STUDIES TOWARDS THE DEVELOPMENT OF A MULTI-PURPOSE HOME SELF-TEST KIT FOR THE DETECTION OF URINARY TETRAHYDROCORTISONE AND TESTOSTERONE METABOLITES

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in Chemistry at Massey University

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Abstract

The development of homogeneous enzyme immunoassays (HEIA) for testosterone glucuronide (TG) and tetrahydrocortisone glucuronide (THEG) in urine are described. The proposed test system is based on the Ovarian Monitor homogeneous immunoassay system, established by J.B Brown and L.F. Blackwell *et al.*[1] as a simple, laboratory accurate, monitoring device for the measurement of estrone glucuronide (E1G) and pregnanediol glucuronide (PdG) as markers of the fertile phase during a womans menstrual cycle. This information can be used readily by women to identify their cyclical periods of fertility and infertility.

The major testosterone metabolite in the urine of males, testosterone β-glucuronide, was synthesised by firstly preparing the glycosyl donor α-bromosugar and conjugating this with testosterone under standard Koenigs-Knorr conditions. ^{1}H nmr studies confirmed that the synthetic steroid glucuronide had the same stereochemistry as the naturally occurring urinary testosterone glucuronide.

Testosterone glucuronide and tetrahydrocortisone glucuronide conjugates of hen egg white lysozyme were prepared using the active ester coupling method in good yield. Unreacted lysozyme was successfully removed from the reaction mixture by a combination of cation-exchange chromatography in 7 M urea and hydrophobic-interaction chromatography. S-Sepharose chromatography allowed two of the major conjugation products in each steroid-glucuronide reaction mixture to be isolated and characterised by MALDI mass spectrometry. All of the selected conjugates were found to be mono-acylated species, with the exception of one of the testosterone glucuronide conjugates, which was found to contain a mixture of mono- and di-substituted hen egg white lysozyme conjugates. Conjugates prepared in this way were tested for their suitability as signal generators in a homogenous immunoassay system for measurement of testosterone glucuronide and tetrahydrocortisone glucuronide in urine samples based on the already established Ovarian Monitor home fertility assay.

Immunogens required for raising anti-steroid antibodies were also prepared using the active ester method to conjugate testosterone glucuronide and tetrahydrocortisone glucuronide to the carrier protein, bovine thyroglobulin. These immunogens were then used to raise anti-testosterone glucuronide and anti-tetrahydrocortisone glucuronide antibodies in four New Zealand White rabbits. The antisera obtained over a number of months from each rabbit were screened for their ability to inhibit the lytic activity of the corresponding steroid glucuronide-lysozyme conjugate. Although all the antisera showed the immunogens had stimulated the

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[1] Brown JB, Blackwell LF, Cox RI, Holmes JM and Smith MA (1988). Chemical and homogenous enzyme immunoassay methods for the measurement of estrogens and pregnanediol and their glucuronides in urine. *Progress in Clinical and Biological Research* **285**:119-38.

production of anti-steroid glucuronide antibodies, the antiserum titre was low, and meant that the volume of antiserum required to inhibit the lytic activity was high. Nevertheless, despite the low antiserum titres, the selected antisera could be used to produce good standard curves for testosterone with sensitivity close to that required for TG and an excellent standard curve for THEG.

Potential applications of home assays for urinary testosterone glucuronide are in self-monitoring of testosterone levels in men undergoing long term testosterone supplementation for a diagnosed androgen deficiency or steroidogenic abnormality. Tetrahydrocortisone glucuronide is a major metabolite of cortisol, the main glucocorticoid produced by the body in response to stress and disease, and has potential as a biomarker for assessing therapies designed to reduce stress and for individuals suffering from stress related conditions such as hypertension and heart disease.

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Table of Contents

Chapter Two: Synthesis and purification of biomaterials required for the immunoassay of testosterone glucuronide (TG) and tetrahydrocortisone glucuronide (THEG)

List of Figures

List of Schemes

List of Tables

Abbreviations

A_{280}	Absorbance at 280 nm
A_{595}	Absorbance at 595 nm
Ab	Antibody
Ag	Antigen
BSA	Bovine serum albumin
DCC	*N',N'*-dicyclohexylcarbodiimide
DMF	Dimethylformamide
E1G	Estrone glucuronide
EC_{50}	Analyte midpoint of Ovarian Monitor standard curve
ELISA	Enzyme linked immunosorbent assay
ESMS	Electrospray mass spectroscopy
Fab	Antigen binding fragment
FPLC	Fast protein liquid chromatography
GC	Gas chromatography
HEIA	Homogenous enzyme immunoassay
HEWL	Hen egg white lysozyme
HPLC	High performance liquid chromatography
IgG	Immunoglobulin type G
KLH	Keyhole Limpet Hemocyanin
m.p.	Melting point
MALDI	Matrix assisted laser desorption and ionisation spectroscopy
MW	Molecular weight (molar mass)
MWCO	Molecular weight cut-off
NHS	*N*-hydroxysuccinimide
NMR	Nuclear magnetic resonance
PdG	Pregnanediol glucuronide
RIA	Radioimmunoassay
SDS	Sodium dodecyl sulphate
ΔT	Change in transmission
TG	Testosterone-17β-glucuronide
THEG	Tetrahydrocortisone-3α-glucuronide
THY	Thyroglobulin
TLC	Thin layer chromatography
TNBS	2,4,6-trinitrobenzene 1-sulphonic acid
tris	Tris(hydroxymethyl)aminomethane
WHO	World Health Organisation

CHAPTER ONE

Measurement of steroid hormones and the development of a multi-purpose home or on-site assay system based on the Ovarian Monitor

1.1 Background to the study

There is a growing realisation that non-invasive home, or point-of-care (for example at the patients medical centre), devices capable of delivering laboratory-accurate data for biomarkers of health and disease to lay individuals and health professionals will revolutionise medical and health practices. A biomarker can be defined as "any substance or process that could be monitored in tissues or body fluids that predicts or influences health or assesses the incidence or biological behaviour of disease" [1]. The best known biomarker is probably blood glucose for which there are already many semi-quantitative devices available used by diabetics to determine their blood glucose levels as a measure of control and immediate insulin requirements. Numerous other important biomarkers of health and disease states are also known and many of these are small molecule intermediates in biosynthetic pathways essential for maintaining homeostasis. During metabolism detoxification of such compounds occurs in the liver, often by hydroxylation reactions involving cytochrome P450 enzymes and subsequent conjugation to glucuronic acid, and to a lesser extent sulphuric acid, in preparation for excretion via the renal system. This detoxification pathway also applies to many xenobiotic compounds such as ingested drugs. Thus, many of the important biomarkers are products of metabolism and are excreted in urine as glucuronide or sulphate conjugates. In principle the relatively high levels of urinary glucuronide metabolites constitute a battery of biomarkers that can be incorporated into non-invasive home or point-of-care urine tests if suitable methodology can be developed.

All glucuronides have the same sugar structure that contains a reactive carboxyl functional group (shown in Figure 1.1) which can be conjugated with various reagents or proteins in

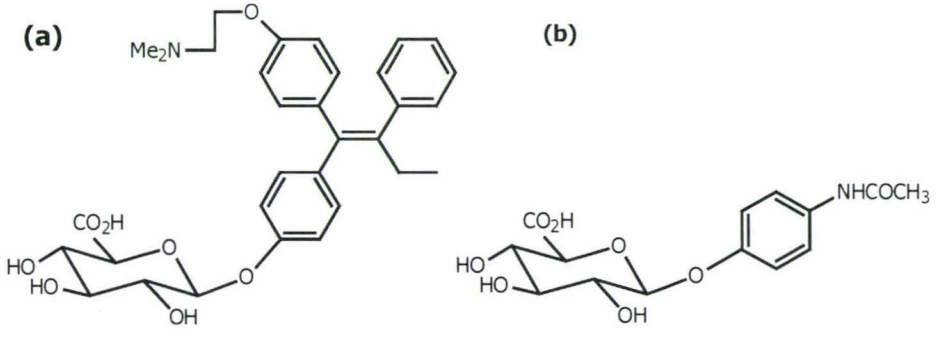


Figure 1.1 Examples of glucuronide metabolites: (a) tamoxifen-4-glucuronide [2], a major urinary metabolite of the potent antiestrogen tamoxifen which is routinely used in the treatment of breast cancer and; (b) paracetamol glucuronide [3].

chemical reactions to produce biomaterials for assays. Clearly, reactions developed for one glucuronide will be applicable to all glucuronides albeit with some modifications, thus success with one system opens the way for a wide range of tests. This thesis is concerned therefore with developing a multi-purpose assay system based on biomarkers containing the generic glucuronide structure.

1.2 Steroids as biomarkers of health and disease

Apart from the monitoring of blood glucose to monitor insulin requirements by diabetics one of the most widely used class of biomarkers has been the steroids, particularly those that function as indicators of the key events in the human menstrual cycle. As well as their role in determining gender and fertility, there is increasing evidence that steroids have other important roles in health and disease. For example it has long been known that the human response to fear, stress and anxiety is also mediated by steroid hormones, and evidence of the potentially deleterious effects of chronic stress on our long term health is increasing. Individual susceptibility to disease may also be monitored using steroid biomarkers since many diseases may result in an overproduction, or underproduction of steroid hormones.

Much interest also exists in developing new approaches to the identification of patients at high risk of disease recurrence particularly by using point-of-care or home testing. For instance, it has been established that androgens play a role in the pathogenesis of breast cancer [4,5]. In a large study of women with operable breast cancer (n = 113), urinary total testosterone levels in 24 hour samples were measured before surgery using gas chromatography to determine the "normal" excretion rate [6]. Further 24 hour samples were taken 40 days after surgery and thereafter once every six months at a specific time during the menstrual cycle for five years. In these women recurrence of breast cancer was associated with supra-normal urinary testosterone secretion. Thus, testosterone is a likely target as an important biomarker and monitoring of testosterone levels in women postmastecomy is especially important in the first five years following surgery since this has been identified as an important time frame during which relapse may occur [6].

A second interesting possibility is the major glucocorticoid hormone cortisol. The concentration of cortisol in the blood plasma is widely used as an indicator of stress [7,8,9], and cortisol metabolites in the urine have been used as a measure of stress in the workplace. In a recent example the level of hypothalamic-pituitary axis (HPA) activation in doctors working the night shift at a hospital who typically experienced stress, fatigue and sleep deprivation was measured [10]. When off-duty, the excretion of the major cortisol urinary metabolites (tetrahydrocortisone + allotetrahydrocortisone + tetrahydrocortisol + allotetrahydrocortisol) in physicians was approximately the same as the non-physician controls. However, while on-duty

the sum of the major cortisol metabolites excreted in the urine increased by a factor of two compared to the off-duty excretion levels as shown in table 1.1 [10].

Table 1.1 Excretion rates (mg 24 hr^{-1}) of cortisol metabolites in on- and off-duty male physicians and in healthy men (controls)

	Physicians (n=8)			Controls (n= 16)	
Glucocorticoid	**Off-Duty**	**On-Duty**	***P* Value***		***P* Value‡**
	Mean ± SD			**Mean ± SD**	
Tetrahydrocortisone	1.8 ± 0.7	4.0 ± 0.9	0.0005	1.4 ± 0.6	0.05
Tetrahydrocortisol	0.9 ± 0.3	2.0 ± 0.5	0.0005	0.8 ± 0.3	0.30
Allotetrahydrocortisol	0.4 ± 0.3	0.7 ± 0.4	0.001	0.5 ± 0.3	0.15
Allotetrahydrocortisone	0.1 ± 0.6	0.1 ± 0.7	0.002	0.2 ± 0.3	0.30
Main glucocorticoid metabolites§	3.1 ± 1.2	6.8 ± 1.6	0.0005	2.9 ± 1.1	0.30
Ratio of cortisone/cortisol metabolites	1.5 ± 0.2	1.6 ± 0.2	0.10	1.3 ± 0.4	0.05

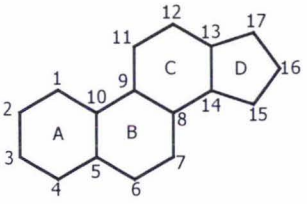
*Comparing on- with off-duty physicians
‡Comparing off-duty physicians with controls
§ Tetrahydrocortisone + allotetrahydrocortisone + tetrahydrocortisol + allotetrahydrocortisol

Cortisol metabolites may also be excreted and quantified in the feces of animals [8]. Recently this has been used as a more practical non-invasive alternative to urine testing as an indicator of stress in animal welfare studies and wildlife breeding programs [11,12].

1.3 General pathway for the biosynthesis of steroid hormones

For the development of non-invasive techniques to monitor steroid production, basic knowledge of the biosynthetic and metabolic pathways by which they are formed and excreted into biological fluids is necessary to aid in the choice of specific biomarkers. For example, many steroids are released from biosynthetic tissues in a pulsatile fashion, and the plasma concentration may increase by as much as 50 to 100% depending on the time of sampling. Steroids circulating in the plasma may be either quickly cleared from the circulation and appear in the urine in the form of glucuronide and sulphate conjugates or metabolised into more potent forms by the target tissue. For example, cortisone may be metabolised to cortisol in the liver; testosterone may be metabolised to dihydrotestosterone in the prostate, or metabolised further to estrogens in reproductive tissues.

The naturally occurring steroid hormones are all based on a fused tetracyclic hydrocarbon ring structure. Substituents, (e.g. carbonyl, hydroxy, methyl) located at specific positions within the steroid skeleton allow the steroids to be classified into three main chemical categories based on androstane, pregnane or oestrane ring structures (Figure 1.2). However the steroid hormones are more commonly classified by their biological activities into androgens, oestrogens, progestogens, corticosteroids and mineralocorticoids [13].



Tetracyclic steroid ring structure

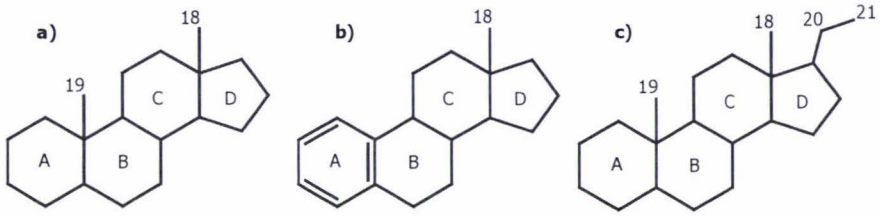


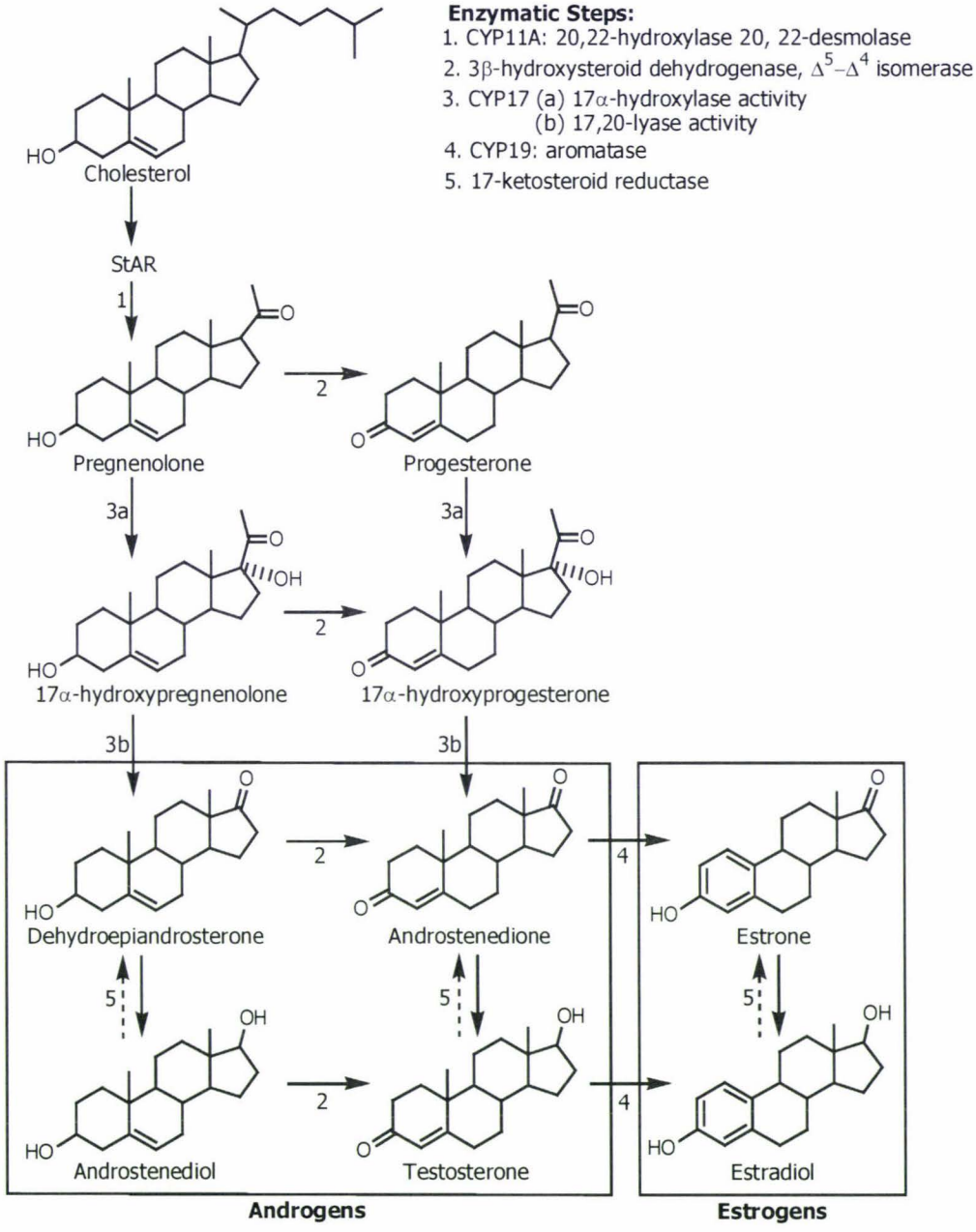
Figure 1.2 Structures of (a) androstane, (b) oestrane and (c) pregnane ring systems.

The major sites of steroidogenesis are the gonads (estrogens and androgens) and adrenal gland (corticosteroids, mineralocorticoids and androgens), although other organs such as the brain, liver and adipose tissue also play a significant role [14]. Recent experiments also suggest the nervous system produces steroid hormones [15]. The overall pathway for the synthesis of all steroid hormones is similar and tissue specific, cell-specific and even subcellular compartment specific differences in the expression of particular steroidogenic enzymes regulate the types and relative amounts of particular steroid hormones synthesised. All steroids are derived from pregnenolone which is itself synthesised from the precursor steroid cholesterol, as shown in Scheme 1.1. Cholesterol may be synthesised from acetate or derived from pools of cholesterol esters in steroidogenic tissues, but the preferred route for around 80% of the cholesterol used for steroid hormone production is via absorption of low density lipoprotein (LDL) cholesterol from the diet [15].

The first step in steroid biosynthesis involves the transfer of cholesterol to the inner mitochondrial membrane where it undergoes side chain cleavage of the $C_{20\text{-}22}$ carbon bond by the cytochrome (CYP) P450 enzyme CYP11A (cholesterol desmolase) to form the 21 carbon steroid pregnenolone [16]. Delivery of cholesterol to the inner mitchondrial membrane is mediated by steroidogenic acute regulatory (StAR) protein, and forms the rate-limiting step of the reaction.

The second subsequent side-chain cleavage of pregnenolone to form testosterone can occur by two distinct pathways. In the Δ^5 pathway, CYP17 catalysed hydoxylation of C_{17} and cleavage of the C_{17}-C_{20} bond occurs before reduction of the C_{17} ketone group by 17β-hydroxysteroid dehydrogenase (17β-HSD) and oxidation of the A-ring by 3β-hydroxysteroid dehydrogenase Δ^5-Δ^4 isomerase (3β-HSD Δ^5-Δ^4 isomerase). In the Δ^4 pathway this sequence is reversed; A-ring oxidation occurs before side chain cleavage and reduction of the C_{17} ketone group. The point in the pathway at which A-ring oxidation occurs depends upon the relative amounts of the

enzymes for the various substrates present and their compartmentalisation within the cell [17]. The predominant pathway in the testes and ovarian follicles, appears to be the Δ^5 pathway (pregnenolone, dehydroepiandrosterone, androstenediol) whereas as the Δ^4 pathway (pregnenolone, progesterone, androstenedione) is dominant in the corpus luteum [18].



Scheme 1.1 Biosynthesis of androgens and estrogens from cholesterol [19]

Androstenedione and testosterone can be converted to their respective estrogens, estrone and estradiol by the cytochrome P450 aromatase enzymes in testes, ovary and extraglandular tissues of both sexes [17]. These conversions are irreversible and require the reduction of the C_3 ketone functional group, removal of the C_{19} methyl group and aromatisation of the A-ring [20]. Although there is more androstenedione produced by the ovary than testosterone the

equilibrium of the 17β-hydroxysteroid dehydrogenase catalysed reaction ensures that most of the resulting ovarian estrogen is converted to estradiol [21].

1.4 Steroids as markers of the fertile window in the human menstrual cycle

The central problem of fertility detection in humans, who lack an estrus symptom, is to identify the fertile window in a menstrual cycle. This is a variable period of days surrounding ovulation when an act of coitus may result in a pregnancy. Since the position of the day of ovulation is usually quite variable even within cycles from the same woman the fertile window will vary from cycle to cycle and from woman to woman. The length of the fertile window will also vary from cycle to cycle and from woman to woman. It is generally agreed that the ovum only survives for 8 - 12 hours after ovulation [22] but sperm survival in the female genital tract extends the fertile window by an amount determined by the sperm survival time. This is generally believed to average about 3 days [22] however pregnancies have been documented that seem to require a rare survival time of up to 6 or 7 days. Hence the maximum length of the fertile window can be 7 - 8 days (Brown *et al* personal communication). What is needed to detect and monitor the fertile window is a biochemical marker for the beginning and end of this time.

To achieve this goal it is first necessary to review the basic physiology of the human menstrual cycle. The menstrual cycle is controlled by a coordinated flow of chemical information between the ovary and the pituitary gland as shown in Figure 1.3.

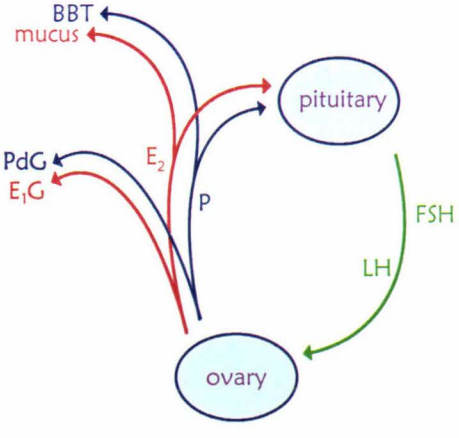


Figure 1.3 Schematic diagram of pituitary-ovary communication. Abbreviations: BBT, basal body temperature; P, progesterone; PdG, pregnanediol glucuronide; E2, estradiol; E1G, estrone glucuronide; LH, luteinising hormone; FSH, follicle stimulating hormone.

The major chemicals which carry the information are the pituitary hormones, follicle stimulating hormone (FSH) and luteinising hormone (LH) which carry information to the ovary and the ovarian hormones, estradiol (E2) and progesterone (P) which signal ovarian responses to the pituitary. Progesterone secreted from the ovary is rapidly cleared from the circulation by conversion to various metabolites. The major conjugated metabolite of progesterone excreted

in the urine is pregnanediol glucuronide (PdG) (about 20%) [23] which may be used as an index of ovarian progesterone production [24]. Once estradiol leaves the ovarian environment, it is oxidised by hydroxysteroid dehydrogenase enzymes in the liver to the less bioactive estrone. Estrone may either then re-enter the circulation or be rapidly metabolised further to estriol or conjugated in the liver to estrone glucuronide (E1G), which is excreted in large amounts in the urine. As well as being present in high quantities, estrone glucuronide is excreted rapidly and levels of E1G appear to be directly correlated to circulating levels of estradiol. This orchestrated chemical communication flow provides a means for "eavesdropping" non-invasively on the ovary to ascertain the state of follicular and corpus luteum development (and hence the state of fertility) at any time throughout the menstrual cycle.

Although the best biochemical markers for the beginning and end of the fertile window are serum estradiol and progesterone their analysis requires several blood samples to be taken during the menstrual cycle which is generally considered an uncomfortable and invasive procedure. As noted above the urinary metabolites estrone glucuronide (E1G) and pregnanediol glucuronide (PdG) (shown in Figure 1.4) are widely accepted as alternative biomarkers of ovarian estradiol and progesterone production respectively. The measurement of urinary metabolites over serum analyses has the advantage that frequent specimen collection is easier and non-invasive with less stress to the subject [25]. In addition the urinary excretion rates are more closely related to ovarian secretion rates and integrate the pulsatile secretion of these hormones [25].

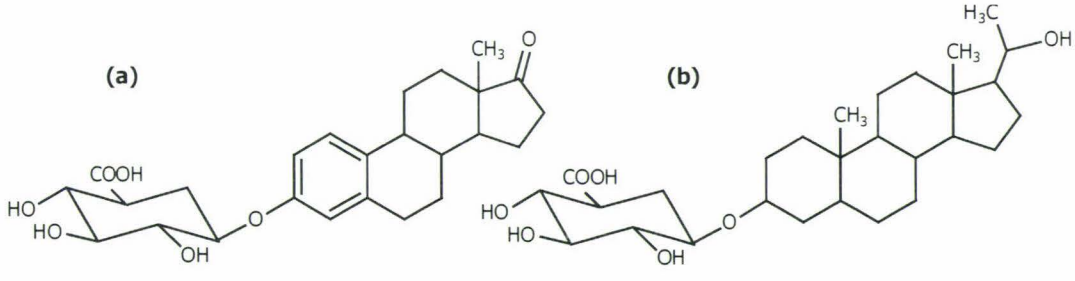


Figure 1.4 Structure of **(a)** estrone glucuronide (E1G) and **(b)** pregnanediol glucuronide (PdG).

All of the available or foreseeable methods of detecting and predicting fertility depend on access to a procedure that detects one or more of the signals shown in Figure 1.3. It should be noted that no matter which method is being used the meaning of the underlying information remains the same.

1.5 Analysis of steroids and their glucuronides

Although some hormones may be measured directly in biological fluids by a readily available assay, some information can be obtained only by measuring the ratio of biosynthetic precursors or hormone metabolites. Information of this type is necessary for the diagnosis of endocrinological diseases which result from disruption of the normal pathways of steroid biosynthesis (e.g. adrenal hyperplasia [26]) or metabolism (e.g. polycystic ovaries [27] and apparent mineralocorticoid excess [28]). Blocks in biosynthesis or metabolism brought about by enzyme deficiencies or enzyme mutations can be readily identified by increased levels of steroids intermediate in the biosynthetic pathway which would normally be present at low levels. Many assays used currently for the identification and quantification of steroid hormones are based on either binding assays or chromatographic methods. Typically immunoassays are used for the analysis of single steroids, measured routinely in a clinical setting and allow rapid, high-throughput, simple analyses. Assays for multiple or unusual steroids normally rely on chromatography methods. While affording high specificity and comparable sensitivity these techniques are often more time consuming, labour intensive and often require specialised technicians to operate.

1.5.1 Chromatography techniques

Chromatography based methods used for the analysis of urinary steroids can be divided into several categories, the most common being paper, thin layer (TLC), high performance liquid (HPLC) and gas chromatography (GC). HPLC and GC are the most common methods applied in routine assays, whereas paper and thin layer chromatography, although popular in the past are now only used in research and organic synthesis laboratories. The preferred detection system for steroid profiling by GC is a mass spectrometer, which allows highly sensitive detection with excellent specificity. Recent reports also suggest that super-critical fluid (SCF) chromatography may have potential for the separation of steroids [29]. This method is based in the fact that some compounds, for example carbon dioxide, have a "critical" temperature and pressure above which their gaseous and liquid phases are no longer distinguishable. The fluids formed under these conditions have enhanced separating capacity by allowing a greater degree of interaction between stationary and mobile phases. However the instrumentation for SCF analysis is not yet widely available for routine analyses.

While intact steroids and steroid conjugates can be assayed directly by GC using high temperature programmes, samples often require lengthy pre-treatment prior to analysis. For example, some steroids particularly corticosteroids, are thermolabile and must be chemically derivatised to prevent losses prior to analysis by GC. Steroid conjugates and columns are also subject to degradation under the high temperatures (350 °C) required to elute the steroid glucuronides [13]. As a result steroid conjugates are often hydrolysed using enzymes such as β-glucuronidase, to yield the free steroid which is then derivatised prior to analysis and the total

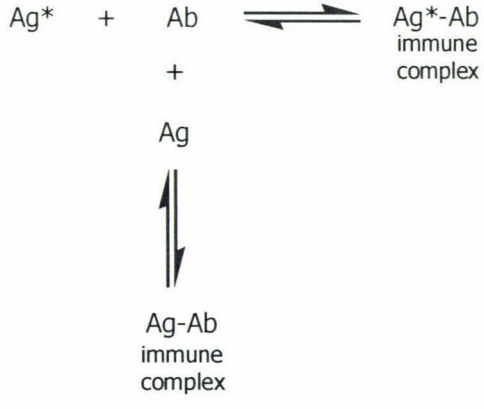
levels of steroid are reported [30]. This was the basis of the total estrogen method of Brown *et al.* which revolutionised our understanding of the human menstrual cycle in the second half of last century [31]. HPLC-MS systems can also be applied to the direct analysis of steroids in urine and plasma. The speed of analysis is often much faster since no derivatisation is required and the technique can be performed at room temperature allowing analysis of thermally unstable molecules, for example intact glucuronide and sulphate conjugates [32,33]. However HPLC-MS systems may not be as sensitive as GC-MS and radioimmunoassay (RIA) systems (discussed in section 1.5.2.1) and some buffered mobile phases may be incompatible with the mass spectrometer. Buffer salts may precipitate out around the interface, resulting in increased noise and necessitate frequent cleaning of the ionisation cone.

1.5.2 Immunoassay techniques

The binding of antibodies to specifically labeled antigens forms the basis of a range of assay techniques known collectively as immunoassays. The antigen may be attached to various labels including radioactive isotopes, as in radioimmunoassay (RIA), fluorescent compounds (in fluorescence polarization assays, FPIA) chemiluminescent molecules (CLIA) or rely on enzymes which form the basis of a battery of tests known as enzyme immunoassays or enzyme-linked immunosorbent assays (ELISA).

1.5.2.1 Radioimmunoassay

A large number of journal reviews and texts have been published on the development of immunoassay techniques [34,35,36]. The basic principle of all immunoassays is that a labeled antigen (i.e. the compound of interest) competes with unlabeled antigen present in the sample (e.g. serum or urine) for a limited number of binding sites on an antibody. Radioimmunoassay (RIA) was the first immunoassay approach developed in the late 1950's [37] and employs radioactive isotopes, usually iodine or tritium, as the antigen label. The principal reactions involved in typical RIA systems are illustrated in Scheme 1.2.



Scheme 1.2 The principle reactions involved in radioimmunoassay (RIA). Abbreviations: Ag*, labeled antigen; Ab, antibody specific for the antigen; Ag, unlabeled antigen in the sample or standard.

Appropriate volumes of antibody (Ab, which is specific for the antigen of interest), radiolabeled antigen (Ag*) and sample containing free antigen (Ag) are incubated together. Because the antibody concentration is limited, the antigen in the test sample must compete with the labeled antigen for the limited number of binding sites provided by the antibody molecules. Hence when equilibrium is reached, the greater the concentration of antigen present in the sample, the less labeled antigen that will be bound by the antibody. The next step in the assay involves separation of the antibody bound labeled antigen from the free labeled antigen. This is necessary since it is not possible to distinguish between free and bound radioactive label by the usual counting techniques. Separation is normally achieved by using procedures which separate the antigen-antibody complex while leaving the unbound antigen, both labeled and unlabeled, in solution. In practice, precipitation of the antigen-antibody complex from solution is most often achieved by adding a second antibody (which has been raised against the first antigen-binding antibody) to the reaction mixture. After separation, the radioactivity in either the free or antibody bound fraction of antigen is measured by radioactive counting. The amount of radioactivity observed is then compared to a standard curve constructed by assaying several samples containing known concentrations of unlabeled antigen.

The major advantage of RIA is the high sensitivity and specificity obtainable with radiolabeled compounds. Since the radioactive label provides a distinct signal that shows little interference from other compounds in a biological fluid, radioimmunoassays have the capacity to detect compounds at levels of picomoles per litre or less. Although these systems are still in use for the detection of low levels of testosterone and cortisol in plasma [38], urine [39] and saliva [40] there are a number of disadvantages associated with the use of radiolabeled antigens. These include the disposal of the radioactive waste material, the limited half life and stability of some labeled antigens and the need for a specially equipped laboratory and expensive counting equipment to measure radioactivity at the assay end point. In addition the need to physically separate the bound and free labeled antigen makes the automation of RIA systems difficult and increases the cost of RIA analysis. However, radioimmunoassays can be established for any analyte providing a suitable specific antibody can be raised against the analyte. Many examples of radioimmunoassays for steroids and in particular estrone glucuronide and pregnanediol glucuronide have been reported [42,43,44].

1.5.2.2 Non-isotopic immunoassay techniques

Since the introduction of RIA a variety of immunoassay systems using non-isotopic labels have been developed for the measurement of steroids and other small analytes in biological fluids. The most successful non-isotopic systems applied to the analysis of steroids include chemiluminescence and fluorescence immunoassay systems which employ either a luminescent or fluorescent tag (e.g. fluorescein or isoluminol) to label and measure the amount of bound and free antigen present in the sample. Both these methods have been applied to the

measurement of steroid and drug metabolites in body fluid [45,46,47]. The main advantages of chemiluminescent and fluorogenic substrates are their low cost, high sensitivity, long shelf-life of the lyophilised conjugates and the absence of radiation hazards [48]. The main disadvantages are associated with high background (signal to noise ratio) interference from endogenous substances in the sample matrix and light scattering, the need to use maximally purified water, and problems with temperature and pH variations which can affect the wavelength and intensity of the emitted light [49].

1.5.2.3 Enzyme immunoassays

The development of immunoassay systems employing enzymes as the non-isotopic signal generating substances has proven to be a particularly popular alternative to RIA. Enzyme immunoassays (EIA's) utilise a) an enzyme labeled with antigen (called an enzyme conjugate), b) the ability of antibodies to discriminate between structurally similar antigens and c) the specificity and catalytic nature of the enzyme towards its substrate which facilitates signal amplification. Direct comparisons have shown that EIA's can be as sensitive or even more so than the corresponding RIA's, without most of the disadvantages associated with radiolabeled antigens [48,50].

Enzyme immunoassays can be divided into two broad groups, heterogeneous immunoassays and homogeneous immunoassays. Heterogeneous enzyme immunoassays (which include the enzyme linked immunosorbent assays or ELISA) are those in which the enzyme labeled with antigen is equally active in the antibody-bound and free state and therefore must be separated before any measurements can be made - as is the case with standard RIA. In homogeneous enzyme immunoassay systems the activity of the enzyme conjugate is extensively inhibited when antibody binds to the antigen. Thus, the amount of antibody bound enzyme label (which depends on the concentration of free antigen present) can be easily measured in solution by a simple kinetic measurement without the need for time consuming separation procedures.

The first homogeneous enzyme immunoassay (HEIA) was performed by Rubenstein and co-workers in 1972 [51] as an alternative to RIA and other heterogeneous immunoassays of the time. Rubenstein demonstrated that polyclonal antibodies specific to morphine could bind to a hen egg white lysozyme (HEWL) – carboxy methylmorphine conjugate and inhibit the enzyme activity by up to 98%. Lysozyme is one of the few enzymes that exhibits such extensive inhibition after binding to an anti-analyte antibody but enzymes such as malate dehydrogenase have been used in commercial EMIT (enzyme-multiplied immunoassay technique) by the SYVA company in California. Other homogeneous assay techniques exist which make use of a change in some property of the system, such as a change in fluorescence polarisation on binding to an antibody, and assays for estrone glucuronide and pregnanediol glucuronide have been described using these techniques [52,53].

1.5.3 Immunoassays for home monitoring – The Ovarian Monitor for estrone glucuronide and pregnanediol glucuronide

It is clear that for home monitoring of any analyte, classical chromatographic assays and radioimmunoassays, and most non-isotopic assays are completely unsuitable. Most reported home assay systems for monitoring fertility have involved either lateral flow membrane assays (or immunochromatography assays) and the Ovarian Monitor which is based on Rubenstein's HEIA technique [51]. For the Ovarian Monitor, a lysozyme-E1G or lysozyme-PdG conjugate must be prepared by combining hen egg white lysozyme (HEWL) with an appropriate activated carboxyl group reagent derived from the steroid glucuronide and isobutyl chloroformate (mixed anhydride method [51,54]) or dicycohexyl carbodiimide (active ester method [55]). The lysozyme conjugate is prepared by coupling of E1G or PdG (*via* the carboxyl groups of the glucuronide moiety) with the amino groups of lysine residues on the surface of the enzyme. The lytic activity of the resulting conjugates in the immunoassay is determined by changes in light transmission of a suspension of the bacterial substrate *Micrococcus lysodeikticus* (*M. lysodeikticus*).

The synthetic steroid glucuronides are also used to prepare immunogens to raise the appropriate antibodies by coupling them (separately) to a protein carrier such as bovine serum albumin or thyroglobulin before injection into sheep or rabbits. A minimum of six months is usually required for the generation of high titre antibodies.

The Ovarian Monitor home fertility system consists of two parts; the assay monitor and a specially designed plastic assay tube (Figure 1.5). The monitor itself consists of a cell holder maintained at 40 °C by a built-in thermostat, an automated timer, a 650 nm light source (a light emitting diode, LED) and a detector. The assay tube is placed inside the cell holder and maintained at 40 °C during the assay. The LED transmits light through the sample to a detector on the opposite side, which then displays the transmission result on the screen as a digital readout. The built-in timer is triggered by a magnetic switch activated by closing the cell holder lid and indicates the completion of each incubation step by a series of beeps.

The plastic assay tube contains the assay components freeze dried at different levels on the tube wall. The antiserum and buffer salts are freeze dried at the bottom of the tube with a small glass bead to facilitate mixing, the lysozyme-hapten conjugate is immobilised near the centre of the tube and the *Micrococcus lysodeikticus* is immobilised near the top. This allows the assay components to be mixed in the appropriate order when sequential additions of urine and water are made. Separate assay tubes are required when assaying for E1G and PdG; E1G tubes contain anti-E1G antibodies and E1G-HEWL conjugate, while the PdG tubes contain anti-PdG antibodies and PdG-HEWL conjugate.

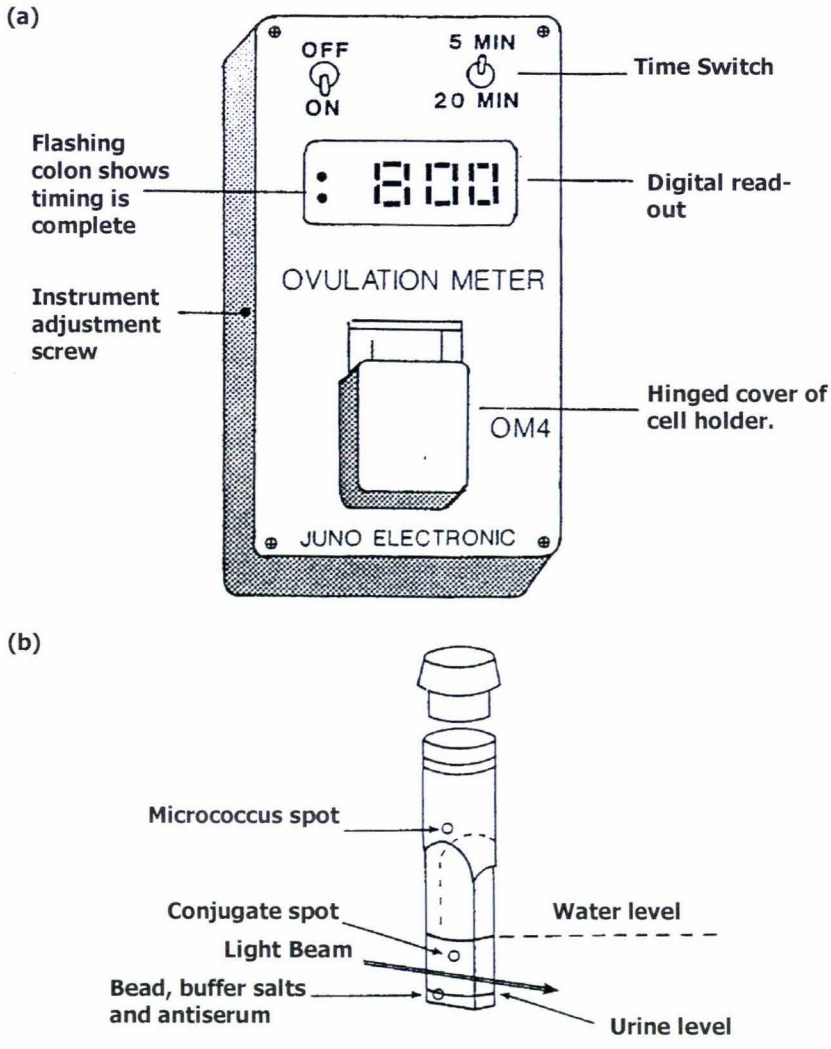


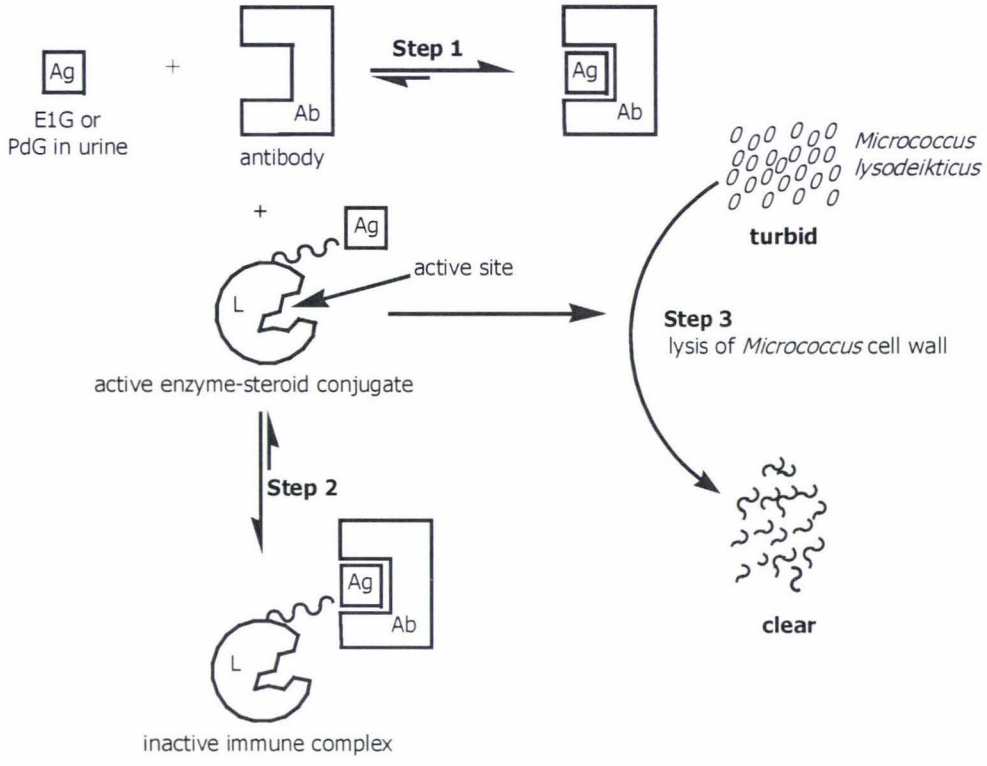
Figure 1.5 The Ovarian Monitor **(a)** and an Ovarian Monitor assay tube **(b)**, showing the position of the assay components freeze-dried to the tube.

The Ovarian Monitor assay is based on the classic enzyme-multiplied immunoassay technique (EMIT) and takes place in three discrete steps as shown in Scheme 1.3.

Step one: The antigen antibody reaction

In the first step, 50 μL of time diluted urine is carefully added (via syringe) to the bottom of the assay tube. The tube is then placed inside the cell holder and allowed to incubate for five minutes to allow time for the buffer salts and antibody (Ab) freeze-dried on the bottom of the tube to completely dissolve. The Monitor signals the end of the incubation period by a series of beeps, after which the tube is removed and gently shaken horizontally for a count of twenty seconds to allow the steroid antigen (Ag, i.e urinary estrone glucuronide or pregnanediol glucuronide) to mix freely with the corresponding anti-steroid antibody (Ab).

Binding of the steroid to the antibody is rapid and effectively irreversible within the time frame of the assay [56]. Thus the level of antibody neutralisation, and hence the amount of free antibodies remaining at the end of the first step is directly proportional to the level of steroid in the urine.



Scheme 1.3 Principle reactions in the Ovarian Monitor homogeneous enzyme immunoassay. Abbreviations used: Ag, antigen i.e urinary estrone glucuronide (E1G) or pregnanediol glucuronide (PdG); L, hen egg white lysozyme; Ab, anti-E1G or anti-PdG antibody.

Step 2: The antibody steroid glucuronide-lysozyme conjugate reaction

In the second step, the lysozyme conjugate spot is dissolved by careful addition of 300 μL of distilled water by syringe to the assay tube. The tube is then gently shaken horizontally for another count of twenty seconds and returned to the Ovarian Monitor cell holder to incubate for a further period (PdG, 5 min; E1G, 20 min).

During the incubation step, the excess antibody which was not bound by urinary steroid in the first step is allowed to bind (again effectively irreversibly on the time scale of the assay) to the steroid-lysozyme conjugate. Binding of the steroid-lysozyme conjugate to its corresponding antibody inactivates the enzyme due to the antibody sterically blocking the access of the bacterial substrate to the active site and also partly by steric restriction of access to the transition state [57]. In this step the level of antibody binding is directly proportional to the amount of free antibody left at the end of the first step. Thus, the level of free enzyme conjugate remaining at the end of step two is proportional to the level of steroid glucuronide in the original sample of urine.

Step 3: The Micrococcus lysodeikticus reaction

In the third and final step, the free conjugate remaining after the end of step two is given access to its natural substrate, the gram positive bacterium *Micrococcus lysodeikticus*. At the end of the incubation period in step 2 (signaled by a second series of beeps) the tube is removed from the cell holder and vigorously shaken for a count of twenty seconds to uniformly suspend the freeze dried *M. lysodeikticus* cells and returned to the cell holder. Closing the cell holder signals the start of the enzyme assay and triggers the Monitor to record the initial transmission value, $T_{0\ min}$. In solution the suspended *M. lysodeikticus* cells are large enough to scatter light and the solution appears opaque. However as the conjugate proceeds to lyse the *Micrococcus* cells by breaking the glycosidic bonds between sugar units in the polysaccharide component of the cell wall, the initially turbid solution begins to clear as the strength of the cell wall decreases and the cell collapses.

At the end of the assay period (PdG, 5 mins; E1G, 20 mins), the timer sounds and the difference between the final and initial transmission values (E1G, $\Delta T\ 5\ min^{-1}$; PdG, $\Delta T\ 20\ min^{-1}$) is left permanently flashing on the LED screen. This change in transmission is a measure of the rate of lysis over the predefined assay period. Because only the free enzyme conjugate retains its lysis activity, the change in transmission (or the rate of clearing) is directly proportional to the amount of unbound conjugate remaining at the end of step two, which is in turn determined by the amount of steroid in the initial urine sample.

Thus when the level of steroid glucuronide in the urine is low, most of the antibody is available for binding and inactivating the enzyme conjugate, resulting in a low rate of clearing of the turbid *Micrococcus lysodeikticus* suspension. However as the concentration of steroid in the urine increases and becomes antibody bound, the proportion of antibody available for inhibiting the conjugate activity decreases resulting in increased rates of lysis activity and higher rates of clearing. Thus, low levels of urinary steroid are associated with only small changes in light transmission (ΔT), whereas a high level of steroid generates a large change in transmission. Using a set of known concentrations a standard curve can be generated which relates the steroid glucuronide concentration to the observed change in transmission. A typical example of an optimised estrone glucuronide (E1G) curve relating the 24 hr molar excretion rates to the change in transmission over a twenty minute assay period ($\Delta T\ 20\ min^{-1}$) is illustrated in Figure 1.6. The curve shows the direct relationship between the lysis rate and the urinary steroid levels. The working range, i.e. the range of E1G excretion rates over which the curve is steepest and thus best able to discriminate between small changes in concentration is 25 – 410 nmol E1G $24\ hr^{-1}$ (as indicated by the arrows). This range includes the physiological range of E1G concentrations expected in the urine of normally cycling women. The midpoint or point of

inflection (EC_{50}) of the curve corresponds to the steroid concentration in the middle of the physiological range at ~140 nmol E1G 24 hr^{-1}.

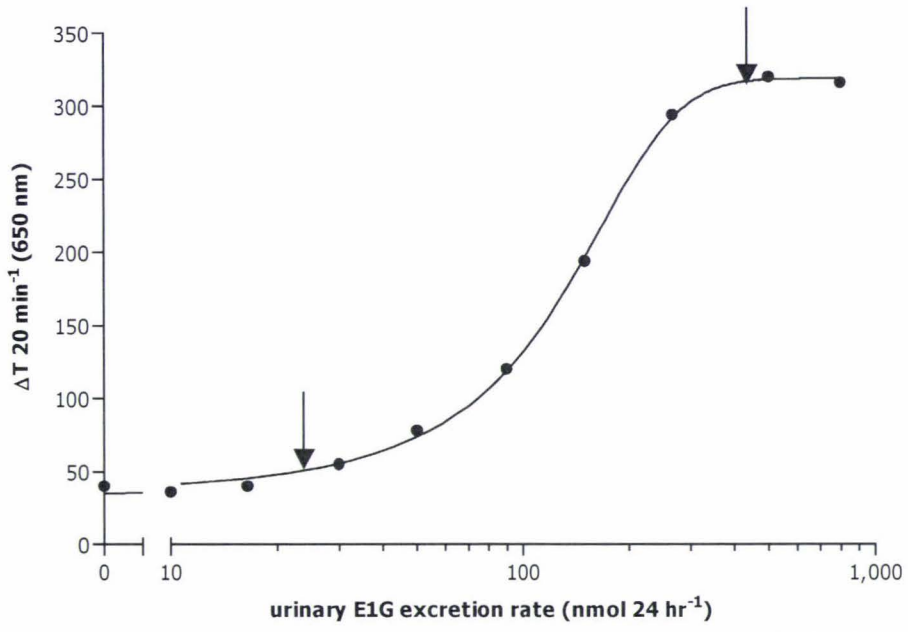


Figure 1.6 A standard curve for estrone glucuronide (E1G) generated using the Ovarian Monitor. The steepest section and thus most sensitive region of the curve corresponds to the range of E1G concentrations expected in the female urine during a normal menstrual cycle.

The standard curve generated with the pre-dried assay tubes for any batch of antiserum and lysozyme steroid conjugate is extremely reproducible and eliminates the need to establish a new standard curve for each new analysis (unlike radioimmunoassay where a new standard curve must be generated for each new batch of samples analysed). Since the standard curve is programmed into the Monitor, the tubes are colour coded to the match the standard curve to which they belong. Using such a curve, the Ovarian Monitor (Mark IV) can convert the change in transmission value to a 24 hour molar excretion rate. Conversion to the twenty-four hour molar excretion rate is not absolutely necessary for home use (unless quantitative data is required) as it is the pattern of hormone excretion that is important for the determination of fertility status. For example each batch of PdG tubes is labeled with the ΔT 5 min^{-1} value equivalent to a threshold value of 6.3 μmol PdG 24 hr^{-1}. Thus the woman need only check if she has exceeded this change in transmission to know she has reached the late infertile days of the menstrual cycle.

To monitor the changes in fertility status over the menstrual cycle the women can also plot their data as a change in transmission ΔT vs. cycle day (Figure 1.7). Hence a simple rate measurement gives a direct measure of the concentration of steroid glucuronide in the urine which allows couples either trying for, or trying to avoid pregnancy to determine what stage in the fertility cycle the women is. For example, Figure 1.7 shows that the beginning of fertility is

most likely on day 4 (6 days prior to the peak E1G day) as judged by the first rise in E1G above the preceding baseline [58]. The most fertile day is on day 11 (following the mid-cycle E1G peak day) and the end of her fertility for this cycle is given by the PdG rise on day 14 when the PdG threshold value is exceeded [25].

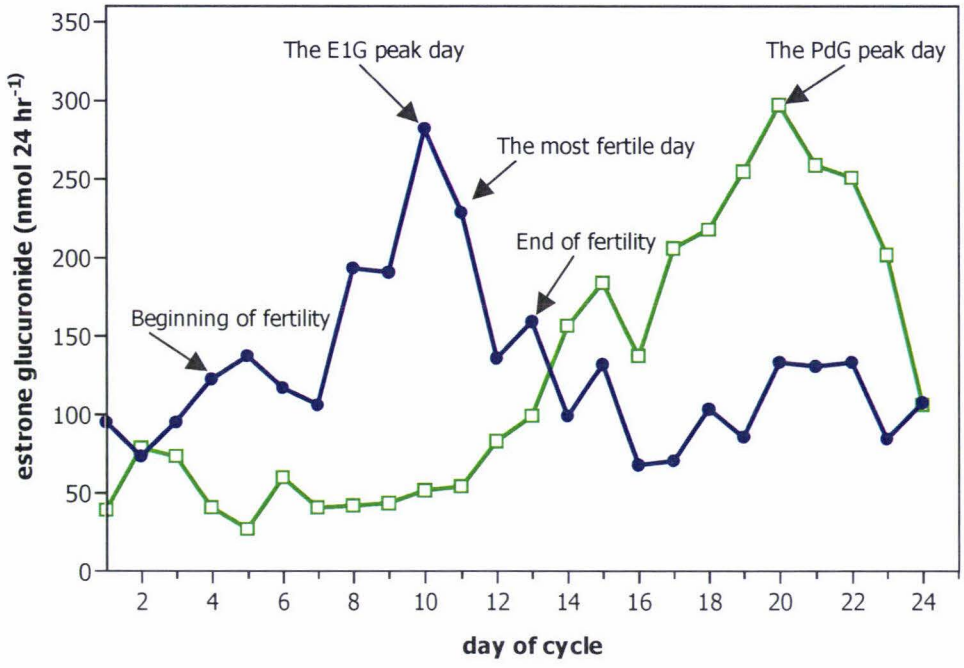


Figure 1.7 Normal menstrual cycle obtained by a women using the Ovarian Monitor.

Timed-diluted specimens of urine are essential for the assay to correct for fluctuations in urine volume which degrade the information and may render it uninterpretable in extreme cases. The urine may be collected overnight or during the day, provided that the period of collection is at least three hours [56]. The urine is diluted with tap water in a specifically graduated jug, such that the final dilution corresponds to the voiding of 150 mL hr^{-1}. This value is chose to represent the mean urine volume excreted of 3600 mL excreted in a 24 hour period. A portion of the diluted urine is then placed in a plastic tube and stored frozen if the test is not to be done immediately.

The validity of the Ovarian Monitor home test system has been verified by numerous trials, including a World Health Organisation trial (WHO) [59], and has been accredited by the Australian National Association of Technical Authorities (NATA) for routine monitoring of the gonadotrophin treatment of infertile women [60] at the Royal Woman's Hospital, and the Mercy Hospital in Melbourne. The E1G assay has also been used since 1987 for monitoring estrogen responses during hyperstimulation on a large IVF program.

1.6 A multi-purpose self monitor for other urinary hormone metabolites

It is obvious that the work carried out with the Ovarian Monitor should be applicable to a whole range of alternative steroid glucuronides providing that the required conjugates and antibodies can be synthesised. A range of assay tubes could then be developed using exactly the same techniques for other analytes of clinical or commercial interest. If the Monitor system were adapted so that it could recognise any assay tube introduced into it by appropriate coding then the same Monitor could measure a range of analytes in a home environment or in a point-of-care setting. For testing the multi-Monitor proposal, the major difficulty is to select an analyte for which there is likely to be a sufficient interest and demand for the test.

1.6.1 Testosterone as a biomarker

Male hypogonadism may be defined as a failure of the testes to produce testosterone, spermatozoa or both. The most common causes of hypogonadism are associated with failure of the anterior pituitary gland, deficiencies in enzymes responsible for testosterone biosynthesis, and chromosomal abnormalities such as Kleinfelter's syndrome (47,XXY karyotype) which affects ~1 in 400 males [61]. Chemotherapy or radiation therapy may also result in long term testicular damage and reduced production of testosterone. Other conditions associated with a low testosterone in men include acute stress, alcoholism, obesity and chronic or acute illness including HIV, leukemia and cirrhosis of the liver [47,62]. Plasma levels of free testosterone also show a steady decline with age, by approximately 1.2% per year starting at around age 40 [63]. Age related testosterone decline may result in the loss of the diurnal rhythm of plasma testosterone secretion and cause a range of physical symptoms including a progressive decrease in muscle mass, loss of libido, erectile dysfunction, reduced sperm count and osteoporosis [61]. In adult females the opposite occurs; an excess of testosterone production is indicative of a large number of abnormalities including polycystic ovaries, (as a result of defects in the enzymes that metabolise testosterone and cortisol) and ovarian tumours [64,65]. Increased levels of testosterone in women are also associated with hirsutism, and adrenal hyperplasia [47].

Over 4 million men in the USA diagnosed with a testosterone deficiency undergo replacement therapy, mostly by self-injection of testosterone every one to three weeks [66]. One of the major problems with this method of self-administration is that it is difficult to mimic the bodies production and management of testosterone levels. Injection introduces a high dose of testosterone, maintained for only the first few days after injection, returning to basal levels at the end of the treatment period. Testosterone administered by mouth is absorbed into the portal blood stream and promptly degraded by the liver so that only a small portion reaches the systemic circulation. More effective alternatives to androgen therapy involve administration of testosterone in a slowly absorbed form (dermal patches or a micronised oral preparation) or the administration of chemically modified analogs [67]. Recently a gel based formula containing

testosterone known as Androgel has become available in the USA [68]. To be administered once or twice a day, Androgel is thought to produce a more even plateau of testosterone concentration, thus avoiding the fluctuation in plasma levels resulting from injection.

The safe use of testosterone replacement therapy relies on the monitoring of testosterone levels in biological fluid to assess the response to treatment, requiring multiple visits to the hospital or specialist. Such frequent visits may involve major disruption to family and working life, and may cost a considerable amount in travel and time away from work. In a clinical setting, testosterone is most frequently measured in the plasma. However since it is released from the testes in episodic spikes during the day, and is 25% higher in the morning than at night, at least 3 blood samples, taken at 20- to 40-minute intervals during the morning are required for measurement [69].

Home monitoring of testosterone production, performed by the patient, has the potential to overcome may of these problems by limiting the number of trips to a specialist clinic but still allow their dose regimes to be monitored safely.

1.6.1.1 Testosterone biosynthesis

In men, over 95 % of the testosterone is produced by the Leydig or interstitial cells of the testes and is mainly controlled in adulthood by lutenising hormone produced by the pituitary gland [47]. In normal females, approximately half the testosterone produced is made by the ovaries; the remainder originates from the adrenal glands and conversion from estrogen in the periphery [70]. The pathway of testosterone biosynthesis from cholesterol has been illustrated already in Scheme 1.1.

In normal males an average of 5 - 6 mg of testosterone is secreted into the plasma per day [71]. For females this level is much lower at around 1.5 mg day [72]. In the blood, only 1 - 2% of the total testosterone circulates in the free state (unbound), the rest is bound to plasma binding proteins. Approximately 45% is bound with high affinity to a β-globulin called sex hormone-binding globulin (SHBG), 50% is loosely bound to albumin, and other 1 - 2% is bound to an α_2-globulin called corticosteroid binding globulin (CBG) [73]. SHBG has about a 1000 fold higher affinity for testosterone than albumin, but the concentration of albumin is so much higher that the binding capacities of both proteins is considered to be similar [17]. For many years, only the free testosterone fraction was regarded as the biologically active portion available for entry into cells and binding to androgen receptors. It is now widely accepted that protein-bound testosterone can dissociate in the capillary bed and nearly all the albumin bound testosterone is available for tissue uptake *in vivo* so that the bioavailable testosterone in men is about half the total (equal to the free plus albumin bound fraction) [74,75].

In adult males testosterone production shows diurnal variation, with the highest levels in the early morning, followed by a progressive fall throughout the day, reaching the lowest levels in the evening and during the first few hours of sleep (Figure 1.8). Peak and lowest values may differ by approximately 15%, although more pronounced differences are sometimes observed [76].

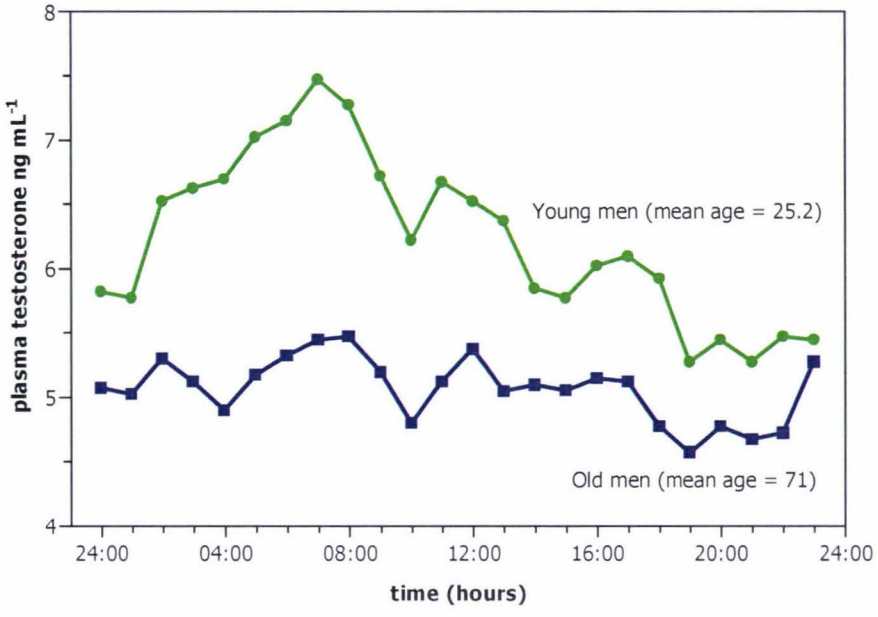


Figure 1.8 Diurnal pattern of testosterone levels in plasma [77].

1.6.1.2 Biological activity of testosterone

Testosterone that is not bound to plasma proteins diffuses out of capillaries and into target as well as non-target cells. Despite being the major steroid hormone produced by the testes, testosterone has few direct actions; instead, it serves as a circulating precursor (or pro-hormone) for formation of the more potent 5α-reduced metabolite dihydrotestosterone (DHT). In the target cells testosterone and DHT bind to the same high-affinity androgen receptor, and the hormone-receptor complexes attach to DNA response elements to initiate biologic responses (shown schematically in Figure 1.9). These include the regulation of gonadotropin secretion by the hypothalamic-pituitary system, initiation and maintenance of spermatogenesis, and control of the male sex-drive [17].

Alternatively, a variety of tissues including the testes, brain, breast and adipose tissue can metabolise testosterone and its immediate precursor androstenedione into estrogens. Estrogen formation is catalysed by the cytochrome P450 enzyme CYP19 (aromatase), which converts testosterone into estradiol and androstenedione into estrone (see Scheme 1.1). These metabolites, particularly estradiol, are biologically active and may act locally by binding to estrogen receptors in the tissues in which they are formed or re-enter the plasma.

The role of estrogens in male physiology is complex and not completely understood, but is thought to include acceleration of the pubertal growth spurt, accrual and maintenance of bone density, a role in male sexual drive, and an influence on gonadotropin secretion [17]. In addition, estrogens may enhance androgen activity by increasing the number of androgen receptors in the prostate. In turn androgens appear to act as weak antiestrogens by preventing the binding of estrogen to the estrogen receptor [17].

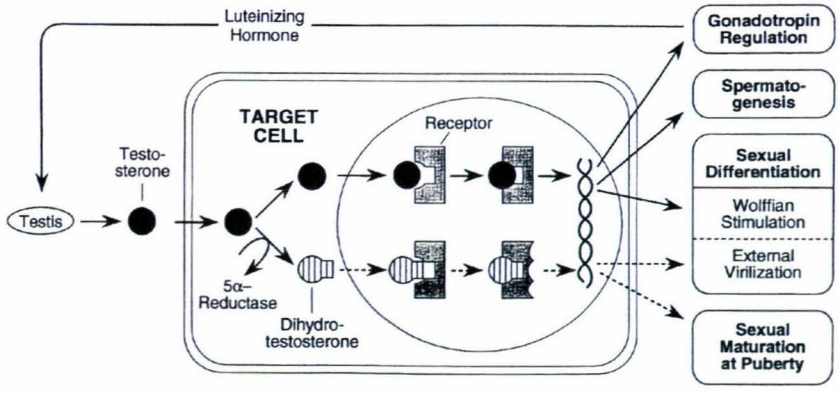


Figure 1.9 Schematic diagram of androgen action. Testosterone, secreted by the testis, binds to the androgen receptor in a target cell either directly or after conversion to dihydrotestosterone (DHT). DHT binds more tightly than testosterone. The major actions of androgens (shown on the right of the diagram) are either mediated by testosterone (solid lines) or by DHT (broken lines) [17].

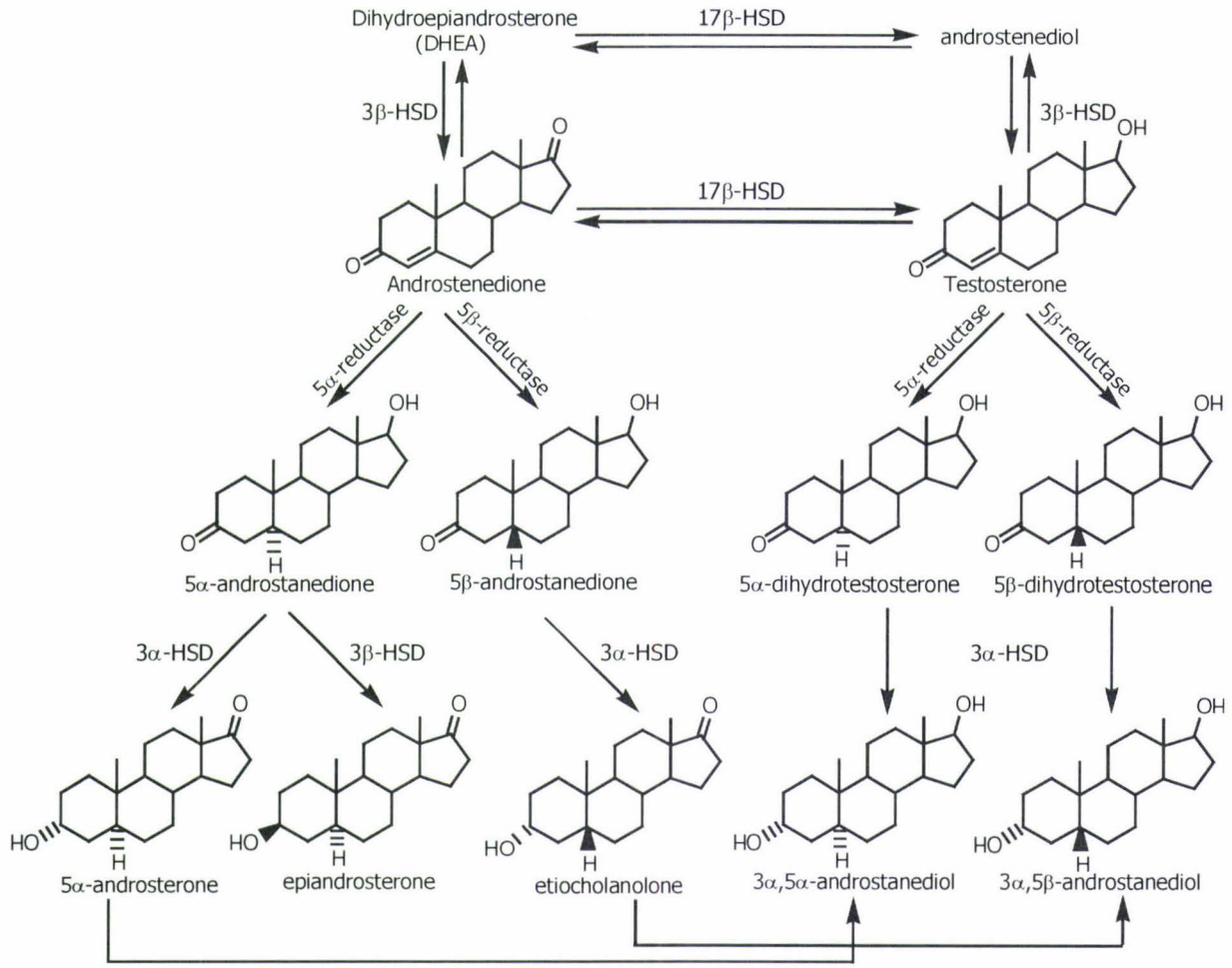
1.6.1.3 Hepatic metabolism of testosterone

As for estradiol and progesterone, inactivation and excretion of testosterone and DHT occurs primarily in the liver, although metabolic enzymes are also present in other organs including the gut and kidneys [13]. Hepatic metabolism of testosterone also serves to increase the water solubility of the steroids by conjugation to glucuronic acid or sulphates, which facilitates their excretion in the urine as explained for E2 and P4 (section 1.4). Unconjugated steroids that are filtered by the kidney are largely reabsorbed [19].

The enzymatic reactions involved in hepatic metabolism are the same for androgens and corticosteroids, all containing a conjugated Δ4,3-keto moiety on the A-ring [13]. Oestrogens on the other hand, have an aromatic A-ring and are not substrates for the enzymes in these pathways [78]. The major reactions involved in the hepatic metabolism of testosterone include: (a) irreversible reduction of the A-ring across the 4-5 double bond from either the α (catalysed by 5α-reductase) or β (catalysed by 5β-reductase) side of the A-ring. This step yields two isomers which differ in the orientation of the C_5 hydrogen between the fused A and B rings. The 5α-metabolites have *trans* fused rings, which are essentially planar and similar to the parent steroids whereas the *cis* 5β fused rings are more skewed. (b) The 3α,5α/β reduced steroids are substrates for 3α-hydroxysteroid dehydrogenases which catalyse reduction of the

3-keto group to form 3α,5α- and 3α,5β-tetrahydrosteroid metabolites which are then (c) rapidly conjugated to sulphates or glucuronic acid and excreted. The conjugates are more soluble in water and have a much lower affinity for plasma binding proteins than did the parent steroid, with glucuronides having the lowest affinity of all [13]. The 3α,5β-reduced products of androstenedione are also substrates for 17β-hydroxsteroid dehydrogenase (17β-HSD) enzymes as shown in Scheme 1.4.

In principle any of the range of testosterone metabolites present in the urine as glucuronides could serve as a urinary biomarker for serum testosterone production in humans. However, the stereospecific reductions which occur *in vivo*, while not impossible *in vitro,* nevertheless require a significant amount of synthetic effort. In the absence of information on the relative levels of these possible biomarkers in urine it seems sensible, at least at this stage of development, to focus on testosterone glucuronide as the urinary marker. It is relatively easy to synthesise and is expected to be excreted into the urine of males in significant amounts.



Scheme 1.4 Metabolism of androgens [13]

1.6.2 Cortisol and its metabolites as biomarkers of stress and disease

Although there is some variation in the way that "stress" is defined, common among these definitions is that stress is a process in which environmental demands tax or exceed individuals' adaptive capacities, contributing to biological and psychological changes that may place them at risk for illness [79].

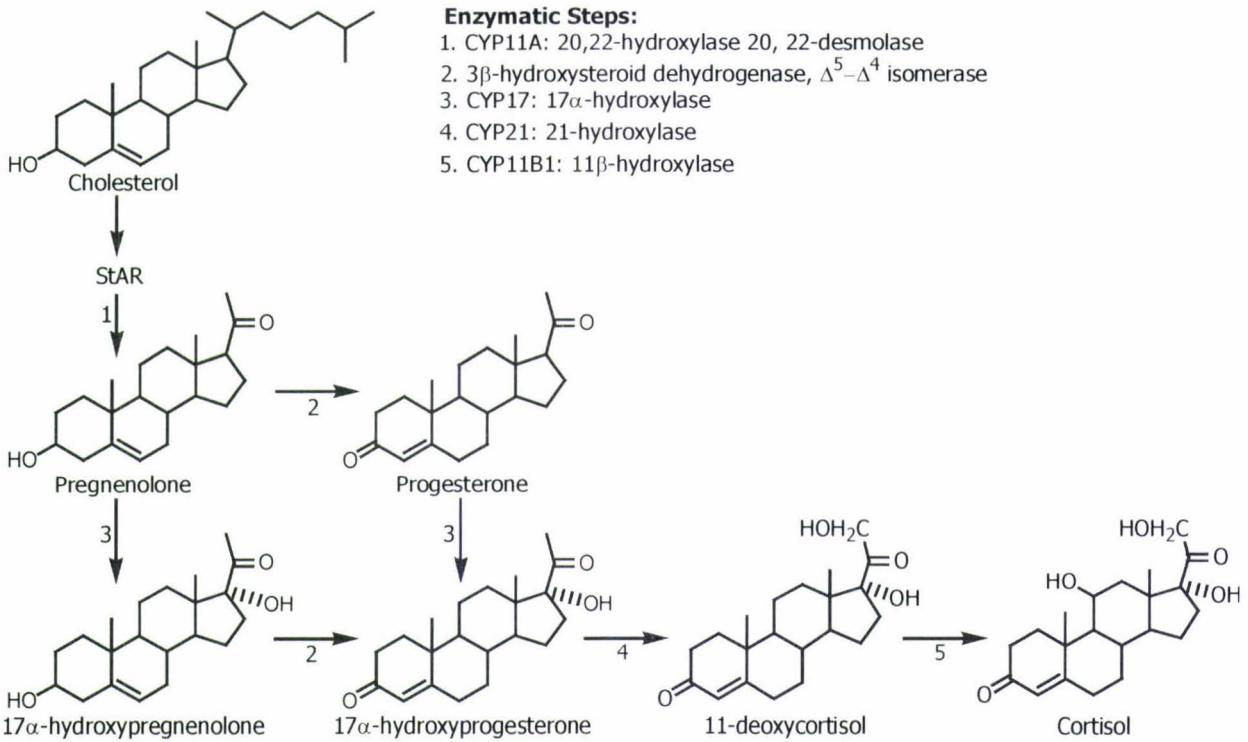
While cortisol activation in response to stress is protective in the short term, the chronic or extreme activation may have long-term negative consequences. Cortisol stimulates the immune system and counteracts inflammatory and allergic reactions at normal levels but can suppress the immune system at excessive levels or when prescribed therapeutically in a synthetic drug form [80]. Whereas hypercorticolism is associated with diseases characterised by immunosuppression and reduced ability to fight infections, including HIV [81,82,83] it may also increase susceptibility of developing chronic inflammatory disease, autoimmune disease and other diseases characterised by inflammation [80]. Hypercortisolism has also been associated with arthritis, multiple sclerosis and all types of cancer [83] and is the primary symptom of Cushing's disease [84]. Cortisol excess may also lead to insulin resistance, hypercholesterolemia and hypertriglyceridemia, thereby contributing to the development of adult onset diabetes, hypertension and heart disease [85,86].

Recently, the suggestion has been that elevated cortisol levels are in fact the causative agent of disease and the results have been presented at an International Conference [83]. For example, it has been proposed that HIV stimulation induces the adrenal gland to produce elevated levels of cortisol and that elevated cortisol is, by itself, capable of inducing all of the symptoms and opportunistic infections encountered in AIDS [87]. Also, evidence has been presented which suggests that high levels of cortisol play a role in Alzheimer's disease, memory loss, shrinking of the brain and aging [88]. The more conventional view is that the elevated cortisol levels are associated with other underlying mechanisms and are indicative rather than causative. For the purposes of this discussion it does not matter which view is correct since the high levels of cortisol can be used as a diagnostic tool in either case and appropriate strategies taken to alleviate the underlying conditions. There is also evidence that cortisol levels are of diagnostic importance for athletes; in particular elite women athletes who may face menstrual cycle disturbances [89].

1.6.2.1 Cortisol biosynthesis

The synthesis of cortisol from cholesterol shown in Scheme 1.5 occurs exclusively in the adrenal cortex via a series of reactions catalysed by cytochrome P450 (CYP) and various short chain dehydrogenase enzymes. These enzymes are located in either the lipophillic membranes of the smooth endoplasmic reticulum (ER) or the inner mitochondrial membrane. As the series of enzymatic reactions to convert cholesterol into cortisol proceeds, the steroid intermediates shuttle between the membranes of the two organelles [19]. In humans, the necessary transformations from cholesterol to cortisol proceed as follows; cholesterol is imported into mitochondria and converted to pregnenolone by the cytochrome P450 enzyme, CYP11A as described for E1G and PdG in section 1.3. Pregnenolone is then transferred to the ER, where a 17α-hydroxylase (CYP17) converts it to 17-hydroxypregnenolone. This is then a substrate for 3β-hydroxysteroid dehydrogenase Δ^5-Δ^4 isomerase which catalyses oxidation at C3, resulting in a ketone functional group, and isomerisation of the double bond to form 17-hydroxyprogesterone. A further hydroxylation in the smooth ER by a 21-hydroxylase (CYP21) converts 17-hydroxyprogesterone to 11-deoxycortisol. The latter is transferred back to the mitochondria and is converted to cortisol by an 11β-hydroxylase (CYP11B1). The final step is highly efficient, resulting in 98% conversion of 11-deoxycortisol to cortisol, which diffuses rapidly into the circulation without significant storage in the adrenal cortex [19]. An alternative pathway for cortisol biosynthesis exists in the mitochondria whereby oxidation of pregnenolone at C3 followed by isomerisation of the double bond forming progesterone occurs before the series of hydroxylations at C17 (17-OH progesterone), C21 (11-deoxycortisol) and finally at C11 in the ER terminating in cortisol [90].

Cortisol secretion under basal (i.e. non-stressed) conditions ranges from 8 mg/day to 25 mg/day (22 - 69 μmol/day) with a mean of ~9.2 mg/day (25 μmol/day) [91]. In the plasma only 3 - 10% of circulating cortisol is in the free state. Approximately 80 - 90% is bound with high affinity to corticosteroid binding protein (CBG) and the remaining 5 to 10% is bound to albumin [19]. As for testosterone (see section 1.6.1.1) albumin has a much lower affinity for cortisol than CBG, but a much higher capacity. The dissociation of cortisol from albumin is more rapid, thus the albumin bound steroid is thought to be more readily available to the tissues than that bound by CBG. A third binding protein, α_1-acid glycoprotein can also bind cortisol, but appears to be only of minor importance to cortisol transport since it preferentially binds progesterone [19].



Scheme 1.5 Biosynthesis of cortisol from cholesterol [19].

Normally about 10 - 15 pulsatile bursts of cortisol are secreted into the plasma in a 24 hour period in adults [19,92]. Like testosterone, cortisol levels in the blood show a clear diurnal rhythm. The levels are lowest around midnight and peak about half an hour after awakening as shown in Figure 1.10 [93]. Peak levels on awakening may be 50 – 100% higher than the lowest levels. Hence this factor must be taken into account when designing assay protocols for cortisol or its metabolites.

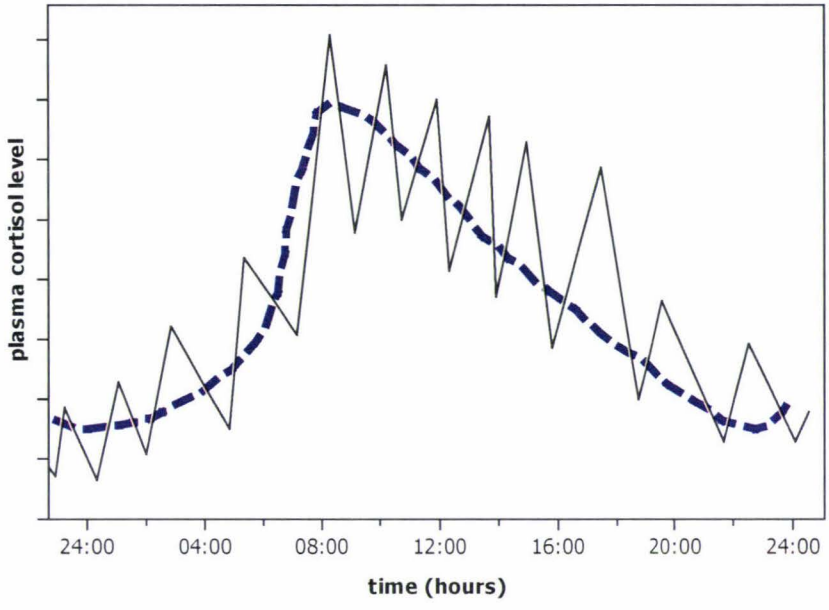


Figure 1.10 Diurnal pattern of cortisol levels in plasma [78].

1.6.2.2 Biological activity of cortisol

Under basal conditions, cortisol interacts mostly with high affinity mineralocorticoid receptors, which are important for normal homeostatic control of metabolic processes and fluid balance. However, when the hypothalamic-pituitary-adrenal (HPA) axis (those glands responsible for integrating the central nervous system and endocrine system) is activated during a stressful experience, cortisol levels can increase ten-fold and at these levels cortisol may bind to lower affinity glucocorticoid receptors in target tissues [94].

Through its interaction with glucocorticoid receptors cortisol is hypothesised to promote short term survival in an acutely stressful event by: (1) increasing glucose and oxygen supply to skeletal muscles and heart and brain to facilitate flight and sharpen cognition to allow an appropriate behavioural response; 2) suppressing reproductive, immune and digestive functions to conserve energy; 3) promoting analgeisa and 4) activating the autonomic nervous system [95].

Increasing levels of cortisol also act as a negative feedback signal to suppress further release of the hormones which stimulate cortisol production; corticotropin-releasing hormone (CRH) released from the hypothalamus and adrenocorticotropin-releasing hormone (ACTH) released from the pituitary (as shown in Figure 1.11).

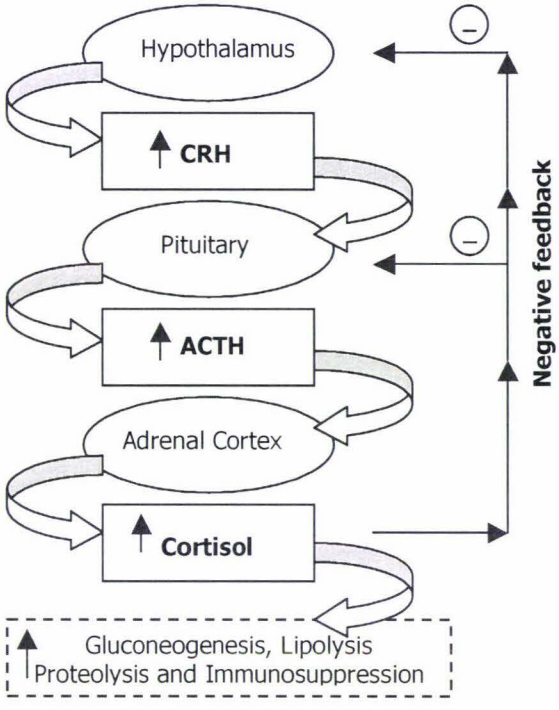


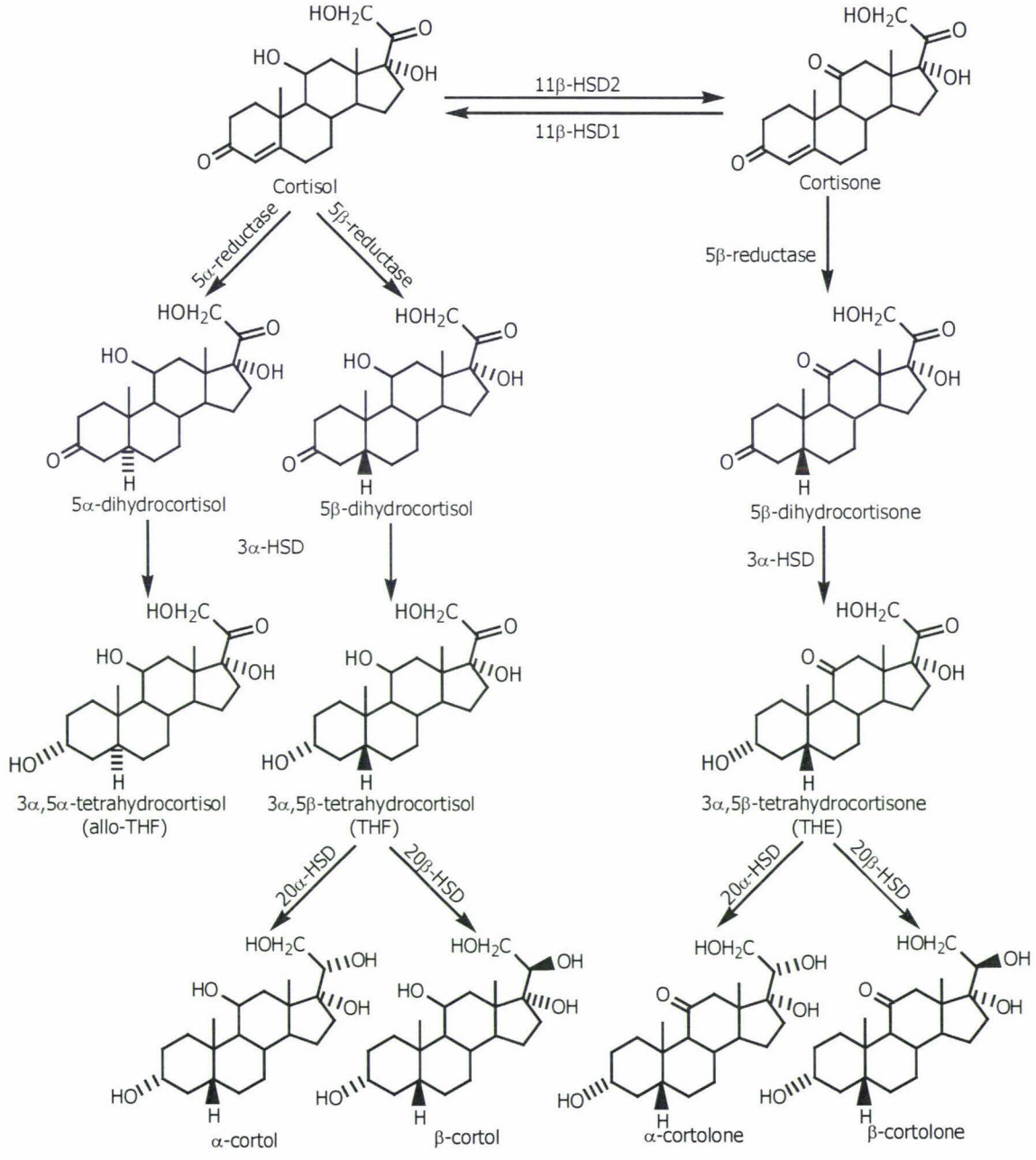
Figure 1.11. Hypothalamic-pituitary axis (HPA) activation. When activated the HPA leads to the sequential release of hormones at each level of the axis, culminating in the release of cortisol. Through negative feedback inhibition, increasing levels of cortisol supress further CRH and ACTH release at the level of the hypothalamus and pituitary. Abbreviations: HPA, hypothalamic-pituitary-adrenal; ACTH, adrenocorticotropin-releasing hormone; CRH, corticotropin-releasing hormone [78].

1.6.2.3 Hepatic metabolism of cortisol

As noted earlier, the androgens and corticosteroids both contain a conjugated Δ4,3-keto moiety on the A-ring and are metabolised by common pathways in the liver. The metabolic steps a), b) and c) described for hepatic metabolism of testosterone in section 1.6.1.3 also apply to cortisol, and the resulting cortisol metabolites are illustrated in Scheme 1.6.

In addition, cortisol is a substrate for the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) which catalyses the oxidation of the 11β-hydroxy group to form the inactive metabolite cortisone. Inactivation of cortisol by 11β-HSD2 occurs in the kidneys and other mineralocorticoid sensitive tissues [96] to protect mineralocorticoid receptors from illicit occupation by cortisol, which is present in concentrations in excess of the preferred ligand,

aldosterone [97]. Cortisone may then be metabolised by steps a), b) and c) as outlined for cortisol or reactivated back to cortisol by 11β-HSD1 in several metabolic tissues including the liver, muscles, adipose tissue and brain [98,99]. Further reduction at the C_{20} position in the side-chain of 3α,5β-tetrahydro corticosteroids, by 20-dehydroxysteroid dehydrogenases can give rise to the corresponding 20α and 20β isomers, namely cortols by reduction of cortisol and cortolones by reduction of cortisone [13]. Finally cleavage of the C_{17} side chain may take place, producing 19 carbon metabolites that have a ketone group at the C_{17} position, such as 11β-hydroxyandrostenedione and 11-ketoetiocholanolone [19].



Scheme 1.6 Metabolism of cortisol and cortisone [13].

In the urine of normal subjects the 5β metabolites predominate (5β:5α-tetrahydrocortisol 2:1). Approximately 50% of the secreted cortisol appears in the urine as tetrahydrocortisol (THF), allo-THF and tetrahydrocortisone (THE); 25 % as cortols and cortolones; 10% as 19 carbon steroids and 10% as cortolic and cortolonic acids. The remaining metabolites exist in the urine as unconjugated steroids; cortisol, cortisone, 6β- and 20α/β-metabolites of THF and THE [100,101]. Marked differences in the amount of total urinary cortisol metabolites excreted by age-matched males and females also exist. The reason for the difference is thought to reflect differences in the hypothalamic-pituitary adrenocorticol axis or primary differences in adrenal function [102]. Normally less than 1% of cortisol is excreted unchanged in the urine of humans. Although this can be measured using RIA the levels are only of the order of 50 μg per day [15].

In a recent study by Yergey *et al.* [103], the metabolites of cortisol in urine were examined by a novel mass spectrometric procedure known as chemical reaction interface mass spectrometry or CRIMS. In this procedure the hydrolysed urinary metabolites of radiolabeled cortisol (9,12,12-$^{2}H_{3}$ cortisol) were measured in six human subjects and it was found that 5β-tetrahydrocortisone (THE) was the major metabolite constituting 42% of the total cortisol metabolites. Tetrahydrocortisone has three available hydroxy groups and hence this compound may appear in urine as a variety of glucuronides, sulphates or mixed sulphates and glucuronides. The 3α-glucuronide is the major metabolite and is excreted in amounts corresponding to about 5 mg per day [103] similar to pregnanediol-3α-glucuronide [25]. These levels are sufficient to make it possible to produce a rapid (5 minute) home and laboratory test for tetrahydrocortisone-3α-glucuronide, as a measure of total free cortisol by adaptation of our presently existing home test for the steroidal markers of the fertile phase of the human menstrual cycle [56,60].

1.7 Aims of the present study

In summary the aims of the present study are:

1. To expand the range of urinary steroid metabolites that can be measured using the Ovarian Monitor homogeneous immunoassay format currently used as a home monitoring device for determining the fertile period during a women's menstrual cycle.

2. To prepare biomaterials for a testosterone glucuronide assay that can be used as a device for application to the urine of males as a biomarker of testosterone in men undergoing androgen replacement therapy.

3. To prepare biomaterials for a tetrahydrocortisone glucuronide assay that could be used in a device for measuring stress.

4. To establish standard curves for the two urinary metabolites which are capable of measuring the physiological ranges of testosterone glucuronide and tetrahydrocortisone glucuronide.