Immunoprocedures for detecting human chorionic gonadotropin: clinical aspects and doping control

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The pregnancy hormone human chorionic gonadotropin (hCG) is also present at low concentrations in plasma and urine of men and nonpregnant women. hCG immunoreactivity occurs in various molecular forms: Besides the intact hCG heterodimer, considerable amounts of proteolytically cleaved forms, free subunits, and fragments are found in plasma and urine. Especially in urine, proteolytic fragments constitute a major part of the hCG immunoreactivity. The different forms of hCG cross-react to various degrees in immunoassays and constitute a problem for standardization of specific hCG determinations. After injection of hCG (10 000 IU of Pregnyl®; Organon), above-normal concentrations of hCG can be detected in serum and urine for 7–11 days. Most immunoassays for hCG also measure hCGβ. Quantitative hCG determinations are mainly performed on serum samples, and very few commercial hCG determinations have been validated for determination of urine samples. Considerable care must therefore be exercised when utilizing such assays to analyze urines for doping control.

INDEXING TERMS: standardization • steroid hormones • urine • reference values • pregnancy • cancer • luteinizing hormone • estrogen

The gonadotropins luteinizing hormone (LH) and chorionic gonadotropin (hCG) are therapeutically used to stimulate gonadal steroid production, both in women and men. hCG is also used by some athletes to stimulate testosterone production and to normalize the testosterone/epitestosterone ratio during or after administration of testosterone and other anabolic steroids. The International Olympic Committee (IOC) imposed a ban on hCG administration in 1987 [1], but no official decision limit for hCG in urine has been defined. IOC prescribes that hCG should be determined by two different assays, but neither the decision limit nor the specificity of the assays has been defined. Assay of hCG in serum and urine can now be performed with extremely sensitive and specific methods, which can determine the low basal concentrations found in nonpregnant women and men. From the hCG concentrations in urine from 1400 men, Lairdell et al. have recommended that a cutoff value 10 IU/L should be used for doping control [1]. However, this recommendation may be considered valid only for the method used. Different assay methods for determining hCG in serum are poorly standardized—e.g., the results obtained by various methods may vary by a factor of five [2]—and different methods show large variation in reactivity with various forms of hCG [3]. Furthermore, given the more extensive heterogeneity of hCG in urine than in serum, larger differences will be observed in urine. Therefore, the various forms of hCG in circulation and urine and the design of the assays used will affect the results. Little is known about the extent of this effect, because quantitative assays for hCG in urine have very little clinical use, and kit manufacturers have therefore not validated their methods for assay of urine. Thorough knowledge of the forms of hCG detected in urine by various methods under normal conditions and after hCG administration are prerequisites for the use of hCG determinations for doping control.

Molecular Forms of hCG and Its Subunits

The following nomenclature for well-characterized molecular forms of hCG has been suggested by the Working Group for standardization of hCG determinations appointed by the IFCC [2].

Human chorionic gonadotropin (hCG) is the intact heterodimer, consisting of an α- and a β-subunit. hCG is
the major form of hCG immunoreactivity during pregnancy.

The free beta subunit (hCGβ) consists of a 22-kDa polypeptide containing 145 amino acid residues, with two N-linked and four O-linked carbohydrate chains. hCGβ is secreted by the placenta during pregnancy, and the serum concentrations are ~1% of those of hCG.

The free alpha subunit (hCGα) consists of a glycosylated 14-kDa polypeptide containing 92 amino acid residues and two N-linked carbohydrate chains. This subunit is common to hCG, LH, follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH). hCGα is produced in excess of the β-subunits by both the pituitary and the placenta [4, 5].

The core fragment of hCGβ (hCGβcf) is a 10-kDa two-chain polypeptide lacking the amino acid sequences 1–5, 41–54, and 93–145. The two chains are held together by disulfide bonds. hCGβcf is probably formed from hCG and hCGβ by proteolytic degradation in the kidneys.

Nicked hCG (hCGn) and nicked hCGβ (hCGβn) are partially degraded forms of hCG and hCGβ, in which the peptide chain of hCGβ is cleaved in the region 43–49 (usually between amino acids 47 and 48 and less frequently between 44 and 45).

**Assay Methods**

**Serum assays.** Specific determination of hCG in serum from nonpregnant subjects was first performed by RIA with antiserum SB6 [6]. This assay also measures hCGβ. Specific measurement of hCG in serum became possible with the development of ultrasensitive immunometric assays that were based on monoclonal antibodies [7, 8]. This technology has also made it possible to develop “sandwich”-type assays for measuring hCGβ in serum [9] and hCGβcf in urine [10, 11]. Only a few commercially available methods are capable of measuring the physiologic concentrations of hCG in men and nonpregnant women, and the concentrations of hCGβ and hCGβcf can only be measured by some research methods.

Many hCG immunoassays are designed to measure both hCG and hCGβ. Such methods are based on the use of two or three antibodies specific for hCGβ or of a monoclonal antibody and polyclonal antiserum. A common problem with these assays is that they tend to overestimate hCGβ [2]. On the other hand, some assays based on a combination of anti-hCG and anti-β antibodies may underestimate hCGn [3]. In pregnancy, urine hCG may be extensively “nicked,” but whether this form occurs in urine of nonpregnant subjects is not known. By utilizing one antibody to hCGβ and another one to hCGα or the αβ dimer, one can design highly specific assays reacting with hCG but not with hCGβ. Cross-reaction with LH, which used to be a problem with conventional RIA methods, is usually negligible. However, denaturation of LH during storage has been shown to increase its cross-reactivity, even in highly specific assays based on monoclonal antibodies [7].

**Urine assays.** Commercial hCG assays have usually not been validated for determination of hCG in urine. The reactivity with hCGβcf may be a problem in assays designed to measure both hCG and hCGβ. Another potential problem is the cross-reaction with denatured LH. The core fragment of LH (LHβcf) is the predominant form of LH immunoreactivity in urine and may interfere in hCG assays. Assays for total hCG that overestimate hCGβ cannot be used to specifically measure hCG in urine, which may contain much hCGβ and hCGβcf.

Given the close structural similarity between hCGβcf and LHβcf, some assays for hCGβcf also measure LHβcf [12], which is present in urine from nonpregnant subjects at much higher concentrations than hCGβcf [13]. However, assays based on two monoclonal antibodies with negligible cross-reaction with LHβcf (<2%) have been described [11].

Because of the large differences in assay design and specificity for different forms of hCG, the concentrations measured are highly dependent on the assay used [2, 3]. The concentrations measured in quality-control samples may vary fivefold [2]. The relative amounts of various hCG forms also depend on the clinical situation of the subject tested. All these factors contribute to the large variation in reported hCG concentrations.

hCG can be detected in urine by highly specific mass-spectrometric methods. However, there is still at least a 100-fold difference in detection limit between mass-spectrometric and immunological methods [14].

The concentrations of protein hormones in urine are usually corrected for variations in urine flow. Mostly the concentrations are normalized by dividing the hormone concentrations with the creatinine concentration, but correction to mean urine density is also used [15]. In healthy subjects the mean density is 1.015 mg/L (Alfthan and Stenman, unpublished); however, normal urine may range from 10-fold more dilute to 3-fold more concentrated than average. Although creatinine correction is widely used, in our experience the concentrations of gonadotropins are not directly correlated to creatinine in very dilute or concentrated urines [16], and better ways of normalizing urine concentrations of protein hormones in urine need to be developed. This appears to be especially important in athletes, whose urine output after strenuous exercise may be very low, which may be expected to increase the concentrations of protein hormones.

**Concentrations of hCG in Serum and Urine in Health and Disease**

**IN NONPREGNANT WOMEN AND MEN**

**Serum.** The concentrations of hCG in serum of nonpregnant women are measurable by sensitive assays. These concentrations are dependent on gonadal function, showing a clear increase around the menopause, when the upper reference limit increases from 3 to 5 IU/L (from 8.6 to 15.5 pmol/L) [17]. Concentrations up to 8–10 IU/L are occasionally observed in healthy women.
In men, the concentrations are lower but mostly are measurable by highly sensitive assays. In those <50 years old, the upper reference limit is 0.7 IU/L (2.1 pmol/L); in those >50, it is 2 IU/L (6.1 pmol/L) [17]. Concentrations as great as 3–4 IU/L are occasionally observed in healthy men.

The concentrations of hCGβ are lower than those of hCG. Only a slight increase is observed with age and the sex difference is small. The upper reference limit in women <50 years is 1.6 pmol/L and in men 1.9 pmol/L. The corresponding values for those >50 years are 2.0 and 2.1 pmol/L, respectively. The concentrations of hCGβcf in serum are below the detection limit of present assays [17].

Urine. The median concentrations of hCG and hCGβ are similar in serum and urine, whereas those of hCGβcf are much higher in urine [17]. This suggests that, as in pregnancy, hCGβcf is produced by proteolytic degradation of hCG and hCGβ in the kidneys. The concentrations of hCG and hCGβ in urine of women and men increase with age but not as sharply as those in serum. The upper reference limits for hCG in women <50 and ≥50 years are 3 and 4 IU/L (8.8 and 11.5 pmol/L), respectively. The corresponding values for men are 1 and 3 IU/L (2.9 and 8.4 pmol/L) [17].

The concentrations of hCGβcf in urine are similar in women and men [17], increasing at age ~50. By our method, the upper reference limits for pre- and postmenopausal women are 8.1 and 9.5 pmol/L, respectively, and those for men <50 and ≥50 are 6.7 and 8.5 pmol/L, respectively [17].

In nonpregnant subjects, the common glycoprotein hormone α subunit (hCGα) is mainly derived from the pituitary, and its serum concentrations do not reflect hCG production. The concentrations in urine are three- to fivefold those in serum [18, 19].

IN WOMEN WITH NORMAL PREGNANCY
The concentration of hCG in serum starts to increase 7–11 days after ovulation, corresponding to 21–25 days after the last menstrual period [20]. The increase is nearly exponential during the first 5 weeks after implantation [21]. As measured by our method, the concentrations peak at ~110 000 IU/L during week 8–10 after the last menstrual period (i.e., 5–7 weeks after implantation), after which they decrease and reach a nadir of 36 000 IU/L at the beginning of the second trimester [9]. (These values are method dependent [2, 31] There is a small increase before delivery [22], and the concentrations of hCG and its subunits are higher in multiple pregnancies than in singleton ones [23]. Individual variation in hCG concentrations is large [9].

After normal delivery, the hCG concentrations decrease with a half-time of about 24–32 h [24] and normalize within 1–3 weeks [25, 26]. Serum hCG concentrations after a first-trimester abortion may take 4–5 weeks to return to normal values [27]. Mean serum concentrations of hCGβ during pregnancy vary from 0.5% to 1.6% (range 0.1–3.6%) of the hCG concentrations [9, 28].

hCGβcf can be detected in serum during pregnancy in quantities corresponding to 0.03% of the hCG content. The concentrations in urine are ~4000-fold those in serum [29]. hCGβcf is the major form of hCG immunoreactivity in pregnancy urine except during the first month of pregnancy, when hCG predominates [30]. The concentrations of hCG immunoreactivity in paired serum and urine samples are similar and correlate strongly [31]. Total hCG immunoreactivity is higher in urine [20, 32] apparently attributable to hCGβcf.

IN PREGNANCY-RELATED DISORDERS
Early fetal loss. About 20–25% of all conceptions end in an early spontaneous abortion [33, 34]. Often the only indication of pregnancy is an increased concentration of hCG in serum or urine; hence, the condition is also called subclinical abortion or biochemical pregnancy [35]. Spontaneous abortions occur most often during the first trimester. Initially, the concentration of hCG in serum may increase normally before it starts falling [36].

Ectopic pregnancy. In ectopic pregnancy, the concentrations of hCG in serum tend to be lower than during normal pregnancy [36, 37], but they may also be normal for a long time.

IN MALIGNANT DISEASE
Gestational trophoblastic disease. The concentrations of hCG in serum and urine are strongly increased in choriocarcinoma and molar disease [38]. In benign trophoblastic disease, the concentrations of hCGβ are usually <5% of those of hCG, but in choriocarcinoma the proportion of hCGβ is higher [39, 40]. The proportion of hCGn is higher in serum of patients with choriocarcinoma than in pregnancy [41].

Testicular cancer. About 50% of the nonseminomatous testicular cancers cause an increase of hCG in serum and urine, often to very high concentrations. hCG is seldom increased in seminomas, but these tumors relatively often produce hCGβ [42].

Nontrophoblastic tumors. hCG is very rarely produced by nontrophoblastic tumors, but low-level expression of hCGβ is quite common. Because the serum concentrations of hCGβ in cancer patients are usually only moderately increased, hCGβ is a useful marker only if measured by an ultrasensitive assay [43]. The strong correlation between hCGβ in serum and hCGβcf in urine suggests that, when excreted into urine, hCGβ is degraded to hCGβcf. However, by immunohistochemistry staining with monoclonal hCGβcf antibodies, positive staining has frequently been observed in nontrophoblastic cancer cells, suggesting that some hCGβ (and hCG) may be degraded within
the cell before secretion [44]. The relative contribution of the various sources of hCGβ cf remains to be determined.

**Effect of Drugs**

**Gonadotropin-releasing Hormone and Estrogens**

In men and nonpregnant women, gonadotropin-releasing hormone causes a threefold increase in the serum concentrations of hCG, whereas treatment with estrogen and progestagen lowers the serum hCG by ~50%. These results suggest that hCG in serum is derived from the pituitary [7].

**HCG Administration**

Intramuscular injection of partially purified urinary hCG is used to induce ovulation in hormone-stimulated menstrual cycles. We have measured the concentrations of hCG in serum and urine and of hCGβ cf in urine after injection of 10 000 IU of Pregnyl® to women during stimulated cycles. The assays used have been described in detail [9, 11, 17]. The procedures followed were in accordance with the Helsinki Declaration of 1975, revised in 1983. Peak serum hCG concentrations of 200–300 IU/L are observed 1 day after injection, after which they decline with a mean (±SD) half-time of 1.56 ± 0.12 days (Fig. 1A). hCG concentrations in serum stay above the upper reference limit for 7–11 days after injection and reach baseline values within 8–13 days. Similar results have been obtained in another study [45].

The hCG concentrations in urine correlate fairly closely with those in serum, and there is very little delay in excretion. The concentrations remain increased for the same time as those in serum, and their elimination half-times are similar, 1.45 ± 0.10 day (Fig. 1A). The pattern of hCGβ cf in urine is slightly different. Concomitant with the hCG peak in serum is a sharp peak of hCGβ cf in urine. The hCGβ cf concentration decreases rapidly within 1 day, after which it falls at the same rate as the concentration of hCG. The urine concentrations of hCGβ cf drop below the
upper reference limit 2–3 days earlier than does hCG (Fig. 1A). Pregnyl contains equal amounts of hCG and hCGβcf, and the first hCGβcf peak is explained by rapid excretion of hCGβcf into urine [46].

In women who conceive after hCG stimulation, the hCG concentrations in serum and urine start increasing again before the injected hCG has reached baseline values in serum. The hCG concentrations in urine are similar to those in serum, but the hCGβcf concentrations start increasing after a delay of 3–4 days (Fig. 1B). This delay is similar to the delay in excretion of LHβcf after the midcycle LH surge [13]. On the basis of this, we expected to also see a delayed excretion of hCGβcf into urine, but this was not the case. The metabolism of purified hCG may therefore differ from that of endogenous LH. Interestingly, after conception, the excretion of hCGβcf is delayed (Fig. 1) in a way similar to that of LHβcf [13].

Conclusions

hCG is a normally occurring hormone, and its concentrations in serum and urine can be measured in men and in nonpregnant women by sensitive methods. The determination is complicated by the occurrence of many different forms of hCG immunoreactivity, which exert a variable response in different assays. The urine concentrations are also affected by variations in urinary flow rate. To be useful for doping control, the reference values for hCG need to be determined on a sufficiently large number of samples from women and men of the proper age group. Furthermore, the reference values need to be determined by the same method as used for doping control. The results obtained by various hCG assays vary by as much as fivefold because of differences in specificity and calibration. Therefore, some methods may give false-positive results whereas others may miss doping cases. It is also essential that the effect of exercise be determined.

The disappearance curves for hCG in serum and urine after injection of urinary hCG in combination with appropriately established reference values provide the basis for detection of hCG administration. Intramuscular administration of hCG causes a prolonged increase of serum hCG and of the urinary excretion of hCG and hCGβcf. The serum and urine concentrations decrease to less than the upper reference limit in 6–9 days. Generally, however, decision limits higher than the upper reference limit are used for doping control.

If appropriately standardized and validated methods are used, investigators should be able to detect selfadministration of hCG in men as reliably as anabolic steroids and testosterone are now being detected by mass-spectrometric methods. However, although the methods for such determinations are available, very few have been appropriately validated. Therefore, the present recommendation by the IOC that hCG should be determined by two different immunoassays needs to be more specifically defined before an assay is used to sentence athletes.

References


