British Journal of Pharmacology (2008) 154, 569–583

0007–1188/08 $30.00
www.brjpharmacol.org

REVIEW

Gonadotropins in doping: pharmacological basis and detection of illicit use

U-H Stenman, K Hotakainen and H Alfthan

Department of Clinical Chemistry, Helsinki University Central Hospital, HUS, Finland

Parenteral administration of human chorionic gonadotropin (hCG) or luteinizing hormone (LH) stimulates the production of testosterone in males and these gonadotropins can therefore be used by athletes to enhance muscle strength. However, they are more expensive and less efficient than testosterone and anabolic steroids. Therefore their main use is probably to stimulate gonadal testosterone production during and after self-administration of testosterone or anabolic steroids. A positive effect of hCG on muscle strength has not been demonstrated in women and elevated concentrations of hCG in females are often caused by pregnancy. The use of gonadotropins is therefore prohibited only in males but not in females. HCG occurs at low but measurable concentrations in plasma and urine of healthy males and can be measured by sensitive methods. However, the characteristics of the method to be used for doping control have not been defined. Virtually all commercially available hCG assays have been designed for determination of hCG in serum rather than urine, which is used for doping control. Methods based on mass spectrometric detection of fragments derived from hCG extracted from urine by immunoadsorption have been developed but their suitability for doping control remains to be determined. The concentrations of LH in serum and urine are variable and more then 10-fold higher than those hCG. It is therefore difficult to detect illicit use of LH. The characteristics and reference values for hCG and LH assays used in doping control and the cutoff values need to be defined.
British Journal of Pharmacology (2008) **154,** 569–583; doi:10.1038/bjp.2008.102; published online 14 April 2008

Keywords: gonadotropins; hCG; LH; immunoassay; mass spectrometry; doping control; doping

Abbreviations: CTP, C-terminal peptide; ESI, electro spray ionization; FSH, follicle stimulating hormone; hCG, human chorionic gonadotropin; hCGn, nicked human chorionic gonadotropin; hCGβ, free β subunit of hCG; hCGβn, nicked free β subunit of hCG; hCGα, free α subunit of hCG; hCGh, hyperglycosylated hCG; hCGβcf, core fragment of hCGβ; GnRH, gonadotropin releasing hormone; GPH, glycoprotein hormone; GPHα, free α subunit of glycoprotein hormones; hCGα, free α subunit of hCG; IU, international unit; LH, luteinizing hormone; MALDI–TOF, matrix-assisted laser desorption and ionization—time of flight; MS, Mass spectrometry; rhLH, recombinant human LH; rhCG, recombinant human chorionic gonadotropin; TSH, thyroid-stimulating hormone

Introduction

Human chorionic gonadotropin (hCG) is a glycoprotein produced at high concentrations by the trophoblasts of the placenta (Stenman *et al*., 2006). HCG and luteinizing hormone (LH) exert the same activity, that is, they stimulate the production of testosterone in the testicles of males and progesterone and estradiol in the female ovaries. The half-life of hCG is longer than that of LH and pharmaceutical preparations are more readily available. It can therefore be used by male athletes to increase testosterone production and to normalize testicular testosterone production that is suppressed during and after prolonged use of anabolic steroids. In women, hCG has not been shown to provide a clear beneficial effect on athletic performance, and furthermore detection of hCG is considered an intrusion on the privacy of a pregnant athlete. Thus the use of hCG and LH is illegal in male but not in female athletes (Handelsman, 2006). Determination of hCG in urine is used to detect illegal use in male athletes and cutoffs between 10 and 25 IU/l have been recommended, (Kicman *et al*., 1991; Laidler *et al*., 1994; Gam *et al*., 2003). World Anti-Doping Agency states that the hCG assay used should have a detection limit of at least 5 IU/l, but recommendations regarding assay specificity have not been issued. The pituitary produces hCG at low concentrations that can be detected in serum and urine of men and nonpregnant women by sensitive methods. Furthermore, about half of the hCG immunoreactivity in urine consists of the so-called core fragment (hCGβcf) of the β subunit of hCG (hCGβ), which most hCG assays do not detect. There are no

Correspondence: Professor U-H Stenman, Department of Clinical Chemistry, Helsinki University Central Hospital, HUS FIN-00027, Finland.
E-mail: ulf-hakan.stenman@helsinki.fi
Received 23 November 2007; revised 15 February 2008; accepted 4 March 2008; published online 14 April 2008

recommendations regarding the use of LH determinations for doping control.

Secretion of pituitary LH and hCG (and FSH) is regulated by gonadotropin releasing hormone (GnRH), which is available as a pharmaceutical preparation. GnRH and its analogues can be used to restore testicular function after the use of anabolic steroids and they could also be used to increase gonadotropin and testosterone secretion for doping purposes. This would be difficult to detect and there are no reports on such use.

The physiological basis for the use of hCG and LH in doping has been extensively reviewed (Kicman *et al*., 1991; Kicman and Cowan, 1992; Handelsman, 2006). This review will focus on the pharmacological basis for the use of gonadotropins for doping and on analytical requirements for the methods used to measure them as well as reference and cutoff values.

Biochemistry of hCG and LH

HCG belongs to the glycoprotein hormone (GPH) family that also comprises LH, follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH). The GPHs are heterodimers consisting of an α (GPHα) and a β subunit. The α subunit contains 92 amino acids and is identical in all GPHs whereas the β subunits are different and confer biological specificity. The β subunits display considerable homology; that between hCG and LH being about 80% whereas that with FSHβ and TSHβ is lower. HCGβ contains 145 and LHβ 121 amino acids, the difference being due to a 24 amino acid extension on hCGβ, the so-called C-terminal peptide (CTP) (Pierce and Parsons, 1981).

HCG is highly glycosylated, one third of the mass being attributed to eight carbohydrate moieties. Each subunit contains two N-linked and the CTP on hCGβ four O-linked oligosaccharides (Kessler *et al*., 1979a, b; Elliott *et al*., 1997). Owing to variation in the content of terminal sialic acid on the carbohydrate chains, hCG displays extensive charge heterogeneity with pI values ranging from three to seven. HCG produced in early pregnancy and by trophoblastic cancer contains more complex carbohydrates than that produced later in pregnancy (Mizuochi *et al*., 1983; Elliott *et al*., 1997; Birken, 2005). The proportion of this so-called hyperglycosylated hCG (hCGh) decreases to a few per cent after 10 weeks of pregnancy (Kovalevskaya *et al*., 2002). Thus little hCGh occurs in commercial hCG preparations prepared from pregnancy urine, which are commonly used for doping.

The MW of the major form of pregnancy hCG calculated on the basis of the sum of the peptide and carbohydrate moieties is 38 931 but because of heterogeneity of the carbohydrates moieties the MW displays a wide spectrum of values (Valmu *et al*., 2006). The average MWs of hCGα, hCGβ and intact hCG isolated from a commercial hCG preparation are 14 000 and 23 500 and 37 500, respectively (Birken *et al*., 2003). The lower MW of this hCG is explained by partial degradation of the carbohydrate moieties.

Part of hCG and hCGβ in urine is nicked (abbreviated hCGn and hCGβn), that is, the peptide chain is cleaved at various positions between amino acids 44 and 48 (Cole *et al*., 1991). Nicked forms of hCG may also occur in serum especially from patients with an hCG-secreting tumour (Valmu *et al*., 2006).

When excreted through the kidneys, much of hCG is degraded proteolytically and about half of the immunoreactive hCG in urine consists of a fragment of hCGβ called the beta-core fragment (hCGβcf). It consists of amino acids 6–40 and 55–92 linked by disulphide bridges and two truncated carbohydrate moieties on Asn 13 and 30 (Birken *et al*., 1988; Stenman *et al*., 1993).

LH is a 31 kDa glycoprotein containing N-linked glycosylation sites on asparagines 49 and 75 on the α subunit and on asparagine 30 on the β subunit. The processing of N-linked carbohydrates is tissue- and hormone-specific. LH and TSH carry sulphated oligosaccharides and *N*-acetylglucosamine at their carbohydrate termini whereas the carbohydrates on hCG (and FSH) carry terminal sialic acid and galactose. These differences are thought to determine the longer half-life of hCG in circulation (Baenziger *et al*., 1992; Manna *et al*., 2002). However, sulphated LH isolated from urine and recombinant LH, which was sialylated, have been found to have identical half-lives (le Cotonnec *et al*., 1998a). Like hCG, a major part of the LH immunoreactivity in urine consists of a fragment (LHβcf) resembling hCGβcf (O'Connor *et al*., 1998).

Standards for various forms of hCG and LH

WHO issues standards for hCG that are calibrated in international units (IU). So far all standards have been calibrated against the preceding one by bioassay. The presently used 3rd and the identical 4th international standards (3rd and 4th IS) were initially available from NIH as CR119 (1st IRP) (Canfield and Ross, 1976) and were adopted as WHO standards in 1980 (Storring *et al*., 1980). In the standard for hCG (75/537), 1 μg of hCG corresponds to about 9.3 IU. The free subunits lack hCG activity and therefore the standards for hCGβ (75/551) and hCGα (75/569) were assigned values based on mass with 1 μg corresponding to 1 IU (Storring *et al*., 1980). Thus their units are not comparable to those of hCG. Table 1 shows the relationship between the units for hCG and its subunits. Most commercial hCG assays measure hCG and hCGβ together and the results are expressed in IU/l based on the units for hCG.

Table 1 Comparison of WHO units (IU), mass units (μg) and substance concentrations (pmol)

Abbreviation	*MW*	*IU*	*μg*	*pmol*
hCG	37 500	1	0.11	2.9
hCGβ	23 500	1	1	42.5
hCGα	14 000	1	1	71.4
hCGβcf	12 000		1	83.3

The WHO unit for hCG is based on its bioactivity while the corresponding values for hCGβ and hCGα are based on mass. Modified from (Stenman *et al*., 2006).

Table 2 IFCC nomenclature and WHO designations for the 1st International Reference Reagents for hCG and major hCG-related molecules occurring in serum and urine

IFCC Nomenclature for hCG and related molecules			*Ampoule code*
Molecule	*Symbol*	*Description*	
Human chorionic gonadotropin, intact	hCG	hCG devoid of nicked forms and free subunits	99/688
Human chorionic gonadotropin, nicked	hCGn	Partially degraded hCG, missing peptide bonds in the hCGβ-40-50 region	99/642
Human chorionic gonadotropin, α-subunit	HCGα	Purified hCGα, dissociated from hCG	99/720
Human chorionic gonadotropin, β-subunit	hCGβ	Purified hCGβ, dissociated from hCG	99/650
Human chorionic gonadotropin, nicked β-subunit	hCGβn	Partially degraded hCGβ, missing peptide bonds in the hCGβ-40-50 region	99/692
Human chorionic gonadotropin, β-core fragment	hCGβcf	Residues hCGβ-6-40, joined by disulphide bonds to hCGβ-55-92	99/708

Modified from (Stenman *et al.*, 2006).

New standards for hCG and clinically important hCG-related molecules have been prepared and approved by the WHO as reference reagents (RR) for immunoassay (Birken *et al.*, 2003; Bristow *et al.*, 2005). Immunoassays reflect molar concentrations of protein rather than bioactivity. Therefore, use of substance concentrations, that is, mol/l, is the only appropriate way of comparing the concentrations of two or several analytes (for example, hCG, hCGβ and hCGβcf) of different MW (Stenman *et al.*, 1993). Furthermore, carbohydrate composition has rarely an effect on immunoreactivity but it strongly affects bioactivity. Therefore, the RRs have been assigned values in molar concentrations (Birken *et al.*, 2003; Bristow *et al.*, 2005) (Table 2). The RRs are purer than the 3rd IS and thus the bioactivity of the hCG preparation is about 50% higher than that of the 3rd IS (Birken *et al.*, 2003). When the RRs are used as standards for immunoassay of hCG, hCGβ, hCGα and hCGβcf, the concentrations are expressed in pmol/l.

The new RRs for nicked forms of hCG (hCGn) and hCGβ (hCGβn) are important for characterization of assay specificity. Some antibodies do not recognize hCGn, which occurs especially in urine (Cole *et al.*, 1991; Hoermann *et al.*, 1994). Assays recognizing hCGn are therefore preferable for doping control. As hCGβcf represents a major part of the hCG immunoreactivity in urine, its standard is important for characterization of assays used to determine hCG immunoreactivity in urine.

Some studies suggest that some hCG assays underestimate hyperglycosylated hCG (hCGh) (Cole and Kohorn, 2006) but we have not been able to detect any essential differences in recognition between 14 commercial hCG assays (Stenman U H and Alfthan H, unpublished). HCGh is anyhow a very minor component in pharmaceutical urinary hCG preparations and recognition of this form is not important for doping control.

A pharmaceutical preparation of recombinant hCG (rhCG) produced in Chinese hamster ovary cells is now available and is probably used for doping. For ovulation induction, a dose of 250 μg of rhCG is equivalent to 10 000 IU of urinary hCG (Chang *et al.*, 2001). In other studies 250 μg of rhCG has been found to have the same biological activity as 5000 IU of urinary hCG (Gervais *et al.*, 2003; Al-Inany *et al.*, 2005). The hCG content of this preparation is expressed on the basis of the mass of the peptide moiety disregarding the carbohydrate content (Gervais *et al.*, 2003). Thus 250 μg of rhCG correspond to approximately 360 μg of urinary hCG. It can thus be calculated to have a specific activity of 13 900 IU/mg (Stenman *et al.*, 2006), which is similar to the potency of about 15 000 IU/mg of the new WHO hCG RR 98/688 (Birken *et al.*, 2003).

The O-linked carbohydrates on the CTP of rhCG differ somewhat from those in pregnancy hCG (Gervais *et al.*, 2003). This can probably be used to differentiate between native and recombinant hCG by mass spectrometry.

WHO has issued various standards for LH. Most immunoassays are calibrated against WHO IRP 78/549 or the more recent IRP 80/552, which both have been purified from pituitary extracts and calibrated in IU based on biological activity. Standards for the subunits and core fragment of LH are not available.

Function and metabolism

The secretion of LH is regulated by the gonadotropin-releasing hormone (GnRH) that is a 10 amino acid peptide produced by the hypothalamus. It is secreted through a portal venous system leading to the anterior pituitary. GnRH is secreted in a pulsatile fashion with 60–120 min intervals. LH is therefore also secreted in pulses with the same frequency. Boys have higher serum LH and testosterone concentrations than girls during their first 6 months of life after which they decrease to similar concentrations in both genders. Puberty is initiated by increasing LH concentrations. When measured by sensitive assays, the LH concentrations are below 0.05 IU/l before puberty and a concentration above 0.2 U/l is a reliable sign of early puberty (Apter *et al.*, 1989). The start of puberty can also be detected by measuring LH in urine and especially morning urine. An increase in LH indicating the start of puberty can be detected earlier in urine than in serum (Demir *et al.*, 1996).

LH and hCG mediate their action through the LH receptor (Alexander *et al.*, 2007). LH regulates testicular testosterone production in males and estradiol and progesterone production in women. The main physiological function of hCG is to maintain progesterone production of corpus luteum during early pregnancy. When administered to males, hCG and LH stimulate testosterone production in the Leydig cells

of the testis, which is the basis for their use for doping. The LH receptor is also present in a large number of tissues other than the ovary and thus hCG (and LH) may have hitherto unknown functions (Filicori *et al*., 2005). The hyperglycosylated form of hCG (hCGh) produced in early pregnancy is thought to mediate implantation of the early trophoblast (Cole *et al*., 1999) but no receptor other than the LH receptor has been identified (Stenman *et al*., 2006).

The free subunits lack hCG activity but hCGβ may exert growth-promoting activity in bladder cancer cells (Butler and Iles, 2003) and prevent apoptosis in choriocarcinoma cells (Hamada *et al*., 2005). It has been suggested that this effect is mediated by interference with the growth-inhibiting effect of TGFβ, PDGF-β and NGF (Butler and Iles, 2004). HCGα (GPHα) has been shown to stimulate prolactin production in decidual cells (Blithe *et al*., 1991; Moy *et al*., 1996) and to mediate decidualization of these (Nemansky *et al*., 1998).

The metabolism of hCG has been studied after delivery, termination of pregnancy and after intramuscular injection of hCG purified from pregnancy urine. The terminal half-life of hCG in circulation is 1–1.5 days (Stenman *et al*., 2006) and that of rhCG is similar (Gervais *et al*., 2003). After intramuscular injection of 10 000 IU of urinary hCG the serum concentration increase to around 500–1000 pmol/l (170–300 IU/l) the first day and decrease exponentially to below 10 IU/l within 7–12 days (Korhonen *et al*., 1997; Stenman *et al*., 1997).

Early studies on the metabolism of LH in man indicated a much shorter half-life (less than an hour) than for hCG (Yen *et al*., 1968). However, recent studies with pharmaceutical preparations of urinary and recombinant LH injected intravenously show an initial (distribution) half-life of about 1 h and a terminal half-life 10 h. Recombinant and urinary LH have very similar metabolism and biological activity (le Cotonnec *et al*., 1998b). The short half-life of LH in earlier studies has been ascribed to sulphation of the carbohydrates (Manna *et al*., 2002); however, the carbohydrate chains of recombinant LH are not sulphated but sialylated (Amoresano *et al*., 1996; le Cotonnec *et al*., 1998b).

The hCG concentrations in urine are similar to those in serum (Figure 1) and after intramuscular injection the peak is reached some hours later in urine than in plasma. Urine additionally contains hCGβcf at molar concentrations similar to those of hCG (Stenman *et al*., 1997). With an assay measuring hCG and hCGβcf together in an equimolar fashion the concentrations measured will be approximately twofold those of intact hCG. The urine concentrations of hCGβ are low and have little effect on the hCG results (Alfthan *et al*., 1992b).

The urine concentrations of hCG vary due to variation in urinary flow rate. Thus, the protein concentration of urine regularly varies more than 10-fold, which is reflected in urine densities between 1.002 and 1.030. To compensate for this, the urine concentrations of hCG and LH are often normalized to a density of 1.015 or 1.020 or divided by the concentration of urine creatinine (Demir *et al*., 1994).

Therapeutic use of LH and hCG

The therapeutic use of hCG and LH in assisted reproductive treatment provides information on the relative biological effects of these hormones. Partially purified urinary hCG preparations are used as a surrogate for LH to achieve final oocyte maturation and ovulation in controlled ovarian hyperstimulation protocols. Urinary hCG has mainly been used but recombinant human LH and hCG (rhCG) are now available (le Cotonnec *et al*., 1998b; Gervais *et al*., 2003).

The two- to threefold more rapid clearance of LH than of hCG is reflected in the dose required to induce ovulation in assisted reproduction. A single dose of 15 000–30 000 IU of recombinant human LH is comparable to 5000 IU of urinary hCG. After intramuscular or subcutaneous injection of such doses, the LH concentrations return to baseline concentrations in about 3 days (le Cotonnec *et al*., 1998b) as compared with 6–7 days for hCG (Figure 1) (Stenman *et al*., 1997).

In males, hCG is used to treat infertility associated with hypogonadism caused by defects in the hypothalamic—pituitary axis, that is, hypogonadotropic hypogonadism. The androgen deficiency of this condition is usually treated with testosterone, which does not normalize spermatogenesis. If the patient wants to father a child, testosterone is replaced by gonadotropin treatment, usually a combination of FSH and hCG (or LH), which usually restores fertility within 7–10 months but longer treatment may be needed (Zitzmann and Nieschlag, 2000; Liu *et al*., 2002). Cryptorchidism in boys has

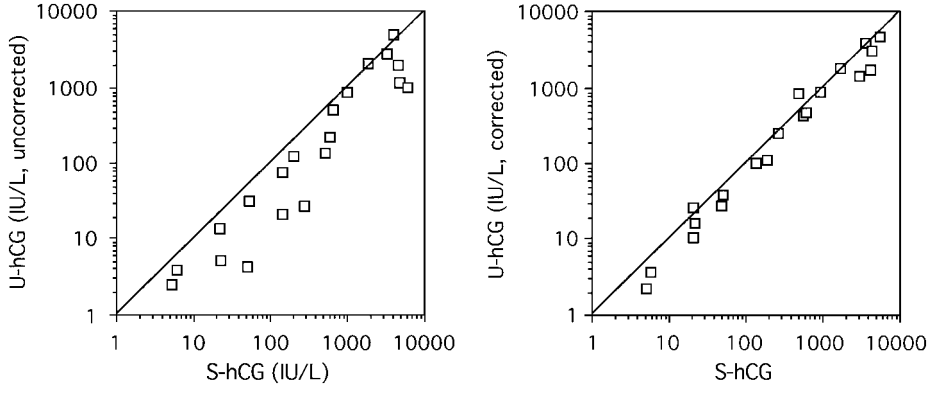

Figure 1 Comparison of hCG values in serum urine before (left) and after (right) correction of the urine hCG concentrations for urine density. Reproduced from (Alfthan *et al*., 1993) with permission.

also been treated with hCG, but because of side effects, this treatment is presently not recommended (Thorsson *et al*., 2007). HCG alone is not used for treatment of primary hypogonadism but human menopausal gonadotropin has been successfully used to treat hypogonadism caused by use of anabolic steroids (Menon, 2003).

If used for doping in male athletes, LH can be expected to stimulate gonadal testosterone production at normal or only moderately elevated plasma and urine concentrations. After intramuscular or subcutaneous injection of 5000 IU of recombinant LH the peak serum concentration 1 day later is about 20 IU/l (European Recombinant LH Study Group, 2001). Thus detection of illegal use of LH will be more difficult than detection of hCG. Principally, recombinant LH (but not urinary LH) can be distinguished from natural LH by analysis of the carbohydrate side chains.

Gonadotropin-releasing hormone

Pulsatile injection of GnRH increases LH and FSH secretion and stimulates gonadal steroid production. Idiopathic hypogonadism is often due to defective secretion of GnRH and this condition can be treated by delivery of GnRH with a pump attached to a subdermal needle (Delemarre-van de Waal, 2004). This treatment can also be used to normalize testicular function that has been suppressed by long-term use of anabolic steroids (van Breda *et al*., 2003). There is no published information on the misuse of GnRH for doping and it is not mentioned in the list of prohibited substances. However, based on its activity its use can be considered illegal but detection of GnRH would be very difficult because this small peptide has a very short half-life (reviewed in (Bergendahl *et al*., 1996)). When given in large doses, GnRH analogues suppress gonadotropin secretion and continuous delivery from subcutaneous capsules is widely used to suppress testosterone secretion in men with prostate cancer. Initially, this causes a transient increase in serum testosterone. Thus is obvious that intermittent delivery of GnRH analogues in suitable doses could be used to induce prolonged stimulation of gonadotropin and testosterone secretion.

Effect of gonadotropin administration on the testosterone—epitestosterone ratio in urine

Testosterone is one of the most commonly used drugs for doping. It can be reliably measured in urine by mass spectrometry, but because untimed samples are used for doping control, the concentration as such is not a sensitive indicator of illicit use. However, determination of the testosterone—epitestosterone (T/E) ratio in urine is a reliable sign of testosterone administration. Testosterone and epitestosterone are secreted at equal concentrations by the testicles and the average T/E ratio in urine is close to 1. Administration of testosterone causes suppression of gonadotropin secretion leading to reduced testicular production of testosterone and epitestosterone. Thus exogenous testosterone leads to an increased T/E ratio and a ratio exceeding six is used to indicate testosterone doping (Kicman *et al*., 1990). Simultaneous use of gonadotropins and testosterone can be expected to reduce the T/E ratio by increasing the testicular secretion of epitestosterone (and testosterone). Administration of hCG alone does not affect the T/E ratio (Cowan *et al*., 1991) but repeated administration of testosterone and hCG has been shown to suppress the increase in T/E ratio seen when testosterone was given alone. This study comprised only a single male athlete, but the results are expected and probably representative. However, the effect of hCG on the T/E ratio is limited and, depending on timing and dosage of the hormone preparations, the ratio may not be normalized (de Boer *et al*., 1991).

Effect of oestrogen blockers

Clomiphene citrate has been used for decades to increase gonadotropin secretion and induce ovulation in women with anovulatory infertility. Several oestrogen blockers with different mechanisms of action have been developed especially for treatment of oestrogen receptor-positive breast cancer. As a result of their oestrogen blocking effect, they also inhibit the negative feedback of estradiol on the hypothalamic–pituitary axis. Use of oestrogen blockers has not been shown to increase testosterone concentrations in women (reviewed in (Handelsman, 2006)), but in men, clomiphene citrate has successfully been used to reverse premature andropause caused by steroid abuse (Tan and Vasudevan, 2003) and to cure hypogonadotropic hypogonadism in a male runner (Burge *et al*., 1997). Thus it is possible that oestrogen blockers are used to stimulate gonadotropin and testosterone secretion for doping purposes in males.

Nomenclature for hCG assays

The first assays for hCG using antisera raised against intact hCG also measured LH, but a specific radioimmunoassay was developed using a rabbit antiserum (SB6) prepared by immunization with hCGβ (Vaitukaitis *et al*., 1972). This explains why the expressions 'β-hCG assay' or 'hCG-beta assay' have become erroneously used to describe hCG assays that do not cross-react with LH. Most commercially available hCG assays measure hCG and hCGβ together. The International Federation of Clinical Chemistry recommends that assays should be exactly defined according to what they measure, for example, hCG and hCGβ separately or together (Stenman *et al*., 1993).

Determination of hCG

For clinical purposes, hCG is determined by immunoassays but methods based on immunoextraction and mass spectrometry have been developed especially for doping control. These are intended for use as confirmatory tests but there are no reports on their actual use. Immunoassays are therefore used and the only requirement of World Anti-Doping

Agency is that the assay should have a detection limit of at least 5 IU/l and that two different assays should be used (Kicman *et al*., 1991).

Effect of assay design on specificity and sensitivity

Virtually all presently used commercial hCG assays are based on the sandwich principle, that is, one antibody coupled to a solid phase captures hCG from the sample and another antibody labeled with an enzyme, a fluorophor or a luminescent marker is used to measure the amount of bound antigen. Most assays use two monoclonal antibodies whereas a few use a combination of a monoclonal antibody and a polyclonal antiserum. The use of monoclonal antibodies with known epitope specificity facilitates design of assays specific for each form of hCG (Bidart *et al*., 1985; Alfthan *et al*., 1992a). The assays used by various doping control laboratories have not been described and no assays have been designed specifically for doping control.

Several antigenic regions on hCG have been characterized; five epitopes can be discerned on hCGα (α_1–α_5) and seven on hCGβ in intact hCG (β_1–β_5, β_8–β_9). Epitopes β8 and β9 on CTP recognize hCG and hCGβ. Four epitopes (C_1–C_4) occur only on intact hCG and two (C_1 and C_2) are lost in nicked hCG (Hoermann *et al*., 1994). Two epitopes (α_6 and α_7) are specific for free hCGα, whereas β_6 and β_7 occur only on free hCGβ (Berger *et al*., 1996). The specificity of an assay can be deduced from information on the epitopes recognized by the monoclonal antibodies used (Berger *et al*., 2002) but exact information on assay specificity can only be obtained by analyzing well-defined forms of hCG with the final assay (Birken *et al*., 2003).

Although the variation in carbohydrate composition has limited effects on immunoreactivity (Schwartz *et al*., 1991; Lottersberger *et al*., 2003) two monoclonal antibodies recognizing certain carbohydrate variants have been developed. Antibodies B152 and CTP 104, which were prepared against hCG isolated from the urine of a choriocarcinoma patient, recognize carbohydrate epitopes (Kovalevskaya *et al*., 1999). Epitope of B152 consists of a biantennary core 2 *o*-glycan on Ser132 and adjacent peptide structures on hCGβ whereas CTP 104 reacts with a sialylated glycan on Ser138 (Birken, 2005). The expression 'hyperglycosylated hCG' was initially used to denote hCG with larger than normal carbohydrate chains, but presently it usually denotes the forms of hCG detected by assays using antibody B152 (Birken, 2005).

Design of hCG immunoassays for various purposes

Virtually all commercial hCG assays are designed for determination of serum samples whereas urine is used for doping control. Urine samples are also used for pregnancy tests and for identification of false-positive results in serum samples. In pregnancy, hCG is the main form in serum but hCGβ often occurs in patients with trophoblastic, testicular and nontrophoblastic cancers. Most serum assays are therefore designed to measure hCG and hCGβ together (Stenman *et al*., 2004). Only a few commercial hCG assays detect hCGβcf but they tend to underestimate that variant. This is not a problem for assay of serum, which contains very little hCGβcf (Alfthan and Stenman, 1990). Many assays utilize an antibody to CTP in combination with another antibody to hCGβ and these do not detect hCGβcf, which lacks CTP. Furthermore, CTP antibodies have only moderate affinity (Berger *et al*., 2002), which limits assay sensitivity. Although most assays measure both hCG and hCGβ, these are not always detected equally (Cole and Sutton, 2003). Assays using one antibody to hCGβ to capture hCG together with an antibody to hCGα as a tracer are specific for hCG, and some of the most sensitive assays for hCG are based on this principle (Pettersson *et al*., 1983; Alfthan *et al*., 1992a). Separate assay of the various forms of hCG provide valuable information in many clinical conditions, but few laboratories maintain more than one assay (Stenman *et al*., 2006).

Assays detecting hCG, hCGβ and hCGβcf together would be advantageous for measurement of hCG immunoreactivity in urine, which mostly contains hCG and hCGβcf whereas the content of hCGβ is low (Stenman *et al*., 2006). Immunoassays based on the binding inhibition principle, that is, classical radioimmunoassays, usually recognize most forms of hCG. Determination of hCG in urine with such assays has certain advantages for detection of a relapse after treatment of trophoblastic tumours (Mitchell, 1999), but they are generally less sensitive than sandwich assays. It is complicated to design assays detecting all forms of hCG equally with the sandwich principle (Stenman *et al*., 2006).

Sensitive assays that are specific for hCGβ have been developed in several laboratories (Ozturk *et al*., 1987; Alfthan *et al*., 1988) but commercially available hCGβ assays are usually intended for maternal screening of Down's syndrome and not suited for determination of the low concentrations occurring in serum of cancer patients (Stenman *et al*., 2004). Because most of hCGβ is broken down to hCGβcf when excreted through the kidneys, specific assay of hCGβ is of limited utility for assay of urine samples (Alfthan *et al*., 1992b).

Determination of hCGβcf and other degraded forms of hCG in urine (also called urinary gonadotropin fragments, or urinary gonadotropin peptides) has been shown to be useful for detection of several forms of nontrophoblastic cancer (O'Connor *et al*., 1988; Alfthan *et al*., 1992a; Cole, 1998). Although determination of hCGβcf is potentially useful for doping control, no assays are presently commercially available. Assay of hCG immunoreactivity in urine is further complicated by large variations in urinary flow rate, which is partially eliminated by normalization against urinary creatinine or density (Alfthan *et al*., 1993; Ngan *et al*., 1995). The correlation between hCG concentrations before and after correction for urinary density is shown in Figure 2.

Mass spectrometry

The introduction of soft ionization techniques has had a considerable impact on the analysis of large biomolecules, including the analysis of gonadotropins and related molecules (Kicman *et al*., 2007). Two ionization methods are

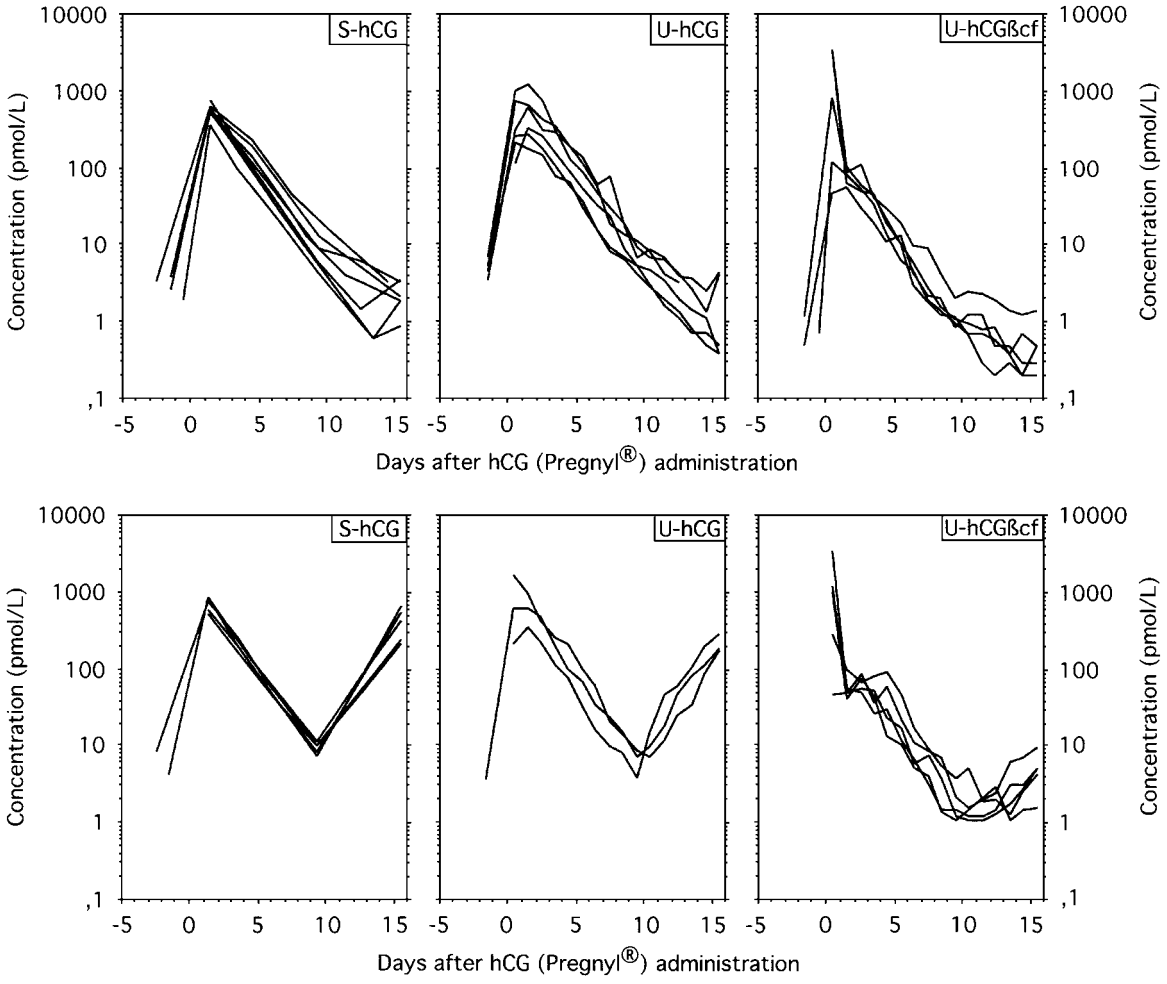

Figure 2 Serum and urine concentrations of hCG (Pregnyl) and urine concentrations of hCGβcf after intramuscular injection of 10 000 IU of hCG (Pregnyl) to women for induction of ovulations. The upper panel shows concentrations of women who did not and the lower one of women who conceived. Modified from (Stenman *et al.*, 1997) with permission.

widely used, matrix assisted laser desorption and ionization—time of flight (MALDI–TOF) and electro spray ionization (ESI). MALDI–TOF has a large mass range comprising most serum proteins and it mainly produces singly charged ions of proteins and peptides, which simplifies interpretation. However, the sensitivity of this technique decreases rapidly with increasing molecular weight and it is not a quantitative technique (Hortin, 2006). Mass spectrometers using ESI are usually coupled to a liquid chromatograph facilitating separation of peptides and proteins by reverse phase chromatography before MS analysis. The mass-to-charge (M/Z) range is limited to about 3000 Da, but because ESI produces multiply charged ions, it is possible to accurately determine the mass of proteins. For analysis by ESI techniques, proteins are usually digested enzymatically to peptides, which can be accurately quantitated. Triple quadrupole instruments, which are widely used for doping control of drug abuse, are also suited for quantitative determination of peptides. Hybrid mass spectrometers with both quadrupoles and a TOF unit are used for structural analysis of peptides but they are less suitable for quantitation (Valmu *et al.*, 2006). High-resolution MS methods, for example, Fourier transform ion cyclotron resonance and Orbitrap, provide a substantial improvement in resolution and detection limit, but they are expensive and not widely available.

HCG and its subunits are very heterogeneous due to variation in carbohydrate structure, which complicates quantitation, but various forms of hCG in urine have successfully been characterized by MS (Laidler *et al.*, 1995; Liu and Bowers, 1997; Valmu *et al.*, 2006; Kicman *et al.*, 2007). The concentrations of proteins in urine are about 1000-fold lower than in plasma, approximately 1 μmol/l, which is still more than 100 000-fold that of hCG in urine of healthy males, that is less than 10 pmol/l. Thus direct analysis of hCG in urine is not feasible, but hCG can be extracted from urine by immunopurification and then reduced, alkylated and digested with trypsin. Most of the resulting peptides can be detected by mass spectrometry and the nonglycosylated ones used for quantitation. This requires the use of an internal standard labeled with a stable isotope, but accurate quantitation is possible only if the protein is quantitatively extracted and completely digested. HCG occurring at concentrations of 5–25 IU/l has been detected by mass spectrometry after immunoextraction (Liu and Bowers, 1996; Gam *et al.*, 2003). However, without the

use of an internal standard, the determination is not quantitative. Isotope-labeled hCG would facilitate monitoring of both extraction and digestion, but such a standard can presently be obtained only by biosynthesis and has not yet been produced. Furthermore, part of the immunoreactive hCG in urine is present as fragments, which may or may not be extracted by the immunoaffinity methods. These problems need to be solved before mass spectrometry is a viable alternative to immunoassay for determination of hCG in urine.

Mass spectrometry is a potentially useful reference method for protein determinations. Such a method has recently been developed for insulin using an isotope-labeled synthetic insulin internal standard. The standard was added to plasma and extracted together with native insulin by immuno adsorption after which insulin was determined with a triple quadrupole instrument. Recovery of the internal standard added to plasma was 30–40% (Van Uytfanghe *et al*., 2007). This demonstrates the necessity to use an internal standard corresponding to the protein to be measured rather than a standard consisting of the peptide(s) used for quantitation.

Assay methods for LH

Luteinizing hormone is also determined by immunoassay, but assay characteristics and decision limits to be used for doping control have not been issued. A recent report aiming at defining reference values for LH in urine samples from top-level female and male athletes was recently published (Robinson *et al*., 2007).

Presently all commercial LH immunoassays are based on the sandwich principle using two monoclonal or in some cases one monoclonal capture antibody and a polyclonal detector antibody. The choice of reagents is critical because some antibodies do not detect a fairly common variant of LH (Pettersson and Soderholm, 1991). The anomalous variant has two point mutations in the gene for LHβ, Trp8Arg and Ile15Thr and 0–50% of various populations are homo- or heterozygous for these mutations (Raivio *et al*., 1996). Some monoclonal antibodies do not recognize the LH variant while other antibodies underestimate it. This explains the considerable variation in LH results obtained in earlier used assays (Vermes *et al*., 1991). Antibodies detecting wild-type and variant LH equally are available, and the problem is well known. However, based on data from quality assessment programs, there are still two-fold differences in the results obtained by some LH assays (Alfthan H and Stenman UH, unpublished).

Like hCG, LH is excreted into urine at concentrations similar to those in serum. A substantial part of LH is degraded into LHβcf during passage through the kidneys. This fragment is detected by some but not by other immunoassays. In menstruating women, the peak of LH immunoreactivity in urine often appears 2–3 days after the LH peak in serum and this peak is caused by delayed excretion of LHβcf (O'Connor *et al*., 1998). It is therefore important to know the specificity of the LH assays used for doping control.

Reference values for various forms of hCG

The concentrations of hCG in healthy men have to be considered when using hCG determinations in urine for doping control. The presence of hCG in urine from nonpregnant subjects was first demonstrated with an immunoassay utilizing an antibody to CTP (Chen *et al*., 1976) and hCG was later isolated from pituitary extracts (Birken *et al*., 1996). Low concentrations of hCG occur in serum from men and nonpregnant women and the concentrations increase after the menopause in women whereas a smaller increase is observed in men after 60 years of age (Alfthan *et al*., 1992a). The secretion of pituitary hCG is regulated by GnRH in the same way as LH secretion, and in postmenopausal women the concentrations can be suppressed by oestrogen treatment (Stenman *et al*., 1987). The serum concentrations fluctuate in a pattern similar to that of LH (Odell and Griffin, 1987). The hCG concentrations expressed in IU/l are about 3–10% of those of LH. These results indicate that hCG in serum from men and nonpregnant women is mainly derived from the pituitary (Odell and Griffin, 1987; Stenman *et al*., 1987). Low-level expression of the genes for both hCG subunits occurs in the testis, breast, prostate and skeletal muscle (Bellet *et al*., 1997) but it is unlikely that these tissues secrete measurable amounts of hCG into circulation. The pituitary produces free α subunit (GPHα) in excess of the β subunits and concentrations up to 100 IU/l occur in men and menstruating women and they increase further after the menopause (Stenman *et al*., 2006).

Serum from healthy men and nonpregnant women contains hCGβ at low concentrations that do not increase with age (Alfthan *et al*., 1992a). Elevated serum concentrations occur in 30–70% of patients with non-trophoblastic cancer (Marcillac *et al*., 1992) and this is associated with similar concentrations of hCGβcf in urine (Alfthan *et al*., 1992a). This is a potential cause of increased hCG immunoreactivity in urine.

The reference values for hCG, hCGβ and hCGβcf in urine are important when determination of hCG is used for doping control (Table 3). To facilitate comparison of the concentrations of various forms, they are expressed in pmol/l and for

Table 3 Reference limits for serum and urine concentrations of hCG, hCGβ and hCGβcf in nonpregnant women and in men

	Women				*Men*			
	Menstruating		*Postmenopausal*		*< 50 years*		*⩾ 50 years*	
	(pmol/l)	*IU/l*	*(pmol/l)*	*IU/l*	*(pmol/l)*	*IU/l*	*(pmol/l)*	*IU/l*
Serum								
hCG	8.6	3.0	15.5	5.4	2.1	0.7	6.1	2.1
hCGβ	1.6		2.0		1.9		2.1	
Total hCG	9.0		17.0		3.2		7.1	
Urine								
hCG	8.8	3.1	11.5	4.0	2.9	1.0	8.4	2.9
hCGβ	1.7		4.3		1.3		3.6	
hCGβcf	8.1		9.5		6.7		8.5	
Total hCG	13.6		20.4		8.0		21.5	

Modified from (Alfthan *et al*., 1992a).

hCG also in IU/l. One IU of hCG corresponds to 0.11 μg and 2.9 pmol, that is, the values in pmol/l are roughly threefold those in IU/l (Table 1). The concentrations of hCGβ are seldom expressed in IU/l based on its own standard (the 3rd IS) but rather in pmol/l (Stenman *et al*., 1993).

We have determined the reference values for hCG in serum and urine on the basis of values in 1029 serum and 432 urine samples from men and women of various ages and calculated the upper reference limit using the 97.5th percentile. For serum hCG the limits are 3 and 5.4 IU/l in fertile and postmenopausal women, respectively, whereas those for men below and above 60 years of age are 0.7 and 2.1 IU/l, respectively (Table 3). Occasional values up to 8 IU/l may be observed in women and up to 5 IU/l in men (Figure 3). The upper reference limit for hCGβ is 2 pmol/l both in men and women and it is not dependent on age. The concentrations of hCG and hCGβ in urine are slightly higher than those in serum. Importantly, urine contains hCGβcf at concentrations similar to those of hCG (Figure 4). Therefore, the concentration of total hCG immunoreactivity in urine (calculated by adding the values of hCG, hCGβ and hCGβcf in each subject) is about 30% higher than in serum in women and two times as high in men (Table 3). This has to be taken into account if assays recognizing all these forms are used to measure hCG in urine. Reference values for hCG in urine have been established for only one commercial assay whereas values for hCGβ and hCGβcf are based on in-house methods (Alfthan *et al*., 1992a). Reliable information on the reference values and recognition of the various forms of hCG in urine by other commercial assays is not available.

The values in Table 3 were determined by highly sensitive immunofluorometric assays and are strictly valid only for these (Alfthan *et al*., 1992a). The calibration of assays varies and in a study on the suitability of two commercial hCG assays for doping control with urine samples, a 30% difference in calibration was observed (Shahzad *et al*., 2007) and twofold differences are observed in quality assessment schemes (Alfthan H and Stenman UH, unpublished). An upper reference limit for serum hCG of 5 IU/l is recommended in most textbooks but higher upper reference limits need to be used for many commercial methods. Serum concentrations up to 14 IU/l were observed in a recent study on 720 women (Snyder *et al*., 2005). It is necessary that reference values for urine are determined for each assay. Very few assays are approved for determination of hCG in urine and it is actually surprising if athletes can be banned on the basis of results that have been determined with methods that have not been validated for assay of hCG in urine. The salt concentration in urine may vary more than 10-fold which affects the affinity of some antibodies. Validation of assays used for urine samples is therefore important.

Chemotherapy used to treat malignant tumours often induces gonadal suppression and elevation of serum hCG to 'postmenopausal' concentrations (Stenman *et al*., 2004). This

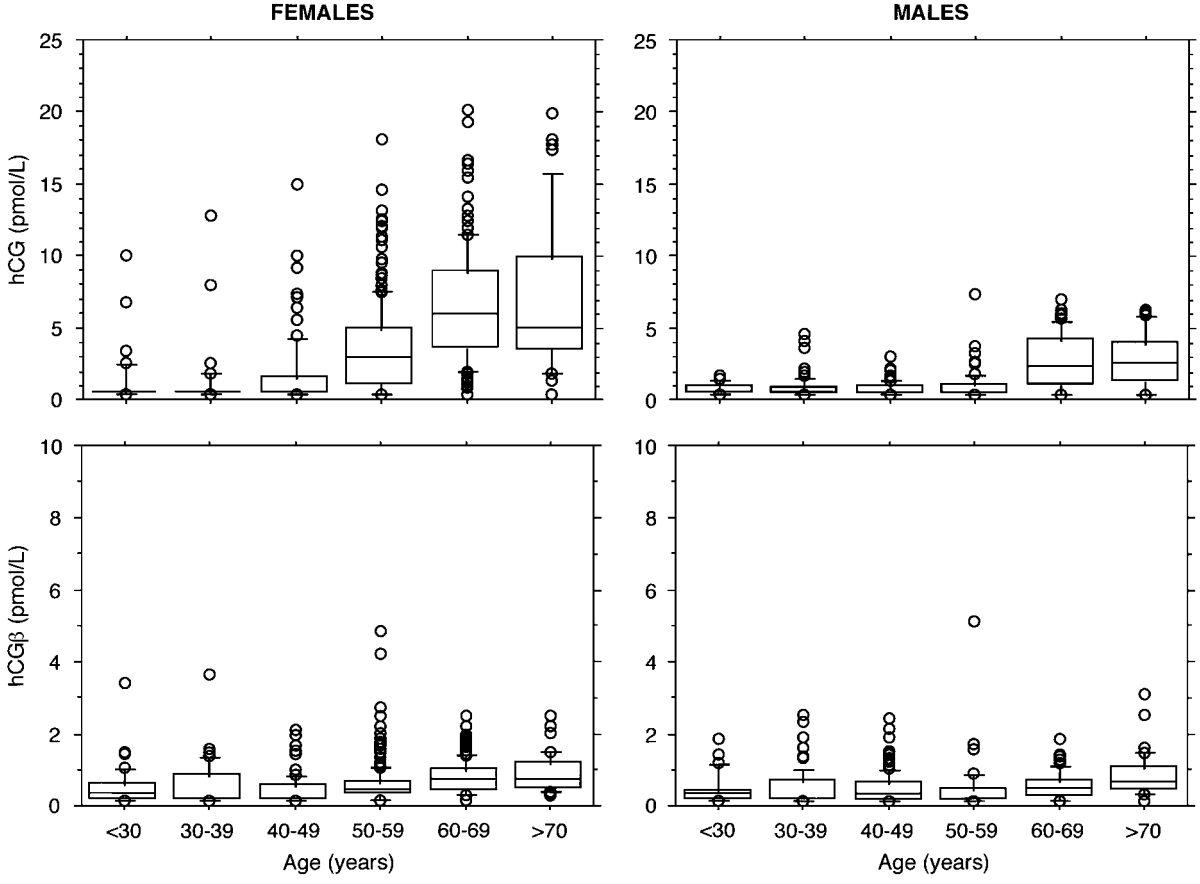

Figure 3 Distribution of serum concentrations of hCG (upper panel) and hCGβ (lower panel) in 423 men and 606 women. Reproduced from (Alfthan *et al*., 1992a) with permission.

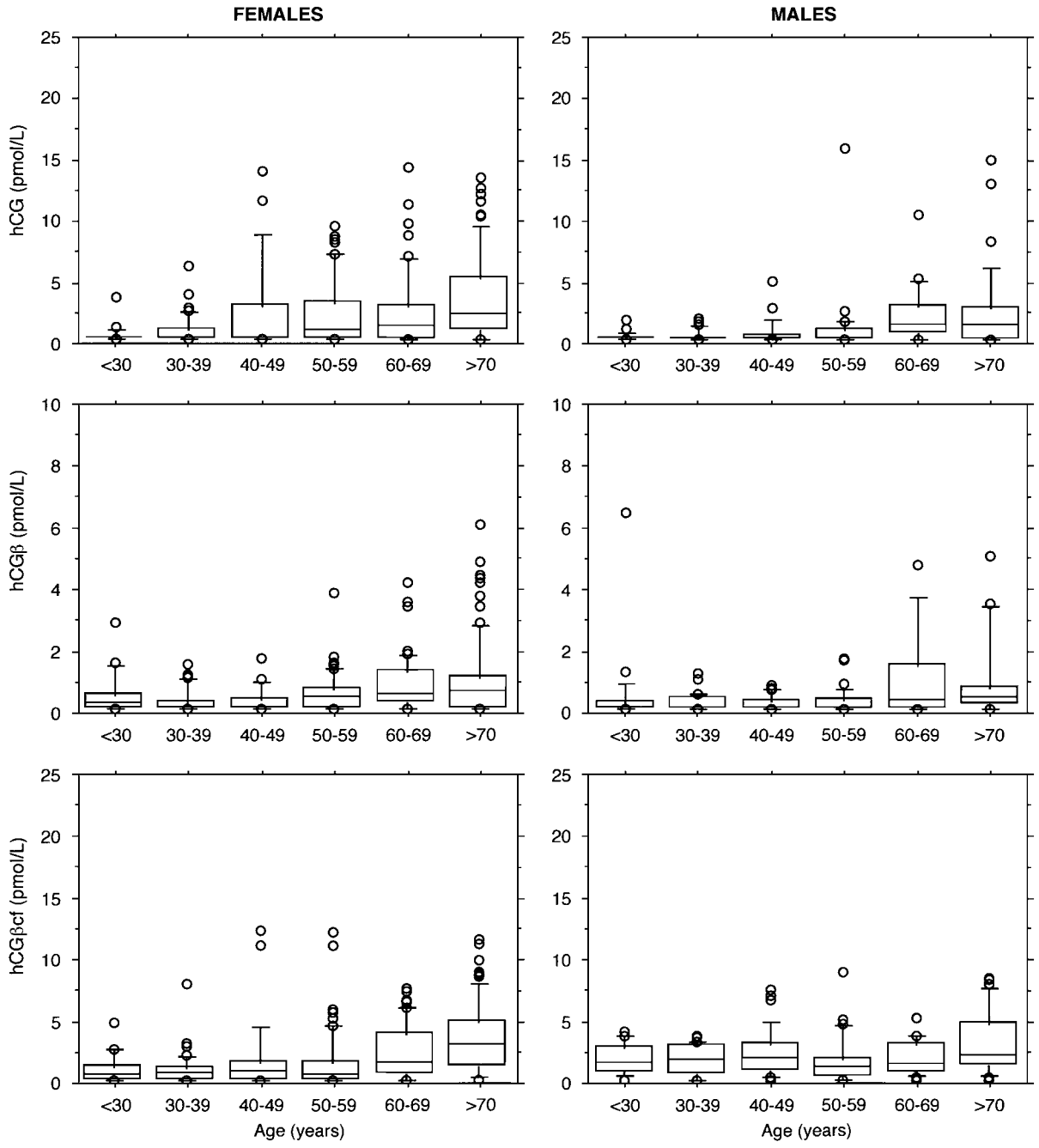

Figure 4 Distribution of urine concentrations of hCG (upper panel) and hCGβ (lower panel) in 172 men and 423 women. (Alfthan *et al.*, 1992a) Reproduced with permission from Clinical Chemistry.

is important when young men treated for testicular cancer are monitored by determination of hCG. We recently observed serum concentrations of hCG increasing from less than 1 to 4 IU/l in a young man who had gonadal malfunction after unilateral orchiectomy for a seminoma. The hCG concentration increased over several months when he stopped using testosterone replacement. The condition was identified on the basis of highly elevated serum concentrations of LH and FSH, and these and the hCG concentration were suppressed by testosterone replacement (Lempiainen *et al.*, 2007).

Reference values for LH

GnRH is released from the hypothalamus in intermittent secretory bursts resulting in a pulsatile secretion of LH and FSH. The pulsatile mode of GnRH release is critical for physiological function of the gonadotrophs and a prerequisite for normal reproductive function. Normally, the peak interval is 90–120 min and the difference between nadir and peak concentrations is often more then threefold (Bergendahl *et al.*, 1996). In fertile women the concentrations additionally vary during the menstrual cycle, although

they are fairly stable in males. Reference values determined by the Abbott Architect method for LH in serum of ovulatory women were recently published. During the follicular and luteal phases the LH concentrations varied between 3 and 10 IU/l and during the LH surge from 21 to 74 IU/l (Stricker *et al*., 2006). These values are valid only for this assay, and comparison of the result in the Finnish Labquality quality control programme show that the results for other assays are between 60 and 150% of those obtained with the Architect assay (Alfthan H and Stenman UH, unpublished). Values for LH in urine corrected for urinary creatinine have been determined with the Perkin-Elmer Wallac Delfia assay. There was larger variation between individuals and the concentrations tend to increase between early and late follicular phase being in the range 1–25 IU/g creatinine. During the LH peak values of 30–100 IU/g creatinine were reached and after the peak the concentrations slowly decreased to less than 10 IU/g creatinine by the end of the luteal phase. It is notable that this assay detected LHβcf (Park *et al*., 2007), but this form is not detected by the LH assay presently available from this manufacturer. To establish cutoff levels to be used for doping control, LH was determined by the Access LH assay from Beckman Coulter and the Elecsys LH from Roche in urine samples from 1066 male and 552 female athletes sent to a doping control laboratory. The correlation between the assays was acceptable ($R = 0.953$) and the slope of the regression line was 1.04 but up to 50% differences were observed in some samples. The 99.9 percentile was 37 IU/l in women and 15 IU/l in men. The results were not corrected for variation in urinary flow rate (Robinson *et al*., 2007).

Sample handling

Gonadotropins are much less stable in urine than in serum and care should be taken to avoid loss of immunoreactivity. Addition of glycerol to a concentration of 7% and storage of the samples at −25 °C has been shown to retain LH immunoreactivity (Livesey *et al*., 1983). However, it is not known whether the assay used recognized LH fragments, which might have formed during storage. In our experience, LH is stable for several weeks when stored at +4 °C with sodium azide (0.02%) to prevent bacterial growth (Demir *et al*., 1994). When stored at −20 °C a highly variable loss of immunoreactivity may occur, in some samples more than 90% is lost. The loss depends on storage time, but in some samples it occurs after a single round of freezing and thawing.

HCG is somewhat more stable than LH, but freezing at −20 °C may cause rapid loss of immunoreactivity. In some samples the loss of hCG is accompanied by a corresponding increase in hCGβ, indicating dissociation of the subunits, but in some samples all forms of immunoreactivity are reduced. Fresh urine contains rather little hCGβ and the presence of this form in urine is usually a sign of inappropriate storage. HCGβcf is somewhat more stable than hCG, but there is large variation between samples. Like LH, hCG is quite stable when stored at +4 °C with 0.02% azide (Stenman *et al*., 2006). Nonspecific adsorption of urinary proteins to the walls of certain plastic tubes is a further preanalytical problem. In concentrated samples the loss is small but in very dilute ones it may be substantial.

Unusual causes of positive hCG results

An elevated hCG concentration in serum of a man is a strong indication of testicular cancer although nontrophoblastic tumours may produce only hCGβ, which is detected by most hCG assays. Therefore, patients with a falsely elevated hCG value caused by nonspecific interference have inadvertently been treated as cancer patients (Rotmensch and Cole, 2000). Heterophilic antibodies are the most common causes of falsely elevated immunoassay results in serum (Stenman *et al*., 2006), but immunoglobulins are not excreted into urine and therefore this is not a problem in doping control. False-positive results in serum can actually be identified by measuring hCG in urine (Stenman *et al*., 2004).

Production of hCG by a testicular cancer is a potential cause of elevated hCG values in urine of a young man, whereas tumours producing hCGβ are likely to cause elevation of hCGβcf in urine, which may lead to a positive result with some assays. However, it is unlikely that this condition is misinterpreted to indicate doping. If a tumour is suspected, the hCG concentrations should be measured in a serum sample.

Evidential analysis—what is fit for the purpose?

Gonadotropins are probably widely used to normalize testosterone secretion after administration of anabolic steroid hormones, but demonstration of illicit use is complicated by the presence of both hCG and LH in plasma and urine. The physiological concentrations LH vary considerably due to ultradian and circadian fluctuation and the concentrations in urine are additionally dependent on more than 10-fold variations in urinary flow rate. Furthermore, part of LH and hCG occur in urine as fragments, which are differently detected by various assay methods. Mass spectrometry is not expected to solve these problems soon. Detection of gonadotropins in urine is furthermore complicated by poor stability when urine is frozen at −20 °C. To make detection of illicit use of gonadotropins reliable it is therefore necessary to optimize the whole chain of events from sample collection and handling, selection of assay methods, establishment of reference values and definition of decision limits.

The methods used to determine hCG have changed since the studies, on which the present recommendations are based, were performed. Therefore, there is an urgent need to validate the assays presently used for doping control. The radioimmunoassay initially used have been replaced by more sensitive immunometric assays based on the sandwich principle and some automated assays can reliably detect hCG concentrations below 1 IU/l, that is, well below decision limits that are relevant for doping control (Alfthan *et al*., 1992a; Stenman *et al*., 2006). This eliminates the need to concentrate urine before immunoassay, which was considered necessary with some earlier assays (Cowan *et al*., 1991;

Laidler *et al*., 1994). Cross-reaction with LH, which was a problem in early hCG assays, is not an issue any more.

The variable concentrations of salts and metabolites in urine are a potential source of immunoassay interference. Because most assays have been designed for assay of serum, the calibrators are usually serum-based. The high salt concentration that may occur in urine reduces the affinity of some antibodies, which might cause a false low result in sandwich assays. This problem can be avoided by the use of inherently sensitive assays in which the sample volume is small in relation to the assay volume. This should be ascertained by spiking urine samples of various densities with hCG standards.

Owing to the occurrence of fragmented forms of hCG in urine, it is necessary to determine the specificity of the assay used. Specific determination of the three major forms of hCG in urine shows that the upper reference limit for 'total hCG immunoreactivity' (hCG, hCGβ and hCGβcf) is 2 to threefold that of hCG alone (Table 3). The assay specificity should be determined using the WHO reference reagents (Birken *et al*., 2003). Furthermore, the decision limits of each assay should be determined using a sufficient number of urine samples collected under the same conditions as used for doping control. Due to variation in assay standardization and specificity (Stenman, 2004) the decision limits indicating illicit use of hCG are likely to be assay specific. The decision limit of 10 IU/l proposed by Laidler *et al*. (1994) is probably fairly safe, but this has to be verified. As a first step, the assays and decision limits used in different doping laboratories should be reported. A limited number of methods should be extensively validated and those found suitable selected for routine use. Ideally, the methods used for doping control should be identically standardized, but this goal is unrealistic if commercial methods are used (Stenman, 2004). Establishment of hCG assays specifically designed for doping control is possible, but it is unlikely that all doping laboratories would be able to maintain such methods.

Determination of LH and the testosterone/LH ratio has been shown to provide additional evidence for self-administration of testosterone (Kicman *et al*., 1990) but LH assays are not included in the testing protocol. If adopted for use in doping control, the assays used should be evaluated in the same way as hCG assays.

Sample handling also needs to be standardized. The presently used handling protocols are likely to cause loss of hCG and LH immunoreactivity. Although this does not cause false-positive results, it renders the use of stored samples for establishment of reference values questionable. Although glycerol is a potentially useful additive, it is incompatible with the mass spectrometric methods used to analyze hormones and drugs. This problem needs to be studied and optimal methods developed.

Acknowledgements

Support from the Finnish Academy of Sciences, The Sigrid Juselius Foundations, The Finnish Cancer Society and Finska Läkaresällskapet is gratefully acknowledged.

Conflict of interest

Ulf-Håkan Stenman has served as a consultant for Perkin-Elmer Wallac, Abbott Diagnostics and Orion Diagnostica.

References

Al-Inany H, Aboulghar MA, Mansour RT, Proctor M (2005). Recombinant versus urinary gonadotrophins for triggering ovulation in assisted conception. *Hum Reprod* **20**: 2061–2073.

Alexander SP, Mathie A, Peters JA (2007). Guide to receptors and channels, 2nd edition (2007 Revision). *Br J Pharmacol* **150** (Suppl 1): S1–S168.

Alfthan H, Bjorses UM, Tiitinen A, Stenman UH (1993). Specificity and detection limit of ten pregnancy tests. *Scand J Clin Lab Invest Suppl* **216**: 105–113.

Alfthan H, Haglund C, Dabek J, Stenman U-H (1992a). Concentrations of human chorionic gonadotropin, its β-subunit and the core fragment of the β-subunit in serum and urine of men and nonpregnant women. *Clin Chem* **38**: 1981–1987.

Alfthan H, Haglund C, Roberts P, Stenman UH (1992b). Elevation of free beta subunit of human choriogonadotropin and core beta fragment of human choriogonadotropin in the serum and urine of patients with malignant pancreatic and biliary disease. *Cancer Res* **52**: 4628–4633.

Alfthan H, Schröder J, Fraser R, Koskimies A, Halila H, Stenman U-H (1988). Choriogonadotropin and its β subunit separated by hydrophobic-interaction chromatography and quantified in serum during pregnancy by time-resolved immunofluorometric assays. *Clin Chem* **34**: 1758–1762.

Alfthan H, Stenman U-H (1990). Pregnancy serum contains the beta-core fragment of human choriogonadotropin. *J Clin Endocrinol Metab* **70**: 783–787.

Amoresano A, Siciliano R, Orru S, Napoleoni R, Altarocca V, De Luca E *et al*. (1996). Structural characterisation of human recombinant glycohormones follitropin, lutropin and choriogonadotropin expressed in Chinese hamster ovary cells. *Eur J Biochem* **242**: 608–618.

Apter D, Cacciatore B, Alfthan H, Stenman UH (1989). Serum luteinizing hormone concentrations increase 100-fold in females from 7 years to adulthood, as measured by time-resolved immunofluorometric assay. *J Clin Endocrinol Metab* **68**: 53–57.

Baenziger JU, Kumar S, Brodbeck RM, Smith PL, Beranek MC (1992). Circulatory half-life but not interaction with the lutropin/chorionic gonadotropin receptor is modulated by sulfation of bovine lutropin oligosaccharides. *Proc Natl Acad Sci USA* **89**: 334–338.

Bellet D, Lazar V, Bieche I, Paradis V, Giovangrandi Y, Paterlini P *et al*. (1997). Malignant transformation of nontrophoblastic cells is associated with the expression of chorionic gonadotropin beta genes normally transcribed in trophoblastic cells. *Cancer Res* **57**: 516–523.

Bergendahl M, Evans WS, Veldhuis JD (1996). Current concepts on ultradian rhythms of luteinizing hormone secretion in the human. *Hum Reprod Update* **2**: 507–518.

Berger P, Bidart JM, Delves PS, Dirnhofer S, Hoermann R, Isaacs N *et al*. (1996). Immunochemical mapping of gonadotropins. *Mol Cell Endocrinol* **125**: 33–43.

Berger P, Sturgeon C, Bidart JM, Paus E, Gerth R, Niang M *et al*. (2002). The ISOBM TD-7 workshop on hCG and related molecules. Towards user-oriented standardization of pregnancy and tumor diagnosis: assignment of epitopes to the three-dimensional structure of diagnostically and commercially relevant monoclonal antibodies directed against human chorionic gonadotropin and derivatives. *Tumour Biol* **23**: 1–38.

Bidart JM, Ozturk M, Bellet DH, Jolivet M, Gras-Masse H, Troalen F *et al*. (1985). Identification of epitopes associated with hCG and the beta hCG carboxyl terminus by monoclonal antibodies produced against a synthetic peptide. *J Immunol* **134**: 457–464.

Birken S (2005). Specific measurement of o-linked core 2 sugar-containing isoforms of hyperglycosylated human chorionic gonadotropin by antibody b152. *Tumour Biol* **26**: 131–141.

Birken S, Armstrong EG, Kolks MA, Cole LA, Agosto GM, Krichevsky A *et al*. (1988). Structure of the human chorionic gonadotropin beta-subunit fragment from pregnancy urine. *Endocrinology* **123**: 572–583.

Birken S, Berger P, Bidart JM, Weber M, Bristow A, Norman R *et al*. (2003). Preparation and characterization of new WHO reference reagents for human chorionic gonadotropin and metabolites. *Clin Chem* **49**: 144–154.

Birken S, Maydelman Y, Gawinowicz MA, Pound A, Liu Y, Hartree AS (1996). Isolation and characterization of human pituitary chorionic gonadotropin. *Endocrinology* **137**: 1402–1411.

Blithe DL, Richards RG, Skarulis MC (1991). Free alpha molecules from pregnancy stimulate secretion of prolactin from human decidual cells: a novel function for free alpha in pregnancy. *Endocrinology* **129**: 2257–2259.

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

Burge MR, Lanzi RA, Skarda ST, Eaton RP (1997). Idiopathic hypogonadotropic hypogonadism in a male runner is reversed by clomiphene citrate. *Fertil Steril* **67**: 783–785.

Butler SA, Iles RK (2003). Ectopic human chorionic gonadotropin beta secretion by epithelial tumors and human chorionic gonadotropin beta-induced apoptosis in Kaposi's sarcoma: is there a connection? *Clin Cancer Res* **9**: 4666–4673.

Butler SA, Iles RK (2004). The free monomeric beta subunit of human chorionic gonadotrophin (hCG beta) and the recently identified homodimeric beta–beta subunit (hCG beta–beta) both have autocrine growth effects. *Tumour Biol* **25**: 18–23.

Canfield RE, Ross GT (1976). A new reference preparation of human chorionic gonadotrophin and its subunits. *Bull World Health Organ* **54**: 463–472.

Chang P, Kenley S, Burns T, Denton G, Currie K, DeVane G *et al*. (2001). Recombinant human chorionic gonadotropin (rhCG) in assisted reproductive technology: results of a clinical trial comparing two doses of rhCG (Ovidrel) to urinary hCG (Profasi) for induction of final follicular maturation in *in vitro* fertilization-embryo transfer. *Fertil Steril* **76**: 67–74.

Chen HC, Hodgen GD, Matsuura S, Lin LJ, Gross E, Reichert Jr LE *et al*. (1976). Evidence for a gonadotropin from nonpregnant subjects that has physical, immunological, and biological similarities to human chorionic gonadotropin. *Proc Natl Acad Sci USA* **73**: 2885–2889.

Cole LA (1998). hCG, its free subunits and its metabolites. Roles in pregnancy and trophoblastic disease. *J Reprod Med* **43**: 3–10.

Cole LA, Kardana A, Ying FC, Birken S (1991). The biological and clinical significance of nicks in human chorionic gonadotropin and its free beta-subunit. *Yale J Biol Med* **64**: 627–637.

Cole LA, Kohorn EI (2006). The need for an hCG assay that appropriately detects trophoblastic disease and other hCG-producing cancers. *J Reprod Med* **51**: 793–811.

Cole LA, Shahabi S, Oz UA, Bahado-Singh RO, Mahoney MJ (1999). Hyperglycosylated human chorionic gonadotropin (invasive trophoblast antigen) immunoassay: a new basis for gestational down syndrome screening. *Clin Chem* **45**: 2109–2119.

Cole LA, Sutton JM (2003). HCG tests in the management of gestational trophoblastic diseases. *Clin Obstet Gynecol* **46**: 523–540.

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.

de Boer D, de Jong EG, van Rossum JM, Maes RA (1991). Doping control of testosterone and human chorionic gonadotrophin: a case study. *Int J Sports Med* **12**: 46–51.

Delemarre-van de Waal HA (2004). Application of gonadotropin releasing hormone in hypogonadotropic hypogonadism—diagnostic and therapeutic aspects. *Eur J Endocrinol* **151** (Suppl 3): U89–U94.

Demir A, Alfthan H, Stenman UH, 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 A, Voutilainen R, Juul A, Dunkel L, Alfthan H, Skakkebaek NE *et al*. (1996). Increase in first morning voided urinary luteinizing hormone levels precedes the physical onset of puberty. *J Clin Endocrinol Metab* **81**: 2963–2967.

Elliott MM, Kardana A, Lustbader JW, Cole LA (1997). Carbohydrate and peptide structure of the alpha- and beta-subunits of human chorionic gonadotropin from normal and aberrant pregnancy and choriocarcinoma. *Endocrine* **7**: 15–32.

European Recombinant LH Study Group (2001). Human recombinant luteinizing hormone is as effective as, but safer than, urinary human chorionic gonadotropin in inducing final follicular maturation and ovulation in *in vitro* fertilization procedures: results of a multicenter double-blind study. *J Clin Endocrinol Metab* **86**: 2607–2618.

Filicori M, Fazleabas AT, Huhtaniemi I, Licht P, Rao Ch V, Tesarik J *et al*. (2005). Novel concepts of human chorionic gonadotropin: reproductive system interactions and potential in the management of infertility. *Fertil Steril* **84**: 275–284.

Gam LH, Tham SY, Latiff A (2003). Immunoaffinity extraction and tandem mass spectrometric analysis of human chorionic gonadotropin in doping analysis. *J Chromatogr B Analyt Technol Biomed Life Sci* **792**: 187–196.

Gervais A, Hammel YA, Pelloux S, Lepage P, Baer G, Carte N *et al*. (2003). Glycosylation of human recombinant gonadotrophins: characterization and batch-to-batch consistency. *Glycobiology* **13**: 179–189.

Hamada AL, Nakabayashi K, Sato A, Kiyoshi K, Takamatsu Y, Laoag-Fernandez JB *et al*. (2005). Transfection of antisense chorionic gonadotropin beta gene into choriocarcinoma cells suppresses the cell proliferation and induces apoptosis. *J Clin Endocrinol Metab* **90**: 4873–4879.

Handelsman DJ (2006). Clinical review: the rationale for banning human chorionic gonadotropin and estrogen blockers in sport. *J Clin Endocrinol Metab* **91**: 1646–1653.

Hoermann R, Berger P, Spoettl G, Gillesberger F, Kardana A, Cole LA *et al*. (1994). Immunological recognition and clinical significance of nicked human chorionic gonadotropin in testicular cancer. *Clin Chem* **40**: 2306–2312.

Hortin GL (2006). The MALDI-TOF mass spectrometric view of the plasma proteome and peptidome. *Clin Chem* **52**: 1223–1237.

Kessler MJ, Mise T, Ghai RD, Bahl OP (1979a). Structure and location of the *O*-glycosidic carbohydrate units of human chorionic gonadotropin. *J Biol Chem* **254**: 7909–7914.

Kessler MJ, Reddy MS, Shah RH, Bahl OP (1979b). Structures of *N*-glycosidic carbohydrate units of human chorionic gonadotropin. *J Biol Chem* **254**: 7901–7908.

Kicman AT, Brooks RV, Collyer SC, Cowan DA, Nanjee MN, Southan GJ *et al*. (1990). Criteria to indicate testosterone administration. *Br J Sports Med* **24**: 253–264.

Kicman AT, Brooks RV, Cowan DA (1991). Human chorionic gonadotrophin and sport. *Br J Sports Med* **25**: 73–80.

Kicman AT, Cowan DA (1992). Peptide hormones and sport: misuse and detection. *Br Med Bull* **48**: 496–517.

Kicman AT, Parkin MC, Iles RK (2007). An introduction to mass spectrometry based proteomics-detection and characterization of gonadotropins and related molecules. *Mol Cell Endocrinol* **260–262**: 212–227.

Korhonen J, Alfthan H, Ylostalo P, Veldhuis J, Stenman U-H (1997). Disappearance of human chorionic gonadotropin and its alpha- and beta- subunits after term pregnancy. *Clin Chem* **43**: 2155–2163.

Kovalevskaya G, Birken S, Kakuma T, O'Connor JF (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, Kakuma T, Ozaki N, Sauer M, Lindheim S *et al*. (2002). Differential expression of human chorionic gonadotropin (hCG) glycosylation isoforms in failing and continuing pregnancies: preliminary characterization of the hyperglycosylated hCG epitope. *J Endocrinol* **172**: 497–506.

Laidler P, Cowan DA, Hider RC, Keane A, Kicman AT (1995). Tryptic mapping of human chorionic gonadotropin by matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun Mass Spectrom* **9**: 1021–1026.

Laidler P, Cowan DA, Hider RC, Kicman AT (1994). New decision limits and quality-control material for detecting human chorionic gonadotropin misuse in sports. *Clin Chem* **40**: 1306–1311.

le Cotonnec JY, Loumaye E, Porchet HC, Beltrami V, Munafo A (1998a). Pharmacokinetic and pharmacodynamic interactions between recombinant human luteinizing hormone and recombinant human follicle-stimulating hormone. *Fertil Steril* **69**: 201–209.

le Cotonnec JY, Porchet HC, Beltrami V, Munafo A (1998b). Clinical pharmacology of recombinant human luteinizing hormone: Part I. Pharmacokinetics after intravenous administration to healthy female volunteers and comparison with urinary human luteinizing hormone. *Fertil Steril* **69**: 189–194.

Lempiainen A, Hotakainen K, Blomqvist C, Alfthan H, Stenman UH (2007). Increased human chorionic gonadotropin due to hypogonadism after treatment of a testicular seminoma. *Clin Chem* **53**: 1560–1561.

Liu C, Bowers LD (1997). Mass spectrometric characterization of the beta-subunit of human chorionic gonadotropin. *J Mass Spectrom* **32**: 33–42.

Liu CL, Bowers LD (1996). Immunoaffinity trapping of urinary human chorionic gonadotropin and its high-performance liquid chromatographic-mass spectrometric confirmation. *J Chromatogr B Biomed Appl* **687**: 213–220.

Liu PY, Gebski VJ, Turner L, Conway AJ, Wishart SM, Handelsman DJ (2002). Predicting pregnancy and spermatogenesis by survival analysis during gonadotrophin treatment of gonadotrophin-deficient infertile men. *Hum Reprod* **17**: 625–633.

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.

Lottersberger C, Hoermann R, Mann K, Schwarz S, Berger P (2003). Tumor- and pregnancy-derived isoforms of human chorionic gonadotropin: biological and diagnostic relevance. *Horm Res* **59**: 125–134.

Manna PR, Joshi L, Reinhold VN, Aubert ML, Suganuma N, Pettersson K *et al*. (2002). Synthesis, purification and structural and functional characterization of recombinant form of a common genetic variant of human luteinizing hormone. *Hum Mol Genet* **11**: 301–315.

Marcillac I, Troalen F, Bidart JM, Ghillani P, Ribrag V, Escudier B *et al*. (1992). Free human chorionic gonadotropin beta subunit in gonadal and nongonadal neoplasms. *Cancer Res* **52**: 3901–3907.

Menon DK (2003). Successful treatment of anabolic steroid-induced azoospermia with human chorionic gonadotropin and human menopausal gonadotropin. *Fertil Steril* **79** (Suppl 3): 1659–1661.

Mitchell H (1999). Analysis of hCG: clinical applications and assay requirements. *Ann Clin Biochem* **36** (Part 2): 259–260.

Mizuochi T, Nishimura R, Derappe C, Taniguchi T, Hamamoto T, Mochizuki M *et al*. (1983). Structures of the asparagine-linked sugar chains of human chorionic gonadotropin produced in choriocarcinoma. Appearance of triantennary sugar chains and unique biantennary sugar chains. *J Biol Chem* **258**: 14126–14129.

Moy E, Kimzey LM, Nelson LM, Blithe DL (1996). Glycoprotein hormone alpha-subunit functions synergistically with progesterone to stimulate differentiation of cultured human endometrial stromal cells to decidualized cells: a novel role for free alpha-subunit in reproduction. *Endocrinology* **137**: 1332–1339.

Nemansky M, Moy E, Lyons CD, Yu I, Blithe DL (1998). Human endometrial stromal cells generate uncombined alpha-subunit from human chorionic gonadotropin, which can synergize with progesterone to induce decidualization. *J Clin Endocrinol Metab* **83**: 575–581.

Ngan HY, Yeung WS, Cheng GT, Wong LC, de Madeiros SF, Norman RJ *et al*. (1995). Problems in the use of urinary hCG-beta-core as a tumor marker in gynecologic cancer. *Int J Gynecol Cancer* **5**: 15–19.

O'Connor JF, Kovalevskaya G, Birken S, Schlatterer JP, Schechter D, McMahon DJ *et al*. (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 JF, Schlatterer JP, Birken S, Krichevsky A, Armstrong EG, McMahon D *et al*. (1988). Development of highly sensitive immunoassays to measure human chorionic gonadotropin, its beta-subunit, and beta core fragment in the urine: application to malignancies. *Cancer Res* **48**: 1361–1366.

Odell WD, Griffin J (1987). Pulsatile secretion of human chorionic gonadotropin in normal adults. *N Engl J Med* **317**: 1688–1691.

Ozturk M, Bellet D, Manil L, Hennen G, Frydman R, Wands J (1987). Physiological studies of human chorionic gonadotropin (hCG), alpha hCG, and beta hCG as measured by specific monoclonal immunoradiometric assays. *Endocrinology* **120**: 549–558.

Park SJ, Goldsmith LT, Skurnick JH, Wojtczuk A, Weiss G (2007). Characteristics of the urinary luteinizing hormone surge in young ovulatory women. *Fertil Steril* **88**: 684–690.

Pettersson K, Siitari H, Hemmila I, Soini E, Lovgren T, Hanninen V *et al*. (1983). Time-resolved fluoroimmunoassay of human chorio-gonadotropin. *Clin Chem* **29**: 60–64.

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.

Raivio T, Huhtaniemi I, Anttila R, Siimes MA, Hagenas L, Nilsson C *et al*. (1996). The role of luteinizing hormone-beta gene polymorphism in the onset and progression of puberty in healthy boys. *J Clin Endocrinol Metab* **81**: 3278–3282.

Robinson N, Saudan C, Sottas PE, Mangin P, Saugy M (2007). Performance characteristics of two immunoassays for the measurement of urinary luteinizing hormone. *J Pharm Biomed Anal* **43**: 270–276.

Rotmensch S, Cole LA (2000). False diagnosis and needless therapy of presumed malignant disease in women with false-positive human chorionic gonadotropin concentrations. *Lancet* **355**: 712–715.

Schwartz S, Krude H, Merz WE, Lottersberger C, Wick G, Berger P (1991). Epitope mapping of the receptor-bound agonistic form of human chorionic gonadotropin (hCG) in comparison to the antagonistic form (deglycosylated hCG). *Biochem Biophys Res Commun* **178**: 699–706.

Shahzad K, Kim DH, Kang MJ (2007). Analytic evaluation of the beta-human chorionic gonadotropin assay on the Abbott IMx and Elecsys2010 for its use in doping control. *Clin Biochem* **40**: 1259–1265.

Snyder JA, Haymond S, Parvin CA, Gronowski AM, Grenache DG (2005). Diagnostic considerations in the measurement of human chorionic gonadotropin in aging women. *Clin Chem* **51**: 1830–1835.

Stenman U-H, Bidart JM, Birken S, Mann K, Nisula B, O'Connor J (1993). Standardization of protein immunoprocedures. Choriogonadotropin (CG). *Scand J Clin Lab Invest Suppl* **216**: 42–78.

Stenman UH (2004). Standardization of assays for human chorionic gonadotropin. *Clin Chem* **50**: 798–800.

Stenman UH, Alfthan H, Hotakainen K (2004). Human chorionic gonadotropin in cancer. *Clin Biochem* **37**: 549–561.

Stenman UH, Alfthan H, Ranta T, Vartiainen E, Jalkanen J, Seppälä M (1987). Serum levels of human chorionic gonadotropin in nonpregnant women and men are modulated by gonadotropin-releasing hormone and sex steroids. *J Clin Endocrinol Metab* **64**: 730–736.

Stenman UH, Tiitinen A, Alfthan H, Valmu L (2006). The classification, functions and clinical use of different isoforms of HCG. *Hum Reprod Update* **12**: 769–784.

Stenman UH, Unkila-Kallio L, Korhonen J, Alfthan H (1997). Immunoprocedures for detecting human chorionic gonadotropin: clinical aspects and doping control. *Clin Chem* **43**: 1293–1298.

Storring PL, Gaines-Das RE, Bangham DR (1980). International Reference Preparation of Human Chorionic Gonadotrophin for Immunoassay: potency estimates in various bioassay and protein binding assay systems; and International Reference Preparations of the alpha and beta subunits of human chorionic gonadotrophin for immunoassay. *J Endocrinol* **84**: 295–310.

Stricker R, Eberhart R, Chevailler MC, Quinn FA, Bischof P, Stricker R (2006). Establishment of detailed reference values for luteinizing hormone, follicle stimulating hormone, estradiol, and progesterone during different phases of the menstrual cycle on the Abbott ARCHITECT analyzer. *Clin Chem Lab Med* **44**: 883–887.

Tan RS, Vasudevan D (2003). Use of clomiphene citrate to reverse premature andropause secondary to steroid abuse. *Fertil Steril* **79**: 203–205.

Thorsson AV, Christiansen P, Ritzen M (2007). Efficacy and safety of hormonal treatment of cryptorchidism: current state of the art. *Acta Paediatr* **96**: 628–630.

Vaitukaitis JL, Braunstein GD, Ross GT (1972). A radioimmunoassay which specifically measures human chorionic gonadotropin in the presence of human luteinizing hormone. *Am J Obstet Gynecol* **113**: 751–758.

Valmu L, Alfthan H, Hotakainen K, Birken S, Stenman UH (2006). Site-specific glycan analysis of human chorionic gonadotropin beta-subunit from malignancies and pregnancy by liquid chromatography—electrospray mass spectrometry. *Glycobiology* **16**: 1207–1218.

van Breda E, Keizer HA, Kuipers H, Wolffenbuttel BH (2003). Androgenic anabolic steroid use and severe hypothalamic-pituitary dysfunction: a case study. *Int J Sports Med* **24**: 195–196.

Van Uytfanghe K, Rodriguez-Cabaleiro D, Stockl D, Thienpont LM (2007). New liquid chromatography/electrospray ionisation tandem mass spectrometry measurement procedure for quantitative analysis of human insulin in serum. *Rapid Commun Mass Spectrom* **21**: 819–821.

Vermes I, Bonte HA, v d Sluijs Veer G, Schoemaker J (1991). Interpretations of five monoclonal immunoassays of lutropin and follitropin: effects of normalization with WHO standard. *Clin Chem* **37**: 415–421.

Yen SS, Llerena O, Little B, Pearson OH (1968). Disappearance rates of endogenous luteinizing hormone and chorionic gonadotropin in man. *J Clin Endocrinol Metab* **28**: 1763–1767.

Zitzmann M, Nieschlag E (2000). Hormone substitution in male hypogonadism. *Mol Cell Endocrinol* **161**: 73–88.