Injection of Depot Nandrolone Decanoate Using Dried Blood Spots Sampling Coupled With LC-MS/MS Assays

The content in this chapter has been published in The Journal of Clinical Endocrinology and Metabolism 2014, 99: 2592-2598

Chapter 3 Pharmacokinetic-Pharmacodynamic Study of Subcutaneous Injection of Depot Nandrolone Decanoate Using Dried Blood Spots Sampling Coupled With LC-MS/MS Assays

3.1 Introduction

Since the first clinical use of T in 1937 (Hamilton 1937), two years after its Nobel Prize-winning discovery as the primary mammalian androgen (Butenandt and Hanisch 1935, David et al. 1935, Ruzicka and Wettstein 1935), its sole unequivocal therapeutic indication remains as replacement therapy for T deficiency states due to usually life-long hypothalamic, pituitary or testicular disorders (Handelsman 2010). Although exogenous T can be administered by implantable, transdermal or oral products, depot injectable formulations have long been the most widely used and affordable products for delivering T treatment aiming to maintain long-term adherence and sustained effective T replacement therapy for many years.

Depot injectable T products consist of T esterified to fatty acid side-chains with the length increasing hydrophobicity for partitioning into the vegetable oil vehicle as a depot for deep intramuscular (im) injection. These injections require skilled medical personal for slow administration, usually into the upper outer quadrant of gluteal muscle. While self-injection is feasible for the dexterous, most patients consider it too difficult so self-administration is rare. Im injections cause local pain or discomfort (Mackey et al. 1995, Sartorius et al. 2010), risk local injection site infection or bruising and, rarely, pulmonary oil micro-embolisation (Gu et al. 2009, Mackey et al. 1995, Svendsen and Aaes-Jorgensen 1979, Svendsen et al. 1980). Among patients with bleeding disorders or those taking anti-coagulants or antiplatelet therapy, deep im injections may be less suitable due to risk of im hematoma. In those situations daily-use oral or transdermal T products can be substituted; however, they have

high rates of discontinuation (Schoenfeld et al. 2013) as they lack the convenience of low-demand reliability of long-acting depot T products. Hence, a more convenient, self-administered method to inject T esters would be advantageous and cost-saving.

Clinical pharmacological studies to define drug pharmacokinetics and pharmacodynamics requires frequent blood sampling to generate sufficient data to define the time of peak and peak concentrations as well as circulating half-times and other derived pharmacological variables (Gibaldi and Perrier 1982, Minto et al. 1997). For long-acting depot products, this makes necessary many clinic visits for venesection followed by sample processing to separate serum for frozen storage until assay. These features add greatly to cost and complexity of conducting such pharmacological studies. We previously validated DBS technology for field studies of steroid pharmacology (Howe and Handelsman 1997) and now apply this remote sampling technology for repeated blood sampling at convenient times in the participants own home. This eliminate the need for medical professional to take blood samples, process and freeze samples while reducing the total blood sampled. As the time course of subcutaneous (sc) injections of steroids in an oil vehicle is not reported and may differ from the im route of administration (Handelsman et al. 1995, Minto et al. 1997), we investigated the pharmacokinetics of a single sc injection of nandrolone decanoate (ND) and the pharmacodynamics of its effects on endogenous serum T. As a generic androgen ester in an oil vehicle, this may demonstrate the feasibility of sc injection of T esters in an oil vehicle with potential to simplify injectable T replacement therapy. Nandrolone (N), also known as 19-nortestosterone has structural similarities with T but lacking a methyl group at the 19^{th} position (Hemmersbach and Grobe 2009) (Figure 3.1). ND consists of a long aliphatic side chain (10 carbons) (Figure 3.1) that provides sustained release of this steroid into the circulation. ND is further hydrolyzed into its active steroid, N which inhibits the pituitary

gonadotropin release through the negative feedback loop resulting in decrease of endogenous T production (Minto et al. 1997).

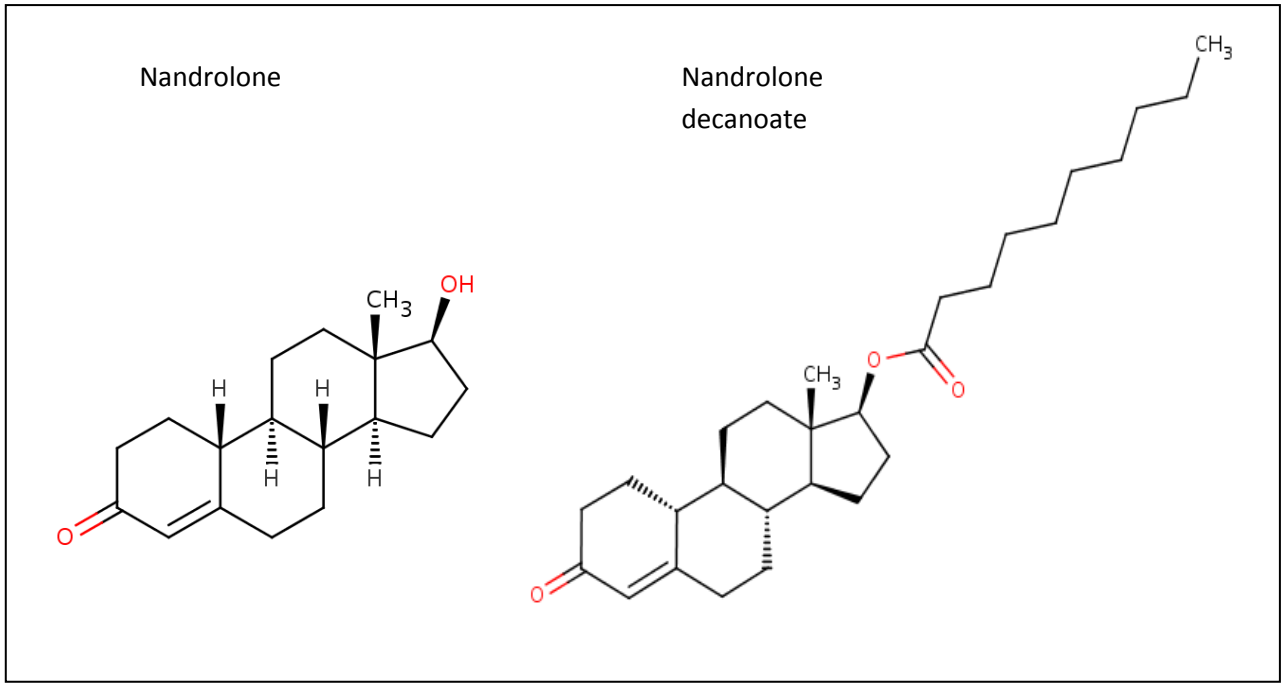


Figure 3.1 Structures of nandrolone and nandrolone decanoate.

Nandrolone decanoate, a synthetic anabolic steroid has a long duration of action due to its 10-carbon aliphatic side chain. Nandrolone decanoate is released into the extracellular fluid gradually with oil/water partitioning of the steroid ester from the oil vehicle with rapid hydrolysis of the ester to form active steroid (nandrolone) in the bloodstream (Minto et al. 1997).

3.2 Study Design

Healthy eugonadal volunteer men (n=8, age 31 ± 10 (SD) year, height 175 ± 7 cm, weight 80.7 ± 0.8 kg, BMI 26.3 ± 3.0 kg/m^2) were administered 100 mg ND in 2 mL arachis oil vehicle (MSD, Australia) by sc injection (21 gauge needle (0.8x25mm) Becton Dickinson Medical) into a single subdermal abdominal site. Participants were instructed in collecting capillary blood by finger prick using a single-use lancet (BD Microtainer, Contact-Activated Lancet 2.0x1.5mm; Becton Dickinson, Franklin Lakes NJ 07417) and applying blood onto filter cards (McDade 2014). Blood spots were applied to the 1st, 3rd and 5th of the five pre-

marked rings allowing for a gap to avoid overlap of blood samples. Capillary blood was obtained before and daily for 21 days recording the exact time of sampling. Venous blood was also sampled before and at weekly intervals to store serum and to spot venous blood directly onto filter cards. Serum samples were stored at -20°C until assayed. The dried blood spots were allowed to dry overnight at room temperature and were then stored in a sealed paper bag at room temperature until extracted. The participants provided written informed consent and the study was approved by the Sydney Local Health District Human Ethics Committee within National Health and Medical Research Council Guidelines for Human Experimentation (NHMRC) guideline for human experimentation. Volunteers received $200 for their time and effort on completing the full study (5 visits, 22 finger-prick blood samples).

3.3 Data analysis

Comparison of T and N concentrations in different fluids (serum, capillary blood, venous blood) was performed by non-parametric (Passing-Bablok) regression, deviance analysis of differences (modified Bland-Altman) and Spearman rank correlation using NCSS and MedCalc software. Peak or nadir concentrations and time of peak or nadir were determined directly from the serial daily hormone concentrations as well as from population pharmacokinetic/pharmacodynamic modelling using the exact time of finger-prick sampling (rather than nominal day) by non-linear curve fitting (Gibaldi and Perrier 1982, Minto et al. 1997). Data were expressed as mean and standard error of mean (SEM) for data with a Gaussian distribution and median (range) otherwise.

3.4 Results

All scheduled samples (finger-prick, venous) were collected without complaints about finger-prick blood collection. There were no complaints of discomfort following the injections. Hematocrit was unchanged by participation in the study (pre 0.45 ± 0.01, post 0.45 ± 0.01, difference 0.0 ± 0.01 or -0.5 ± 2.4 % of baseline, paired t-test $p>0.8$).

Capillary T and N concentration displayed a high correlation ($r=0.95$, 0.96, respectively) and was unbiased (mean deviation -2.5 ± 60(SD) %, -27 ± 39 %) compared with measurements in serum for T (Figure 3.2) and for N (Figure 3.3). Similar findings were obtained with high correlation ($r=0.97$ for all) and lack of bias between venous whole blood spot and serum or capillary T and N.

Serum N rose to a peak concentration of 2.50 ± 0.25 (SEM) ng/mL at a median (range) of 6 (4-13) days (Figure 3.4). Serum T declined from 3.50 ± 0.57 ng/mL at baseline to a nadir of 0.38 ± 0.13 (SEM) ng/mL representing 89 ± 3% maximal suppression at a median (range) of 8 (5-16 days) (Figure 3.5). Using non-linear curve fitting, N peaked at 1.72 ng/ml at 6.4 days while serum T reached a nadir of 0.53 ng/ml (suppressed by 2.9 ng/ml) at 10.3 days (Figure 3.5). Capillary T and N displayed virtually identical patterns following sc ND injection (Figure 3.6). Serum LH and FSH were both suppressed maximally at day 7 (median 7 days, range 7-14 days) to reach a nadir of 1.2 IU/L (LH) and 1.1 IU/L (FSH) after injection, representing a median 60% and 49% suppression from baseline, respectively, and recovered towards, but not full reaching, baseline by day 21 (Figure 3.6).

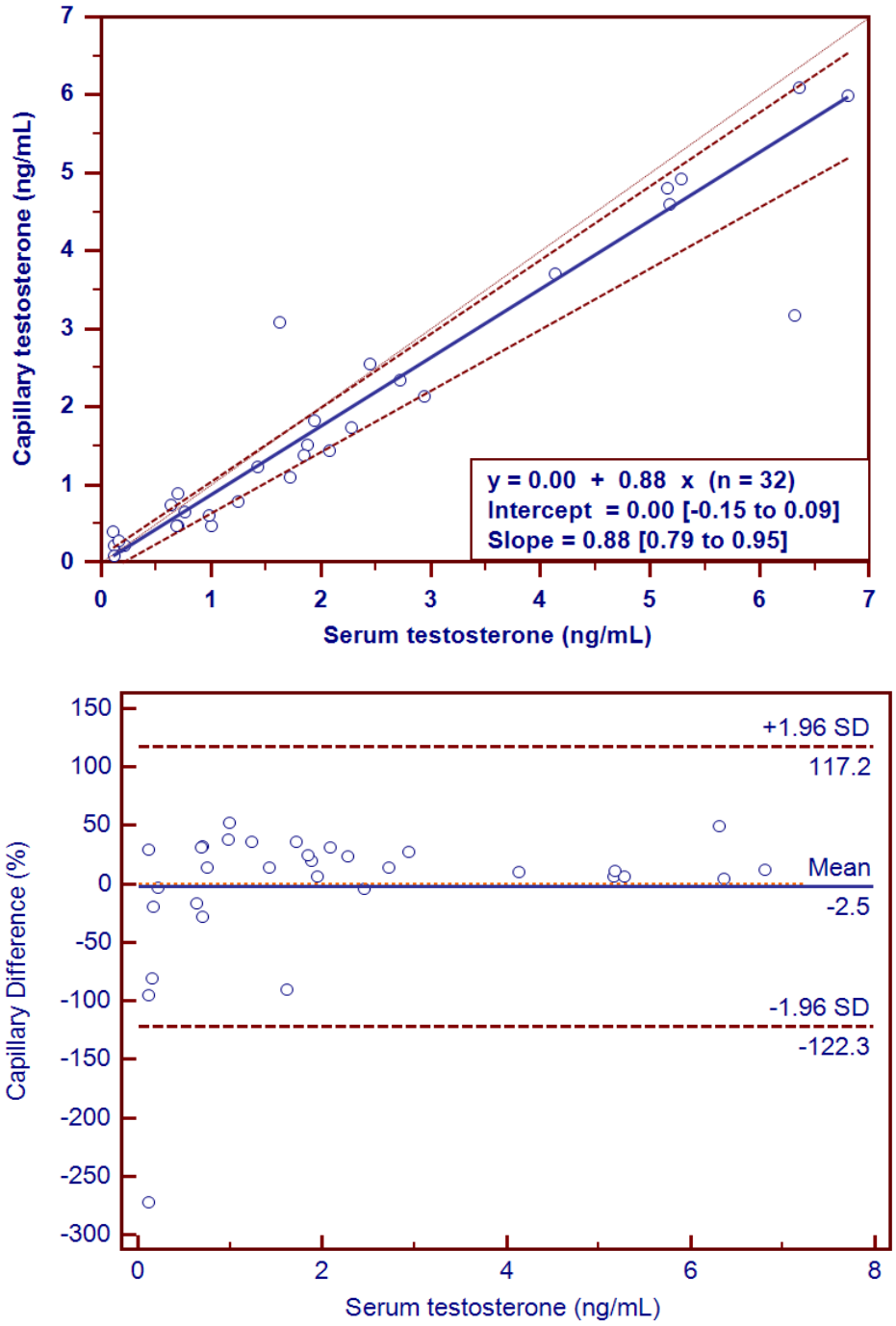


Figure 3.2 Plot of correlation (Passing-Bablok-upper panel) and deviance (modified Bland-Altman-lower panel) of individual capillary and serum testosterone in 32 samples obtained from 8 participants before and weekly for 3 weeks after sc injections of ND

Line of best fit is shown as a solid line with upper and lower 95% confidence intervals in dashed lines and the line of identity shown as a dotted line. Inset are the (Passing-Bablok) regression formula and 95% confidence limits on intercept and slop. The deviance (modified Bland-Altman) plot show the deviation of capillary from serum testosterone expressed as a percentage. For further details see text.

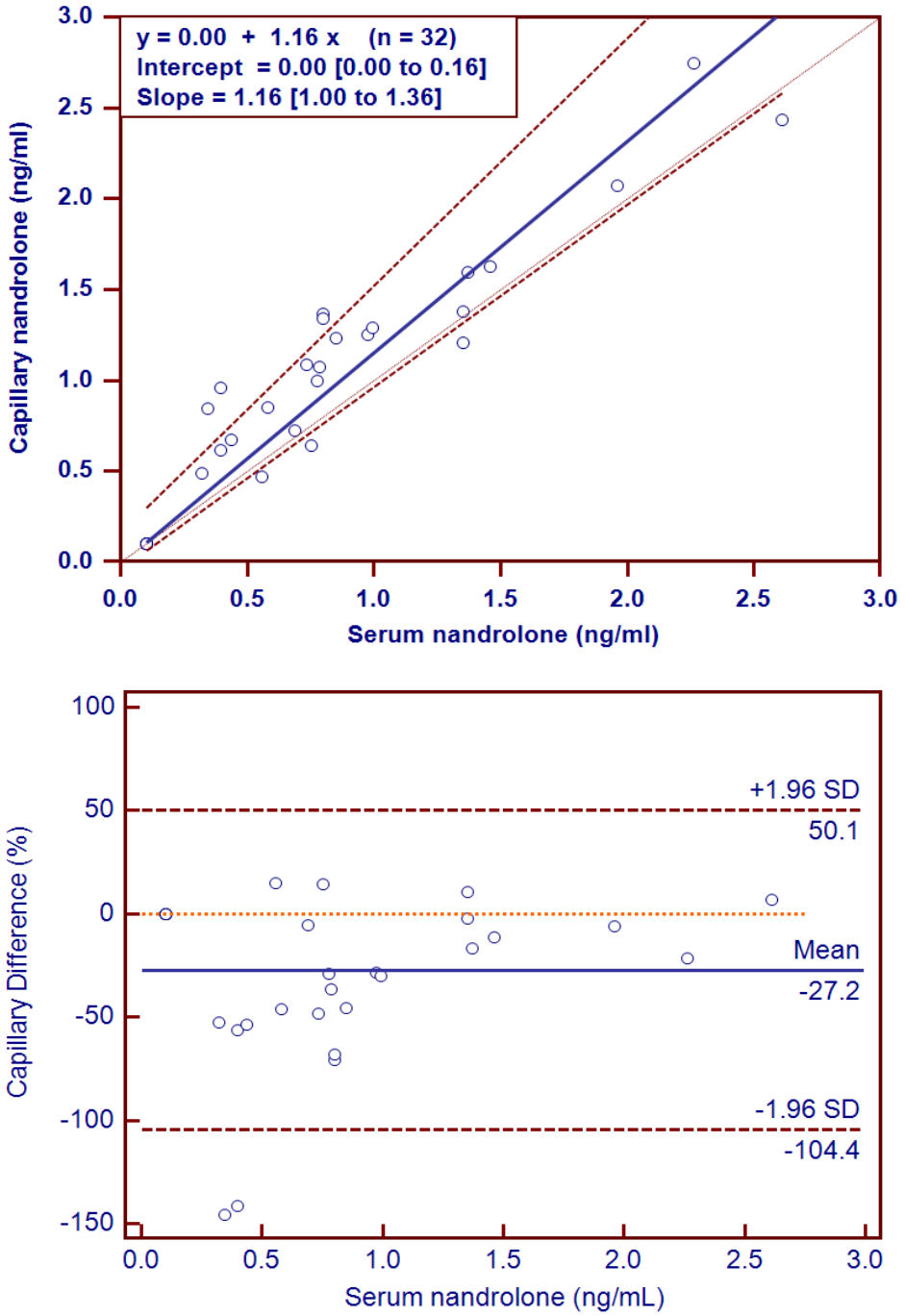


Figure 3.3 Plot of correlation (Passing-Bablok-upper panel) and deviance (modified Bland-Altman-lower panel) of individual capillary and serum nandrolone in 32 samples obtained from 8 participants before and weekly for 3 weeks after sc injections of ND.

Line of best fit is shown as a solid line with upper and lower 95% confidence intervals in dashed lines and the line of identity shown as a dotted line. Inset are the (Passing-Bablok) regression formula and 95% confidence limits on intercept and slop. The deviance (modified Bland-Altman) plot showing the deviation of capillary from serum nandrolone expressed as a percentage. For further details see text.

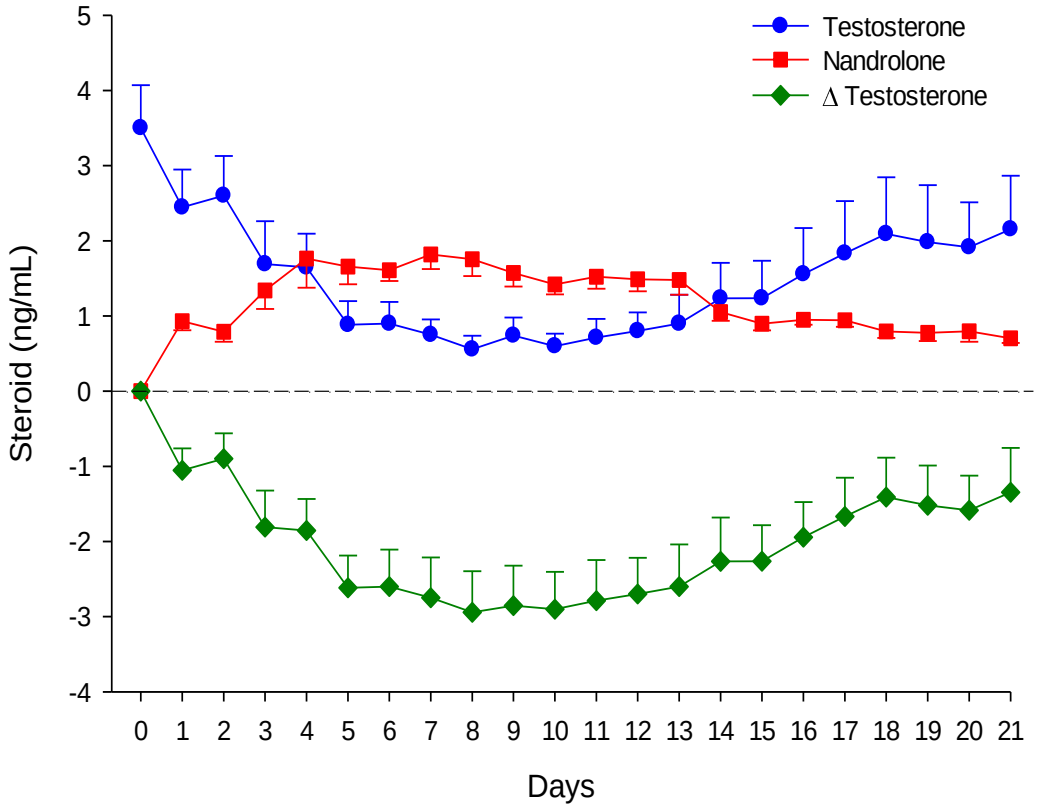


Figure 3.4 Plot of mean and SEM of capillary nandrolone, testosterone and change in testosterone from pre-injection baseline in 8 participants before and daily for 21 days after subcutaneous injections of 100 mg nandrolone decanoate in 2 mL arachis oil.

Note: Some SEM are not visible when smaller than the data symbol.

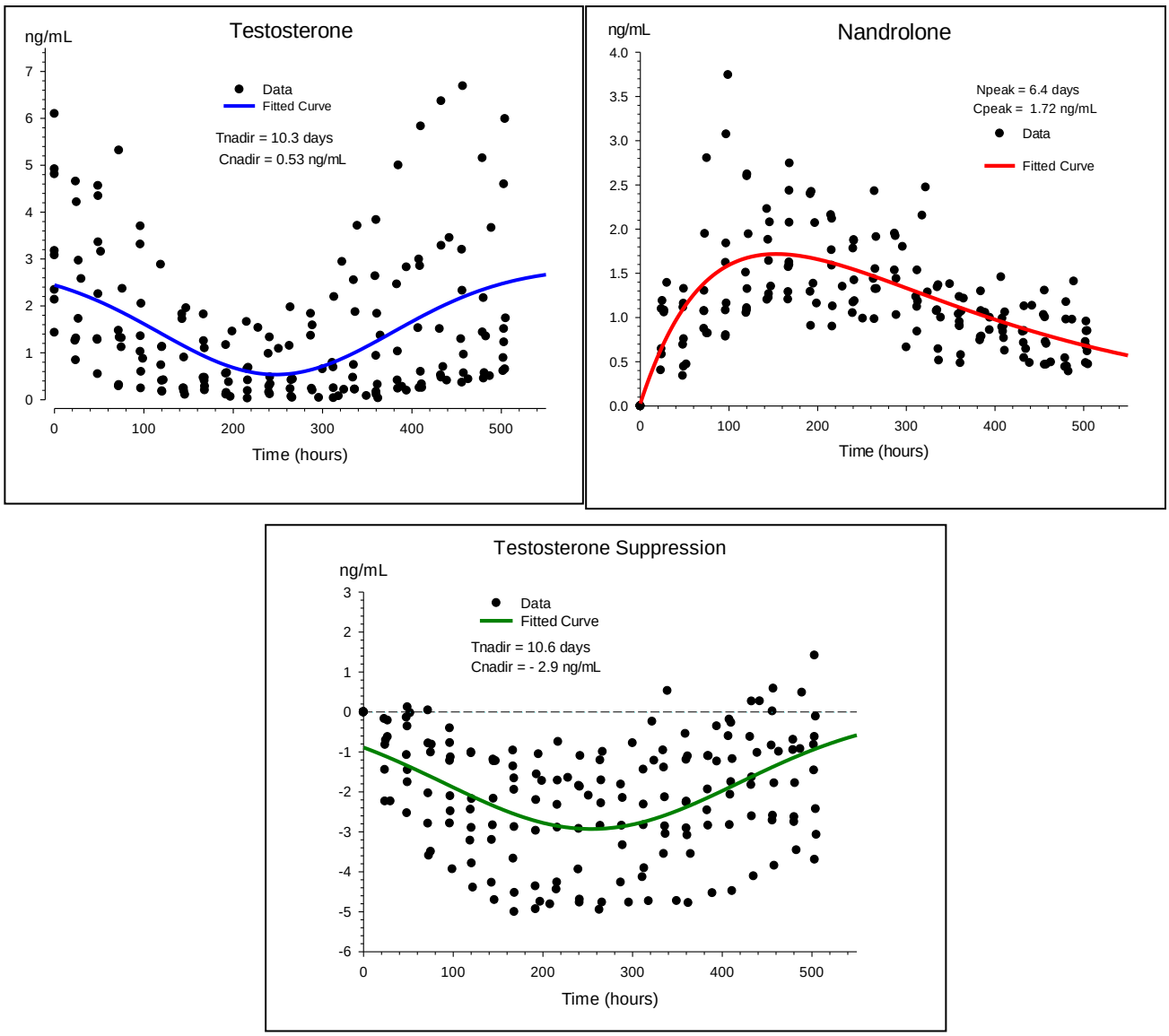


Figure 3.5 Plots of non-linear curve fitting of T (left panel), N (right panel) and T suppression (lower panel) of capillary blood collection from 8 participants before and daily according to exact individual collection time points after SC injection of ND

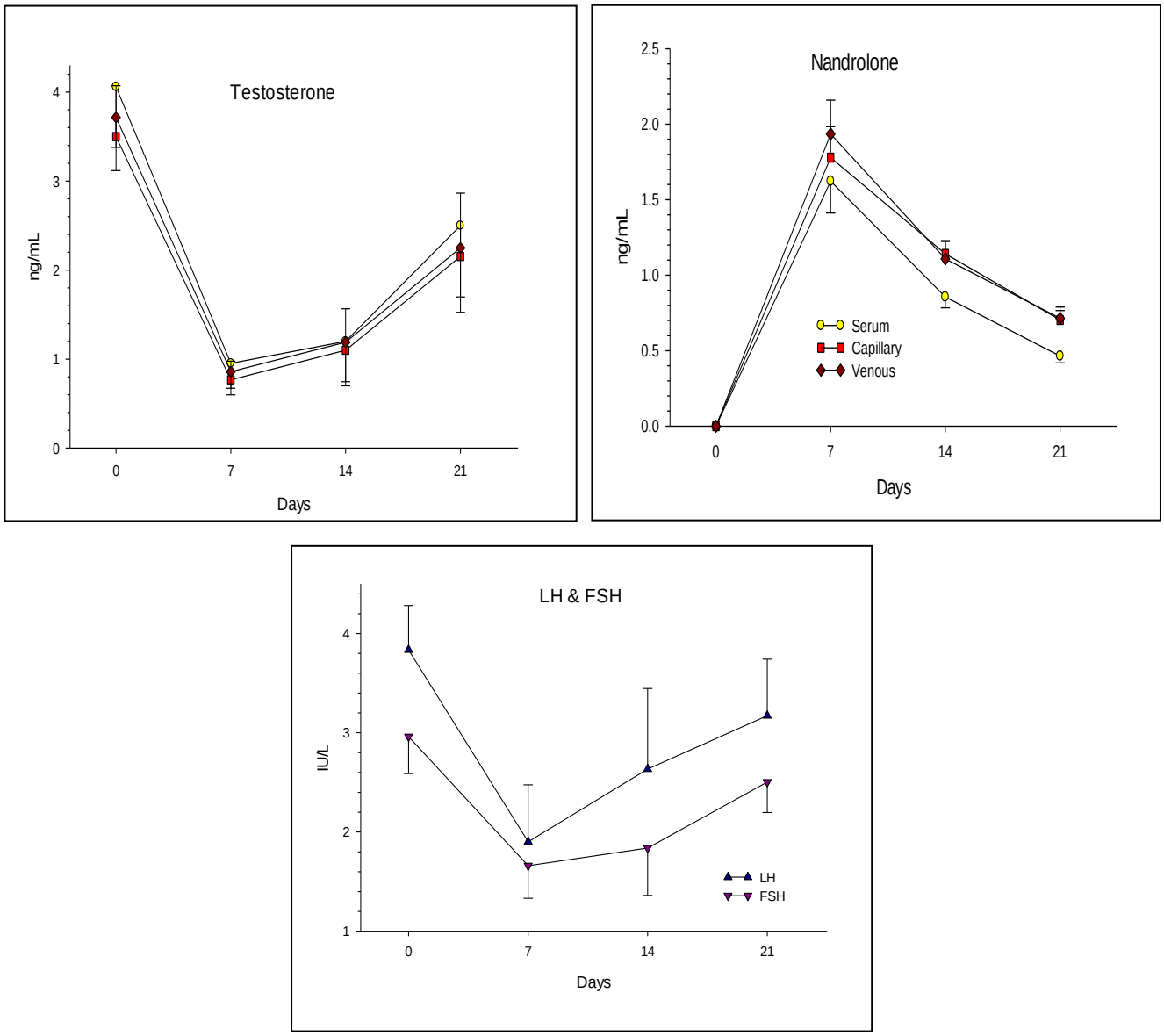


Figure 3.6 Plot of mean and SEM of capillary, serum and venous nandrolone (left panel), testosterone (right panel) and serum LH and FSH (lower panel) in 8 participants before and weekly for 3 weeks after subcutaneous injections of 100 mg ND

3.5 Discussion

DBS technology has been used for over 50 years mostly to measure individual analytes at a single time-point in screening assays or cross-sectional studies (McDade 2014, McDade et al. 2007) for its simplified sample collection, processing and storage. It has been extended to measuring multiple analytes from a single filter spot (Edelman et al. 2007, Magnisali et al. 2011, Worthman and Stallings 1997) but few studies have reported serial DBS sampling of ambulatory, community-dwelling individuals (Howe and Handelsman 1997) and its feasibility has been doubted (Kissinger 2011). This study shows that this advantageous feature is feasible for pharmacological studies of depot steroids, where remote sampling at home is more convenient than requiring repeated clinic visits for venipuncture as well as centrifugation for separating serum or plasma and bulky, frozen sample storage until assay and decreases in hemoglobin from frequent venesection. Furthermore, the use of population PK/PD modelling employing the exact time of finger-prick sampling also relaxed the stringency of time of day requirements for remote sampling. By reducing cost and complexity, thereby features facilitate community-based studies involving steroid measurements such as for the pharmacology of long-acting depot steroids and drugs.

For over 6 decades, injectable T esters have been administered in a vegetable oil vehicle marketed solely for im injection (Junkman 1957). The pharmacokinetics and pharmacodynamics are primarily determined by ester chain side-length, volume of oil vehicle and injection site, all determining the hydrophobic physicochemical partitioning between the oil vehicle and aqueous extracellular fluid wherein ubiquitous esterases liberate the free steroids from their esters (Minto et al. 1997). However, there are no reports of the pharmacology of sc injections of androgen esters in an oil vehicle, other than a pilot study of 22 hypogonadal men where blood T was measured by immunoassay a day before and a day after sc injection of low dose of T enanthate (mean 55 mg weekly, <0.3 ml oil) (Al-Futaisi et

al. 2006). No adverse effects or discontinuations were reported but the data provides minimal insight into the pharmacology of sc injection of androgen esters. In the present study using DBS technology to facilitate intensive and yet simplified blood collection from participants at home, we provide the first detailed pharmacological analysis of sc injection of ND in arachis oil vehicle. The present finding of sustained depot-like effects is expected given the known pharmacology of im N esters (Bagchus et al. 2005, Howe and Handelsman 1997, Minto et al. 1997) together with the observations that most im injections are actually sc (see below). Using prior pharmacological data of im ND injections with steroids measured by immunoassays, sc injection of 100 mg in 2 mL oil most closely resembles gluteal injection of 100 mg in 4mL (rather than gluteal or deltoid in 1 mL) in pharmacokinetics (time of peak 6.0 vs 5.0 days; peak concentration 2.5 vs 3.7 ng/mL) and pharmacodynamics (time of nadir 8.0 vs 9.2 days; nadir concentration 0.38 vs 0.69 ng/mL) (Minto et al. 1997). Another study of gluteal im injection of 100 mg ND in 1 mL oil reported higher peak N concentrations (4.9 ng/mL) and earlier (1.25 days) peak time (Bagchus et al. 2005) consistent with the reported effects of injection volume (Minto et al. 1997).

Nevertheless, inadvertent and unrecognized sc injection of T and N esters in oil vehicle is common. It has been known for decades that sc fat is so thick that gluteal im injections frequently deposit the injectate into sc fatty tissue rather than muscle tissue (Cockshott et al. 1982). Using computed tomography or ultrasound scanning to measure sc fat thickness and placement of injectate or comparison with length of injection needles, it is estimated that 12-85% of im injections in men and 55-95% in women are actually sc (Boyd et al. 2013, Burbridge 2007, Chan et al. 2006, Cockshott et al. 1982, Haramati et al. 1994, Joo and Sohng 2010, Nisbet 2006), the higher estimate in women corresponding to their greater sc fat thickness. This may also explain our observation that im T injections were less painful in overweight or obese men (Sartorius et al. 2010). Experimental studies in pigs show that sc

and im injections of drugs in an oil vehicle have similar pharmacokinetics (Perry et al. 1997, Svendsen et al. 1985). Although injection site granulomata have been reported more frequently in sc fat compared with muscle (Baumann 2012, Cockshott et al. 1982), this observation is derived from hospital-based im injections, usually non-steroids in aqueous solutions, rather than the smaller minority of less toxic, oil vehicle based im injections typically administered in ambulatory care settings (Svendsen 1983, Svendsen et al. 1985).

Although the tolerability of sc injections of androgen esters in an oil vehicle requires more extensive validation through further pharmacological studies of T esters as well as experience in its use, it is likely to be safe and tolerable. Extensive experience with T pellets indicates that sc implantation of pure crystalline T is safe and acceptable for months to years, marred only by a low rate of extrusions (Handelsman et al. 1997) due to a sterile reaction to implantation which is unrelated to mechanical factors (Kelleher et al. 2001, Kelleher et al. 1999) or prevented by antibiotic cover (Kelleher et al. 2002). Similarly, the vegetable oils used as injection vehicles (e.g. sesame, arachis, castor, cottonseed and mellaleuca oils) for androgen esters are well tolerated clinically when administered intramuscularly (Mackey et al. 1995). They are better tolerated than mineral oils which are much more irritating when injected subdermally (Darsow et al. 2000, McWillams 1927, Rollins et al. 1997, Symmers 1955) whereas vegetable oils also reduce local muscular damage compared with aqueous injections (Svendsen 1983, Svendsen et al. 1985). Previous studies have already shown that the pharmacology of androgen esters depends on site and volume of injection (Minto et al. 1997) and further studies using T esters in men with differing body weight and after repeated injections are required to evaluate duration of action and usefulness in clinical practice of sc relative to im injections.

The present study suggests that sc injections of T esters may prove safe and tolerable. If so, this would make self-injection of T esters feasible with the potential benefits of reducing

medical care costs, demand on medical personnel time and injection site pain or bruising as well as allowing freer use among those with bleeding disorders or on anticoagulants to use this more convenient long-acting depot form of T delivery.

Chapter 4

Immunoreactive LH in long-term frozen human urine samples

The content in this chapter has been published in Drug Testing and Analysis 2014, 6: 336-341

Chapter 4 *Immunoreactive LH in long-term frozen human urine samples.*

4.1 Introduction

LH together with FSH are principal pituitary hormones exerting dynamic neuroendocrine control of the initiation and maintenance of mature reproductive functions. In addition to initiating puberty, in women LH drives follicle maturation and ovulation while in men it governs Leydig cell production and secretion of T. Consequently measuring LH is fundamental to understanding of reproductive neuroendocrinology. Urine has always been the preferred sampling format for anti-doping testing as well as for long-term and field studies such as neonatal (Kuijper et al. 2006), adolescent (Hayes and Johanson 1972, McNeilly et al. 2012), occupational (Kesner et al. 1999, Whelan et al. 2002) or toxicological (Kesner et al. 1999, Kuijper et al. 2006, Whelan et al. 2002) cohort studies where urine collections avoid the need for repeated venipuncture. Urinary LH measurement has a role in confirming androgen doping because exogenous androgens suppress urinary LH (Cowan et al. 1991, Goebel et al. 2009, Handelsman et al. 2009, Kicman et al. 1990). Furthermore, abuse of anti-estrogens, GnRH analogs and recombinant LH (Handelsman 2006) may also be detected by increased urinary LH levels.

One limitation is that modern commercial human LH immunoassays have been established and validated solely for blood samples and not urine sample, unlike hCG immunoassays which were always developed for qualitative urine pregnancy testing as well as for blood samples although quantitative urinary hCG assays are described (Cole and Khanlian 2009). Urine LH immunoreactivity may depend on urine sample storage conditions as well as immunoassay epitope specificity. For example, pre-storage extraction by acetone precipitation (Reiter et al. 1973, Saketos et al. 1994) or by addition of glycerol or albumin

(Kesner et al. 1995, Livesey et al. 1983, Saketos et al. 1994) stabilize immunoreactivity. Furthermore, whereas two-site assays with epitopes on different subunit of the heterodimer optimizes detection of intact LH (Alonso-Whipple et al. 1988, Nilsson et al. 2001, Pettersson et al. 1991), dissociation of LH into its subunits, influenced by exposure to high urinary concentrations of urea (Lempiainen et al. 2012), may be reflected in loss of immunoreactivity whether or not the subunit remain intact especially at -20 °C storage (Lempiainen et al. 2012). This is consistent with the stability of a urinary LH assay based on a different strategy of pre-assay dissociation into subunits with detection aimed at the LHβ subunit (Brindle et al. 2006), an assay displaying prolonged stability during frozen storage (Brindle et al. 2006, O'Connor et al. 2006). However, it is simpler for field studies to implement urine LH immunoassays that do not require pre-storage manipulation. In addition, as glycerol is on the World Anti-Doping Prohibited List as a banned chemical for athletes, it cannot be used with urine samples collected for anti-doping testing. Therefore, this study aimed to evaluate the stability of urinary LH immunoreactivity using two commercially available immunoassays, an immunofluorometric (IF, Delfia, Perkin-Elmer) and an immunochemiluminometric (ICL, Immulite, Siemens) assay, before and after 4 years of storage at -20 °C without addition of any preservatives.

4.2 Study Design

Urine samples were obtained from a previous study that evaluated the potential of recombinant hCG (rhCG, Ovidrel, MerckSerono Pty Ltd) as an indirect androgen doping agent (Handelsman et al. 2009). The effects of two doses of rhCG, with or without concomitant administration of ND (Deca-Durabolin, Organon Australia Pty Ltd, Lane Cove, Sydney) to suppress endogenous LH and T (Minto et al. 1997), on urine and serum LH and testosterone were examined in 24 healthy young men aged 18-45 years who received a single sc injection of rhCG (250 or 750 μg). Spot urine samples were collected before and daily for

8 days after dosing and stored frozen at -20 °C until analysis in a single batch at the end of study (2008) and then again (2012) after 4 years of unthawed storage at -20 °C. At both times, the full batch was analysed in duplicate by ICL (Immulite 1000 LH, Siemens) and IF (Delfia hLH assay, Perkin Elmer, Rowville, Melbourne, Victoria) assays (refer Chapter 2 for assay details). Urinary SG was measured by a refractometer and urine LH values were adjusted to a standard SG of 1.020. Prior to assay, urine samples were thawed and warmed at 37°C in a sealed container for 10-15 minutes with shaking to redissolve sediments.

4.3 Data analysis

Results are expressed as mean ± SEM. The LH concentrations measured in either assay at each time were compared by Passing-Bablok non-parametric regression and deviance (Bland-Altman plot) analysis using MedCalc software.

4.4 Results

The comparison of urinary LH concentrations measured using IF and ICL immunoassays, adjusted to standard SG, when run in either 2008 or after 4 years frozen storage (2012) is shown in Figure 4.1. The comparison between the two LH assays when both were run in 2008 and then again when both were run in 2012 after 4 years frozen storage, is shown in Figure 4.2. The details of the Passing-Bablok regression equations to compare urinary LH using the IF and ICL at both times is given in Table 4.1.

The urine LH concentrations measured using the ICL assay when comparing measurements in 2008 and 2012 shows good correlations (Figure 4.1, Table 4.1). By contrast, the IF assay showed marked reduction (~70%) in LH concentration when measured in 2012 compared to 2008 (Figure 4.1, Table 4.1).

When comparing LH assays performed in 2008, and then again after 4 years frozen storage, the two LH assays were discrepant. In 2008 the IF assay provided LH measurements which

were ~50% of those in the concurrent ICL LH assay (Figure 4.2, Table 4.1). In 2012, the IF LH assay was reduced to ~30 % compared with the contemporaneous ICL assay (Figure 4.2, Table 4.1).

The mean urinary LH concentration (adjusted for urine SG) in the original study measured with IF and ICL in year 2008 and 2012 is depicted in Figure 4.3. The pattern of the urinary LH profile was very similar but not identical using either immunoassays at the end of study (2008) and again after 4 years of frozen (-20 °C) storage. Despite loss of immunoreactivity according to one (IF) but not the other (ICL) assays in all series the suppression of urine LH was clearly evident.

The details of the Passing-Bablok regression equations to compare urinary (ICL and IF) and serum LH (IF) is given in Table 4.2. The intercept of all the four comparisons was not significantly different from 0 although there were variations in the slope and correlations (Table 4.2).

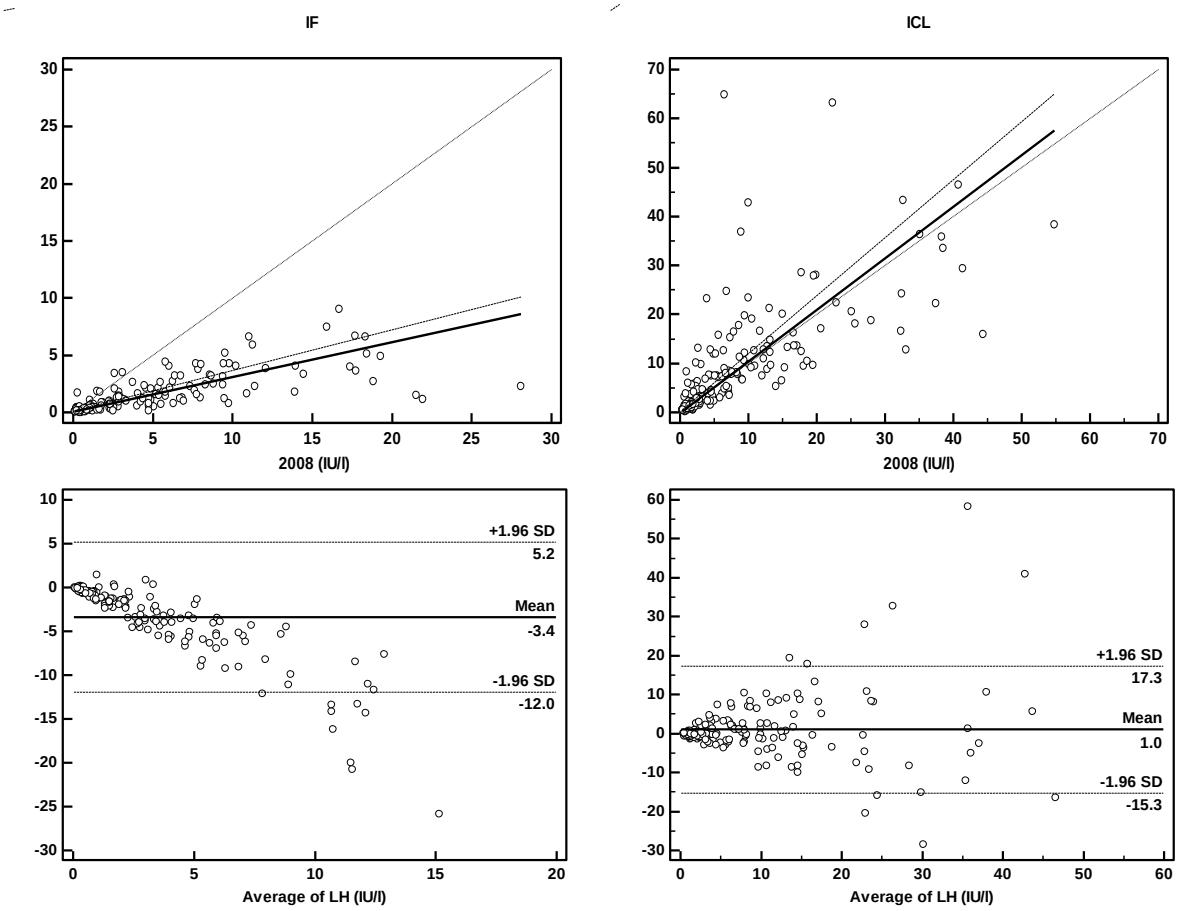


Figure 4.1 Comparison of urinary LH concentrations obtained using IF (left panels) and ICL (right panels) measured before and after four years of storage at -20 °C.

Levels of urinary LH between the same assays were compared using Passing and Bablok regression analysis (upper panels). The slope (solid line) is calculated with 95% confidence bands (dotted lines) and line of identity (fine dotted line). The Bland-Altman plots (lower panels) represent urinary LH differences against averages of the two measurements. The solid line and the dotted line represent the observed average agreement and 95% limit of confidence respectively.

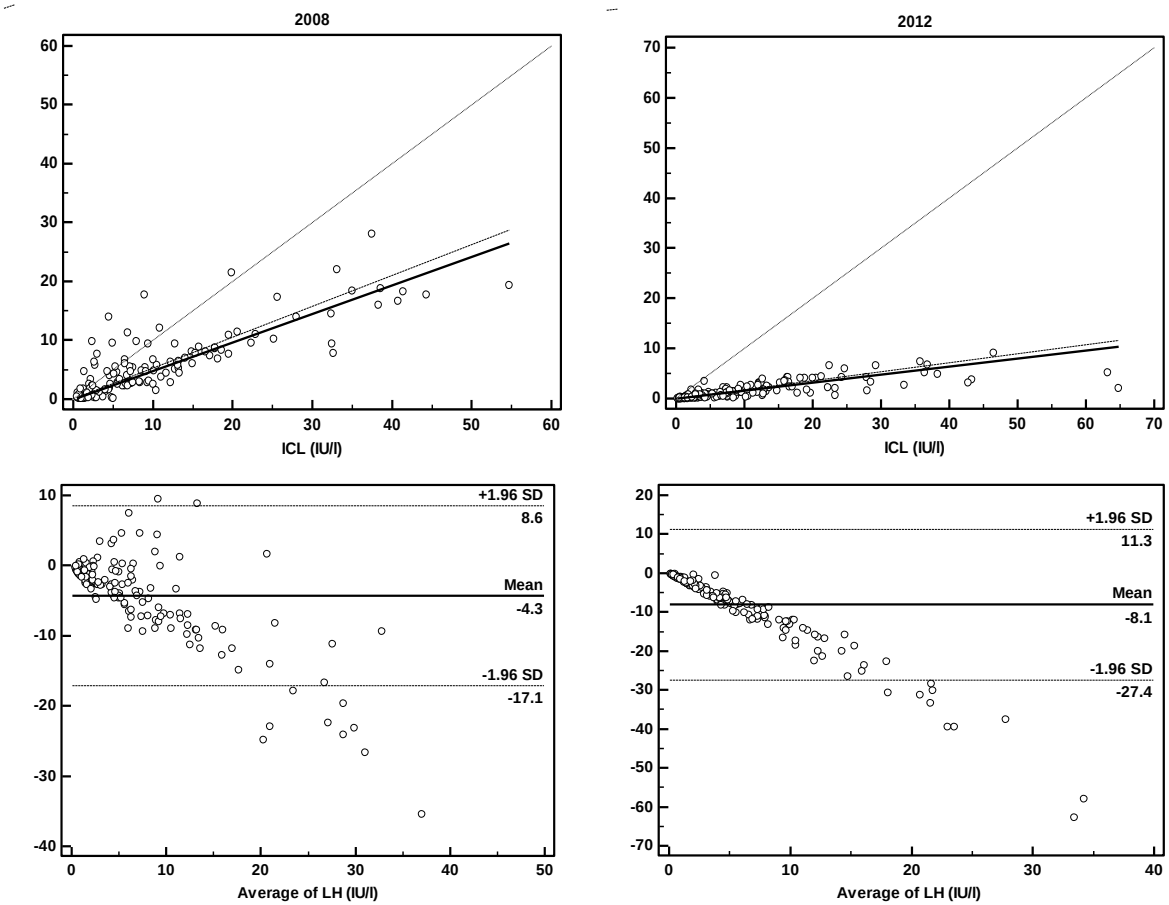


Figure 4.2 Comparison of urinary LH concentrations measured by IF and ICL in 2008 (left panels) and 2012 (right panels).

Levels of urinary LH between the two assays were compared using Passing and Bablok regression analysis (upper panels). The slope (solid line) is calculated with 95 % confidence bands (dotted lines) and line of identity (fine dotted line). The Bland-Altman plots (lower panels) represent urinary LH differences against averages of the two measurements. The solid line and the dotted line represent the observed average agreement and 95 % limit of confidence respectively.

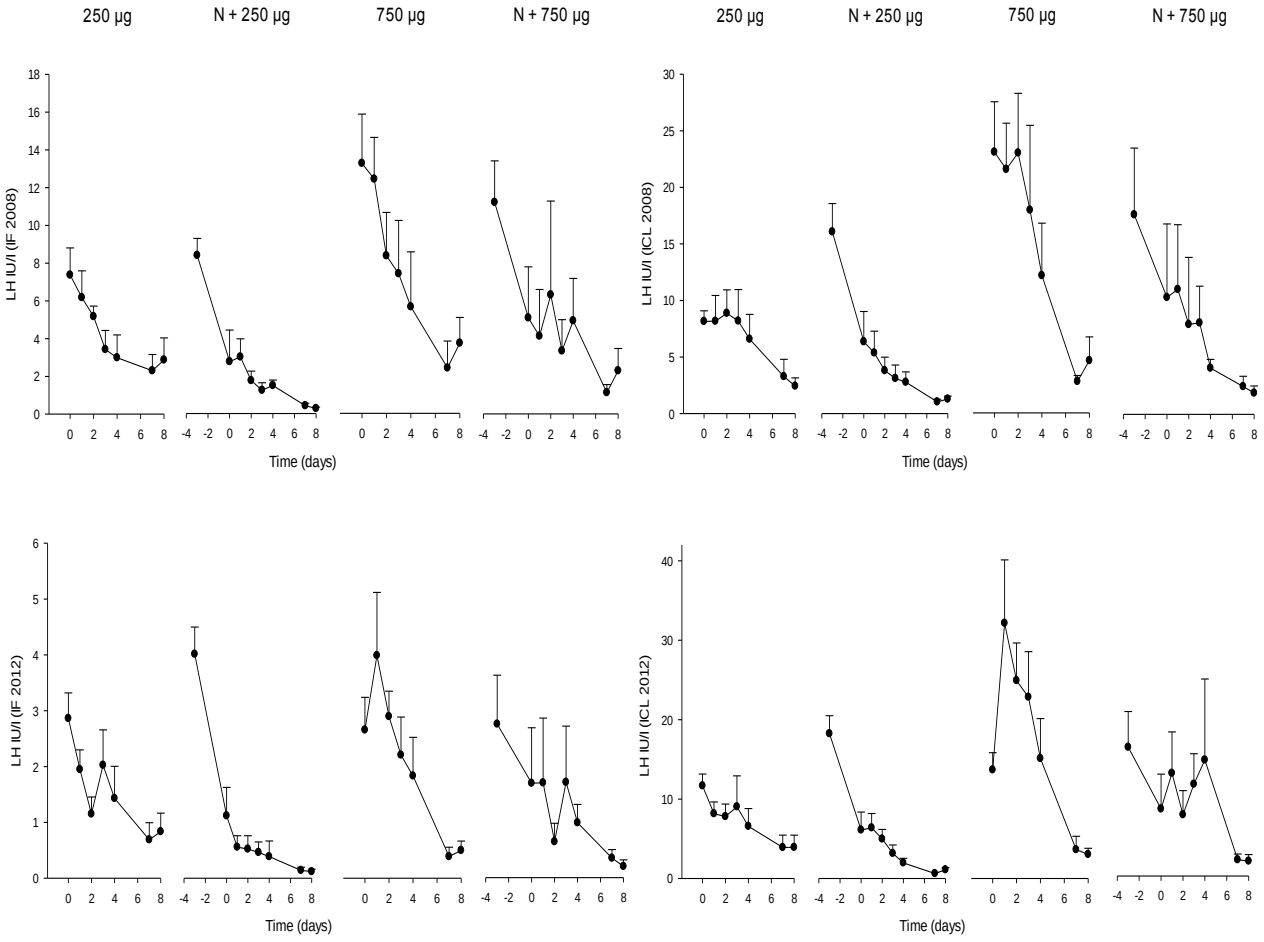


Figure 4.3 Plot of urine LH assayed with Delfia (left panels) and Immulite (right panels) for year 2008 (upper panels) and 2012 (lower panels).

Data represents the mean and SEM of spot urine collected before and after dosing of hCG (with or without nandrolone suppression). Note all LH concentrations are adjusted to standard urine SG of 1.020.

Table 4.1 Comparison of urine LH determined using two immunoassays.

The number of urine samples (n) with the slope, intercept and correlation coefficient (r) and their 95% confidence intervals in parentheses determined by Passing-Bablok regression.

Assays compared	n	Slope	Intercept	r
Delfia 2008 vs 2012	156	0.30 (0.27, 0.36)	0.04 (-0.01, 0.10)	0.72 (0.63, 0.78)
Immulite 2008 vs 2012	180	1.06 (0.94, 1.19)	-0.25 (-0.57, 0.15)	0.70 (0.61, 0.76)
Immulite vs Delfia 2008	156	0.49 (0.45, 0.53)	-0.11 (-0.41, -0.02)	0.84 (0.79, 0.88)
Immulite vs Delfia 2012	156	0.16 (0.14, 0.18)	-0.03 (-0.07, 0.00)	0.78 (0.71, 0.83)

Table 4.2 Comparison of urine LH determined using Immulite and Delfia (2008 and 2012) versus serum LH determined using Delfia (2008).

The number of samples (n) with the slope, intercept and correlation coefficient (r) and their 95% confidence intervals in parentheses determined by Passing-Bablok regression.

Urine LH vs Serum LH (Delfia 2008)	n	Slope	Intercept	r
* Delfia 2008	156	0.34 (0.28, 0.40)	0.02 (-0.05, 0.09)	0.70 (0.61,0.77)
Delfia 2012	180	1.22 (1.04, 1.41)	-0.03 (-0.11, 0.04)	0.61 (0.51, 0.69)
*Immulite 2008	180	0.16 (0.13, 0.19)	-0.03 (-0.08,0.02)	0.64 (0.54, 0.72)
Immulite 2012	180	0.15 (0.11, 0.19)	0.03 (-0.05,0.07)	0.43 (0.30, 0.54)

* Note: Data obtained from (Handelsman et al. 2009)

4.5 Discussion

The present study demonstrated that two available commercial LH immunoassays optimized for blood samples can also be used for urinary LH measurement. However, the performance of both LH assay with urine samples differed markedly. The ICL immunoassay showed quantitatively reproducible LH measurement even after prolonged storage at -20 °C for 4 years without addition of preservatives. By contrast, the IF immunoassay demonstrated consistently but proportionately lower LH measurements relative to the ICL assay both initially and then exhibited a further decrease after 4 years of frozen storage. Yet, despite the reduced absolute LH measurements by the IF immunoassay, both LH immunoassays displayed sufficiently similar patterns of urine LH measurements to confirm the findings of the original study that hCG administration produces LH suppression (Handelsman et al. 2009). Hence either assay used before or after prolonged frozen storage may be sufficient for a research study whereas only one provides quantitative results after frozen storage as might be required for look-back of stored anti-doping samples, an application which requires reproducible quantification of absolute levels. Similar findings were reported for serum LH measurements where the performance of the ICL was reported to be more sensitive and accurate compared to the IF immunoassay for the determination of reference range during sexual development in normal children (Resende et al. 2007).

Commercially available immunoassays designed for serum or plasma LH measurements require careful validation for use with urine samples particularly when inference is to be drawn from suppressed urine LH values (Cowan et al. 1991, Goebel et al. 2009, Kicman et al. 1990, Mareck et al. 2010, Palonek et al. 1995, Perry et al. 1997). In addition to the two LH immunoassays in this study, thorough validation has been published for two LH immunoassays (Access and Elecsys) (Robinson et al. 2007) and indirectly for a third (Axsym) (Llouquet et al. 2013, Perry et al. 1997). Other studies reported good correlation of

urine and serum LH in children and cycling women as determined by IF immunoassay (Demir et al. 1994, Saketos et al. 1994). Validation after prolonged frozen conditions has not been evaluated by available commercial immunoassays.

These discrepancies between LH immunoassays most probably reflect the diversity of immunoreactive LH species in human urine compared with the bloodstream (Birken et al. 2001, Birken et al. 2007, Stenman et al. 2008). This diversity reflects the metabolism of the gonadotropins which produces nicked dimers and free subunits including the core fragment. Like hCG where the metabolism is more fully characterized (Bristow et al. 2005), the homologous LH heterodimer is metabolized and excreted in the urine in various fragments which are largely in the forms of free subunits and fragmented forms of LHβ (Birken et al. 1996, Kovalevskaya et al. 1995). The differences between the two LH assays presumably reflect on the capability of the two-site immunoassays to detect various LH epitopes (O'Connor et al. 1998). Both the assays detect intact LH and some fragmented forms probably including the core beta fragment. It is possible that the ICL immunoassay detects other unknown fragment(s) which are not detected by the IF immunoassay. This could explain the consistently lower LH levels measured by the IF assay than the ICL assay. Earlier studies have also reported IF immunoassays meant to detect intact LH failed to detect variant forms either in urine or serum samples that lead in discrepancies in measurements of LH using different immunoassays (Nilsson et al. 2001, Pettersson et al. 1991). LH immunoassays with both β-directed monoclonal antibodies are reported to give higher urinary LH concentrations compared to assays that only detect intact LH (Nilsson et al. 2001). Assays that have highly specific antibodies may also be problematic in that they may not measure all forms of LH (Pettersson et al. 1991, Pettersson et al. 1992, Pettersson and Soderholm 1991).

Loss in urinary LH and hCG immunoreactivity after 7 to 70 days of storage at -20 °C (unpreserved) measured with the IF immunoassay (Lempiainen et al. 2012, Saketos et al. 1994), RIA (Livesey et al. 1983) and immunoenzymatic assay (Robinson et al. 2007) was also reported previously which was attributed to the dissociation of the heterodimeric glycoprotein into its subunits in the presence of urinary urea in specimens (Lempiainen et al. 2012, Livesey et al. 1983). Loss of immunoreactivity of urinary LH (measured with IF immunoassay) was also reported after 24 weeks of -80 °C storage (Kesner et al. 1995). Acetone extraction and addition of preservatives such as bovine serum albumin and glycerol was reported to stabilize LH immunoreactivity in frozen storage (Kesner et al. 1995, Livesey et al. 1983, Saketos et al. 1994). However, using sample preservatives for research sample collection is inconvenient and adds to time and expense (Brindle et al. 2006) and similarly some preservatives such as glycerol are unsuitable for anti-doping purpose. Previous studies have reported that plasma LH is stable after storage for 8 and 14 days after refrigeration (4 °C) (Kubasik et al. 1982, Livesey et al. 1980), for 14 days stored frozen (-6 °C) (Kubasik et al. 1982) and for up to 9 months after -20 or -70 °C storage (Tsatsoulis et al. 1988).

The quantitative reproducibility of the ICL immunoassay indicates it is more robust for measurements of urinary LH for anti-doping purposes compared with the IF assay. Nevertheless despite the quantitative reduction in urine LH by the IF assay, the reduction was proportionate over the four years frozen storage. Consequently the IF assay remains applicable in research studies where, rather than absolute quantification, relative measurements confined to within-study inference, may still be useful.

Chapter 5

Requirement for Specific Gravity and Creatinine Adjustments for Urinary Steroids and Luteinizing Hormone Concentrations in Adolescents

The content in this chapter has been accepted for publication in Annals of Clinical Biochemistry 2015

Chapter 5 *Requirement for Specific Gravity and Creatinine Adjustments for Urinary Steroids and Luteinizing Hormone Concentrations in Adolescents*

5.1 Introduction

Measurements of urinary gonadotropins and steroids in children and adolescents emerged as methods to estimate pubertal development and gonadal function early in the immunoassay era (Kulin and Santner 1977, Raiti et al. 1969, Vestergaard et al. 1966). Subsequently, urine sampling was replaced by serum or plasma immunoassays due to preferences for blood collection over cumbersome and time-consuming urine collections (e.g. 24 hr sampling) and extraction procedures required to assess urine solute concentrations. In recent years, however, measurements of urinary gonadotropins and steroids have reappeared with the development of highly sensitive assays that can measure urinary gonadotropins without extraction (Brindle et al. 2006).

Urine may be collected either as untimed spot collection at random times, first morning void or timed (e.g. 24 hours urine collection) collections. Urine sampling provides an integrated measurement especially for hormones secreted in pulsatile manner such as LH (Kulin et al. 1975) or diurnally like sex steroids in early puberty, and is more acceptable to children and adolescents than venipuncture. Further, for longitudinal studies, spot urine hormone measurements are more convenient and/or less intrusive than blood or saliva collections, a simplification which enhances the feasibility and participant compliance with repeated sampling over time. The relatively high hormone concentrations in urine compared with blood or saliva, together with the ability to concentrate urine, is advantageous for assays with low sensitivity or analytes at low concentrations. However, an inherent problem of using urine is the wide and unregulated variation reflecting the individual's fluid status.

Urine dilution or concentration creates corresponding changes in urine solute concentrations so that adjustment of urine concentration may be required to avoid misinterpreting hormone excretion due to variation in hydration (Cone et al. 2009). Osmolality, SG and creatinine measurements are used to adjust for hydration (Barr et al. 2005). Although measurement of osmolality by freezing point depression is considered the reference method (Chadha et al. 2001), it is laborious, time consuming and expensive so is usually replaced by SG and creatinine measurements particularly for large scale, field studies. Urinary SG is measured using a refractometer to compare light refraction of a urine sample against pure water standard or by reagent strips which measure the ionic strength of urine by color changes. Urine SG of sample is normalized to a population reference value. While SG measurement has been largely superseded by urine creatinine adjustment in clinical laboratories, SG adjustment for urine dilution remains standard in anti-doping laboratories and is used in some toxicology studies (Aylward et al. 2014). Creatinine adjustment is based on the assumption that (a) this end-product formed endogenously from muscle creatine is released into the bloodstream and excreted in urine at a constant rate depending only on total muscle mass (Boeniger et al. 1993) and (b) endogenous hormones and creatinine undergo renal excretion at the same rate (Barr et al. 2005). Yet, creatinine excretion rate may be influenced by the growing muscle mass during puberty leading to potential systemic errors in using creatinine adjustments (Heavner et al. 2006).

Some (Denari et al. 1981, Haddow et al. 1994) but not other (Alessio et al. 1985, Berlin et al. 1985, Jatlow et al. 2003, Thompson et al. 1990, Zacur et al. 1997) studies suggest creatinine or SG adjustment for measurement of urinary substances although such adjustments may be either unnecessary or even introduce additional measurement errors. Furthermore, none have focused on situations where creatinine is changing systematically due to somatic growth. Thus the present study aimed to determine whether the first morning void hormonal

assessments carried out in growing young adolescents at various stages of pubertal progression require adjustments and, if so, to determine whether creatinine or SG adjustment was better.

5.2 Study Design

Urine samples were from the Adolescent Rural Cohort, Hormones, Health, Education and Relationships (ARCHER) study, a three year longitudinal study aiming to determine how temporal changes in pubertal hormones influence the physical status, behavior and mental health of adolescents. The details of the study design have been published previously (Steinbeck et al. 2012). Briefly, the study involves a total of 342 adolescents from the regional towns Dubbo, Orange and their surrounding rural region in western NSW, Australia. The samples reported in the present study were from those collected in the first year of the study (n=644; age distribution ranging between 10.1 and 14.3 years at initial sampling). Ethical approval was obtained from the Human Research Ethics Committee, University of Sydney (HREC 13094). Fasting morning blood samples were collected at 0 and 12 months (n=343) and first morning urine collected three monthly after 12 hr fasting at home by the adolescents between 7.00 am and 8.30 am (n=644). Post-menarcheal girls provided samples in the mid-follicular phase (day 7-10) with the assumption of 28-32 day cycle. Serum and urine samples were stored at -80 C until analysis. The adolescents also provided a self-rating of puberty using line drawings based on the Tanner stages at 0 and 12 months.

The LH and steroid concentrations were adjusted to standard SG of 1.020 according to the formula [hormone concentration$_{sample}$ X (1.020-1)/(SG$_{sample}$-1)] (Wallis et al. 1985) and to standard CR measurement of the present study adolescent population (12.40 mmol/L [n=644; F 331]) using the formula [hormone concentration$_{sample}$ X (Creatinine$_{population}$/Creatinine$_{sample}$)] where Creatinine$_{population}$ was defined as the mean of the urinary creatinine of the whole sample.

5.3 Data Analysis

The steroid and LH concentrations unadjusted and adjusted for SG or creatinine were compared by Deming (orthogonal) regression and deviance (Bland-Altman) analysis using MedCalc software. Based on using the same analyte with different adjustments, the variance ratio in the Deming regression was assumed to be unity. Non-independence according to variations in the number of samples provided by each individual was ignored in this analysis. Descriptive statistics including mean, SD and SEM were performed by SPSS version 21. The F- ratios of age and Tanner stage group comparison for each hormone were calculated by one-way ANOVA separately for each gender.

5.4 Results

In first morning urine void samples (n=644), the mean (SD, range) creatinine concentration was 12.4 (4.5, 1.4 - 31.5) mmol/L with an overall gender difference being higher in males ($P<0.05$). The SG was 1.020 (0.0054, 1.005 - 1.030) without significant gender difference ($P=0.054$). Urine creatinine concentrations were progressively increased according to chronological age and to Tanner stage (Figure 5.1) for both genders. Pooling genders, there were significant differences in urine creatinine concentrations by age and Tanner stage ($P<0.05$, two-way ANOVA) but not for urine SG according to age ($P=0.29$) or Tanner stages ($P=0.22$).

Urinary LH, E_2, T, DHT and DHEA concentrations, adjusted for either SG or creatinine, are compared according to Deming regression line and the deviance plots are shown in Figure 5.2. For each urinary hormone concentration, there was a good correlation between the SG and creatinine adjusted concentrations (correlation of determination-R^2: 0.69 - 0.85) free from proportional bias between adjustment methods.

Similarly, Deming regression and Bland-Altman comparison between the unadjusted and adjusted hormone concentrations with either SG or creatinine (Table 5.1) also demonstrate lack of bias whether adjusted or not by either creatinine or SG.

The mean, SD and F-ratios of the unadjusted, SG adjusted and creatinine adjusted hormones according to Tanner stages and age groups in females and males are shown in Tables 5.2-5.3 and Table 5.4-5.5, respectively. The results show consistent estimates and progression according to age and Tanner stage of unadjusted, SG adjusted and creatinine adjusted urine LH and steroid concentrations. The mean, SD and F-ratios of serum hormones according to Tanner stage and age groups are shown in Table 5.6 and 5.7.

The r of paired urinary and serum hormone concentrations is given in Table 5.8. The unadjusted and adjusted (creatinine and SG) urinary LH, E_2, T, DHT and DHEA concentration showed similar correlation against serum. The samples were also grouped into three creatinine and SG percentile ranges (up to 25th percentile, between 25th to 75th percentile and above 75th percentile) and regression analysis was performed between unadjusted/adjusted urine hormone concentrations against serum hormone concentrations. There were no improvements in the r values within the groups. Dividing the same percentiles according to gender also did not improve the correlations between the urine unadjusted/adjusted hormones against serum hormones concentrations.

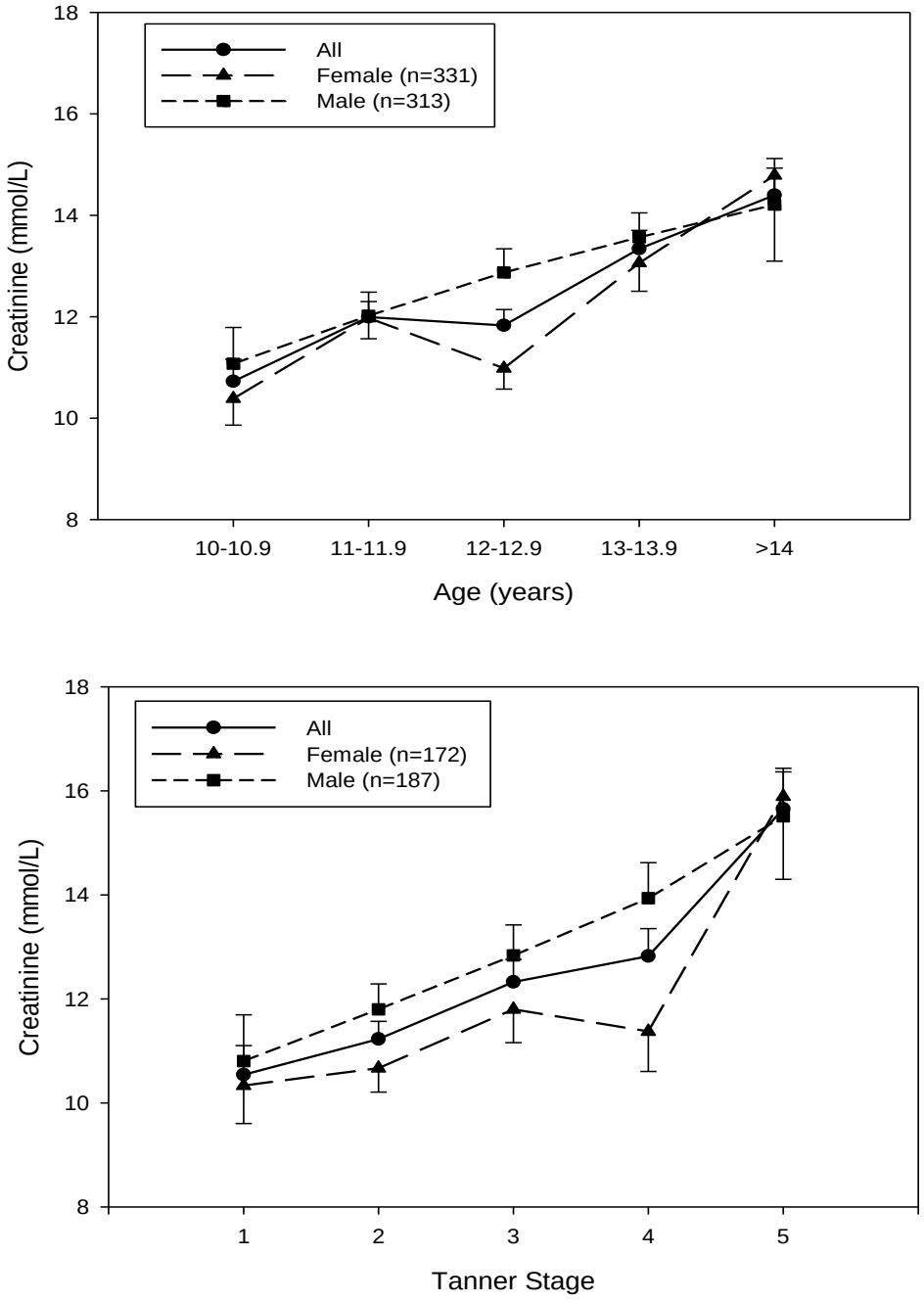


Figure 5.1 Plot of urinary creatinine measurements of adolescents groups according to age (upper panel) and Tanner stage (lower panel).

Data represents the creatinine mean and SEM. For the age plot, samples were from 3-monthly intervals (n=644) whereas for the Tanner stage plot, samples were from 0 and 12 months (n=359).

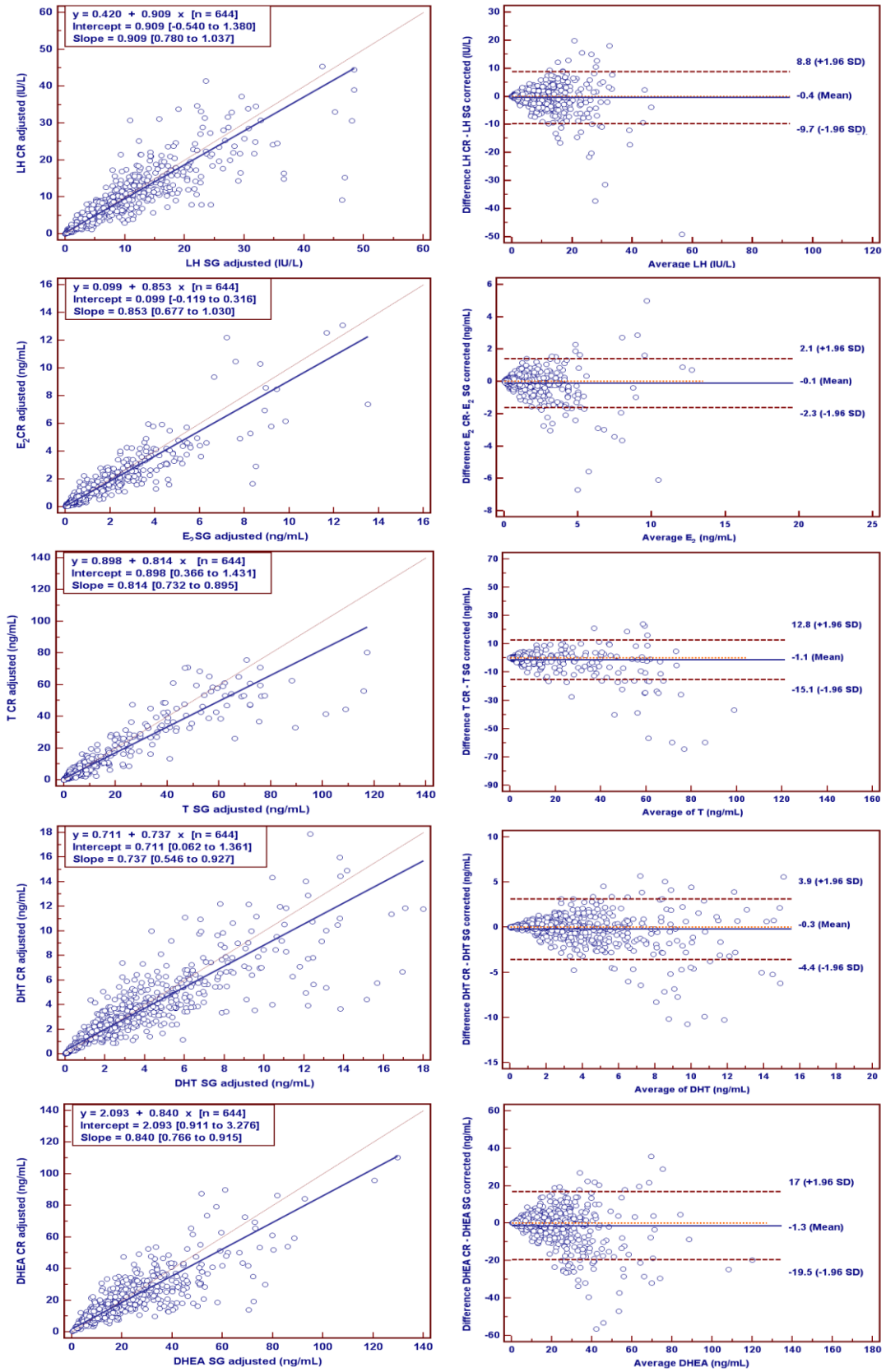


Figure 5.2 Comparison of urinary LH, E2, T, DHT and DHEA concentrations adjusted by SG and creatinine.

Comparisons were made according to Deming regression analysis (left panels) and Bland-Altman plots (right panels). For the Deming plots, the slope is shown as a solid line and line of identity in fine dotted line. Insets are the regression formula and 95% confidence limits on the intercept and slope. The Bland-Altman plots represent the differences between creatinine and SG adjusted hormone concentrations against the averages of the hormone concentrations adjusted with the two-correction method. The solid line and the dashed lines represent the observed average and the 95% limit of confidence (± 1.96 SD), respectively. Note: A small number (n=6) of outliers were removed for graphical purposes. For the Deming regression, the variance ratio was assumed to be unity. To convert steroids from ng/mL to nmol/L multiply 3.47 for T and DHEA, 3.67 for E2 and 3.44 for DHT

Table 5.1 Comparison of unadjusted against SG and CR adjusted urinary hormone measurements.

The slope, intercept and 95% confidence interval (CI) were determined by Deming regression. The mean and 95% CI (± 1.96 SD) were derived from the Bland-Altman plots.

	UA vs SG					UA vs CR				
	Deming Regression			Bland-Altman		Deming Regression			Bland-Altman	
	Slope (95% CI)	Intercept (95% CI)	R^2	Mean	95% CI	Slope (95% CI)	Intercept (95% CI)	R^2	Mean	95% CI
LH (IU/L)	0.94 (0.84 to 1.03)	0.03 (-0.67 to 0.73)	0.83	0.6	8.3, -7.2	1.03 (0.94 to 1.11)	-0.39 (-0.98 to 0.19)	0.80	0.1	8.1, -7.8
E_2 (ng/mL)	0.92 (0.75 to 1.09)	0.02 (-0.19 to 0.22)	0.86	0.1	2.0, -1.8	1.08 (0.93 to 1.22)	-0.09 (-0.24 to 0.07)	0.85	0.0	1.8, -1.8
T (ng/mL)	0.98 (0.89 to 1.06)	-0.17 (-0.75 to 0.42)	0.90	0.4	11.5, -10.7	1.20 (1.08 to 1.31)	-1.23 (-1.99 to –0.47)	0.88	-0.7	11.7, -13.2
DHT (ng/mL)	1.12 (0.74 to 1.50)	-0.63 (-1.94 to 0.68)	0.89	0.2	3.5, -3.2	1.53 (0.71 to 2.35)	-1.75 (-4.44 to 0.94)	0.82	-0.1	4.8, -5.0
DHEA (ng/mL)	0.90 (0.81 to 0.99)	0.65 (-0.92 to 2.22)	0.75	1.5	17.9, -14.9	1.07 (0.94 to 1.20)	-1.58 (-3.67 to 0.50)	0.67	0.2	17.6, -17.2

Note: UA- unadjusted; SG- specific gravity adjusted; CR- creatinine adjusted; R^2-correlation of determination. For the Deming regression, the variance ratio was assumed to be unity.

Table 5.2 Comparison of mean urinary hormone measurements (unadjusted, SG adjusted and CR adjusted) and F- ratios according to Tanner stage in female.

Mean (SD) (n)[#]	Tanner Stage 1 (24)[#]	2 (54)[#]	3 (50)[#]	4 (30)[#]	5 (14)[#]	F Ratio*
Unadjusted						
LH (IU/L)	3.59 (6.20)	6.90 (9.69)	9.84 (8.67)	11.59 (6.71)	10.52 (9.28)	4.01
E_2 (ng/mL)	0.67 (1.12)	1.21 (1.30)	2.06 (1.59)	2.96 (2.33)	5.72 (4.24)	19.59
T (ng/mL)	1.95 (2.49)	2.99 (2.85)	4.70 (3.54)	5.70 (4.45)	6.57 (3.60)	7.75
DHT (ng/mL)	2.05 (1.98)	2.62 (1.88)	2.20 (1.39)	2.84 (2.73)	2.85 (2.52)	0.95 (P=0.44)
DHEA (ng/mL)	11.51 (8.67)	16.62 (14.97)	18.98 (11.95)	24.56 (17.79)	29.34 (11.69)	5.39
SG adjusted						
LH (IU/L)	3.64 (5.09)	7.67 (10.01)	11.08 (9.59)	12.63 (7.54)	12.54 (12.62)	4.57
E_2 (ng/mL)	0.67 (0.90)	1.36 (1.28)	2.34 (1.90)	3.12 (2.28)	6.65 (5.53)	19.92
T (ng/mL)	2.11 (2.24)	3.49 (3.36)	5.36 (5.00)	5.94 (4.58)	7.22 (4.07)	5.95
DHT (ng/mL)	2.22 (1.74)	3.09 (2.42)	2.38 (1.61)	3.03 (3.06)	2.88 (2.19)	1.11 (P=0.35)
DHEA (ng/mL)	14.30 (14.06)	20.00 (19.67)	20.94 (15.52)	26.59 (20.23)	32.58 (16.97)	3.08
Creatinine adjusted						
LH (IU/L)	3.70 (5.33)	7.64 (9.65)	10.83 (8.87)	13.52 (6.94)	9.73 (9.21)	5.43
E_2 (ng/mL)	0.67 (0.84)	1.34 (1.17)	2.23 (1.62)	3.27 (2.25)	4.68 (3.06)	18.50
T (ng/mL)	2.13 (1.82)	3.46 (2.92)	4.86 (3.27)	6.28 (4.61)	5.91 (4.23)	7.02
DHT (ng/mL)	2.42 (2.04)	3.09 (2.01)	2.42 (1.41)	3.16 (3.45)	2.63 (2.89)	0.95 (P=0.44)
DHEA (ng/mL)	13.73 (8.78)	19.55 (15.07)	20.45 (12.44)	28.75 (23.33)	26.18 (18.53)	3.58

*All the P values were <0.05 unless indicated in parentheses
(n)[#] Indicates number of samples

Table 5.3 Comparison of mean urinary hormone measurements (unadjusted, SG adjusted and CR adjusted) and F- ratios according to Tanner stage in male.

Mean (SD) (n)[#]	Tanner Stage 1 (19)[#]	2 (53)[#]	3 (52)[#]	4 (39)[#]	5 (24)[#]	F Ratio*
			Unadjusted			
LH (IU/L)	2.33 (2.77)	4.26 (4.69)	4.79 (4.32)	11.24 (8.14)	11.24 (8.36)	15.46
E_2 (ng/mL)	0.15 (0.17)	0.20 (0.26)	0.29 (0.39)	0.96 (0.90)	1.26 (0.85)	24.30
T (ng/mL)	2.00 (2.26)	3.86 (7.33)	6.33 (9.23)	25.55 (21.59)	42.14 (34.07)	31.25
DHT (ng/mL)	2.30 (1.58)	2.35 (2.18)	2.24 (2.01)	5.26 (3.46)	6.19 (3.78)	16.73
DHEA (ng/mL)	10.13 (8.68)	10.25 (7.47)	13.68 (10.86)	21.00 (12.99)	25.15 (13.60)	12.15
			SG adjusted			
LH (IU/L)	2.39 (2.68)	4.48 (4.68)	5.21 (5.03)	11.21 (8.09)	11.52 (8.76)	13.90
E_2 (ng/mL)	0.16 (0.16)	0.20 (0.23)	0.32 (0.40)	0.95 (0.83)	1.25 (0.83)	25.31
T (ng/mL)	2.04 (2.00)	3.87 (6.75)	7.59 (13.26)	27.21 (25.31)	43.06 (33.50)	27.57
DHT (ng/mL)	2.44 (1.45)	2.84 (3.36)	2.51 (2.31)	5.29 (3.77)	6.31 (4.18)	10.01
DHEA (ng/mL)	11.98 (11.63)	11.86 (11.07)	15.18 (12.42)	20.42 (11.70)	24.44 (12.97)	6.56
			Creatinine adjusted			
LH (IU/L)	2.59 (2.75)	4.50 (4.88)	4.64 (4.56)	10.29 (6.57)	9.17 (6.24)	12.77
E_2 (ng/mL)	0.16 (0.15)	0.20 (0.21)	0.28 (0.32)	0.79 (0.55)	1.04 (0.72)	27.99
T (ng/mL)	1.99 (1.74)	4.00 (6.95)	6.52 (9.90)	22.97 (19.26)	34.45 (24.35)	30.45
DHT (ng/mL)	2.67 (1.59)	2.48 (2.71)	2.32 (2.23)	4.61 (2.83)	5.02 (2.95)	8.85
DHEA (ng/mL)	11.78 (8.24)	10.87 (6.86)	13.60 (10.56)	18.27 (8.10)	20.78 (12.06)	7.13

*All the P values were <0.05 unless indicated in parentheses
(n)[#] Indicates number of samples

Table 5.4 Comparison of mean urinary hormone measurements (unadjusted, SG adjusted and creatinine adjusted) and F- ratios according to age groups in female.

Mean (SD) (n)#	Age (years) 10-10.9 (21)#	11-11.9 (57)#	12-12.9 (50)#	13-13.9 (36)#	>14 (10)#	F Ratio*
			Unadjusted			
LH (IU/L)	4.61 (6.25)	6.76 (8.57)	9.23 (9.37)	11.66 (9.14)	8.80 (4.68)	3.00
E_2 (ng/mL)	0.94 (1.22)	1.26 (1.32)	1.80 (1.84)	2.99 (1.85)	6.74 (4.74)	21.71
T (ng/mL)	2.23 (1.84)	3.48 (3.26)	4.35 (3.51)	5.39 (4.38)	5.57 (4.31)	3.61
DHT (ng/mL)	1.89 (1.43)	2.53 (1.87)	2.69 (1.98)	2.31 (2.18)	2.97 (2.99)	0.82 (P=0.52)
DHEA (ng/mL)	12.97 (7.98)	15.73 (11.41)	18.41 (15.63)	23.60 (14.95)	36.98 (14.35)	7.52
			SG adjusted			
LH (IU/L)	5.42 (7.01)	6.70 (7.58)	10.55 (10.25)	13.88 (11.48)	9.87 (5.56)	4.57
E_2 (ng/mL)	1.04 (1.16)	1.32 (1.40)	2.04 (1.77)	3.43 (2.40)	7.57 (6.05)	21.38
T (ng/mL)	2.66 (2.16)	3.61 (3.31)	5.14 (4.44)	6.12 (5.58)	5.77 (4.16)	3.57
DHT (ng/mL)	2.47 (2.43)	2.62 (2.00)	3.13 (2.39)	2.53 (2.26)	3.11 (2.95)	0.62 (P=0.65)
DHEA (ng/mL)	17.15 (14.01)	16.47 (12.75)	22.57 (21.65)	26.16 (17.16)	41.04 (20.44)	5.48
			Creatinine adjusted			
LH (IU/L)	5.68 (8.00)	7.00 (7.90)	10.52 (9.68)	12.59 (8.93)	9.21 (6.64)	3.49
E_2 (ng/mL)	1.09 (1.26)	1.36 (1.27)	1.96 (1.63)	3.02 (1.73)	5.86 (3.67)	19.98
T (ng/mL)	2.76 (2.36)	3.77 (2.86)	4.90 (3.95)	5.21 (3.74)	5.34 (5.52)	2.50
DHT (ng/mL)	2.30 (1.72)	2.83 (1.74)	3.09 (2.43)	2.38 (2.18)	3.31 (4.46)	0.89 (P=0.47)
DHEA (ng/mL)	15.76 (9.76)	18.07 (11.18)	21.56 (19.65)	23.92 (13.44)	37.84 (27.70)	4.34

*All the P values were <0.05 unless indicated in parentheses.
(n)# indicates number of samples

Table 5.5 Comparison of mean urinary hormone measurements (unadjusted, SG adjusted and creatinine adjusted) and F- ratios according to age groups in male.

Mean (SD) (n)[#]	Age (years) 10-10.9 (29)[#]	11-11.9 (54)[#]	12-12.9 (45)[#]	13-13.9 (42)[#]	>14 (17)[#]	F Ratio*
Unadjusted						
LH (IU/L)	2.52 (4.16)	4.07 (4.50)	7.61 (5.55)	10.38 (8.19)	9.21 (8.90)	10.44
E_2 (ng/mL)	0.11 (0.09)	0.21 (0.28)	0.42 (0.42)	1.03 (1.00)	1.15 (0.73)	20.77
T (ng/mL)	1.33 (2.96)	4.60 (9.48)	11.19 (14.46)	27.63 (25.63)	36.99 (34.82)	19.96
DHT (ng/mL)	1.77 (1.29)	2.65 (2.27)	3.09 (2.72)	5.01 (3.71)	5.57 (3.90)	9.37
DHEA (ng/mL)	6.94 (6.33)	11.15 (8.69)	16.60 (11.04)	21.41 (13.33)	24.72 (12.85)	13.63
SG adjusted						
LH (IU/L)	2.66 (3.95)	4.24 (4.45)	7.51 (5.93)	11.36 (8.82)	8.63 (7.34)	11.59
E_2 (ng/mL)	0.12 (0.08)	0.21 (0.23)	0.41 (0.39)	1.08 (0.95)	1.09 (0.68)	24.48
T (ng/mL)	1.29 (2.35)	4.82 (10.45)	11.03 (14.16)	32.25 (31.29)	34.36 (28.89)	20.40
DHT (ng/mL)	1.87 (1.20)	3.30 (3.83)	3.02 (2.67)	5.43 (3.82)	5.25 (3.73)	6.84
DHEA (ng/mL)	7.71 (6.00)	13.01 (12.50)	16.18 (10.69)	23.13 (13.00)	23.69 (12.75)	10.71
Creatinine adjusted						
LH (IU/L)	2.56 (3.41)	4.19 (5.00)	6.92 (5.08)	9.54 (6.89)	8.07 (5.52)	10.05
E_2 (ng/mL)	0.12 (0.08)	0.21 (0.23)	0.38 (0.34)	0.86 (0.71)	1.00 (0.47)	26.39
T (ng/mL)	1.31 (2.19)	4.26 (7.09)	10.26 (14.12)	24.28 (20.14)	33.08 (25.23)	24.36
DHT (ng/mL)	2.00 (1.48)	2.78 (2.88)	2.78 (2.37)	4.46 (2.83)	4.85 (3.23)	6.29
DHEA (ng/mL)	7.68 (5.56)	11.80 (9.21)	14.91 (8.85)	19.37 (9.52)	22.00 (9.20)	12.13

*All the P values were <0.05 unless indicated in parentheses.
$(n)^{\#}$ indicates number of samples

Table 5.6 Comparison of mean serum hormone measurements and F- ratios according to Tanner stage in female and male.

Mean (SD)	Tanner Stage 1	2	3	4	5	F Ratio*
			Female			
(n)#	(24)#	(50)#	(43)#	(32)#	(12)#	
LH (IU/L)	0.88 (1.54)	2.06 (2.12)	3.47 (2.33)	4.39 (2.57)	5.70 (3.14)	14.90
E_2 (pg/mL)	26.26 (23.00)	36.86 (23.48)	54.76 (28.76)	56.58 (22.32)	74.43 (41.57)	10.80
T (ng/mL)	0.10 (0.06)	0.16 (0.10)	0.22 (0.16)	0.82 (1.84)	0.31 (0.13)	4.09
DHT (ng/mL)	0.08 (0.05)	0.10 (0.07)	0.11 (0.07)	0.15 (0.10)	0.16 (0.08)	4.69
DHEA (ng/mL)	2.26 (1.11)	2.94 (1.62)	3.45 (2.17)	4.01 (2.50)	5.10 (1.70)	5.92
			Male			
(n)#	(17)#	(52)#	(48)#	(34)#	(22)#	
LH (IU/L)	0.50 (0.58)	0.81 (0.81)	1.17 (1.02)	2.64 (1.51)	2.73 (1.53)	24.52
E_2 (pg/mL)	11.88 (6.97)	10.54 (5.44)	13.73 (7.56)	22.28 (11.65)	25.52 (11.19)	18.80
T (ng/mL)	0.09 (0.08)	0.64 (0.92)	0.93 (1.26)	3.55 (2.09)	4.58 (2.08)	51.04
DHT (ng/mL)	0.12 (0.08)	0.17 (0.14)	0.26 (0.66)	0.33 (0.16)	0.35 (0.15)	1.85 (P=0.12)
DHEA (ng/mL)	1.74 (1.23)	2.11 (0.99)	2.42 (1.38)	3.37 (1.73)	3.43 (1.46)	8.41

*All the P values were <0.05 unless indicated in parentheses
(n)# indicates number of samples

Table 5.7 Comparison of mean serum hormone measurements and F- ratios according to age groups in female and male.

Mean (SD)	Age (years)					
	10-10.9	11-11.9	12-12.9	13-13.9	>14	F Ratio*
			Female			
(n)#	(20)#	(54)#	(48)#	(36)#	(9)#	
LH (IU/L)	0.90 (1.18)	2.14 (2.44)	3.00 (2.38)	4.38 (2.17)	6.09 (3.40)	13.20
E_2 (pg/mL)	29.10 (22.51)	31.44 (20.44)	48.80 (21.79)	64.87 (27.98)	88.42 (42.14)	19.86
T (ng/mL)	0.12 (0.08)	0.15 (0.10)	0.24 (0.26)	0.56 (1.27)	1.15 (2.44)	4.17
DHT (ng/mL)	0.08 (0.05)	0.12 (0.69)	0.10 (0.06)	0.14 (0.08)	0.20 (0.14)	6.43
DHEA (ng/mL)	2.54 (1.07)	2.82 (1.51)	2.96 (1.84)	4.05 (1.84)	6.81 (3.69)	12.27
			Male			
(n)#	(26)#	(55)#	(40)#	(40)#	(15)#	
LH (IU/L)	0.65 (0.74)	0.79 (0.84)	1.34 (0.86)	2.27 (1.33)	3.57 (1.98)	28.43
E_2 (pg/mL)	11.38 (5.59)	10.37 (6.68)	15.46 (9.35)	21.09 (9.22)	29.76 (10.90)	22.80
T (ng/mL)	0.14 (0.18)	0.52 (1.04)	1.77 (1.83)	3.26 (2.11)	4.67 (2.00)	37.66
DHT (ng/mL)	0.13 (0.08)	0.22 (0.62)	0.23 (0.18)	0.30 (0.15)	0.37 (0.15)	1.44 (P=0.22)
DHEA (ng/mL)	1.89 (1.20)	2.04 (1.29)	2.60 (1.31)	3.13 (1.32)	4.15 (1.69)	10.92

*All the P values were <0.05 unless indicated in parentheses
(n)# indicates number of samples

Table 5.8 Pearson's correlation coefficient and confidence intervals (in parentheses) of paired urinary and serum LH, E2, T, DHT and DHEA (n=343).

Urine versus serum	LH	E_2	T	DHT	DHEA
UA	0.56 (0.48 to 0.63)	0.73 (0.67 to 0.77)	0.79 (0.74 to 0.82)	0.44 (0.35 to 0.52)	0.63 (0.56 to 0.69)
SG	0.57 (0.50 to 0.64)	0.72 (0.66 to 0.77)	0.79 (0.74 to 0.83)	0.42 (0.33 to 0.50)	0.60 (0.52 to 0.66)
CR	0.56 (0.48 to 0.63)	0.79 (0.74 to 0.82)	0.80 (0.76 to 0.84)	0.35 (0.25 to 0.44)	0.66 (0.59 to 0.71)

Note: UA- unadjusted; SG- specific gravity adjusted; CR- creatinine adjusted.

5.5 Discussion

Urinary measurement of reproductive hormones is a convenient means to evaluate pubertal status and gonadal function for field population studies. In clinical settings, adjustment based on the assumption of stable urine creatinine excretion is commonly used to adjust for variations in hydration although other techniques such as regression normalization or log transformation are proposed (Gaines et al. 2010, Heavner et al. 2006). As an end metabolite of muscle creatine, urine creatinine is determined by total muscle mass in addition to other factors such as age, gender, diet (meat consumption), physical activity, and BMI some of which exert their effects via changes in muscle mass (Alessio et al. 1985, Barr et al. 2005, Carrieri et al. 2000, Suwazono et al. 2005). Hence one aim of the present study was to determine for the first time whether creatinine adjustment was valid or required for longitudinal studies of growing adolescents.

Our findings confirm that the first morning urine creatinine concentration increases with age and Tanner stages and was higher among males. However, adjustment for urine creatinine was no better or worse than adjustment for SG or even no adjustment. This may reflect the fact that we studied first morning void urine samples which control hydration, whereas similar interpretation may not apply to urine sampled at random when hydration state may

vary more. Our findings are consistent with previous studies showing prominent intra- and inter-individual variability in creatinine excretion of second morning and 24h urine samples in adults due to variable fluid intake (Alessio et al. 1985). Significantly higher creatinine levels in morning versus afternoon (Colombi et al. 1983), in evening spot samples (Barr et al. 2005) and creatinine loss due to multiple freeze-thaw cycles have also been reported (Garde et al. 2003, Schneider et al. 2002) all of which introduce systematic errors in use of urine creatinine for dilution adjustments. Thus, although studies have suggested alternative adjustment based on SG in adult humans and primates (Miller et al. 2004, White et al. 2010), none have focused on the need for SG adjustments in first morning voids of growing adolescents.

SG is readily measured by reagent strip for field studies without needing a laboratory. Previous studies demonstrate good agreement between SG measurements by reagent strip versus refractometer (Frew et al. 1982, Moore Jr et al. 1997) or osmolality (Dorizzi et al. 1987, Frew et al. 1982, Gounden and Newall 1983). SG measurement by reagent strip is widely used in clinical applications (Burkhardt et al. 1982, Gounden and Newall 1983). Although refractometer urine SG may be influenced by disease states leading to high serum protein or glycosuria,(Chadha et al. 2001, Voinescu et al. 2002) reagent strip SG is not affected by glucose, only minimally by urea and albumin, but may be affected by the rare instances of alkaline urine (Dorizzi et al. 1987). Urine SG reading may also be influenced by diet, environment and the renal reabsorption capacity (Trevisan 1990). Among adolescents, we find that urine SG measured with reagent strips is systematically not influenced by age or gender consistent with previous reports (Nermell et al. 2008, Suwazono et al. 2005).

Limits of acceptable creatinine and SG measurements vary between studies. Generally, urine is considered too dilute when the SG and creatinine levels are lower than 1.010 and 0.5 g/l (4.4 mmol/L), respectively, and too concentrated where SG and creatinine levels higher than

1.030 (or 1.035) and 3 g/l (26.5 mmol/L), respectively (Alessio et al. 1985, Trevisan 1990). However, due to the standardized method of collection and hydration (first morning void), the present study did not discard any samples as too dilute or too concentrated.

The present study demonstrated that the fasting first morning void urine hormone concentrations adjusted by creatinine correlated well with those adjusted by SG in this adolescent population. This is consistent with previous reports that used randomly collected or timed urine collection from children and adults showing good correlations when creatinine and SG adjustments were compared directly (Carrieri et al. 2000, Cone et al. 2009, Gaines et al. 2010, Haddow et al. 1994, Parikh et al. 2002, Trevisan 1990) or with adjustment according to both (Cone et al. 2009, Miller et al. 2004, White et al. 2010) including a reduced variation using these adjustments in some studies (Haddow et al. 1994, Heavner et al. 2006). However, the present study shows that neither of the adjustment methods for first morning void urine sample of adolescents were significantly improved compared to unadjusted hormone concentrations. These observations are consistent with previous reports for creatinine adjustment of urine steroid measurements in adult women (Hakim et al. 1994, Miyakawa et al. 1981).

In studies where the urinary hormone concentrations were correlated with paired circulating serum concentrations, the urinary unadjusted concentration or concentration expressed by volume of urine correlates better than the adjustment based on analyte to creatinine ratios (Demir et al. 1994, Denari et al. 1981, Zacur et al. 1997), although some studies have shown improved correlation with creatinine adjustments (Munro et al. 1991, Seki et al. 1985). The present study demonstrated that the urinary hormone concentrations adjusted with creatinine and SG did not improve the correlation with paired serum concentrations. These samples were also grouped into three creatinine and SG percentile ranges (25th, 25-75th and 75th) to replicate non-fasting conditions with wider variation in hydration status. However, no

improvement was observed in terms of correlation between the unadjusted or adjusted urine hormone and paired serum concentrations. These findings further support that the adjustments may not be necessary for first morning void urine samples.

In conclusion, the present study shows that adjustment of urinary steroid and LH concentration for hydration state may not be required for first morning void specimens of even growing adolescents. If adjustments are required, then either creatinine or SG are equally suitable and provide comparable results. Reagent strip SG measurements are simple and sufficiently reliable, economical and time-saving for large numbers of urine sampling in long-term field studies.

Chapter 6

Urinary Sex Steroids, Luteinizing Hormone and Anthropometric Markers of Puberty

Chapter 6 Urinary Sex Steroids, Luteinizing Hormone and Anthropometric Markers of Puberty

6.1 Introduction

The circulating gonadotropin, T and E_2 changes that drive the external manifestations of puberty are well described from cross-sectional studies according to chronological age or Tanner staging interpreted quasi-longitudinally (Dorn et al. 2006, Lee and Houk 2006). Such cross-sectional analysis artificially smooths longitudinal data due to a low resolution in temporal sampling, markedly underestimating the underlying within-subject variability. Hence the large, normal variability in both time of onset and tempo of completing puberty has been insufficiently considered as important intermediate factors in the marked physical and psychological changes wrought by puberty. Furthermore previous studies have relied upon older methods of often direct (unextracted) sex steroid immunoassays, an inaccurate technology especially at low circulating steroid levels concentrations (Handelsman and Wartofsky 2013), which is now being supplanted by more sensitive and specific MS-based steroid assays (Handelsman and Wartofsky 2013, Sikaris et al. 2005). Similar more sensitive MS-based methods have been recently described for serum sex steroids in pre-pubertal children (Courant et al. 2010).

In clinical settings the usual methods to appraise pubertal development comprise hormone measurements and anthropometry, with emphasis on timing of the height growth spurt, clinical inspection and bone age (Biro et al. 1995, Bordini and Rosenfield 2011). In epidemiological studies the definition of puberty has to be simplified to be based on adolescent self-report or parental report against either Tanner stage line drawings (Marshall and Tanner 1969, Marshall and Tanner 1970) or Petersen's Pubertal Development Scale

(Dorn et al. 2006). Self-rated Tanner staging is less intrusive and more feasible than clinician assessment for epidemiological studies despite some loss of reliability, especially in early puberty, which can be overcome by larger sample size (Bonat et al. 2002, Desmangles et al. 2006, Hergenroeder et al. 1999, Morris and Udry 1980). Menarche (a late pubertal event), spermarche (Ji 2001, Nielsen et al. 1986) which is difficult to evaluate (Sivananthan et al. 2012), semenarche, and voice breakage have all been described in relation to age and/or Tanner stage (Laron 2010, Sun et al. 2012) but as solitary time points provide minimal information on either timing of onset or tempo of puberty. To date there have been no reports describing individual puberty hormone change with sufficient measurement frequency to adequately capture individual variation, which is necessary to adequately describe onset and tempo of puberty for a community-based study.

The first aim of this study is to validate the feasibility of the frequent urine sampling regimen and the urine assay methodology for LH, E_2 and T. The second aim is to validate the changes in urine LH, E_2 and T over 12 months by reference to contemporaneous changes in anthropometry and self-reported Tanner stage.

6.2 Study design

Adolescents between the ages of 10 and 12 years were recruited from local schools of two regional towns in the state of New South Wales (NSW), Australia. Fasting morning blood samples were collected at 0 and 12 months for the measurement of LH, E_2 and T, and first morning (fasting) urine collected three monthly for the same measures. No participants had an endocrine disorder or were on any type of gonadal steroid hormone therapy. Height was measured using a portable stadiometer (to 0.1 cm). Weight was measured in light clothing using a Tanita TBF-300 Pro Body Composition Analyzer (Kettaneh et al. 2005). Height, weight and BMI (kg/m^2) were expressed as z-scores using the Centres for Disease Control

(CDC 2000) and World Health Organization (WHO 2006) standard age and gender reference charts. The adolescents provided a self-rating of puberty using line drawings based on the Tanner stages (Marshall and Tanner 1969, Marshall and Tanner 1970). Self-report of Tanner stage (Bonat et al. 2002, Desmangles et al. 2006, Hergenroeder et al. 1999, Morris and Udry 1980) was the only feasible and ethically acceptable measure of pubertal staging available to the investigators.

First morning urine following a 12 hour fast was collected at home before blood samples were collected between 7:00 am and 8:30 am to minimise the effects of diurnal hormone variation (Bremner et al. 1983, Dorn et al. 2006). Serum and urine were stored at -80 °C. Post-menarcheal girls provided urine and blood specimens in the mid-follicular phase (Day 7-10) of their menstrual cycle. Serum and urine steroids were measured by LC-MS/MS assay (Chapter 2). Blood and urine samples with hormone values less than the LLOQ for E_2 and T were taken as half the LLOQ.

Serum and urine LH were measured by Immulite 1000 LH (Chapter 2). The within-assay coefficients of variation were <10%. Serum and urine LH values below the detection limit (0.1 IU/L) were set at zero. Urine FSH assays (Immulite, Delfia) did not pass validity tests (dilutional linearity, quantitative spike recovery) and were not used in this study. All urine hormone concentrations were adjusted for urine SG measured by reagent strip (Chapter 2) to a standard SG of 1.020.

The study has ethical approval from the Human Research Ethics Committee, University of Sydney (HREC 13094) within the NHMRC, which are consistent with the Declaration of Helsinki. All participants assented, and a parent provided written informed consent prior to commencing the study.

6.3 Data analysis

Two-way ANOVA was used to determine effects of gender and scheduled sampling time and their interaction on anthropometric and hormone outcomes. ANCOVA was used to assess the relationship between serum hormones with changes in anthropometric variables. Mixed models linear regression was used to assess the relationship between longitudinal changes in urinary hormones and changes in anthropometric values. Baseline hormone values and baseline anthropometric measurements were covariates. All statistical analyses were conducted using Stata 12.1 (StataCorp, Texas, USA). Statistical significance was set at the 0.05 level.

6.4 Results

6.4.1 Cohort characteristics

One hundred and four participants were recruited. The mean ages (SD) for the study participants at baseline were 12.5 (0.93) years for males and 11.8 (0.98) years for females. At follow-up, the ages were 13.5 (0.94) years for males and 12.9 (0.97) years for females. For the females, 22 (39%) had menarche prior to the study and one additional girl experienced menarche during the follow-up year. Post-menarcheal girls were significantly older than their pre-menarcheal counterparts (12.9 years vs. 11.9 years, $p<0.001$). A high proportion of scheduled samples were collected for urine (484, 92%) and serum (194, 93%). There was a low loss to follow-up (7, 6.7%).

Mean anthropometric measurements, other than BMI, increased significantly over 12 months (Table 6.1) whereas age- and gender-standardized z-scores did not change over the 12 months follow-up (Table 6.2). Pre-menarcheal girls significantly increased their age-standardized weight ($p<0.001$) and height z-scores ($p = 0.002$), whereas these z-scores did not change for

post-menarcheal girls. Rate of change in anthropometric measurements were similar between genders (interaction p>0.05).

Self-rated Tanner staging increased with fewer in stage 1 and more in stage 5 at 12-month follow-up. During the year, 14 participants (13.5%) progressed two Tanner stages, 43 (41.3%) progressed one stage and 41 (39.4%) did not change in their self-rated Tanner stage (Table 6.3). Six (5.8%) participants (three boys) provided a lower self-rated Tanner stage at follow-up than baseline. One participant (1%, 1 boy) did not provide baseline Tanner staging and seven (6.7%, 1 boy, 6 girls) did not provide follow-up Tanner staging.

Table 6.1 Baseline and 12-month follow-up anthropometry measurements.

	Baseline		**12 Months**		**ANOVA F-statistic (p-value)**		
Mean (SD)	**M (n=47)**	**F (n=57)**	**M (n=47)**	**F (n=57)**	**Gender**	**Time**	**Gender x Time**
Height (cm)	156.8 (9.9)	150.8 (8.5)	163.8 (10.1)	157.1 (7.9)	25.7 (<0.001)	27.6 (<0.001)	0.08 (0.78)
Weight (kg)	49.3 (12.0)	44.1 (10.9)	55.2 (13.2)	50.1 (11.4)	9.7 (0.002)	13.2 (<0.001)	0.00 (0.99)
BMI (kg/m^2)	19.7 (3.5)	20.4 (3.8)	19.3 (3.7)	20.3 (4.0)	0.29 (0.59)	2.84 (0.09)	0.08 (0.77)

Table 6.2 Baseline and 12 month follow-up WHO and CDC z-scores (for age).

Mean (SD)	**Baseline**		**12 months**		**Two-Way ANOVA F-statistic (p-value)**		
	M	**F**	**M**	**F**	**Gender**	**Time**	**Gender x Time**
Height z (CDC)	0.50 (1.05)	0.16 (0.87)	0.50 (1.08)	0.25 (0.91)	4.81 (0.03)	0.13 (0.72)	0.12 (0.73)
Weight z (CDC)	0.43 (1.12)	0.19 (0.96)	0.46 (1.11)	0.35 (0.91)	1.46 (0.23)	0.44 (0.51)	0.19 (0.67)
BMI z (CDC)	0.28 (1.03)	0.18 (1.00)	0.27 (1.04)	0.27 (0.98)	0.12 (0.73)	0.09 (0.76)	0.12 (0.72)
Height z (WHO)	0.56 (1.11)	0.14 (0.92)	0.58 (1.12)	0.30 (0.93)	6.18 (0.02)	0.40 (0.53)	0.25 (0.62)
BMI z (WHO)	0.51 (1.24)	0.29 (1.18)	0.42 (1.21)	0.32 (1.18)	0.91 (0.34)	0.03 (0.87)	0.14 (0.71)

Table 6.3 Self-Rated Tanner staging at baseline and 12 month follow-up.

N	Baseline		12 Months	
Tanner	M	F	M	F
1	6	14	0	3
2	8	17	7	13
3	9	16	6	15
4	18	7	18	12
5	5	3	14	8
Total	46	57	45	51

6.4.2 Hormone measurements

Serum: Of the 194 serum collections, nine (5%) LH assays and two (1%) T (all in separate individuals) were below the LLOQ. No E_2 samples were below LLOQ. Those with LH samples below the limits of detection were significantly younger than their peers (mean difference = 1.47 years 95% CI 0.81-2.14 $p<0.001$). No difference in age was observed for those with serum T or E_2 samples below LLOQ. Hormone values all significantly increased over the 12-month period and were significantly different between genders, but rate of change was not statistically significant between gender (interaction $p>0.05$) (Table 6.4).

Table 6.4 Baseline and 12-month follow-up serum LH, E_2 and T.

Mean (SD)	Baseline		12 months		ANOVA F-statistic (p-value)		
	M (n=47)	F (n=57)	M (n=47)	F (n=57)	Gender	Time	Gender x Time
LH (IU/L)	1.7 (1.3)	2.3 (2.4)	2.5 (1.8)	3.6 (2.7)	9.4 (0.002)	11.2 (0.001)	0.70 (0.402)
E_2 (pg/mL)	19.1 (8.5)	43.4 (29)	24.9 (9)	54.7 (30)	73.5 (<0.001)	6.4 (0.012)	0.74 (0.390)
T (ng/mL)	2.4 (2.3)	0.16 (0.10)	3.4 (2.55)	0.2 (0.12)	130.1 (<0.001)	5.0 (0.027)	3.80 (0.052)

Figure 6.1 shows serum LH, E_2 and T at baseline and follow-up, stratified by Tanner stages. Data stratified by chronological age were similar. Hormone concentrations increased through each Tanner stage and each year of age, although ordinal groupings overlap.

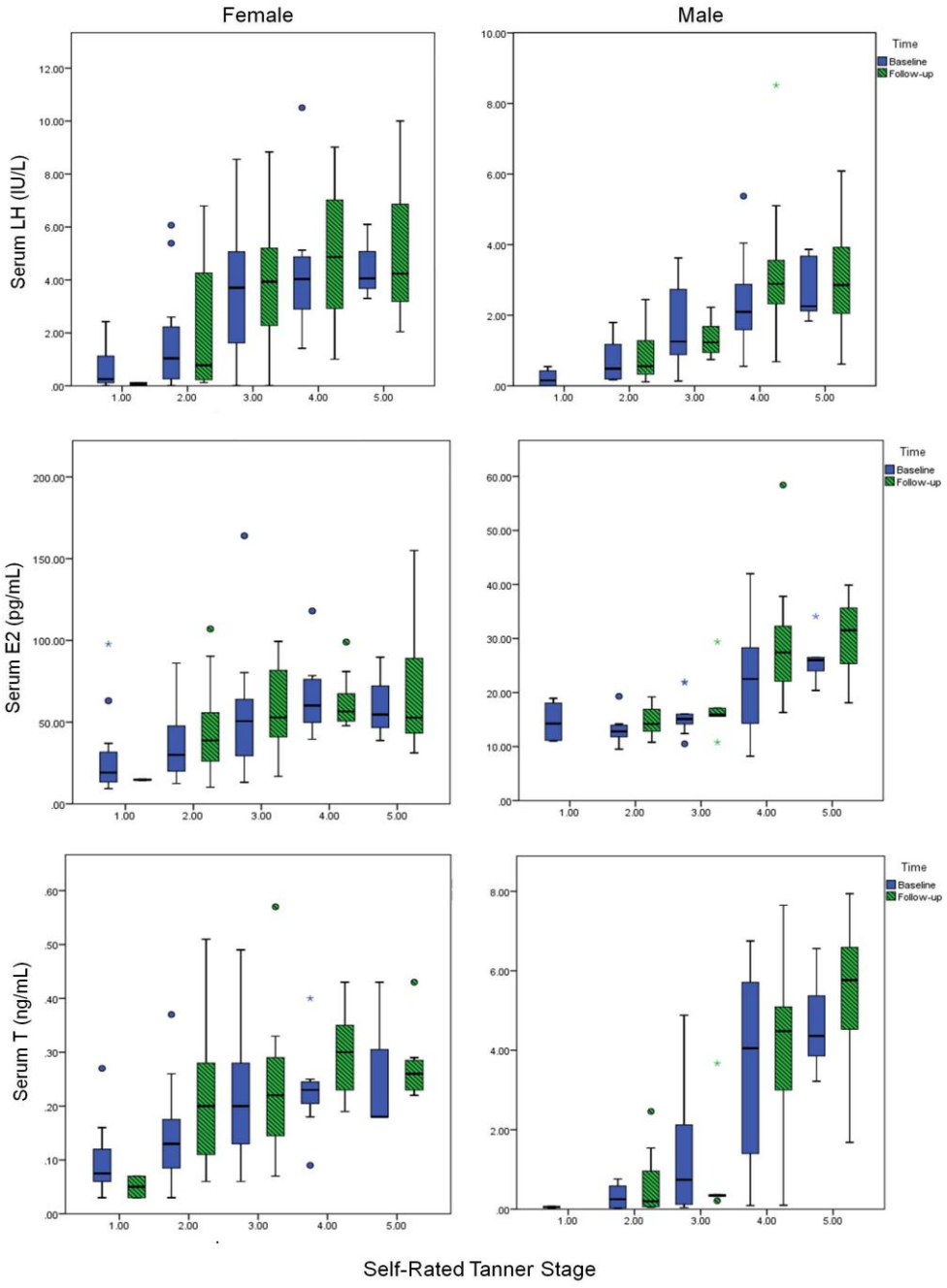


Figure 6.1 Cross-sectional plots of serum LH (top), E2 (centre) and T (bottom) at baseline and 12-month follow-up, by self-rated Tanner stage.

Urine: For the 484 urine collections, five (1%) LH assays, 14 (3%) E_2 and nine (2%) T assays were below the LLOQ. For LH, these participants were younger than the rest of the cohort (mean difference 1.35 years; 95% CI 0.41-2.30; p=0.005), but there was no age difference between those with urine E_2 or T samples below or above the LLOQ. In five such cases (one urine and serum LH, one urine E_2, one urine E_2 and T, one urine T, one serum LH) the participant provided a Tanner stage 1 at baseline.

Figure 6.2 shows within-person changes from baseline over the 12-month collection period and Table 6.5 shows the mean three monthly values for urinary LH, E_2 and T over 12 months. There is a significant increase from baseline to follow-up in E_2, T and LH. The same pattern is seen in females when stratified by menarcheal status (Table 6.6). Urine hormone levels were not strictly progressive and in some instances decreased over time, though there was an overall increase in mean levels for all three hormones for males and females overall. For serum hormones, a decline from baseline to follow-up was observed in 26 adolescents (18 female) for LH, 22 adolescents (11 female) for E_2 and 19 (13 female) for T. Overall declines in urinary LH were observed in 48 adolescents (30 female), 23 (9 female) for urinary E_2, and 24 (14 female) for urinary T.

Urinary LH, E_2 and T all positively correlated with Tanner staging at baseline and 12-month follow-up ($p<0.001$ for all). Urinary LH, E and T all increased across Tanner stages.

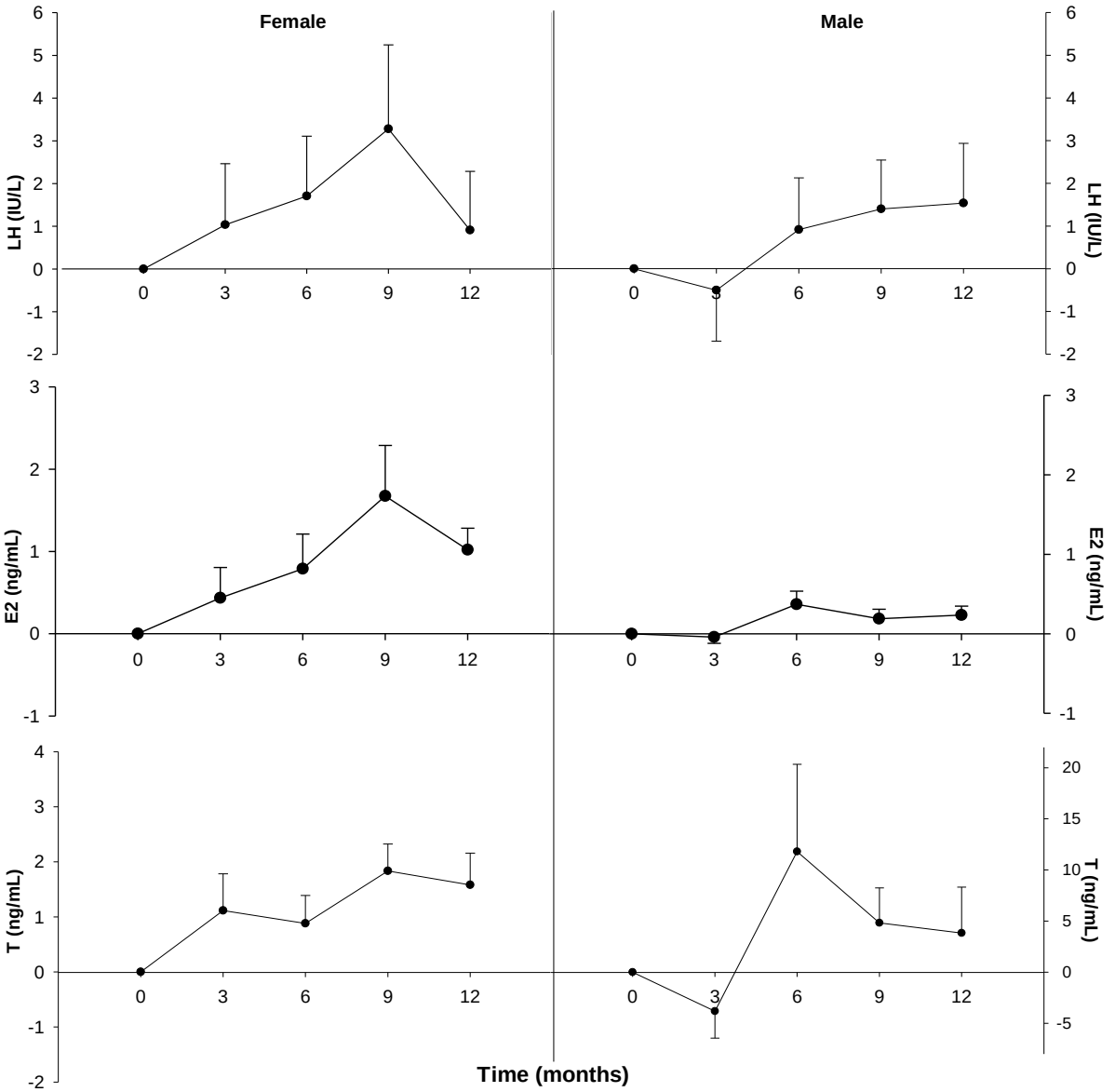


Figure 6.2 Plots of mean and SEM of individual changes from baseline (0) in urine LH, E2 and T concentrations in female (left panels) and male (right panels) at 3, 6, 9 and 12 months.

Note the y-axis scales of the T plots differ between genders.

Table 6.5 Mean urine LH, E2 and T.

Mean (SD)	Baseline		3 Months		6 Months		9 Months		12 Months (Follow-Up)		Two Way ANOVA F-statistic (p-value)		
	M	F	M	F	M	F	M	F	M	F	Gender	Time	Gender x Time
LH (IU/L)	8.7 (7.4)	9.6 (10.0)	8.4 (5.9)	10.2 (10.1)	9.6 (7.4)	11.5 (11.4)	9.8 (6.1)	13.3 (16.6)	10.1 (7.9)	10.1 (9.9)	3.2 (0.074)	0.9 (0.438)	0.4 (0.802)
E_2 (ng/mL)	0.7 (0.9)	1.8 (2.0)	0.7 (0.9)	2.1 (2.8)	1.1 (1.6)	2.5 (3.1)	0.9 (1.1)	3.5 (4.8)	1.0 (1.0)	2.7 (2.8)	52.4 **(<0.001)**	2.3 (0.059)	1.3 (0.255)
T (ng/mL)	23.3 (35)	3.9 (3.8)	19.9 (26.1)	5.0 (6.2)	35.1 (80)	5.0 (4.1)	26.4 (34)	6.0 (4.1)	26.2 (28)	5.4 (5.1)	57.9 **(<0.001)**	0.9 (0.4870)	0.8 (0.548)

Table 6.6 Mean urine hormone measurements for girls by menarcheal status.

Mean (SD)	Baseline		3 Months		6 Months		9 Months		12 Months (Follow-Up)		Two-way ANOVA F-statistic (p-value)		
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Menarcheal Status	Time	Menarcheal Status x Time
LH (IU/L)	6.5 (7.48)	14.6 (11.53)	6.8 (7.55)	16.1 (11.25)	7.6 (7.21)	17.4 (14.10)	9.0 (8.48)	19.2 (22.69)	9.5 (8.90)	11.0 (11.34)	30.34 **(<0.001)**	1.05 (0.384)	1.34 (0.255)
E_2 (pg/mL)	0.7 (0.67)	3.4 (2.19)	0.9 (0.84)	4.1 (3.70)	1.1 (0.80)	4.7 (4.03)	1.5 (1.39)	6.4 (6.32)	1.7 (1.90)	4.3 (3.16)	95.54 **(<0.001)**	3.20 (**0.014**)	1.50 (0.201)
T (ng/mL)	2.4 (2.00)	6.3 (4.70)	2.7 (2.03)	8.9 (8.70)	3.2 (2.72)	7.7 (4.48)	4.5 (2.99)	8.0 (4.53)	4.3 (3.16)	6.9 (4.38)	57.21 **(<0.001)**	1.41 (**0.232**)	1.33 (0.258)

6.4.3 Association between Serum and Urine Hormone Measurements and Anthropometry

Change in serum LH was significantly positively associated with change in height and weight for females, but with neither in males (Table 6.7). Serum T was significantly positively associated with height changes in both sexes. Change in height was positively associated with urinary E_2 and T in females and urinary E_2 in males. No other significant associations were observed between urinary hormones and anthropometry over 12 months (Table 6.7).

Table 6.7 Regression results for serum (left) and urine (right) hormones and anthropometric markers of puberty.

Serum	β	95% CI	F-statistic	p-value	Urine	β	95% CI	p-value
				Change in Height				
				Female				
LH	0.53	0.21, 0.84	11.50	**0.001**	LH	0.01	-0.47, 0.48	0.979
E_2	-0.001	-0.04, 0.03	0.01	0.943	E_2	-0.25	-0.38, -0.12	**<0.001**
T	10.19	0.77, 19.61	4.73	**0.035**	T	-0.25	-0.44, -0.06	**0.011**
				Male				
LH	-0.16	-0.92, 0.61	0.17	0.681	LH	0.31	-0.11, 0.72	0.149
E_2	0.09	-0.02, 0.21	2.61	0.115	E_2	-0.10	-0.16, -0.03	**0.004**
T	0.89	0.48, 1.31	19.26	**<0.001**	T	-1.05	-3.74, 1.64	0.446
				Change in Weight				
				Female				
LH	0.45	0.10, 0.81	6.67	**0.013**	LH	-0.13	-0.55, 0.28	0.535
E_2	-0.01	-0.05, 0.03	0.20	0.656	E_2	-0.02	-0.14, 0.09	0.695
T	11.81	1.15, 22.48	4.96	**0.031**	T	-0.14	-0.30, 0.03	0.110
				Male				
LH	-0.10	-1.74, 1.54	0.02	0.900	LH	0.07	-0.13, 0.27	0.492
E_2	0.07	-0.18, 0.31	0.31	0.583	E_2	0.01	-0.02, 0.04	0.505
T	0.21	-0.86, 1.28	0.16	0.688	T	0.81	-0.48, 2.10	0.218
				Change in BMI				
				Female				
LH	0.03	-0.10, 0.16	0.24	0.625	LH	-0.79	-2.03, 0.45	0.214
E_2	0.001	-0.01, 0.01	0.01	0.921	E_2	0.03	-0.31, 0.37	0.858
T	2.18	-1.73, 6.09	1.25	0.269	T	-0.47	-0.97, 0.02	0.062
				Male				
LH	-0.10	-0.46, 0.26	0.31	0.580	LH	-0.03	-0.89, 0.82	0.940
E_2	-0.02	-0.07, 0.03	0.69	0.412	E_2	-0.02	-0.16, 0.11	0.737
T	-0.01	-0.24, 0.23	0.00	0.963	T	1.33	-4.19, 6.86	0.636
				Change in Self-Rated Tanner Stage				
				Female				
LH	0.07	-0.04, 0.17	1.51	0.225	LH	0.46	-1.19, 2.11	0.583
E_2	0.002	-0.01, 0.01	0.12	0.730	E_2	-0.24	-0.70, 0.22	0.310
T	1.59	-1.25, 4.44	1.28	0.265	T	0.11	-0.56, 0.78	0.757
				Male				
LH	-0.08	-0.33, 0.17	0.44	0.510	LH	-0.10	-1.23, 1.03	0.863
E_2	0.03	-0.003, 0.07	3.38	0.075	E_2	-0.15	-0.33, 0.03	0.100
T	0.16	0.02, 0.29	5.40	**0.026**	T	-0.27	-7.64, 7.09	0.942

6.5 Discussion

The two major findings of this study are the validation of a MS assay for pubertal urine steroids in a young adolescent population and the demonstration that it is feasible to collect urine samples from a community-based adolescent cohort at three-monthly intervals, with high compliance (93% serum and 92% urine collections completed) and low follow-up attrition rate.

In order to demonstrate the specific effects of puberty hormones on any biological aspect of adolescent development and health, a methodology that allows more frequent biological sampling than has been previously reported for epidemiological studies is essential. Urine samples have the advantage over blood samples that these are more acceptable both ethically and practically by adolescents, and each overnight sample provides a more time-integrated hormone measure. This is particularly true in early puberty when pubertal hormones commence pulsatile secretion nocturnally (Dorn et al. 2006), so that a morning overnight urine sample may be more informative than a serum sample at any single time point. Urine collections also allow for more frequent collection than repeated venepuncture would be tolerated (Aksglaede et al. 2009). Salivary samples are potentially easier to collect, but blood contamination and influence of flow-rate on measurements seriously limits validity and accuracy.

Anthropometric and serum hormone changes in our study revealed the anticipated increases over the course of one year in a cohort of young adolescents. The urine data also revealed anticipated hormone increases over the 12-months of observation. However, mean and individual urinary hormone changes were not strictly progressive; suggesting within-subject variability in early and mid-pubertal hormone levels may contain hitherto unexploited information on determinants of biological aspects of pubertal progression. This finding also

suggests that three monthly urine collections over an extended period have the capacity to provide new insights into the biology of puberty.

The cross-sectional associations between chronological age and self-reported Tanner stage and both serum and urinary hormones reveal considerable overlap between hormone levels at each age or stage, and emphasise the need to have better descriptors of puberty hormone change. Both Tanner stage and anthropometric change lag behind hormonal change. Using the former as surrogates for puberty hormone change will less accurately describe the relationship between hormone changes and the resultant physical changes, as well as any other adolescent health or developmental change of interest, such as mood or behaviour. Previous work has questioned the validity of self-rated Tanner staging (Bonat et al. 2002, Desmangles et al. 2006, Dorn et al. 2006, Hergenroeder et al. 1999, Morris and Udry 1980); however, our data support the validity of self-rated Tanner staging as a high proportion of adolescents completed the scales with findings of consistent or advanced Tanner stage at 12 months follow-up in all but 6% of adolescents and with the self-rated Tanner staging corresponding well with conventional anthropometric measures of puberty.

In conclusion, our work has used a robust methodology of urine sex steroid hormone measurement, using LC tandem mass spectrometry measurements for urine sex steroids (Handelsman and Wartofsky 2013), which display the high sensitivity and specificity to detect the lower levels of sex hormones, a particular challenge to the study of pubertal progression (Courant et al. 2010, Rosner et al. 2013). Based on previously recorded longitudinal growth data (Marceau et al. 2011, Steinbeck et al. 2012), it is anticipated that frequently measured urine samples over the two to three year window of puberty will not only provide a firmer biological basis for clinically observed patterns of puberty, such as early or late onset and rapid or slow tempo, but also allow determination of the true biological effects of puberty hormones on adolescent health and development.

Chapter 7 *Conclusions*

In this thesis, we successfully developed suitable sample preparation techniques and LC-MS/MS assays to measure steroids in various biological samples. The methods were further applied for clinical research studies including remote sampling and field studies. We also demonstrated that previously validated LH immunoassays for human blood samples are also suitable with qualifications for urine samples. This thesis consists of four research chapters as summarized in the sections below.

7.1 Blood spot sampling after sc steroid injection

This study was to assess the feasibility and pharmacology of sc injection of androgen ester in healthy men using DBS for frequent sampling. A sensitive and specific LC-MS/MS assay was developed and validated to measure steroids from DBS samples. To avoid common problems of using DBS samples such as non-homogenous distribution of blood on the filter card and hematocrit effect, we developed a novel sampling method which used the whole blood spotted onto the filter card instead of using a subsample (e.g. by a punch). The DBS technology provided intensive and simplified blood collection by the study participants at home without the need of clinic visit. This study provided a detailed pharmacological analysis of sc injection of ND in oil vehicle. We found a sustained release of this androgen ester which suggests that sc injections of T esters may prove to be safe and tolerable. This would make self-injection of long-acting depot form of T delivery more feasible with the potential benefits of reducing medical care costs demand on medical personnel time. Additionally this delivery method may also reduce injection site pain or bruising as well as allowing freer use among those with bleeding disorders or on anticoagulants. Further studies using T esters in men with differing body weight and after repeated injections are required to evaluate duration of action and usefulness in clinical practice of sc relative to im injections.

7.2 Urinary LH immunoassays in stored samples

This study was to assess whether commercially available LH immunoassays (ICL and IF) previously validated for human blood samples is suitable for urine samples kept at prolonged frozen storage (4 years) without addition of any preservative. Urinary LH may be dissociated into its subunits due to high urinary concentrations of urea that may cause loss in immunoreactivity after prolonged frozen storage. Using samples from a clinical study completed in 2008 and aliquots of the same samples stored frozen at -20 °C without added preservatives, this study describes the deviations in performance of ICL and IF immunoassays and their ultimate applicability for reliability clinical research and for anti-doping purposes. The study demonstrated that the two LH immunoassays optimized for blood samples can also be used for urinary LH measurement but the performance of both the assay differed markedly. The ICL immunoassay showed quantitatively reproducible LH measurement even after prolonged storage at -20 °C for 4 years without addition of preservatives. By contrast, the IF immunoassay demonstrated consistently but proportionately lower LH measurements relative to the ICL assay both initially (2008) and then exhibited a further decrease after 4 years of frozen storage (2012). Yet, both the assays displayed similar patterns of the time-course of urine LH measurement both before and after 4 years of frozen storage. In conclusion, we found that both immunoassays are suitable for urinary LH measurements with ICL assay being more robust for quantitative urinary LH measurement such as for anti-doping purpose whereas the IF could be applicable for research studies where urine LH levels are compared within-study but not in absolute terms.

7.3 SG and creatinine adjustments for urinary hormones

The study aimed to determine whether first morning void hormonal assessments carried out in growing young adolescents require adjustments to correct for variation due to hydration, and if so, to determine the best adjustment method based either on urinary creatinine or SG. While previous studies have examined broadly the issue of how to adjust urinary hormonal measurements none have focused on the specific situation where creatinine is changing systematically due to growth, hence this study contains novel information on whether urinary hormonal concentration adjustments are required in the setting of repeated serial sampling of a large adolescent population at various stages of pubertal progression. The present study demonstrated that unadjusted urinary steroids and LH concentration corresponded well with the concentration adjusted either with creatinine or SG when using first morning void urine samples, reflecting an overnight fast, even in growing adolescents. Based on these findings we suggest the possibility that the first morning void urine samples are not heavily influenced by the hydration status of study participants and may not require adjustments compared with randomly collected spot or timed urine collection methods. However, if the dilution adjustment is desired for the urine specimen, both the creatinine and SG appear equally suitable using first morning void urine samples. We also show that reagent strip SG measurements are simpler alternative to a refractometer and sufficiently reliable, economical and time-saving for large numbers of urine sampling in long-term field studies.

7.4 Urinary puberty hormones

The aim of this study was to develop and validate LC-MS/MS assay to measure urinary T and E_2 in adolescents and subsequently to relate the changes in the urinary sex hormones over 12 months to the standard anthropometric markers of puberty (height, weight, BMI, self-rate Tanner stage). Longitudinal relationships of within-individual hormone and anthropometric changes during puberty have not been fully described previous. Urine provides a more time-

integrated hormone measure compared to blood samples and is particularly important as pulsatile secretion during early progression of puberty occurs nocturnally. The study demonstrated feasibility of urine samples collection from a community based-adolescent cohort at three-monthly intervals with high compliance and low follow-up attrition rate. Both urine and serum hormone data showed anticipated increase over 12 months. This study successfully developed a robust LC-MS/MS method to measure urinary steroids that display high sensitivity and specificity. The study preliminary findings demonstrate the feasibility of intensive collection of urine samples together with validated urine assays for sex steroids and LH. These will allow a more accurate and sensitive, individual assessment of puberty timing and tempo. Currently, three monthly urine and a yearly blood collection for the ARCHER longitudinal study over three years is being carried out to better understand the role of puberty hormones on adolescent events. This longitudinal study aims to determine how temporal changes in T and E_2 independently affect physical status, social and emotional well-being, education, sleep, risk behaviours, and mental health in adolescents.

References

Abraham G, Buster J, Lucas L, Corrales P, Teller R 1972 Chromatographic separation of steroid hormones for use in radioimmunoassay. Anal. Lett. 5:509-517

Abraham GE 1969 Solid-phase radioimmunoassay of estradiol-17β. J. Clin. Endocr. Metab. 29:866-870

Abraham GE 1974 Radioimmunoassay of steroids in biological materials. Acta Endocrinol. (Copenh.) 75

Abreu AP, Dauber A, Macedo DB, Noel SD, Brito VN, Gill JC, Cukier P, Thompson IR, Navarro VM, Gagliardi PC 2013 Central precocious puberty caused by mutations in the imprinted gene MKRN3. N. Engl. J. Med. 368:2467-2475

Agarwal AK, Auchus RJ 2005 Minireview: cellular redox state regulates hydroxysteroid dehydrogenase activity and intracellular hormone potency. Endocrinology 146:2531-2538

Aksglaede L, Sorensen K, Petersen JH, Skakkebaek NE, Juul A 2009 Recent decline in age at breast development: the Copenhagen Puberty Study. Pediatrics 123:e932-939

Al-Futaisi AM, Al-Zakwani IS, Almahrezi AM, Morris D 2006 Subcutaneous administration of testosterone. A pilot study report. Saudi Med. J. 27:1843-1846

Alary J 2001 Comparative study: LC-MS/MS analysis of four steroid compounds using a new photoionization source and a conventional APCI source. Proceedings of the 49th ASMS Conference on Mass Spectrometry and Allied Topics, 2001, pp 27-31

Albert C, Vallée M, Beaudry G, Bélanger A, Hum DW 1999 The monkey and human uridine diphosphate-glucuronosyltransferase UGT1A9, expressed in steroid target tissues, are estrogen-conjugating enzymes. Endocrinology 140:3292-3302

Albertsson-Wikland K, Rosberg S, Karlberg J, Groth T 1994 Analysis of 24-hour growth hormone profiles in healthy boys and girls of normal stature: relation to puberty. J. Clin. Endocr. Metab. 78:1195-1201

Albertsson-Wikland K, Rosberg S, Lannering B, Dunkel L, Selstam G, Norjavaara E 1997 Twenty-four-hour profiles of luteinizing hormone, follicle-stimulating hormone, testosterone, and estradiol levels: A semilongitudinal study throughout puberty in healthy boys. J. Clin. Endocr. Metab. 82:541-549

Alessio L, Berlin A, Dell'Orto A, Toffoletto F, Ghezzi I 1985 Reliability of urinary creatinine as a parameter used to adjust values of urinary biological indicators. Int. Arch. Occup. Environ. Health 55:99-106

Allan CM, Handelsman DJ 2005 Transgenic models for exploring gonadotropin biology in the male. Endocrine 26:235-239

Alonso-Whipple C, Couet ML, Doss R, Koziarz J, Ogunro EA, Crowley WF, Jr. 1988 Epitope mapping of human luteinizing hormone using monoclonal antibodies. Endocrinology 123:1854-1860

Alvarez Sanchez B, Capote FP, Jiménez JR, Luque de Castro M 2008 Automated solid-phase extraction for concentration and clean-up of female steroid hormones prior to liquid chromatography–electrospray ionization–tandem mass spectrometry: An approach to lipidomics. J. Chromatogr. A 1207:46-54

Andersson A-M, Toppari J, Haavisto A-M, Petersen JH, Simell T, Simell O, Skakkebæk NE 1998 Longitudinal reproductive hormone profiles in infants: peak of inhibin B levels in infant boys exceeds levels in adult men. J. Clin. Endocr. Metab. 83:675-681

Andrew R 2001 Clinical measurement of steroid metabolism. Best Pract. Res. Clin. Endocrinol. Metab. 15:1-16

Ankarberg-Lindgren C, Norjavaara E 2008 A purification step prior to commercial sensitive immunoassay is necessary to achieve clinical usefulness when quantifying serum 17β-estradiol in prepubertal children. Eur. J. Endocrinol. 158:117-124

Annesley TM 2007 Methanol-associated matrix effects in electrospray ionization tandem mass spectrometry. Clin. Chem. 53:1827-1834

Antignac J-P, Brosseaud A, Gaudin-Hirret I, André F, Bizec BL 2005 Analytical strategies for the direct mass spectrometric analysis of steroid and corticosteroid phase II metabolites. Steroids 70:205-216

Antignac J-P, de Wasch K, Monteau F, De Brabander H, Andre F, Le Bizec B 2005 The ion suppression phenomenon in liquid chromatography–mass spectrometry and its consequences in the field of residue analysis. Anal. Chim. Acta 529:129-136

Apter D, Cacciatore B, Alfthan H, Stenman U 1989 Serum luteinizing hormone concentrations increase 100-fold in females from 7 years of age to adulthood, as measured by time-resolved immunofluorometric assay. J. Clin. Endocr. Metab. 68:53-57

Apter D, Pakarinen A, Hammond G, Vihko R 1979 Adrenocortical function in puberty serum ACTH, cortisol and dehydroepiandrosterone in girls and boys. Acta Paediatr. 68:599-604

Ascoli M, Narayan P 2014 The Gonadotropin Hormones and Their Receptors. In: Strauss III JF, Barbieri RL eds. Yen and Jaffe's reproduction endocrinology: Physiology, pathophysiology and clinical management. 7th ed: Elsevier Health Sciences; 27-44

Auchus RJ 2004 The backdoor pathway to dihydrotestosterone. Trends Endocrinol. Metab. 15:432-438

August G, Grumbach M, Kaplan S 1972 Hormonal changes in puberty: III. Correlation of plasma testosterone, LH, FSH, testicular size, and bone age with male pubertal development. J. Clin. Endocrinol. Metab. 34:319-326

Aylward LL, Hays SM, Smolders R, Koch HM, Cocker J, Jones K, Warren N, Levy L, Bevan R 2014 Sources of variability in biomarker concentrations. J. Toxicol. Environ. Health, Part B 17:45-61

Bagchus WM, Smeets JM, Verheul HA, De Jager-Van Der Veen SM, Port A, Geurts TB 2005 Pharmacokinetic evaluation of three different intramuscular doses of nandrolone decanoate: analysis of serum and urine samples in healthy men. J. Clin. Endocrinol. Metab. 90:2624-2630

Baird D, Horton R, Fongcope C, Tait J, Izak F, Baker B, Yu Y, Tenore A, De Iorio A, Hellman L 1965 Steroid dynamics under steady-state conditions. Recent Prog. Horm. Res. 25:611-664

Bakhtiar R, Majumdar TK 2007 Tracking problems and possible solutions in the quantitative determination of small molecule drugs and metabolites in biological fluids using liquid chromatography–mass spectrometry. J. Pharmacol. Toxicol. Methods 55:227-243

Bakhtiar R, Ramos L, Tse FL 2002 High-throughput mass spectrometric analysis of xenobiotics in biological fluids. J. Liq. Chromatogr. Relat. Technol. 25:507-540

Balen A, Mulders A, Fauser B, Schoot B, Renier M, Devroey P, Struijs M, Mannaerts B 2004 Pharmacodynamics of a single low dose of long-acting recombinant follicle-stimulating hormone (FSH-carboxy terminal peptide, corifollitropin alfa) in women with World Health Organization group II anovulatory infertility. J. Clin. Endocr. Metab. 89:6297-6304

Banerjee S, Mazumdar S 2012 Electrospray ionization mass spectrometry: a technique to access the information beyond the molecular weight of the analyte. Int. J. Anal. Chem. 2012

Barbier O, Bélanger A 2003 The cynomolgus monkey (Macaca fascicularis) is the best animal model for the study of steroid glucuronidation. J. Steroid Biochem. 85:235-245

Barbier O, Bélanger A 2008 Inactivation of androgens by UDP-glucuronosyltransferases in the human prostate. Best Pract. Res. Clin. Endocrinol. Metab. 22:259-270

Bardin CW, Lipsett MB 1967 Testosterone and androstenedione blood production rates in normal women and women with idiopathic hirsutism or polycystic ovaries. J. Clin. Invest. 46:891

Barr DB, Wilder LC, Caudill SP, Gonzalez AJ, Needham LL, Pirkle JL 2005 Urinary creatinine concentrations in the US population: implications for urinary biologic monitoring measurements. Environ. Health Perspect. 113:192-200

Barrow MP, Burkitt WI, Derrick PJ 2005 Principles of Fourier transform ion cyclotron resonance mass spectrometry and its application in structural biology. Analyst 130:18-28

Basu NK, Kubota S, Meselhy MR, Ciotti M, Chowdhury B, Hartori M, Owens IS 2004 Gastrointestinally distributed UDP-glucuronosyltransferase 1A10, which metabolizes estrogens and nonsteroidal anti-inflammatory drugs, depends upon phosphorylation. J. Biol. Chem. 279:28320-28329

Baumann GP 2012 Growth hormone doping in sports: a critical review of use and detection strategies. Endocr. Rev. 33:155-186

Beaulieu M, Lévesque E, Hum DW, Bélanger A 1996 Isolation and characterization of a novel cDNA encoding a human UDP-glucuronosyltransferase active on C19 steroids. J. Biol. Chem. 271:22855-22862

Bélanger A, Brochu M, Cliche J 1986 Levels of plasma steroid glucuronides in intact and castrated men with prostatic cancer. J. Clin. Endocr. Metab. 62:812-815

Bélanger A, Pelletier G, Labrie F, Barbier O, Chouinard S 2003 Inactivation of androgens by UDP-glucuronosyltransferase enzymes in humans. Trends Endocrinol. Metab. 14:473-479

Belchetz P, Plant T, Nakai Y, Keogh E, Knobil E 1978 Hypophysial responses to continuous and intermittent delivery of hypopthalamic gonadotropin-releasing hormone. Science 202:631-633

Berlin A, Alessio L, Sesana G, Dell'Orto A, Ghezzi I 1985 Problems concerning the usefulness of adjustment of urinary cadmium for creatinine and specific gravity. Int. Arch. Occup. Environ. Health 55:107-111

Berrueta L, Gallo B, Vicente F 1995 A review of solid phase extraction: basic principles and new developments. Chromatographia 40:474-483

Birken S, Gawinowicz MA, Maydelman Y, Milgrom Y 2001 Metabolism of gonadotropins: comparisons of the primary structures of the human pituitary and urinary LH beta cores and the chimpanzee CG beta core demonstrate universality of core production. J. Endocrinol. 171:131-141

Birken S, Kovalevskaya G, O'Connor J 1996 Metabolism of hCG and hLH to multiple urinary forms. Mol. Cell. Endocrinol. 125:121-131

Birken S, Kovalevskaya G, O'Connor J 2001 Immunochemical measurement of early pregnancy isoforms of HCG: potential applications to fertility research, prenatal diagnosis, and cancer. Arch. Med. Res. 32:635-643

Birken S, McChesney R, Yershova O, Gaughan J, Pettersson K, Rechenberg G, Wu CH, Taliadouros G 2007 Patterns of LHbetacf among women in health and disease. Mol. Cell. Endocrinol. 260-262:172-182

Birken S, McChesney R, Yershova O, Gaughan J, Pettersson K, Rechenberg G, Wu CH, Taliadouros G 2007 Patterns of LHβcf among women in health and disease. Mol. Cell. Endocrinol. 260:172-182

Biro FM, Dorn LD 2005 Puberty and adolescent sexuality. Psychiatr. Ann. 36:685-690

Biro FM, Lucky AW, Huster GA, Morrison JA 1995 Pubertal staging in boys. J. Pediatr. 127:100-102

Boeniger MF, Lowry LK, Rosenberg J 1993 Interpretation of urine results used to assess chemical exposure with emphasis on creatinine adjustments: a review. Am. Ind. Hyg. Assoc. J. 54:615-627

Bonat S, Pathomvanich A, Keil MF, Field AE, Yanovski JA 2002 Self-assessment of pubertal stage in overweight children. Pediatrics 110:743-747

Bonfiglio R, King RC, Olah TV, Merkle K 1999 The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. Rapid Commun. Mass Spectrom. 13:1175-1185

Bordini B, Rosenfield RL 2011 Normal pubertal development: part II: clinical aspects of puberty. Pediatr. Rev. 32:281-292

Borts DJ, Bowers LD 2000 Direct measurement of urinary testosterone and epitestosterone conjugates using high-performance liquid chromatography/tandem mass spectrometry. J. Mass Spectrom. 35:50-61

Bos SJ, van Leeuwen SM, Karst U 2006 From fundamentals to applications: recent developments in atmospheric pressure photoionization mass spectrometry. Anal. Bioanal. Chem. 384:85-99

Boscato LM, Stuart M 1988 Heterophilic antibodies: a problem for all immunoassays. Clin. Chem. 34:27-33

Boscato LM, Stuart MC 1986 Incidence and specificity of interference in two-site immunoassays. Clin. Chem. 32:1491-1495

Bouloux P, Handelsman D, Jockenhövel F, Nieschlag E, Rabinovici J, Frasa W, De Bie J, Voortman G, Itskovitz-Eldor J 2001 First human exposure to FSH-CTP in hypogonadotrophic hypogonadal males. Hum. Reprod. 16:1592-1597

Bowers LD 1996 Direct measurement of steroid sulfate and glucuronide conjugates with high-performance liquid chromatography-mass spectrometry. J. Chromatogr. B 687:61-68

Boyd AE, DeFord LL, Mares JE, Leary CC, Garris JL, Dagohoy CG, Boving VG, Brook JP, Phan A, Yao JC 2013 Improving the success rate of gluteal intramuscular injections. Pancreas 42:878-882

Braun T, Schofield P, Sprengel R 1991 Amino-terminal leucine-rich repeats in gonadotropin receptors determine hormone selectivity. EMBO J. 10:1885

Bremner WJ, Vitiello MV, Prinz PN 1983 Loss of circadian rhythmicity in blood testosterone levels with aging in normal men. J. Clin. Endocrinol. Metab. 56:1278-1281

Brindle E, Miller RC, Shofer JB, Klein NA, Soules MR, O'Connor KA 2006 Urinary beta-luteinizing hormone and beta-follicle stimulating hormone immunoenzymometric assays for population research. Clin. Biochem. 39:1071-1079

Bristow A, Berger P, Bidart JM, Birken S, Norman R, Stenman UH, Sturgeon C 2005 Establishment, value assignment, and characterization of new WHO reference reagents for six molecular forms of human chorionic gonadotropin. Clin. Chem. 51:177-182

Brown M, Kovanen P, Goldstein J 1978 Receptor-mediated uptake of lipoprotein-cholesterol and its utilization for steroid synthesis in the adrenal cortex. Recent Prog. Horm. Res. 35:215-257

Buiarelli F, Coccioli F, Merolle M, Neri B, Terracciano A 2004 Development of a liquid chromatography–tandem mass spectrometry method for the identification of natural androgen steroids and their conjugates in urine samples. Anal. Chim. Acta 526:113-120

Burbridge BE 2007 Computed tomographic measurement of gluteal subcutaneous fat thickness in reference to failure of gluteal intramuscular injections. Can. Assoc. Radiol. J. 58:72-75

Burger HG 2002 Androgen production in women. Fertil. Steril. 77:3-5

Burkhardt AE, Johnston KG, Waszak CE, Jackson CE, Shafer SR 1982 A reagent strip for measuring the specific gravity of urine. Clin. Chem. 28:2068-2072

Butenandt A, Hanisch G 1935 Uber die Umwandlung des Dehydroandrosterons in Androstenol-(17)-one-(3) (Testosterone); Umweg zur darstellung des testosterons aus Cholsterin (vorlauf mitteilung). Zeischrift Physiologische Chemie 237:89-97

Caltabiano G, Campillo M, De Leener A, Smits G, Vassart G, Costagliola S, Pardo L 2008 The specificity of binding of glycoprotein hormones to their receptors. Cell. Mol. Life Sci. 65:2484-2492

Campo S, Ambao V, Creus S, Gottlieb S, Fernandez Vera G, Benencia H, Bergadá C 2007 Carbohydrate complexity and proportions of serum FSH isoforms in the male: lectin-based studies. Mol. Cell. Endocrinol. 260:197-204

Carel JC, Lahlou N, Roger M, Chaussain JL 2004 Precocious puberty and statural growth. Hum. Reprod. Update 10:135-147

Carel JC, Leger J 2008 Precocious puberty. N. Engl. J. Med. 358:2366-2377

Caron P, Audet-Walsh E, Lépine J, Bélanger A, Guillemette C 2009 Profiling Endogenous Serum Estrogen and Estrogen-Glucuronides by Liquid Chromatography– Tandem Mass Spectrometry. Anal. Chem. 81:10143-10148

Carrieri M, Trevisan A, Bartolucci GB 2000 Adjustment to concentration-dilution of spot urine samples: correlation between specific gravity and creatinine. Int. Arch. Occup. Environ. Health 74:63-67

Carson III C, Rittmaster R 2003 The role of dihydrotestosterone in benign prostatic hyperplasia. Urology 61:2-7

Carvalho VM, Nakamura OH, Vieira JGH 2008 Simultaneous quantitation of seven endogenous C-21 adrenal steroids by liquid chromatography tandem mass spectrometry in human serum. J. Chromatogr. B 872:154-161

Catt K, Dufau M, Tsuruhara T 1972 Radioligand-receptor assay of luteinizing hormone and chorionic gonadotropin. J. Clin. Endocrinol. Metab. 34:123-132

Caulfield MP, Lynn T, Gottschalk ME, Jones KL, Taylor NF, Malunowicz EM, Shackleton CH, Reitz RE, Fisher DA 2002 The diagnosis of congenital adrenal hyperplasia in the newborn by gas chromatography/mass spectrometry analysis of random urine specimens. J. Clin. Endocrinol. Metab. 87:3682-3690

Cawood ML, Field HP, Ford CG, Gillingwater S, Kicman A, Cowan D, Barth JH 2005 Testosterone measurement by isotope-dilution liquid chromatography–tandem mass spectrometry: validation of a method for routine clinical practice. Clin. Chem. 51:1472-1479

CDC 2000 Centers for Disease Control and Prevention, National Center for Health Statistics Growth Charts: United States. In: http://www.cdc.gov/growthcharts/

Cech NB, Enke CG 2001 Practical implications of some recent studies in electrospray ionization fundamentals. Mass Spectrom. Rev. 20:362-387

Chadha V, Garg U, Alon US 2001 Measurement of urinary concentration: a critical appraisal of methodologies. Pediatr. Nephrol. 16:374-382

Chambers E, Wagrowski-Diehl DM, Lu Z, Mazzeo JR 2007 Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. J. Chromatogr. B 852:22-34

Chan VO, Colville J, Persaud T, Buckley O, Hamilton S, Torreggiani WC 2006 Intramuscular injections into the buttocks: are they truly intramuscular? Eur. J. Radiol. 58:480-484

Chang Y-C, Li C-M, Li L-A, Jong S-B, Liao P-C, Chang LW 2003 Quantitative measurement of male steroid hormones using automated on-line solid phase extraction-liquid chromatography-tandem mass spectrometry and comparison with radioimmunoassay. Analyst 128:363-368

Chen F, Ritter JK, Wang MG, McBride OW, Lubet RA, Owens IS 1993 Characterization of a cloned human dihydrotestosterone/androstanediol UDP-glucuronosyltransferase and its comparison to other steroid isoforms. Biochemistry (Mosc.) 32:10648-10657

Chen H-C, Kuo H-W, Ding W-H 2009 Determination of estrogenic compounds in wastewater using liquid chromatography–tandem mass spectrometry with electrospray and atmospheric pressure photoionization following desalting extraction. Chemosphere 74:508-514

Cheng Z, Rios GR, King CD, Coffman BL, Green MD, Mojarrabi B, Mackenzie PI, Tephly TR 1998 Glucuronidation of catechol estrogens by expressed human UDP-glucuronosyltransferases (UGTs) 1A1, 1A3, and 2B7. Toxicol. Sci. 45:52-57

Cheung CC, Thornton JE, Nurani SD, Clifton DK, Steiner RA 2001 A reassessment of leptin's role in triggering the onset of puberty in the rat and mouse. Neuroendocrinology 74:12-21

Chu S, Letcher RJ 2008 Analysis of fluorotelomer alcohols and perfluorinated sulfonamides in biotic samples by liquid chromatography-atmospheric pressure photoionization mass spectrometry. J. Chromatogr. A 1215:92-99

Clark PA, Iranmanesh A, Veldhuis JD, Rogol AD 1997 Comparison of pulsatile luteinizing hormone secretion between prepubertal children and young adults: Evidence for a mass/amplitude-dependent difference without gender or day/night contrasts. J. Clin. Endocrinol. Metab. 82:2950-2955

Clark PA, Rogol AD 1996 Growth hormones and sex steroid interactions at puberty. Endocrinol. Metab. Clin. North Am. 25:665-681

Cockshott WP, Thompson GT, Howlett LJ, Seeley ET 1982 Intramuscular or intralipomatous injections? N. Engl. J. Med. 307:356-358

Cole LA, Khanlian SA 2009 The need for a quantitative urine hCG assay. Clin. Biochem. 42:676-683

Colombi A, Maroni M, Antonini C, Fait A, Zocchetti C, Foà V 1983 Influence of sex, age, and smoking habits on the urinary excretion of D-glucaric acid. Clin. Chim. Acta 128:349-358

Cone EJ, Caplan YH, Moser F, Robert T, Shelby MK, Black DL 2009 Normalization of urinary drug concentrations with specific gravity and creatinine. J. Anal. Toxicol. 33:1-7

Corbier P, Dehennin L, Castanier M, Mebazaa A, Edwards D, Roffi J 1990 Sex differences in serum luteinizing hormone and testosterone in the human neonate during the first few hours after birth. J. Clin. Endocrinol. Metab. 71:1344-1348

Coste J, Ecosse E, Lesage C, Chaussain J-L, Carel J-C 2002 Evaluation of adolescent statural growth in health and disease: reliability of assessment from height measurement series and development of an automated algorithm. Horm. Res. Paediatr. 58:105-114

Coughtrie M 2002 Sulfation through the looking glass—recent advances in sulfotransferase research for the curious. Pharmacogenomics J. 2:297-308

Counts DR, Pescovitz OH, Barnes KM, Hench KD, Chrousos GP, Sherins RJ, Comite F, Loriaux DL, Cutler Jr GB 1987 Dissociation of adrenarche and gonadarche in precocious puberty and in isolated hypogonadotropic hypogonadism. J. Clin. Endocrinol. Metab. 64:1174-1178

Courant F, Aksglaede L, Antignac J-P, Monteau F, Sorensen K, Andersson A-M, Skakkebaek NE, Juul A, Le Bizec B 2010 Assessment of circulating sex steroid levels in prepubertal and pubertal boys and girls by a novel ultrasensitive gas chromatography-tandem mass spectrometry method. J. Clin. Endocrinol. Metab. 95:82-92

Court MH 2014 Phenotyping UDP-glucuronosyltransferases (UGTs) involved in human drug metabolism: An update. In: Caldwell G, Yan Z eds. Optimization in Drug Discovery: In Vitro Methods. 2nd ed. New York, USA: Springer Science and Business Media; 117-136

Cowan DA, Kicman AT, Walker CJ, Wheeler MJ 1991 Effect of administration of human chorionic gonadotrophin on criteria used to assess testosterone administration in athletes. J. Endocrinol. 131:147-154

Dahl KD, Jia X-C, Hsueh A 1988 Granulosa cell aromatase bioassay for follicle-stimulating hormone. Methods Enzymol. 168:414-422

Dahl KD, Sarkissian A 1993 Validation of an improved in vitro bioassay to measure LH in diverse species. J. Androl. 14:124-129

Dai W, Huang Q, Yin P, Li J, Zhou J, Kong H, Zhao C, Lu X, Xu G 2012 Comprehensive and highly sensitive urinary steroid hormone profiling method based on stable isotope-labeling liquid chromatography–mass spectrometry. Anal. Chem. 84:10245-10251

Dams R, Huestis MA, Lambert WE, Murphy CM 2003 Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: influence of ionization type, sample preparation, and biofluid. J. Am. Soc. Mass Spectrom. 14:1290-1294

Darsow U, Bruckbauer H, Worret WI, Hofmann H, Ring J 2000 Subcutaneous oleomas induced by self-injection of sesame seed oil for muscle augmentation. J. Am. Acad. Dermatol. 42:292-294

David K, Dingemanse E, Freud J, Laqueur E 1935 Uber krystallinisches mannliches Hormon aus Hoden (Testosteron), wirksamer als aus Harn oder aus Cholestrin bereitetes Androsteron. Hoppe Seylers Zeischrift Physiologische Chemie 233:281-282

De Bruin N, Van Velthoven K, De Ridder M, Stijnen T, Juttmann R, Degenhart H, Visser H 1996 Standards for total body fat and fat-free mass in infants. Arch. Dis. Child. 74:386-399

de Peretti E, Forest MG 1978 Pattern of plasma dehydroepiandrosterone sulfate levels in humans from birth to adulthood: evidence for testicular production. J. Clin. Endocrinol. Metab. 47:572-577

De Ronde W, Pols HA, Van Leeuwen JP, De Jong FH 2003 The importance of oestrogens in males. Clin. Endocrinol. (Oxf.) 58:529-542

de Roux N, Genin E, Carel J-C, Matsuda F, Chaussain J-L, Milgrom E 2003 Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. P. Natl. Acad. Sci. USA 100:10972-10976

De Zegher F, Devlieger H, Veldhuis JD 1992 Pulsatile and sexually dimorphic secretion of luteinizing hormone in the human infant on the day of birth. Pediatr. Res. 32:605-607

Dehennin L, Lafarge P, Dailly P, Bailloux D, Lafarge J-P 1996 Combined profile of androgen glucuro- and sulfoconjugates in post-competition urine of sportsmen: a simple screening procedure using gas chromatography-mass spectrometry. J. Chromatogr. B 687:85-91

Dehennin L, Matsumoto AM 1993 Long-term administration of testosterone enanthate to normal men: alterations of the urinary profile of androgen metabolites potentially useful for detection of testosterone misuse in sport. J. Steroid Biochem. 44:179-189

Demir A, Alfthan H, Stenman U-H, Voutilainen R 1994 A clinically useful method for detecting gonadotropins in children: assessment of luteinizing hormone and follicle-stimulating hormone from urine as an alternative to serum by ultrasensitive time-resolved immunofluorometric assays. Pediatr. Res. 36:221-226

Demir HA, Stenman U-H, Voutilainen R 1994 A clinically useful method for detecting gonadotropins in children: assessment of luteinizing hormone and follicle-stimulating hormone from urine as an alternative to serum by ultrasensitive time-resolved immunofluorometric assays. Pediatr. Res. 36:221-226

Denari JH, Farinati Z, Casas PRF, Oliva A 1981 Determination of ovarian function using first morning urine steroid assays. Obstet. Gynecol. 58:5-9

Desmangles JC, Lappe JM, Lipaczewski G, Haynatzki G 2006 Accuracy of pubertal Tanner staging self-reporting. J. Pediatr. Endocrinol. Metab. 19:213-221

Dhillo WS, Chaudhri OB, Patterson M, Thompson EL, Murphy KG, Badman MK, McGowan BM, Amber V, Patel S, Ghatei MA 2005 Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. J. Clin. Endocrinol. Metab. 90:6609-6615

Dorizzi R, Pradella M, Bertoldo S, Rigolin F 1987 Refractometry, test strip, and osmometry compared as measures of relative density of urine. Clin. Chem. 33:190

Dorn LD, Biro FM 2011 Puberty and its measurement: A decade in review. J. Res. Adolescence 21:180-195

Dorn LD, Dahl RE, Woodward HR, Biro F 2006 Defining the boundaries of early adolescence: A user's guide to assessing pubertal status and pubertal timing in research with adolescents. Appl. Dev. Sci. 10:30-56

Dösch J, Dörr HG, Wildt L 2001 Exposure to endogenous estrogens during lifetime. In: Endocrine Disruptors–Part I: Springer; 81-99

Ducharme J-R, Forest MG, De Peretti E, Sempé M, Collu R, Bertrand J 1976 Plasma adrenal and gonadal sex steroids in human pubertal development. J. Clin. Endocrinol. Metab. 42:468-476

Dufau ML, Beitins IZ, Mcarthur JW, Catt KJ 1976 Effects of luteinizing hormone releasing hormone (LHRH) upon bioactive and immunoreactive serum LH levels in normal subjects. J. Clin. Endocrinol. Metab. 43:658-667

Dufort I, Labrie F, Luu-The V 2001 Human Types 1 and 3 3α-Hydroxysteroid Dehydrogenases: Differential Lability and Tissue Distribution. J. Clin. Endocrinol. Metab. 86:841-846

Dunn JF, Nisula BC, Rodbard D 1981 Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. J. Clin. Endocrinol. Metab. 53:58-68

Eakman GD, Dallas JS, Ponder SW, Keenan BS 1996 The effects of testosterone and dihydrotestosterone on hypothalamic regulation of growth hormone secretion. J. Clin. Endocrinol. Metab. 81:1217-1223

Ebeling P, Koivisto V 1994 Physiological importance of dehydroepiandrosterone. Lancet 343:1479-1481

Edelman A, Stouffer R, Zava DT, Jensen JT 2007 A comparison of blood spot vs. plasma analysis of gonadotropin and ovarian steroid hormone levels in reproductive-age women. Fertil. Steril. 88:1404-1407

El-Aneed A, Cohen A, Banoub J 2009 Mass spectrometry, review of the basics: Electrospray, MALDI, and commonly used mass analyzers. Appl. Spectrosc. Rev. 44:210-230

El Majdoubi M, Sahu A, Ramaswamy S, Plant T 2000 Neuropeptide Y: a hypothalamic brake restraining the onset of puberty in primates. P. Natl. Acad. Sci. USA 97:6179-6184

Elviri L, Speroni F, Careri M, Mangia A, di Toppi L, Zottini M 2010 Identification of in vivo nitrosylated phytochelatins in Arabidopsis thaliana cells by liquid chromatography-direct electrospray-linear ion trap-mass spectrometry. J. Chromatogr. A 1217:4120-4126

EMA 2011 Draft Guideline on Validation of Bioanalytical Methods. In: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf

Eneroth P, Hellström K, Ryhage R 1964 Identification and quantification of neutral fecal steroids by gas–liquid chromatography and mass spectrometry: studies of human excretion during two dietary regimens. J. Lipid Res. 5:245-262

Euling SY, Herman-Giddens ME, Lee PA, Selevan SG, Juul A, Sørensen TI, Dunkel L, Himes JH, Teilmann G, Swan SH 2008 Examination of US puberty-timing data from 1940 to 1994 for secular trends: panel findings. Pediatrics 121:S172-S191

Falany C 1997 Enzymology of human cytosolic sulfotransferases. FASEB J. 11:206-216

Falany CN, Wheeler J, Oh TS, Falany JL 1994 Steroid sulfation by expressed human cytosolic sulfotransferases. J. Steroid Biochem. 48:369-375

Fares F 2006 The role of O-linked and N-linked oligosaccharides on the structure–function of glycoprotein hormones: Development of agonists and antagonists. Biochim. Biophys. Acta 1760:560-567

Fares FA, Suganuma N, Nishimori K, LaPolt PS, Hsueh A, Boime I 1992 Design of a long-acting follitropin agonist by fusing the C-terminal sequence of the chorionic gonadotropin beta subunit to the follitropin beta subunit. P. Natl. Acad. Sci. USA 89:4304-4308

FDA 2001 Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration In: http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf

Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM 1989 Electrospray ionization for mass spectrometry of large biomolecules. Science 246:64-71

Field HP 2013 Tandem Mass Spectrometry in Hormone Measurement. In: Wheeler MJ, Hutchinson MJ eds. Hormone Assays in Biological Fluids: Springer; 45-74

Fiete D, Baenziger JU 1997 Isolation of the SO4-4-GalNAcβ1, 4GlcNAcβ1, 2Manα-specific receptor from rat liver. J. Biol. Chem. 272:14629-14637

Fiete D, Srivastava V, Hindsgaul O, Baenziger JU 1991 A hepatic reticuloendothelial cell receptor specific for SO_4-4GalNAcβ1, 4GlcNAcβ1, 2Manα that mediates rapid clearance of lutropin. Cell 67:1103-1110

Finkelstein JS, O'dea LSL, Whitcomb RW, Crowley Jr WF 1991 Sex Steroid Control of Gonadotropin Secretion in the Human Male. II. Effects of Estradiol Administration in Normal and Gonadotropin-Releasing Hormone-Deficient Men. J. Clin. Endocrinol. Metab. 73:621-628

Finkelstein JW, Roffwarg HP, Boyar RM, Kream J, Hellman L 1972 Age-related change in the twenty-four hour spontaneous secretion of growth hormone. J. Clin. Endocrinol. Metab. 35:665-670

Flück CE, Meyer-Böni M, Pandey AV, Kempná P, Miller WL, Schoenle EJ, Biason-Lauber A 2011 Why boys will be boys: two pathways of fetal testicular androgen biosynthesis are needed for male sexual differentiation. Am. J. Hum. Genet. 89:201-218

Frew A, McEwan J, Bell G, Heath M, Knapp M 1982 Estimation of urine specific gravity and osmolality using a simple reagent strip. British medical journal (Clinical research ed.) 285:1168

Frieden EH, Patkin JK, Mills M 1968 Effects of follicle stimulating hormone (FSH) upon steroid aromatization in vitro. Exp. Biol. Med. 129:606-609

Gaganis P, Miners JO, Brennan JS, Thomas A, Knights KM 2007 Human renal cortical and medullary UDP-glucuronosyltransferases (UGTs): immunohistochemical localization of UGT2B7 and UGT1A enzymes and kinetic characterization of S-naproxen glucuronidation. J. Pharmacol. Exp. Ther. 323:422-430

Gaines LG, Fent KW, Flack SL, Thomasen JM, Ball LM, Zhou H, Whittaker SG, Nylander-French LA 2010 Effect of creatinine and specific gravity normalization on urinary biomarker 1, 6-hexamethylene diamine. J. Environ. Monit. 12:591-599

Gamage N, Barnett A, Hempel N, Duggleby RG, Windmill KF, Martin JL, McManus ME 2006 Human sulfotransferases and their role in chemical metabolism. Toxicol. Sci. 90:5-22

Garcia-Campayo V, Sato A, Hirsch B, Sugahara T, Muyan M, Hsueh AJ, Boime I 1997 Design of stable biologically active recombinant lutropin analogs. Nat. Biotechnol. 15:663-667

Garde A, Hansen ÅM, Kristiansen J 2003 Evaluation, including effects of storage and repeated freezing and thawing, of a method for measurement of urinary creatinine. Scand. J. Clin. Lab. Invest. 63:521-524

Geissler WM, Davis DL, Wu L, Bradshaw KD, Patel S, Mendonca BB, Elliston KO, Wilson JD, Russell DW, Andersson S 1994 Male pseudohermaphroditism caused by mutations of testicular 17β–hydroxysteroid dehydrogenase 3. Nat. Genet. 7:34-39

Gibaldi M, Perrier D 1982 Pharmacokinetics. 2nd ed. New York: Marcel Dekker

Girard C, Barbier O, Veilleux G, El-Alfy M, Bélanger A 2003 Human uridine diphosphate-glucuronosyltransferase UGT2B7 conjugates mineralocorticoid and glucocorticoid metabolites. Endocrinology 144:2659-2668

Giton F, Guéchot J, Fiet J 2009 New reusable Celite®/ethylene glycol cartridges for selective chromatography of steroids before immunoassay. Clin. Biochem. 42:1735-1738

Glish GL, Vachet RW 2003 The basics of mass spectrometry in the twenty-first century. Nat. Rev. Drug Discovery 2:140-150

Goebel C, Howe CJ, Ho KK, Nelson A, Kazlauskas R, Trout GJ 2009 Screening for testosterone abuse in male athletes using the measurement of urinary LH, a revision of the paradigm. Drug Test. Anal. 1:511-517

Gomez A, Tang K 1994 Charge and fission of droplets in electrostatic sprays. Physics of Fluids (1994-present) 6:404-414

Goran MI, Gower BA, Nagy TR, Johnson RK 1998 Developmental changes in energy expenditure and physical activity in children: evidence for a decline in physical activity in girls before puberty. Pediatrics 101:887-891

Gosetti F, Mazzucco E, Zampieri D, Gennaro MC 2010 Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry. J. Chromatogr. A 1217:3929-3937

Goto T, Shibata A, Sasaki D, Suzuki N, Hishinuma T, Kakiyama G, Iida T, Mano N, Goto J 2005 Identification of a novel conjugate in human urine: bile acid acyl galactosides. Steroids 70:185-192

Gounden D, Newall R 1983 Urine specific gravity measurements: comparison of a new reagent strip method with existing methodologies, as applied to the water concentration/dilution tests. Curr. Med. Res. Opin. 8:375-381

Greaves RF, Jevalikar G, Hewitt JK, Zacharin MR 2014 A guide to understanding the steroid pathway: New insights and diagnostic implications. Clin. Biochem.

Grebe SK, Singh RJ 2011 LC-MS/MS in the Clinical Laboratory–Where to From Here? Clin. Biochem. Rev. 32:5

Greiner MV, Kerrigan JR 2006 Puberty: timing is everything. Pediatr. Ann. 35:916

Grumbach M, Gluckman PD 1994 The human fetal hypothalamus and pituitary gland:The maturation of neuroendocrine mechanisms controlling the secretion of fetal pituitary growth hormone, prolactin, gonadotropin, adrenocorticotropin-related peptides, and thyrotropin. In: Tulchinsky D, Little A eds. Maternal-Fetal Endocrinology. 2nd ed. Philadelphia: WB Saunders; 193-261

Grumbach MM 2004 The neuroendocrinology of human puberty revisited. Horm. Res. Paediatr. 57:2-14

Grumbach MM 2005 A Window of Opportunity: The Diagnosis of Gonadotropin Deficiency in the Male Infant 1. J. Clin. Endocrinol. Metab. 90:3122-3127

Grumbach MM, Auchus RJ 1999 Estrogen: Consequences and Implications of Human Mutations in Synthesis and Action J. Clin. Endocrinol. Metab. 84:4677-4694

Gu Y, Liang X, Wu W, Liu M, Song S, Cheng L, Bo L, Xiong C, Wang X, Liu X, Peng L, Yao K 2009 Multicenter contraceptive efficacy trial of injectable testosterone undecanoate in Chinese men. J. Clin. Endocrinol. Metab. 94:1910-1915

Guo T, Chan M, Soldin SJ 2004 Steroid profiles using liquid chromatography-tandem mass spectrometry with atmospheric pressure photoionization source. Arch. Pathol. Lab. Med. 128:469-475

Guo T, Taylor RL, Singh RJ, Soldin SJ 2006 Simultaneous determination of 12 steroids by isotope dilution liquid chromatography-photospray ionization tandem mass spectrometry. Clin. Chim. Acta 372:76-82

Gwynne JT, Strauss JF 1982 The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. Endocr. Rev. 3:299-329

Haddow JE, Knight GJ, Palomaki GE, Neveux LM, Chilmonczyk BA 1994 Replacing creatinine measurements with specific gravity values to adjust urine cotinine concentrations. Clin. Chem. 40:562-564

Hagen CP, Sørensen K, Mieritz MG, Johannsen TH, Almstrup K, Juul A 2015 Circulating MKRN3 Levels Decline Prior to Pubertal Onset and Through Puberty: A Longitudinal Study of Healthy Girls. J. Clin. Endocrinol. Metab.

Hakim RB, Gray RH, Zacur HA 1994 Is there a need for creatinine adjustment of urinary steroid hormone levels in studies of early fetal loss? Clin. Chim. Acta 230:209-214

Hamilton JB 1937 Treatment of sexual underdevelopment with synthetic male hormone substance. Endocrinology 21:649-654

Hammes A, Andreassen TK, Spoelgen R, Raila J, Hubner N, Schulz H, Metzger J, Schweigert FJ, Luppa PB, Nykjaer A 2005 Role of endocytosis in cellular uptake of sex steroids. Cell 122:751-762

Hammond G, Ruokonen A, Kontturi M, Koskela E, Vihko R 1977 The simultaneous radioimmunoassay of seven steroids in human spermatic and peripheral venous blood. J. Clin. Endocrinol. Metab. 45:16-24

Handelsman DJ 2006 Clinical review: The rationale for banning human chorionic gonadotropin and estrogen blockers in sport. J. Clin. Endocrinol. Metab. 91:1646-1653

Handelsman DJ 2006 The rationale for banning human chorionic gonadotropin and estrogen blockers in sport. J. Clin. Endocrinol. Metab. 91:1646-1653

Handelsman DJ 2010 Androgen Physiology, Pharmacology and Abuse. In: DeGroot LJ, Jameson JL eds. Endocrinology. 6th ed. Philadelphia: Elsevier Saunders; 2469-2498

Handelsman DJ, Goebel C, Idan A, Jimenez M, Trout G, Kazlauskas R 2009 Effects of recombinant human LH and hCG on serum and urine LH and androgens in men. Clin. Endocrinol. (Oxf.) 71:417-428

Handelsman DJ, Mackey MA, Howe C, Turner L, Conway AJ 1997 Analysis of testosterone implants for androgen replacement therapy. Clin. Endocrinol. (Oxf.) 47:311-316

Handelsman DJ, Newman JD, Jimenez M, McLachlan R, Sartorius G, Jones GR 2014 Performance of direct estradiol immunoassays with human male serum samples. Clin. Chem. 60:510-517

Handelsman DJ, Turner L, Boylan LM, Conway AJ 1995 Pharmacokinetics of human follicle-stimulating hormone in gonadotropin-deficient men. J. Clin. Endocrinol. Metab. 80:1657-1663

Handelsman DJ, Wartofsky L 2013 Requirement for mass spectrometry sex steroid assays in the journal of clinical endocrinology and metabolism. J. Clin. Endocrinol. Metab. 98:3971-3973

Haramati N, Lorans R, Lutwin M, Kaleya RN 1994 Injection granulomas. Intramuscle or intrafat? Arch. Fam. Med. 3:146-148

Harris GC, Levine JE 2003 Pubertal acceleration of pulsatile gonadotropin-releasing hormone release in male rats as revealed by microdialysis. Endocrinology 144:163-171

Harwood DT, Handelsman DJ 2009 Development and validation of a sensitive liquid chromatography–tandem mass spectrometry assay to simultaneously measure androgens and estrogens in serum without derivatization. Clin. Chim. Acta 409:78-84

Hauser B, Deschner T, Boesch C 2008 Development of a liquid chromatography–tandem mass spectrometry method for the determination of 23 endogenous steroids in small quantities of primate urine. J. Chromatogr. B 862:100-112

Hayes A, Johanson A 1972 Excretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in urine by pubertal girls. Pediatr. Res. 6:18-25

Hayes FJ, Seminara SB, DeCruz S, Boepple PA, Crowley Jr WF 2000 Aromatase Inhibition in the Human Male Reveals a Hypothalamic Site of Estrogen Feedback J. Clin. Endocrinol. Metab. 85:3027-3035

Heald A, Butterworth A, Kane J, Borzomato J, Taylor N, Layton T, Kilpatrick E, Rudenski A 2006 Investigation into possible causes of interference in serum testosterone measurement in women. Ann. Clin. Biochem. 43:189-195

Heavner DL, Morgan WT, Sears SB, Richardson JD, Byrd GD, Ogden MW 2006 Effect of creatinine and specific gravity normalization techniques on xenobiotic biomarkers in smokers' spot and 24-h urines. J. Pharm. Biomed. Anal. 40:928-942

Hemmersbach P, Grobe J 2009 Nandrolone: A multi-faceted doping agent. In: Thieme D, Hemmersbach P eds. Doping in sports: Springer Science & Business Media; 127-154

Hemmilä I, Dakubu S, Mukkala V-M, Siitari H, Lövgren T 1984 Europium as a label in time-resolved immunofluorometric assays. Anal. Biochem. 137:335-343

Hendriks G, Uges D, Franke J 2007 Reconsideration of sample pH adjustment in bioanalytical liquid–liquid extraction of ionisable compounds. J. Chromatogr. B 853:234-241

Hergenroeder AC, Hill RB, Wong WW, Sangi-Haghpeykar H, Taylor W 1999 Validity of self-assessment of pubertal maturation in African American and European American adolescents. J. Adolesc. Health 24:201-205

Higashi T, Nishio T, Uchida S, Shimada K, Fukushi M, Maeda M 2008 Simultaneous determination of 17α-hydroxypregnenolone and 17α-hydroxyprogesterone in dried blood spots from low birth weight infants using LC–MS/MS. J. Pharm. Biomed. Anal. 48:177-182

Higashi T, Shimada K 2004 Derivatization of neutral steroids to enhance their detection characteristics in liquid chromatography–mass spectrometry. Anal. Bioanal. Chem. 378:875-882

Himmelsbach M 2012 10years of MS instrumental developments–Impact on LC–MS/MS in clinical chemistry. J. Chromatogr. B 883:3-17

Ho C, Lam C, Chan M, Cheung R, Law L, Lit L, Ng K, Suen M, Tai H 2003 Electrospray ionisation mass spectrometry: principles and clinical applications. Clinical Biochemist Reviews 24:3

Hobkirk R 1985 Steroid sulfotransferases and steroid sulfate sulfatases: characteristics and biological roles. Can. J. Biochem. Cell Biol. 63:1127-1144

Holčapek M, Jirásko R, Lísa M 2010 Basic rules for the interpretation of atmospheric pressure ionization mass spectra of small molecules. J. Chromatogr. A 1217:3908-3921

Honour JW 2010 Steroid assays in paediatric endocrinology. J Clin Res Pediatr Endocrinol 2:1

Horton R, Lobo R 1986 6 Peripheral androgens and the role of androstanediol glucuronide. Clin. Endocrinol. Metab. 15:293-306

Howe CJ, Handelsman DJ 1997 Use of filter paper for sample collection and transport in steroid pharmacology. Clin. Chem. 43:1408-1415

Hsieh Y, Chintala M, Mei H, Agans J, Brisson JM, Ng K, Korfmacher WA 2001 Quantitative screening and matrix effect studies of drug discovery compounds in monkey plasma using fast-gradient liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom. 15:2481-2487

Hu Q, Noll RJ, Li H, Makarov A, Hardman M, Graham Cooks R 2005 The Orbitrap: a new mass spectrometer. J. Mass Spectrom. 40:430-443

Huhtaniemi IT, Korenbrot CC, Jaffe RB 1977 HCG binding and stimulation of testosterone biosynthesis in the human fetal testis. J. Clin. Endocrinol. Metab. 44:963-967

Huhtaniemi IT, Yamamoto M, Ranta T, Jalkanen J, Jaffe RB 1987 Follicle-stimulating hormone receptors appear earlier in the primate fetal testis than in the ovary. J. Clin. Endocrinol. Metab. 65:1210-1214

Huhtinen K, Desai R, Ståhle M, Salminen A, Handelsman DJ, Perheentupa A, Poutanen M 2012 Endometrial and endometriotic concentrations of estrone and estradiol are determined by local metabolism rather than circulating levels. J. Clin. Endocrinol. Metab. 97:4228-4235

Hum DW, Bélanger A, Lévesque É, Barbier O, Beaulieu M, Albert C, Vallée M, Guillemette C, Tchernof A, Turgeon D 1999 Characterization of UDP-glucuronosyltransferases active on steroid hormones. J. Steroid Biochem. 69:413-423

Ibáñez L, DiMartino-Nardi J, Potau N, Saenger P 2000 Premature adrenarche—normal variant or forerunner of adult disease? Endocr. Rev. 21:671-696

Isaacs JT 1984 Antagonistic effect of androgen on prostatic cell death. Prostate 5:545-557

Ishimura K, Fujita H 1997 Light and electron microscopic immunohistochemistry of the localization of adrenal steroidogenic enzymes. Microsc. Res. Tech. 36:445-453

Jaakkola T, Ding Y-Q, Kellokumpu-Lehtinen P, Valavaara R, Martikainen H, Tapanainen J, Ronnberg L, Huhtaniemi I 1990 The ratios of serum bioactive/immunoreactive luteinizing hormone and follicle-stimulating hormone in various clinical conditions with increased and decreased gonadotropin secretion: Reevaluation by a highly sensitive immunometric assay. J. Clin. Endocrinol. Metab. 70:1496-1505

Jaffe RB, Midgley Jr AR 1969 Current status of human gonadotropin radioimmunoassay. Obstet. Gynecol. Surv. 24:200-213

Jäntti SE, Tammimäki A, Raattamaa H, Piepponen P, Kostiainen R, Ketola RA 2010 Determination of steroids and their intact glucuronide conjugates in mouse brain by capillary liquid chromatography-tandem mass spectrometry. Anal. Chem. 82:3168-3175

Janzen N, Sander S, Terhardt M, Peter M, Sander J 2008 Fast and direct quantification of adrenal steroids by tandem mass spectrometry in serum and dried blood spots. J. Chromatogr. B 861:117-122

Jatlow P, McKee S, O'Malley SS 2003 Correction of urine cotinine concentrations for creatinine excretion: is it useful? Clin. Chem. 49:1932-1934

Jenner M, Kelch R, Kaplan S, Grumbach M 1972 Hormonal changes in puberty: IV. Plasma estradiol, LH, and FSH in prepubertal children, pubertal females, and in precocious puberty, premature thelarche, hypogonadism, and in a child with a feminizing ovarian tumor. J. Clin. Endocrinol. Metab. 34:521-530

Ji CY 2001 Age at spermarche and comparison of growth and performance of pre- and post-spermarcheal Chinese boys. Am. J. Hum. Biol. 13:35-43

Jia X-C, Hsueh AJ 1986 Granulosa Cell Aromatase Bioassay for Follicle-Stimulating Hormone: Validation and Application of the Method. Endocrinology 119:1570-1577

Jin Y, Penning TM 2001 Steroid 5α-reductases and 3α-hydroxysteroid dehydrogenases: key enzymes in androgen metabolism. Best Pract. Res. Clin. Endocrinol. Metab. 15:79-94

Jones KP 1992 Estrogens and progestins: what to use and how to use it. Clin. Obstet. Gynecol. 35:871-883

Joo G, Sohng KY 2010 Gluteal subcutaneous fat thickness measured by computed tomography as an estimate of proper gluteal intramuscular injections in Korean adults. J. Korean Acad. Nurs. 40:247-254

Joshi L, Murata Y, Wondisford FE, Szkudlinski MW, Desai R, Weintraub BD 1995 Recombinant thyrotropin containing a beta-subunit chimera with the human chorionic gonadotropin-beta carboxy-terminus is biologically active, with a prolonged plasma half-life: role of carbohydrate in bioactivity and metabolic clearance. Endocrinology 136:3839-3848

Junkman K 1957 Long-acting steroids in reproduction. Recent Prog. Horm. Res. 13:380-419

Juul A 2001 The effects of oestrogens on linear bone growth. APMIS 109:S124-S134

Juul A, Bang P, Hertel NT, Main K, Dalgaard P, Jørgensen K, Müller J, Hall K, Skakkebæk NE 1994 Serum insulin-like growth factor-I in 1030 healthy children, adolescents, and adults: relation to age, sex, stage of puberty, testicular size, and body mass index. J. Clin. Endocrinol. Metab. 78:744-752

Kahn SM, Li Y-H, Hryb DJ, Nakhla AM, Romas NA, Cheong J, Rosner W 2008 Sex hormone-binding globulin influences gene expression of LNCaP and MCF-7 cells in response to androgen

and estrogen treatment. In: Li JJ, Li SA, Mohla S, Rochefort H, Mandelonde T eds. Hormonal Carcinogenesis V: Springer; 557-564

Kaiser UB 2011 Gonadotropin hormones. In: Melmed S ed. Pituitary: Elsevier; 205-260

Kalyan N, Bahl OP 1983 Role of carbohydrate in human chorionic gonadotropin. Effect of deglycosylation on the subunit interaction and on its in vitro and in vivo biological properties. J. Biol. Chem. 258:67-74

Keen KL, Burich AJ, Mitsushima D, Kasuya E, Terasawa E 1999 Effects of pulsatile infusion of the GABAA receptor blocker bicuculline on the onset of puberty in female rhesus monkeys. Endocrinology 140:5257-5266

Keevil BG 2013 Novel liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for measuring steroids. Best Pract. Res. Cl. En. 27:663-674

Kelleher S, Conway AJ, Handelsman DJ 2001 Influence of implantation site and track geometry on the extrusion rate and pharmacology of testosterone implants. Clinical Endocrinology. 55:531-536

Kelleher S, Conway AJ, Handelsman DJ 2002 A randomised controlled clinical trial of antibiotic impregnation of testosterone pellet implants to reduce extrusion rate. Eur. J. Endocrinol. 146:513-518

Kelleher S, Turner L, Howe C, Conway AJ, Handelsman DJ 1999 Extrusion of testosterone pellets: a randomized controlled clinical study. Clinical Endocrinology. 51:469-471

Keski-Rahkonen P, Huhtinen K, Desai R, Tim Harwood D, Handelsman DJ, Poutanen M, Auriola S 2013 LC-MS analysis of estradiol in human serum and endometrial tissue: Comparison of electrospray ionization, atmospheric pressure chemical ionization and atmospheric pressure photoionization. J. Mass Spectrom. 48:1050-1058

Kesner JS, Knecht EA, Krieg EF, Jr. 1995 Stability of urinary female reproductive hormones stored under various conditions. Reprod. Toxicol. 9:239-244

Kesner JS, Knecht EA, Krieg EF, Jr. 1999 Measuring endocrine profiles of women in field studies. Scand. J. Work. Environ. Health 25 Suppl 1:17-19

Kettaneh A, Heude B, Lommez A, Borys JM, Ducimetiere P, Charles MA 2005 Reliability of bioimpedance analysis compared with other adiposity measurements in children: the FLVS II Study. Diabetes Metab. 31:534-541

Kicman A 2008 Pharmacology of anabolic steroids. Br. J. Pharmacol. 154:502-521

Kicman AT 2010 Biochemical and physiological aspects of endogenous androgens. In: Thieme D, Hemmersbach P eds. Doping in Sports: Biochemical Principles, Effects and Analysis: Springer; 25-64

Kicman AT, Brooks RV, Collyer SC, Cowan DA, Nanjee MN, Southan GJ, Wheeler MJ 1990 Criteria to indicate testosterone administration. Br. J. Sports Med. 24:253-264

King C, Rios G, Green M, Tephly T 2000 UDP-glucuronosyltransferases. Curr. Drug Metab. 1:143-161

King R, Bonfiglio R, Fernandez-Metzler C, Miller-Stein C, Olah T 2000 Mechanistic investigation of ionization suppression in electrospray ionization. J. Am. Soc. Mass Spectrom. 11:942-950

Kinoshita M, Tsukamura H, Adachi S, Matsui H, Uenoyama Y, Iwata K, Yamada S, Inoue K, Ohtaki T, Matsumoto H 2005 Involvement of central metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. Endocrinology 146:4431-4436

Kissinger PT 2011 Thinking about dried blood spots for pharmacokinetic assays and therapeutic drug monitoring. Bioanalysis 3:2263-2266

Korth-Schutz S, Levine LS, New MI, Chow DM 1976 Serum androgens in normal prepubertal and pubertal children and in children with precocious adrenarche. J. Clin. Endocrinol. Metab. 42:117-124

Kostiainen R, Kauppila TJ 2009 Effect of eluent on the ionization process in liquid chromatography–mass spectrometry. J. Chromatogr. A 1216:685-699

Kostiainen R, Kotiaho T, Kuuranne T, Auriola S 2003 Liquid chromatography/atmospheric pressure ionization–mass spectrometry in drug metabolism studies. J. Mass Spectrom. 38:357-372

Kovalevskaya G, Birken S, Kakuma T, O'connor J 1999 Early pregnancy human chorionic gonadotropin (hCG) isoforms measured by an immunometric assay for choriocarcinoma-like hCG. J. Endocrinol. 161:99-106

Kovalevskaya G, Birken S, O'Connor J, Schlatterer J, Maydelman Y, Canfield R 1995 HLH beta core fragment immunoreactivity in the urine of ovulating women: a sensitive and specific immunometric assay for its detection. Endocrine 3:881-887

Kroboth PD, Salek FS, Pittenger AL, Fabian TJ, Frye RF 1999 DHEA and DHEA-S: a review. J. Clin. Pharmacol. 39:327-348

Krone N, Hughes BA, Lavery GG, Stewart PM, Arlt W, Shackleton CH 2010 Gas chromatography/mass spectrometry (GC/MS) remains a pre-eminent discovery tool in clinical steroid investigations even in the era of fast liquid chromatography tandem mass spectrometry (LC/MS/MS). J. Steroid Biochem. 121:496-504

Kronenberg HM, Melmed S, Larsen PR, Polonsky KS 2011 Principles of Endocrinology. In: Melmed S, Polonsky KS, Larsen PR, Kronenberg HM eds. Williams Textbook of Endocrinology. 12th ed: Elsevier/Saunders; 3-12

Kubasik NP, Ricotta M, Hunter T, Sine HE 1982 Effect of duration and temperature of storage on serum analyte stability: examination of 14 selected radioimmunoassay procedures. Clin. Chem. 28:164-165

Kuijper EA, Houwink EJ, van Weissenbruch MM, Heij HA, Blankenstein MA, Huijser J, Martens F, Lambalk CB 2006 Urinary gonadotropin measurements in neonates: a valuable non-invasive method. Ann. Clin. Biochem. 43:320-322

Kuiri-Hänninen T, Seuri R, Tyrväinen E, Turpeinen U, Hämäläinen E, Stenman U-H, Dunkel L, Sankilampi U 2011 Increased activity of the hypothalamic-pituitary-testicular axis in infancy results in increased androgen action in premature boys. J. Clin. Endocrinol. Metab. 96:98-105

Kulin H, Bell P, Santen R, Ferber A 1975 Integration of pulsatile gonadotropin secretion by timed urinary measurements: an accurate and sensitive 3-hour test. J. Clin. Endocrinol. Metab. 40:783-789

Kulin HE, Santner SJ 1977 Timed urinary gonadotropin measurements in normal infants, children, and adults, and in patients with disorders of sexual maturation. J. Pediatr. 90:760-765

Kulle A, Riepe F, Melchior D, Hiort O, Holterhus P 2010 A novel ultrapressure liquid chromatography tandem mass spectrometry method for the simultaneous determination of androstenedione, testosterone, and dihydrotestosterone in pediatric blood samples: age-and sex-specific reference data. J. Clin. Endocrinol. Metab. 95:2399-2409

Kushnir MM, Rockwood AL, Bergquist J 2010 Liquid chromatography–tandem mass spectrometry applications in endocrinology. Mass Spectrom. Rev. 29:480-502

Kushnir MM, Rockwood AL, Bergquist J, Varshavsky M, Roberts WL, Yue B, Bunker AM, Meikle AW 2008 High-sensitivity tandem mass spectrometry assay for serum estrone and estradiol. Am. J. Clin. Pathol. 129:530-539

Kushnir MM, Rockwood AL, Roberts WL, Yue B, Bergquist J, Meikle AW 2011 Liquid chromatography tandem mass spectrometry for analysis of steroids in clinical laboratories. Clin. Biochem. 44:77-88

Labrie F, Bélanger A, Cusan L, Gomez J-L, Candas B 1997 Marked decline in serum concentrations of adrenal C19 sex steroid precursors and conjugated androgen metabolites during aging. J. Clin. Endocrinol. Metab. 82:2396-2402

Labrie F, Belanger A, Simard J, Luu-The V, Labrie C 1995 DHEA and peripheral androgen and estrogen formation: intracrinology. Ann. N. Y. Acad. Sci. 774:16-28

Laron Z 2010 Age at first ejaculation (spermarche)--the overlooked milestone in male development. Pediatr. Endocrinol. Rev. 7:256-257

Lash R, Desai R, Zimmerman C, Flack M, Yoshida T, Wondisford F, Weintraub B 1992 Mutations of the human thyrotropin-beta subunit glycosylation site reduce thyrotropin synthesis independent of changes in glycosylation status. J. Endocrinol. Invest. 15:255-263

Lazar L, Phillip M 2012 Pubertal disorders and bone maturation. Endocrinol. Metab. Clin. North Am. 41:805-825

Lazaridis I, Charalampopoulos I, Alexaki V-I, Avlonitis N, Pediaditakis I, Efstathopoulos P, Calogeropoulou T, Castanas E, Gravanis A 2011 Neurosteroid dehydroepiandrosterone interacts with nerve growth factor (NGF) receptors, preventing neuronal apoptosis. PLoS Biol. 9:1001-1051

Lee JS, Ettinger B, Stanczyk FZ, Vittinghoff E, Hanes V, Cauley JA, Chandler W, Settlage J, Beattie MS, Folkerd E 2006 Comparison of methods to measure low serum estradiol levels in postmenopausal women. J. Clin. Endocrinol. Metab. 91:3791-3797

Lee PA, Houk CP 2006 Puberty and Its Disorders. In: Lifschitz F ed. Pediatric Endocrinology. New York: Informa Healthcare; 273-303

Leidenberger F, Reichert Jr LE 1972 Evaluation of a Rat Testis Homogenate Radioligand Receptor Assay for Human Pituitary LH 1. Endocrinology 91:901-909

Lempiainen A, Hotakainen K, Alfthan H, Stenman UH 2012 Loss of human chorionic gonadotropin in urine during storage at -20 degrees C. Clin. Chim. Acta 413:232-236

Leung KS-Y, Fong BM-W 2014 LC–MS/MS in the routine clinical laboratory: has its time come? Anal. Bioanal. Chem. 406:2289-2301

Lévesque É, Beaulieu M, Hum DW, Bélanger A 1999 Characterization and substrate specificity of UGT2B4 (E458): a UDP-glucuronosyltransferase encoded by a polymorphic gene. Pharmacogenet. Genomics 9:207-216

Lévesque É, Turgeon D, Carrier J-S, Montminy V, Beaulieu M, Bélanger A 2001 Isolation and characterization of the UGT2B28 cDNA encoding a novel human steroid conjugating UDP-glucuronosyltransferase. Biochemistry (Mosc.) 40:3869-3881

Li Y, Lindsay J, Wang L-L, Zhou S-F 2008 Structure, function and polymorphism of human cytosolic sulfotransferases. Curr. Drug Metab. 9:99-105

Liberato D, Yergey A, Esteban N, Gomez-Sanchez C, Shackleton C 1987 Thermospray HPLC/MS: a new mass spectrometric technique for the profiling of steroids. J. Steroid Biochem. 27:61-70

Lin D, Sugawara T, Strauss Jr, Clark BJ, Stocco DM, Saenger P, Rogol A, Miller WL 1995 Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. Science 267:1828-1831

Livesey JH, Hodgkinson SC, Roud HR, Donald RA 1980 Effect of time, temperature and freezing on the stability of immunoreactive LH, FSH, TSH, growth hormone, prolactin and insulin in plasma. Clin. Biochem. 13:151-155

Livesey JH, Roud HK, Metcalf MG, Donald RA 1983 Glycerol prevents loss of immunoreactive follicle-stimulating hormone and luteinizing hormone from frozen urine. J. Endocrinol. 98:381-384

Llouquet JL, Crepin N, Lasne F 2013 The problem of anti-doping control of luteinizing hormone in boxing. Drug Test. Anal.

Longcope C, Pratt JH, Stephen HS, Fineberg SE 1978 Aromatization of Androgens by Muscle and Adipose Tissue in Vivo. J. Clin. Endocrinol. Metab. 46:146-152

Lövgren T, Hemmilä I, Pettersson K, Eskola JU, Bertoft E 1984 Determination of hormones by time-resolved fluoroimmunoassay. Talanta 31:909-916

Macedo DB, Abreu AP, Reis ACS, Montenegro LR, Dauber A, Beneduzzi D, Cukier P, Silveira LF, Teles MG, Carroll RS 2014 Central precocious puberty that appears to be sporadic caused by paternally inherited mutations in the imprinted gene makorin ring finger 3. J. Clin. Endocrinol. Metab. 99:E1097-E1103

Mackenzie PI, Bock KW, Burchell B, Guillemette C, Ikushiro S-i, Iyanagi T, Miners JO, Owens IS, Nebert DW 2005 Nomenclature update for the mammalian UDP glycosyltransferase (UGT) gene superfamily. Pharmacogenet. Genomics 15:677-685

Mackenzie PI, Owens IS, Burchell B, Bock K, Bairoch A, Belanger A, Gigleux SF, Green M, Hum DW, Iyanagi T 1997 The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. Pharmacogenet. Genomics 7:255-269

Mackenzie PI, Rodbourne L, Stranks S 1992 Steroid UDP glucuronosyltransferases. J. Steroid Biochem. 43:1099-1105

Mackey MA, Conway AJ, Handelsman DJ 1995 Tolerability of intramuscular injections of testosterone ester in an oil vehicle. Hum. Reprod. 10:862-865

Magnisali P, Chalioti MB, Livadara T, Mataragas M, Paliatsiou S, Malamitsi-Puchner A, Moutsatsou P 2011 Simultaneous quantification of 17alpha-OH progesterone, 11-deoxycortisol, Delta4-androstenedione, cortisol and cortisone in newborn blood spots using liquid chromatography-tandem mass spectrometry. J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci. 879:1565-1572

Manasco PK, Umbach DM, Muly SM, Godwin DC, Negro-Vilar A, Culler MD, Underwood LE 1997 Ontogeny of gonadotrophin and inhibin secretion in normal girls through puberty based on overnight serial sampling and a comparison with normal boys. Hum. Reprod. 12:2108-2114

Mao L, Sun C, Zhang H, Li Y, Wu D 2004 Determination of environmental estrogens in human urine by high performance liquid chromatography after fluorescent derivatization with p-nitrobenzoyl chloride. Anal. Chim. Acta 522:241-246

Marceau K, Ram N, Houts RM, Grimm KJ, Susman EJ 2011 Individual differences in boys' and girls' timing and tempo of puberty: modeling development with nonlinear growth models. Dev. Psychol. 47:1389-1409

Marchetti PM, Barth JH 2013 Clinical biochemistry of dihydrotestosterone. Ann. Clin. Biochem. 50:95-107

Mareck U, Geyer H, Fussholler G, Schwenke A, Haenelt N, Piper T, Thevis M, Schanzer W 2010 Reporting and managing elevated testosterone/epitestosterone ratios--novel aspects after five years' experience. Drug Test. Anal. 2:637-642

Marks V 2002 False-positive immunoassay results: a multicenter survey of erroneous immunoassay results from assays of 74 analytes in 10 donors from 66 laboratories in seven countries. Clin. Chem. 48:2008-2016

Marschall H, Egestad B, Matern H, Matern S, Sjövall J 1989 N-acetylglucosaminides. A new type of bile acid conjugate in man. J. Biol. Chem. 264:12989-12993

Marshall WA 1975 Growth and sexual maturity in normal puberty. J. Clin. Endocrinol. Metab. 4:3-25

Marshall WA, Tanner JM 1969 Variations in pattern of pubertal changes in girls. Arch. Dis. Child. 44:291

Marshall WA, Tanner JM 1970 Variations in the pattern of pubertal changes in boys. Arch. Dis. Child. 45:13-23

Martha PM, Rogol JR AD, Veldhuis JD, Kerrigan JR, Goodman DW, Blizzard RM 1989 Alterations in the pulsatile properties of circulating growth hormone concentrations during puberty in boys. J. Clin. Endocrinol. Metab. 69:563-570

Marynick SP, Loriaux DL, Sherins RJ, Pita Jr JC, Lipsett Jr MB 1979 Evidence that testosterone can suppress pituitary gonadotropin secretion independently of peripheral aromatization. J. Clin. Endocrinol. Metab. 49:396-398

Matsui H, Takatsu Y, Kumano S, Matsumoto H, Ohtaki T 2004 Peripheral administration of metastin induces marked gonadotropin release and ovulation in the rat. Biochem. Biophys. Res. Commun. 320:383-388

Matuszewski B 2006 Standard line slopes as a measure of a relative matrix effect in quantitative HPLC–MS bioanalysis. J. Chromatogr. B 830:293-300

Matuszewski B, Constanzer M, Chavez-Eng C 1998 Matrix effect in quantitative LC/MS/MS analyses of biological fluids: a method for determination of finasteride in human plasma at picogram per milliliter concentrations. Anal. Chem. 70:882-889

Matuszewski B, Constanzer M, Chavez-Eng C 2003 Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal. Chem. 75:3019-3030

Matzuk M, Boime I 1988 Site-specific mutagenesis defines the intracellular role of the asparagine-linked oligosaccharides of chorionic gonadotropin beta subunit. J. Biol. Chem. 263:17106-17111

Matzuk M, Hsueh A, Lapolt P, Tsafriri A, Keene J, Boime I 1990 The biological role of the carboxyl-terminal extension of human chorionic gonadotroin β-subunit. Endocrinology 126:376-383

Mauras N 2001 Growth hormone and sex steroids: interactions in puberty. Endocrinol. Metab. Clin. North Am. 30:529-544

McDade TW 2014 Development and validation of assay protocols for use with dried blood spot samples. Am. J. Hum. Biol. 26:1-9

McDade TW, Williams S, Snodgrass JJ 2007 What a drop can do: dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. Demography 44:899-925

McNamara K, Harwood D, Simanainen U, Walters K, Jimenez M, Handelsman D 2010 Measurement of sex steroids in murine blood and reproductive tissues by liquid chromatography–tandem mass spectrometry. J. Steroid Biochem. 121:611-618

McNeilly JD, Mason A, Khanna S, Galloway PJ, Ahmed SF 2012 Urinary gonadotrophins: a useful non-invasive marker of activation of the hypothalamic pituitary-gonadal axis. Int. J. Pediatr. Endocrinol. 2012:10

McWillams CA 1927 Paraffinomas. Am. J. Surg. 2:129

Medvedovici A, Udrescu S, Albu F, Tache F, David V 2011 Large-volume injection of sample diluents not miscible with the mobile phase as an alternative approach in sample preparation for bioanalysis: an application for fenspiride bioequivalence. Bioanalysis 3:1935-1947

Mei H, Hsieh Y, Nardo C, Xu X, Wang S, Ng K, Korfmacher WA 2003 Investigation of matrix effects in bioanalytical high-performance liquid chromatography/tandem mass spectrometric assays: application to drug discovery. Rapid Commun. Mass Spectrom. 17:97-103

Meloche CA, Falany CN 2001 Expression and characterization of the human 3β-hydroxysteroid sulfotransferases (SULT2B1a and SULT2B1b). J. Steroid Biochem. 77:261-269

Mendel CM 1989 The free hormone hypothesis: A physiologically based mathematical model. Endocr. Rev. 10:232-274

Messeri G, Cugnetto G, Moneti G, Serio M 1984 Helix pomatia induced conversion of some 3β-hydroxysteroids. J. Steroid Biochem. 20:793-796

Miller JD, Tannenbaum GS, Colle E, Guyda HJ 1982 Daytime pulsatile growth hormone secretion during childhood and adolescence. J. Clin. Endocrinol. Metab. 55:989-994

Miller RC, Brindle E, Holman DJ, Shofer J, Klein NA, Soules MR, O'Connor KA 2004 Comparison of specific gravity and creatinine for normalizing urinary reproductive hormone concentrations. Clin. Chem. 50:924-932

Miller WL 2005 Minireview: regulation of steroidogenesis by electron transfer. Endocrinology 146:2544-2550

Miller WL, Auchus RJ 2011 The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. Endocr. Rev. 32:81-151

Miller WL, Bose HS 2011 Early steps in steroidogenesis: intracellular cholesterol trafficking. J. Lipid Res. 52:2111-2135

Minto C, Howe C, Wishart S, Conway AJ, Handelsman DJ 1997 Pharmacokinetics and pharmacodynamics of nandrolone esters in oil vehicle: effects of ester, injection site and volume. J. Pharmacol. Exp. Ther. 281:93-102

Miyakawa I, Stanczyk FZ, March CM, March AD, Goebelsmann U 1981 Urinary Estradiol-17 [beta]-Glucuronide Assay for Gonadotropin Therapy. Obstet. Gynecol. 58:142-147

Mo Q, Lu S-f, Simon NG 2006 Dehydroepiandrosterone and its metabolites: differential effects on androgen receptor trafficking and transcriptional activity. J. Steroid Biochem. 99:50-58

Moal V, Mathieu E, Reynier P, Malthièry Y, Gallois Y 2007 Low serum testosterone assayed by liquid chromatography-tandem mass spectrometry. Comparison with five immunoassay techniques. Clin. Chim. Acta 386:12-19

Moeller BC, Stanley SD 2012 The development and validation of a turbulent flow chromatography–tandem mass spectrometry method for the endogenous steroid profiling of equine serum. J. Chromatogr. B 905:1-9

Moens L, Jakubowski N 1998 The only general method for overcoming spectroscopic interference requires double-focusing instrumentation: Double-focusing mass spectrometers in ICPMS. Anal. Chem. 70:251A-256A

Mojarrabi B, Butler R, Mackenzie P 1996 cDNA cloning and characterization of the human UDP glucuronosyltransferase, UGT1A3. Biochem. Biophys. Res. Commun. 225:785-790

Moore Jr RR, Hirata-Dulas CA, Kasiske BL 1997 Use of urine specific gravity to improve screening for albuminuria. Kidney Int. 52:240-243

Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K 1995 Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. J. Clin. Endocrinol. Metab. 80:3689-3698

Morris NM, Udry JR 1980 Validation of a self-administered instrument to assess stage of adolescent development. J. Youth Adolesc. 9:271-280

Mulder J, Levy G, Hein D 1990 Sulfation. In: Mulder J ed. Conjugation reaction in drug metabolism-an integrated approach. Substrates, co-substrates, enzymes and their interaction in vivo and in vitro: Taylor and Francis, London, UK

Munro C, Stabenfeldt G, Cragun J, Addiego L, Overstreet J, Lasley B 1991 Relationship of serum estradiol and progesterone concentrations to the excretion profiles of their major urinary metabolites as measured by enzyme immunoassay and radioimmunoassay. Clin. Chem. 37:838-844

Nagata C, Iwasa S, Shiraki M, Shimizu H 2006 Estrogen and α-fetoprotein levels in maternal and umbilical cord blood samples in relation to birth weight. Cancer Epidemiol. Biomark. Prev. 15:1469-1472

Navarro V, Fernández-Fernández R, Castellano J, Roa J, Mayen A, Barreiro M, Gaytan F, Aguilar E, Pinilla L, Dieguez C 2004 Advanced vaginal opening and precocious activation of the reproductive axis by KiSS-1 peptide, the endogenous ligand of GPR54. J. Physiol. 561:379-386

Nawata H, Tanaka S, Tanaka S, Takayanagi R, Sakai Y, Yanase T, Ikuyama S, Haji M 1995 Aromatase in bone cell: association with osteoporosis in postmenopausal women. J. Steroid Biochem. 53:165-174

Nelson RE, Grebe SK, O'Kane DJ, Singh RJ 2004 Liquid chromatography–tandem mass spectrometry assay for simultaneous measurement of estradiol and estrone in human plasma. Clin. Chem. 50:373-384

Nermell B, Lindberg A-L, Rahman M, Berglund M, Åke Persson L, El Arifeen S, Vahter M 2008 Urinary arsenic concentration adjustment factors and malnutrition. Environ. Res. 106:212-218

Nielsen CT, Skakkebaek NE, Darling JA, Hunter WM, Richardson DW, Jorgensen M, Keiding N 1986 Longitudinal study of testosterone and luteinizing hormone (LH) in relation to spermarche, pubic hair, height and sitting height in normal boys. Acta Endocrinol. Suppl. (Copenh.) 279:98-106

Nikolaev EN, Kostyukevich YI, Vladimirov GN 2014 Fourier transform ion cyclotron resonance (FT ICR) mass spectrometry: Theory and simulations. Mass Spectrom. Rev.:1-40

Nilsson C, Seppala M, Pettersson K 2001 Immunological characterization of human luteinizing hormone with special regard to a common genetic variant. J. Endocrinol. 168:107-116

Nilsson O, Chrysis D, Pajulo O, Boman A, Holst M, Rubinstein J, Ritzen EM, Savendahl L 2003 Localization of estrogen receptors-alpha and-beta and androgen receptor in the human growth plate at different pubertal stages. J. Endocrinol. 177:319-326

Nilsson O, Marino R, De Luca F, Phillip M, Baron J 2005 Endocrine regulation of the growth plate. Horm. Res. Paediatr. 64:157-165

Nisbet AC 2006 Intramuscular gluteal injections in the increasingly obese population: retrospective study. BMJ 332:637-638

Norman AW, Mizwicki MT, Norman DP 2004 Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. Nat. Rev. Drug Discovery 3:27-41

O'Connor JF, Kovalevskaya G, Birken S, Schlatterer JP, Schechter D, McMahon DJ, Canfield RE 1998 The expression of the urinary forms of human luteinizing hormone beta fragment in various populations as assessed by a specific immunoradiometric assay. Hum. Reprod. 13:826-835

O'Connor KA, Brindle E, Miller RC, Shofer JB, Ferrell RJ, Klein NA, Soules MR, Holman DJ, Mansfield PK, Wood JW 2006 Ovulation detection methods for urinary hormones: precision, daily and intermittent sampling and a combined hierarchical method. Hum. Reprod. 21:1442-1452

Odell W, Ross G, Rayford P 1967 Radioimmunoassay for luteinizing hormone in human plasma or serum: physiological studies. J. Clin. Invest. 46:248

Odell WD, Ross GT, Rayford PL 1966 Radioimmunoassay for human luteinizing hormone. Metabolism. 15:287-289

Ohno S, Nakajin S 2009 Determination of mRNA expression of human UDP-glucuronosyltransferases and application for localization in various human tissues by real-time reverse transcriptase-polymerase chain reaction. Drug Metab. Dispos. 37:32-40

Ojeda SR, Lomniczi A 2014 Puberty in 2013: unravelling the mystery of puberty. Nature Reviews Endocrinology 10:67-69

Ojeda SR, Skinner MK 2006 Puberty in the rat. In: Neill J ed. Knobil and Neill's physiology of reproduction 3rd ed: Elsevier; 2061-2126

Olivares A, Cárdenas M, Timossi C, Zarinan T, Diaz-Sanchez V, Ulloa-Aguirre A 2000 Reactivity of different LH and FSH standards and preparations in the world health organization matched reagents for enzyme-linked immunoassays of gonadotrophins. Hum. Reprod. 15:2285-2291

Oonk R, Parker K, Gibson J, Richards J 1990 Rat cholesterol side-chain cleavage cytochrome P-450 (P-450scc) gene. Structure and regulation by cAMP in vitro. J. Biol. Chem. 265:22392-22401

Padmanabhan V, Lang LL, Sonstein J, Kelch RP, Beitins IZ 1988 Modulation of Serum Follicle-Stimulating Hormone Bioactivity and Isoform Distribution by Estrogenic Steroids in Normal Women and in Gonadal Dysgenesis. J. Clin. Endocrinol. Metab. 67:465-473

Palacios S 2007 Androgens and female sexual function. Maturitas 57:61-65

Palonek E, Gottlieb C, Garle M, Bjorkhem I, Carlstrom K 1995 Serum and urinary markers of exogenous testosterone administration. J. Steroid Biochem. Mol. Biol. 55:121-127

Pang KS, Schwab AJ, Goresky CA, Chiba M 1994 Transport, binding, and metabolism of sulfate conjugates in the liver. Chem. Biol. Interact. 92:179-207

Parikh CR, Gyamlani GG, Carvounis CP 2002 Screening for microalbuminuria simplified by urine specific gravity. Am. J. Nephrol. 22:315-319

Park-Chung M, Malayev A, Purdy RH, Gibbs TT, Farb DH 1999 Sulfated and unsulfated steroids modulate γ-aminobutyric acid receptor$_A$ function through distinct sites. Brain Res. 830:72-87

Pasqualini JR 2009 Estrogen sulfotransferases in breast and endometrial cancers. Ann. N. Y. Acad. Sci. 1155:88-98

Payne AH, Hales DB 2004 Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr. Rev. 25:947-970

Payne AH, Youngblood GL 1995 Regulation of expression of steroidogenic enzymes in Leydig cells. Biol. Reprod. 52:217-225

Pelletier G, Li S, Tremblay Y, Belanger A, Labrie F 2001 Immunoelectron microscopic localization of three key steroidogenic enzymes (cytochrome P450 (scc), 3 beta-hydroxysteroid dehydrogenase and cytochrome P450 (c17)) in rat adrenal cortex and gonads. J. Endocrinol. 171:373-383

Pelletier G, Tětu B, Labrie F 1999 Immunocytochemical Localization of Type 5 17 β-Hydroxysteroid Dehydrogenase in Human Reproductive Tissues. J. Histochem. Cytochem. 47:731-737

Penning TM 2011 Human hydroxysteroid dehydrogenases and pre-receptor regulation: insights into inhibitor design and evaluation. J. Steroid Biochem. 125:46-56

Perel E, Killinger D 1979 The interconversion and aromatization of androgens by human adipose tissue. J. Steroid Biochem. 10:623-627

Perry PJ, MacIndoe JH, Yates WR, Scott SD, Holman TL 1997 Detection of anabolic steroid administration: ratio of urinary testosterone to epitestosterone vs the ratio of urinary testosterone to luteinizing hormone. Clin. Chem. 43:731-735

Petersen AC, Crockett L, Richards M 1988 A self-report measure of pubertal status: Reliability, validity, and initial norms. J. Youth Adolesc. 17:117-133

Pettersson K, Ding YQ, Huhtaniemi I 1991 Monoclonal antibody-based discrepancies between two-site immunometric tests for lutropin. Clin. Chem. 37:1745-1748

Pettersson K, Ding YQ, Huhtaniemi I 1992 An immunologically anomalous luteinizing hormone variant in a healthy woman. J. Clin. Endocrinol. Metab. 74:164-171

Pettersson KS, Soderholm JR 1991 Individual differences in lutropin immunoreactivity revealed by monoclonal antibodies. Clin. Chem. 37:333-340

Pierce JG, Parsons TF 1981 Glycoprotein hormones: structure and function. Annu. Rev. Biochem. 50:465-495

Pinyerd B, Zipf WB 2005 Puberty—Timing is everything! J. Pediatr. Nurs. 20:75-82

Pitteloud N, Dwyer AA, DeCruz S, Lee H, Boepple PA, Crowley Jr WF, Hayes FJ 2008 Inhibition of luteinizing hormone secretion by testosterone in men requires aromatization for its pituitary but not its hypothalamic effects: evidence from the tandem study of normal and gonadotropin-releasing hormone-deficient men. J. Clin. Endocrinol. Metab. 93:784-791

Plant T, Gay V, Marshall G, Arslan M 1989 Puberty in monkeys is triggered by chemical stimulation of the hypothalamus. P. Natl. Acad. Sci. USA 86:2506-2510

Plant TM, Barker-Gibb ML 2004 Neurobiological mechanisms of puberty in higher primates. Hum. Reprod. Update 10:67-77

Plant TM, Skinner SM 2006 Puberty in non-human primates and humans. In: Neill J ed. Knobil and Neill's Physiology of Reproduction. 3rd ed: Elsevier; 2177-2230

Pozo OJ, Van Eenoo P, Van Thuyne W, Deventer K, Delbeke FT 2008 Direct quantification of steroid glucuronides in human urine by liquid chromatography–electrospray tandem mass spectrometry. J. Chromatogr. A 1183:108-118

Raiti S, Light C, Blizzard R 1969 Urinary follicle-stimulating hormone excretion in boys and adult males as measured by radioimmunoassay. J. Clin. Endocrinol. Metab. 29:884-890

Rash J, Jerkunica I, Sgoutas D 1980 Lipid interference in steroid radioimmunoassay. Clin. Chem. 26:84-88

Rauh M 2010 Steroid measurement with LC–MS/MS. Application examples in pediatrics. J. Steroid Biochem. 121:520-527

Rauh M, Gröschl M, Rascher W, Dörr HG 2006 Automated, fast and sensitive quantification of 17α-hydroxy-progesterone, androstenedione and testosterone by tandem mass spectrometry with on-line extraction. Steroids 71:450-458

Reed M, Purohit A, Woo L, Newman S, Potter B 2005 Steroid sulfatase: molecular biology, regulation, and inhibition. Endocr. Rev. 26:171-202

Reed M, Purohit A, Woo L, Potter B 1996 The development of steroid sulphatase inhibitors. Endocr. Relat. Cancer 3:9-23

Reimers T, Salerno V, Lamb S 1996 Validation and application of solid-phase chemiluminescent immunoassays for diagnosis of endocrine diseases in animals. Comp Haematol Int 6:170-175

Reiter EO, Kulin HE, Hamwood SM, Hayes A, Johanson A, Johansson ED, Wide L, Gemzell C, Kulin HE, Rifkind AB, Ross GT 1973 Preparation of urine containing small amounts of FSH and LH for radioimmunoassay: comparison of the kaolin-acetone and acetone extraction techniques. J. Clin. Endocrinol. Metab. 36:661-665

Resende EA, Lara BH, Reis JD, Ferreira BP, Pereira GA, Borges MF 2007 Assessment of basal and gonadotropin-releasing hormone-stimulated gonadotropins by immunochemiluminometric and immunofluorometric assays in normal children. J. Clin. Endocrinol. Metab. 92:1424-1429

Richards JS, Pangas SA 2010 The ovary: basic biology and clinical implications. J. Clin. Invest. 120:963

Riedy M, Wang J-Y, Miller AP, Buckler A, Hall J, Guida M 2000 Genomic organization of the UGT2b gene cluster on human chromosome 4q13. Pharmacogenet. Genomics 10:251-260

Ritter JK 2000 Roles of glucuronidation and UDP-glucuronosyltransferases in xenobiotic bioactivation reactions. Chem. Biol. Interact. 129:171-193

Rižner TL, Penning TM 2014 Role of aldo–keto reductase family 1 (AKR1) enzymes in human steroid metabolism. Steroids 79:49-63

Robb DB, Blades MW 2006 Atmospheric pressure photoionization for ionization of both polar and nonpolar compounds in reversed-phase LC/MS. Anal. Chem. 78:8162-8164

Robb DB, Blades MW 2008 State-of-the-art in atmospheric pressure photoionization for LC/MS. Anal. Chim. Acta 627:34-49

Robinson N, Saudan C, Sottas P-E, Mangin P, Saugy M 2007 Performance characteristics of two immunoassays for the measurement of urinary luteinizing hormone. J. Pharmaceut. Biomed. 43:270-276

Robinson N, Saudan C, Sottas P-E, Mangin P, Saugy M 2007 Performance characteristics of two immunoassays for the measurement of urinary luteinizing hormone. J. Pharm. Biomed. Anal. 43:270-276

Rochira V, Granata AR, Madeo B, Zirilli L, Rossi G, Carani C 2005 Estrogens in males: what have we learned in the last 10 years? Asian J. Androl. 7:3-20

Rodriguez G, Samper M, Olivares J, Ventura P, Moreno L, Perez-Gonzalez J 2005 Skinfold measurements at birth: sex and anthropometric influence. Arch. Dis. Child-Fetal 90:F273-FF275

Rodríguez G, Samper MP, Ventura P, Moreno LA, Olivares JL, Pérez-González JM 2004 Gender differences in newborn subcutaneous fat distribution. Eur. J Pediatr. 163:457-461

Rogol AD 2002 Androgens and puberty. Mol. Cell. Endocrinol. 198:25-29

Rogol AD 2010 Sex steroids, growth hormone, leptin and the pubertal growth spurt. Endocr. Dev. 17:77-85

Rogol AD, Roemmich JN, Clark PA 2002 Growth at puberty. J. Adolesc. Health 31:192-200

Rollins CE, Reiber G, Guinee DG, Jr., Lie JT 1997 Disseminated lipogranulomas and sudden death from self-administered mineral oil injection. Am. J. Forensic Med. Pathol. 18:100-103

Roselli CE, Stadelman H, Horton LE, Resko JA 1987 Regulation of androgen metabolism and luteinizing hormone-releasing hormone content in discrete hypothalamic and limbic areas of male Rhesus Macaques. Endocrinology 120:97-106

Rosner W, Hankinson SE, Sluss PM, Vesper HW, Wierman ME 2013 Challenges to the measurement of estradiol: an endocrine society position statement. J. Clin. Endocrinol. Metab. 98:1376-1387

Rothman MS, Carlson NE, Xu M, Wang C, Swerdloff R, Lee P, Goh VH, Ridgway EC, Wierman ME 2011 Reexamination of testosterone, dihydrotestosterone, estradiol and estrone levels across

the menstrual cycle and in postmenopausal women measured by liquid chromatography–tandem mass spectrometry. Steroids 76:177-182

Ruggiero RJ, Likis FE 2002 Estrogen: physiology, pharmacology, and formulations for replacement therapy. J. Midwifery Womens Health 47:130-138

Ruzicka L, Wettstein A 1935 Uber die krystallische Herstellung des Testikelhormons, Testosteron (androsten-3-on-17-ol). Helv. Chim. Acta 18:1264-1275

Saenger P 1996 Turner's syndrome. N. Engl. J. Med. 335:1749-1754

Saez J, Forest M, Morera A, Bertrand J 1972 Metabolic clearance rate and blood production rate of testosterone and dihydrotestosterone in normal subjects, during pregnancy, and in hyperthyroidism. J. Clin. Invest. 51:1226

Saketos M, Sharma N, Adel T, Raghuwanshi M, Santoro N 1994 Time-resolved immunofluorometric assay and specimen storage conditions for measuring urinary gonadotropins. Clin. Chem. 40:749-753

Salido EC, Yen PH, Barajas L, Shapiro LJ 1990 Steroid sulfatase expression in human placenta: Immunocytochemistry and in situ hybridization study. J. Clin. Endocrinol. Metab. 70:1564-1567

Sangster T, Spence M, Sinclair P, Payne R, Smith C 2004 Unexpected observation of ion suppression in a liquid chromatography/atmospheric pressure chemical ionization mass spectrometric bioanalytical method. Rapid Commun. Mass Spectrom. 18:1361-1364

Santa T 2011 Derivatization reagents in liquid chromatography/electrospray ionization tandem mass spectrometry. Biomed. Chromatogr. 25:1-10

Sanzo MA, Reichert L 1982 Gonadotropin receptor binding regulators in serum. Characterization and separation of follitropin binding inhibitor and lutropin binding stimulator. J. Biol. Chem. 257:6033-6040

Sartorius G, Fennell C, Spasevska S, Turner L, Conway AJ, Handelsman DJ 2010 Factors influencing time course of pain after depot oil intramuscular injection of testosterone undecanoate. Asian J. Androl. 12:227-233

Sasano H, Uzuki M, Sawai T, Nagura H, Matsunaga G, Kashimoto O, Harada N 1997 Aromatase in human bone tissue. J. Bone Miner. Res. 12:1416-1423

Schneider U, Schober EA, Streich NA, Breusch SJ 2002 Urinary creatinine instability falsely increases the deoxypyridinoline/creatinine quotient. Clin. Chim. Acta 324:81-88

Schoenfeld MJ, Shortridge E, Cui Z, Muram D 2013 Medication adherence and treatment patterns for hypogonadal patients treated with topical testosterone therapy: a retrospective medical claims analysis. J. Sex. Med. 10:1401-1409

Schreiner F, Gohlke B, Hamm M, Korsch E, Woelfle J 2014 MKRN3 mutations in familial central precocious puberty. Horm. Res. Paediatr.:122-126

Sedlmeyer IL, Palmert MR 2002 Delayed puberty: analysis of a large case series from an academic center. J. Clin. Endocrinol. Metab. 87:1613-1620

Seki K, Seki M, Kato K 1985 Correlation between urinary oestrogen levels determined by haemagglutination inhibition reaction and serum oestradiol levels determined by radioimmunoassay. Acta Endocrinol. (Copenh.) 110:130-134

Seminara SB, Hayes FJ, Crowley Jr WF 1998 Gonadotropin-releasing hormone deficiency in the human (idiopathic hypogonadotropic hypogonadism and Kallmann's syndrome): pathophysiological and genetic considerations. Endocr. Rev. 19:521-539

Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno Jr JS, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG 2003 The GPR54 gene as a regulator of puberty. N. Engl. J. Med. 349:1614-1627

Settas N, Dacou-Voutetakis C, Karantza M, Kanaka-Gantenbein C, Chrousos GP, Voutetakis A 2014 Central precocious puberty in a girl and early puberty in her brother caused by a novel mutation in the MKRN3 gene. J. Clin. Endocrinol. Metab. 99:E647-E651

Shah VP, Midha KK, Findlay JW, Hill HM, Hulse JD, McGilveray IJ, McKay G, Miller KJ, Patnaik RN, Powell ML 2000 Bioanalytical method validation—a revisit with a decade of progress. Pharm. Res. 17:1551-1557

Shibata Y, Arai S, Honma S 2014 Methodological approach to the intracrine study and estimation of DHEA and DHEA-S using liquid chromatography–tandem mass spectrometry (LC–MS/MS). J. Steroid Biochem.

Shibayama Y, Higashi T, Shimada K, Odani A, Mizokami A, Konaka H, Koh E, Namiki M 2009 Simultaneous determination of salivary testosterone and dehydroepiandrosterone using LC–MS/MS: method development and evaluation of applicability for diagnosis and medication for late-onset hypogonadism. J. Chromatogr. B 877:2615-2623

Siervogel RM, Maynard LM, Wisemandle WA, Roche AF, Guo SS, Chumlea WC, Towne B 2000 Annual Changes in Total Body Fat and Fat-free Mass in Children from 8 to 18 Years in Relation to Changes in Body Mass Index: The Fels Longitudinal Study. Ann. N. Y. Acad. Sci. 904:420-423

Sikaris K, McLachlan RI, Kazlauskas R, de Kretser D, Holden CA, Handelsman DJ 2005 Reproductive hormone reference intervals for healthy fertile young men: evaluation of automated platform assays. J. Clin. Endocrinol. Metab. 90:5928-5936

Silveira LF, Teles MG, Trarbach EB, Latronico AC 2010 Role of kisspeptin/GPR54 system in human reproductive axis. Front. Horm. Res. 39:13-24

Simpson ER, Davis SR 2001 Minireview: aromatase and the regulation of estrogen biosynthesis—some new perspectives. Endocrinology 142:4589-4594

Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR, Michael MD 1994 Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr. Rev. 15:342-355

Singh J, O'Neill C, Handelsman DJ 1995 Induction of spermatogenesis by androgens in gonadotropin-deficient (hpg) mice. Endocrinology 136:5311-5321

Sivananthan T, Bathur F, Jimenez M, Conway A, Idan A, Handelsman D 2012 Objective non-intrusive markers of sperm production and sexual activity. Asian J. Androl. 14:476-480

Sjövall J, Vihko R 1968 Analysis of solvolyzable steroids in human plasma by combined gas chromatography-mass spectrometry. Acta Endocrinol. (Copenh.) 57:247-260

Souverain S, Rudaz S, Veuthey J-L 2004 Matrix effect in LC-ESI-MS and LC-APCI-MS with off-line and on-line extraction procedures. J. Chromatogr. A 1058:61-66

Stanczyk FZ 2004 Extraction/chromatographic testosterone RIA can be used as the “gold standard” for determining the reliability of direct testosterone immunoassay measurements. Clin. Chem. 50:2219-2220

Stanczyk FZ, Clarke NJ 2010 Advantages and challenges of mass spectrometry assays for steroid hormones. J. Steroid Biochem. 121:491-495

Stanczyk FZ, Jurow J, Hsing AW 2010 Limitations of direct immunoassays for measuring circulating estradiol levels in postmenopausal women and men in epidemiologic studies. Cancer Epidemiol. Biomark. Prev. 19:903-906

Steelman SL, Pohley FM 1953 Assay of the follicle stimulating hormone based on the augmentation with human chorionic gonadotropin. Endocrinology 53:604-616

Steinbeck K, Hazell P, Cumming RG, Skinner SR, Ivers R, Booy R, Fulcher G, Handelsman DJ, Martin AJ, Morgan G, Starling J, Bauman A, Rawsthorne ML, Bennett DL, Chow CM, Lam MK, Kelly P, Brown NJ, Paxton K, Hawke C 2012 The study design and methodology for the ARCHER study - adolescent rural cohort study of hormones, health, education, environments and relationships. BMC Pediatr. 12:143

Stenman U-H, Unkila-Kallio L, Korhonen J, Alfthan H 1997 Immunoprocedures for detecting human chorionic gonadotropin: clinical aspects and doping control. Clin. Chem. 43:1293-1298

Stenman UH, Hotakainen K, Alfthan H 2008 Gonadotropins in doping: pharmacological basis and detection of illicit use. Br. J. Pharmacol. 154:569-583

Sterner J, Johnston M, Nicol G, Ridge D 2000 Signal suppression in electrospray ionization Fourier transform mass spectrometry of multi-component samples. J. Mass Spectrom. 35:385-391

Strahm E, Kohler I, Rudaz S, Martel S, Carrupt P-A, Veuthey J-L, Saugy M, Saudan C 2008 Isolation and quantification by high-performance liquid chromatography–ion-trap mass

spectrometry of androgen sulfoconjugates in human urine. J. Chromatogr. A 1196:153-160

Strassburg CP, Manns MP, Tukey RH 1998 Expression of the UDP-glucuronosyltransferase 1A locus in human colon identification and characterization of the novel extrahepatic UGT1A8. J. Biol. Chem. 273:8719-8726

Strauss J, Martinez F, Kiriakidou M 1996 Placental steroid hormone synthesis: unique features and unanswered questions. Biol. Reprod. 54:303-311

Strauss JF 2014 The Synthesis and Metabolism of Steroid Hormones. In: Strauss III JF, Barbieri RL eds. Yen and Jaffe's reproduction endocrinology: physiology, pathophysiology and clinical management. 7th ed: Elsevier Health Sciences; 66-92

Styne DM, Grumbach MM 2011 Puberty: Ontogeny, Neuroendocrinology, Physiology, and Disorders. In: Melmed S, Polonsky KS, Larsen PR, Kronenberg HM eds. Williams Textbook of Endocrinology. 12th ed: Elsevier/Saunders; 1054-1201

Sun Y, Tao F, Su PY 2012 National estimates of pubertal milestones among urban and rural Chinese boys. Ann. Hum. Biol. 39:461-467

Suwazono Y, Åkesson A, Alfven T, Järup L, Vahter M 2005 Creatinine versus specific gravity-adjusted urinary cadmium concentrations. Biomarkers 10:117-126

Svendsen O 1983 Local muscle damage and oily vehicles: a study on local reactions in rabbits after intramuscular injection of neuroleptic drugs in aqueous or oily vehicles. Acta Pharmacol. Toxicol. (Copenh.) 52:298-304

Svendsen O, Aaes-Jorgensen T 1979 Studies on the fate of vegetable oil after intramuscular injection into experimental animals. Acta Pharmacol. Toxicol. (Copenh.) 45:352-378

Svendsen O, Blom L, Aaes-Jorgensen T, Larsen JJ 1985 Local toxicity of different drugs after intramuscular or intralipomatous injection in pigs: serum concentrations after three different formulations of cis(Z)-clopenthixol. Acta Pharmacol. Toxicol. (Copenh.) 57:78-87

Svendsen O, Dencker SJ, Fog R, Gravem AO, Kristansen P 1980 Microscopic evidence of lymphogenic absorption of oil in humans receiving neuroleptic oily depot preparations intramuscularly. Acta Pharmacol. Toxicol. (Copenh.) 47:157

Sweeley C, Horning EC 1960 Microanalytical separation of steroids by gas chromatography. Nature 187:144-145

Symmers W 1955 Simulation of cancer by oil granuloma of therapeutic origin. Br. Med. J. 2:1536-1539

Taieb J, Benattar C, Birr AS, Lindenbaum A 2002 Limitations of steroid determination by direct immunoassay. Clin. Chem. 48:583-585

Taieb J, Mathian B, Millot F, Patricot M-C, Mathieu E, Queyrel N, Lacroix I, Somma-Delpero C, Boudou P 2003 Testosterone measured by 10 immunoassays and by isotope-dilution gas chromatography–mass spectrometry in sera from 116 men, women, and children. Clin. Chem. 49:1381-1395

Taylor G 1964 Disintegration of water drops in an electric field. P Roy Soc Lond A Mat 280:383-397

Terasawa E, Guerriero KA, Plant TM 2013 Kispeptin and puberty in mammals. In: Kauffman AS, Smith JT eds. Kisspeptin signalling in reproductive biology. 1st ed: Springer; 253-274

Themmen AP, Huhtaniemi IT 2000 Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary-gonadal function. Endocr. Rev. 21:551-583

Thigpen A, Silver R, Guileyardo J, Casey ML, McConnell J, Russell D 1993 Tissue distribution and ontogeny of steroid 5 alpha-reductase isozyme expression. J. Clin. Invest. 92:903

Thompson S, Barlow R, Wald N, Van Vunakis H 1990 How should urinary cotinine concentrations be adjusted for urinary creatinine concentration? Clin. Chim. Acta 187:289-295

Thotakura NR, Blithe DL 1995 Glycoprotein hormones: glycobiology of gonadotrophins, thyrotrophin and free α subunit. Glycobiology 5:3-10

Topaloglu AK, Reimann F, Guclu M, Yalin AS, Kotan LD, Porter KM, Serin A, Mungan NO, Cook JR, Ozbek MN 2008 TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for Neurokinin B in the central control of reproduction. Nat. Genet. 41:354-358

Trevisan A 1990 Concentration adjustment of spot samples in analysis of urinary xenobiotic metabolites. Am. J. Ind. Med. 17:637-642

Trufelli H, Palma P, Famiglini G, Cappiello A 2011 An overview of matrix effects in liquid chromatography–mass spectrometry. Mass Spectrom. Rev. 30:491-509

Tsatsoulis A, Mavroudis K, Frost J, Lambert A, Shalet SM, Robertson WR 1988 The stability in vitro of bioactive and immunoreactive LH in human blood and plasma. J. Endocrinol. 117:139-145

Tukey RH, Strassburg CP 2000 Human UDP-glucuronosyltransferases: metabolism, expression, and disease. Annu. Rev. Pharmacol. Toxicol. 40:581-616

Turgeon D, Carrier J-Sb, Lévesque Er, Hum DW, Bélanger A 2001 Relative Enzymatic Activity, Protein Stability, and Tissue Distribution of Human Steroid-Metabolizing UGT2B Subfamily Members. Endocrinology 142:778-787

Ulloa-Aguirre A, Maldonado A, Damián-Matsumura P, Timossi C 2001 Endocrine regulation of gonadotropin glycosylation. Arch. Med. Res. 32:520-532

Ulloa-Aguirre A, Timossi C 2000 Biochemical and functional aspects of gonadotrophin-releasing hormone and gonadotrophins. Reprod. Biomed. Online 1:48-62

Ulloa-Aguirre A, Timossi C, Méndez J 2001 Is there any physiological role for gonadotrophin oligosaccharide heterogeneity in humans? I. Gondatrophins are synthesized and released in multiple molecular forms. A matter of fact. Hum. Reprod. 16:599-604

Urban MD, Lee PA, Gutai JP, Migeon CJ 1980 Androgens in pubertal males with Addison's disease. J. Clin. Endocrinol. Metab. 51:925-929

Van Damme M-P, Robertson D, Marana R, Ritzen E, Diczfalusy E 1979 A sensitive and specific in vitro bioassay method for the measurement of follicle-stimulating hormone activity. Acta Endocrinol. (Copenh.) 91:224-237

Van Eeckhaut A, Lanckmans K, Sarre S, Smolders I, Michotte Y 2009 Validation of bioanalytical LC–MS/MS assays: evaluation of matrix effects. J. Chromatogr. B 877:2198-2207

Vanden Heuvel W, Sweeley C, Horning E 1960 Separation of steroids by gas chromatography. J. Am. Chem. Soc. 82:3481-3482

Vanluchene E, Eechaute W, Vandekerckhove D 1982 Conversion of free 3β-hydroxy-5-ene-steroids by incubation with Helix pomatia. J. Steroid Biochem. 16:701-703

Veldhuis JD, Johnson ML, Dufau ML 1989 Physiological attributes of endogenous bioactive luteinizing hormone secretory bursts in man. Am. J. Physiol. Endocrinol. Metab. 256:E199-E207

Veldhuis JD, Metzger DL, Martha Jr PM, Mauras N, Kerrigan JR, Keenan B, Rogol AD, Pincus SM 1997 Estrogen and testosterone, but not a nonaromatizable androgen, direct network integration of the hypothalamo-somatotrope (growth hormone)-insulin-like growth factor I axis in the human: Evidence from pubertal pathophysiology and sex-steroid hormone replacement. J. Clin. Endocrinol. Metab. 82:3414-3420

Veldhuis JD, Roemmich JN, Richmond EJ, Rogol AD, Lovejoy JC, Sheffield-Moore M, Mauras N, Bowers CY 2005 Endocrine control of body composition in infancy, childhood, and puberty. Endocr. Rev. 26:114-146

Venturelli E, Cavalleri A, Secreto G 1995 Methods for urinary testosterone analysis. J. Chromatogr. B 671:363-380

Verhoeven G, Willems A, Denolet E, Swinnen JV, De Gendt K 2010 Androgens and spermatogenesis: lessons from transgenic mouse models. Philos T Roy Soc B 365:1537-1556

Vermeulen A, Kaufman J, Goemaere S, Van Pottelberg I 2002 Estradiol in elderly men. Aging Male 5:98-102

Vessey DA 1978 The biochemical basis for the conjugation of bile acids with either glycine or taurine. Biochem. J. 174:621-626

Vestergaard P, Raabo E, Vedsø S 1966 Determination of urinary testosterone in men, women and children. Clin. Chim. Acta 14:540-552

Vogeser M, Parhofer K 2007 Liquid chromatography tandem-mass spectrometry (LC-MS/MS)-technique and applications in endocrinology. Exp. Clin. Endocrinol. Diabetes 115:559-570

Voinescu GC, Shoemaker M, Moore H, Khanna R, Nolph KD 2002 The relationship between urine osmolality and specific gravity. Am. J. Med. Sci. 323:39-42

Wallis M, Howell S, Taylor K 1985 Hormones of the adenohypophysis: the gonadotropins and thyrotropin (and related placental hormones). The Biochemistry of the Polypeptide Hormones. New York: John Wiley and Sons:147-183

Wang C 1988 Bioassays of follicle stimulating hormone. Endocr. Rev. 9:374-377

Wang C, Catlin DH, Demers LM, Starcevic B, Swerdloff RS 2004 Measurement of total serum testosterone in adult men: comparison of current laboratory methods versus liquid chromatography-tandem mass spectrometry. J. Clin. Endocrinol. Metab. 89:534-543

Watanabe G, Terasawa E 1989 In vivo release of luteinizing hormone releasing hormone increases with puberty in the female rhesus monkey. Endocrinology 125:92-99

Waterman MR, Simpson ER 1985 Regulation of the biosynthesis of cytochromes P-450 involved in steroid hormone synthesis. Mol. Cell. Endocrinol. 39:81-89

Weise M, De-Levi S, Barnes KM, Gafni RI, Abad V, Baron J 2001 Effects of estrogen on growth plate senescence and epiphyseal fusion. P. Natl. Acad. Sci. USA 98:6871-6876

Weykamp C, Penders T, Schmidt N, Borburgh A, Van de Calseyde J, Wolthers B 1989 Steroid profile for urine: reference values. Clin. Chem. 35:2281-2284

Whelan EA, Grajewski B, Wood E, Kwan L, Nguyen M, Schnorr TM, Knecht EA, Kesner JS 2002 Feasibility issues in reproductive biomonitoring of female flight attendants and teachers. J. Occup. Environ. Med. 44:947-955

White BC, Jamison KM, Grieb C, Lally D, Luckett C, Kramer KS, Phillips J 2010 Specific gravity and creatinine as corrections for variation in urine concentration in humans, gorillas, and woolly monkeys. Am. J. Primatol. 72:1082-1091

WHO 2006 World Health Organization: Child Growth Standards. In: http://www.who.int/childgrowth/standards/weight_for_height/en/

Wide L 1985 Median charge and charge heterogeneity of human pituitary FSH, LH and TSH. Acta Endocrinol. (Copenh.) 109:181-189

Wide L 1989 Follicle-stimulating hormones in anterior pituitary glands from children and adults differ in relation to sex and age. J. Endocrinol. 123:519-529

Wide L, Bakos O 1993 More basic forms of both human follicle-stimulating hormone and luteinizing hormone in serum at midcycle compared with the follicular or luteal phase. J. Clin. Endocrinol. Metab. 76:885-889

Wide L, Roos P, Gemzell C 1961 Immunological determination of human pituitary luteinizing hormone (LH). Acta Endocrinol. (Copenh.) 37:445-449

Wietholtz H, Marschall HU, Reuschenbach R, Matern H, Matern S 1991 Urinary excretion of bile acid glucosides and glucuronides in extrahepatic cholestasis. Hepatology 13:656-662

Wildt L, Häusler A, Marshall G, Hutchison J, Plant T, Belchetz P, Knobil E 1981 Frequency and Amplitude of Gonadotropin-Releasing Hormone Stimulation and Gonadotropin Secretion in the Rhesus Monkey. Endocrinology 109:376-385

Wiren KM 2007 Androgens and Skeletal Biology: Basic Mechanisms. Osteoporosis:425

Witchel SF, Plant TM 2014 Puberty: Gonadarche and adrenarche In: Strauss III JF, Barbieri RL eds. Yen and Jaffe's reproduction endocrinology: physiology, pathophysiology and clinical management. 7th ed: Elsevier Health Sciences; 253-274

Worthman CM, Stallings JF 1997 Hormone measures in finger-prick blood spot samples: new field methods for reproductive endocrinology. Am. J. Phys. Anthropol. 104:1-21

Wright AS, Thomas LN, Douglas RC, Lazier CB, Rittmaster RS 1996 Relative potency of testosterone and dihydrotestosterone in preventing atrophy and apoptosis in the prostate of the castrated rat. J. Clin. Invest. 98:2558

Wu C-H, Lundy LE 1971 Radioimmunoassay of plasma estrogens. Steroids 18:91-111

Wu F, Butler G, Kelnar C, Stirling H, Huhtaniemi I 1991 Patterns of pulsatile luteinizing hormone and follicle-stimulating hormone secretion in prepubertal (midchildhood) boys and girls and patients with idiopathic hypogonadotropic hypogonadism (Kallmann's syndrome): a study using an ultrasensitive time-resolved immunofluorometric assay. J. Clin. Endocrinol. Metab. 72:1229-1237

Wu X, Wan S, Lee MM 2007 Key factors in the regulation of fetal and postnatal Leydig cell development. J. Cell. Physiol. 213:429-433

Xian F, Hendrickson CL, Marshall AG 2012 High resolution mass spectrometry. Anal. Chem. 84:708-719

Xu F, Zou L, Liu Y, Zhang Z, Ong CN 2011 Enhancement of the capabilities of liquid chromatography–mass spectrometry with derivatization: general principles and applications. Mass Spectrom. Rev. 30:1143-1172

Xu F, Zou L, Ong CN 2010 Experiment-originated variations, and multi-peak and multi-origination phenomena in derivatization-based GC-MS metabolomics. TrAC-Trend Anal. Chem. 29:269-280

Xu RN, Fan L, Rieser MJ, El-Shourbagy TA 2007 Recent advances in high-throughput quantitative bioanalysis by LC–MS/MS. J. Pharm. Biomed. Anal. 44:342-355

Xu X, Veenstra TD, Fox SD, Roman JM, Issaq HJ, Falk R, Saavedra JE, Keefer LK, Ziegler RG 2005 Measuring fifteen endogenous estrogens simultaneously in human urine by high-performance liquid chromatography-mass spectrometry. Anal. Chem. 77:6646-6654

Yalow RS, Berson SA 1959 Assay of plasma insulin in human subjects by immunological methods.

Yarrow JF, Beck DT, Conover CF, Beggs LA, Goldberger BA, Borst SE 2013 Invalidation of a commercially available human 5α-dihydrotestosterone immunoassay. Steroids 78:1220-1225

You L 2004 Steroid hormone biotransformation and xenobiotic induction of hepatic steroid metabolizing enzymes. Chem. Biol. Interact. 147:233-246

Zacur H, Kaufman S, Smith B, Westhoff C, Helbig D, Lee Y, Gentile G 1997 Does creatinine adjustment of urinary pregnanediol glucuronide reduce or introduce measurement error? Gynecol. Endocrinol. 11:29-33

Zamek-Gliszczynski MJ, Hoffmaster KA, Nezasa K-i, Tallman MN, Brouwer KL 2006 Integration of hepatic drug transporters and phase II metabolizing enzymes: mechanisms of hepatic excretion of sulfate, glucuronide, and glutathione metabolites. Eur. J. Pharm. Sci. 27:447-486

Zeleznik A, Pohl C 2006 Control of follicular development, corpus luteum function, the maternal recognition of pregnancy, and the neuroendocrine regulation of the menstrual cycle in higher primates. In: Neill J ed. Knobil and Neill's physiology of reproduction. 3rd ed; 2449-2510

Zhu BT, Conney AH 1998 Functional role of estrogen metabolism in target cells: review and perspectives. Carcinogenesis 19:1-27

Appendix

Chapter 3:

Gurmeet KS Singh was responsible to optimize the DBS sampling method, LC-MS/MS assay development and conducting the assays, as well as statistical analyses and involved in writing and editing the manuscript. Leo Turner is a nurse at the Andrology clinic, Concord Hospital that was responsible for recruiting of the volunteers, administrating the drug, monitoring, sample collection and editing the manuscript. Reena Desai was involved in the assay development and editing of the manuscript. Mark Jimenez conducted the gonadotropin assays and was involve in the editing of the manuscript. David J Handelsman was responsible for study design, assay development, statistical analyses, writing and editing the manuscript.

Chapter 4:

Gurmeet KS Singh was responsible for the statistical analyses and involved in writing the first draft and editing the manuscript. Mark Jimenez conducted the LH assays and was involved in the editing of the manuscript. Ron Newman was involved in the editing of the manuscript. David J Handelsman was responsible for study design, statistical analyses, writing and editing the manuscript.

Chapter 5:

Gurmeet KS Singh was responsible for assay development and conducting the assays, as well as statistical analyses, writing the first draft and editing the manuscript. Ben WR Belzer, Reena Desai and Mark Jimenez were involved in the writing and editing of the manuscript. Reena Desai was also involved in the assay development. Katharine S Steinbeck was involved in study design, study oversight, writing, and editing this manuscript. David J

Handelsman was responsible for study design, assay development, statistical analyses, writing and editing the manuscript.

Chapter 6:

Gurmeet KS Singh and Ben WR Balzer share equal authorship of this paper. Gurmeet KS Singh was responsible for assay development and conducting the assays, as well as authorship and editing the manuscript. Ben WR Balzer performed statistical analyses, wrote the first draft and contributed to the editing of the manuscript. Patrick J Kelly performed statistical analyses and contributed to editing the manuscript. Karen Paxton was involved in study design, sample collection, performed anthropometry assessments and edited the manuscript. Catherine I Hawke was involved in study design and oversight, as well as contributing to the writing and editing of the manuscript. David J Handelsman was involved in assay development and conduct, as well as statistical analyses and writing and editing the manuscript. Katharine S Steinbeck is the chief investigator of the ARCHER Study and was involved in study design, study oversight, writing, and editing this manuscript.