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6.1.1.2 Quality Specifications for Determinations of HCG and Related Substances

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BACKGROUND

The use of sandwich immunoprocedures based on monoclonal antibodies has improved the quality of immunological determinations of human chorionic gonadotropin (hCG), but results from external quality assessment schemes (EQAS) show, that there is a need for further improvement. Although the present hCG standard certified by WHO (Third International Standard) is generally used by kit manufacturers, results for different procedures may give highly variable results. This is probably due to differences in antibody specificity and to nonspecific interference. During the last decade, immunoprocedures specifically measuring low concentrations of free α (hCGα) and ß (hCGß) subunits have become available, and these are becoming clinically important [1, 2, 3, 4, 5].

A working group of the International Federation for Clinical Chemistry (IFCC) has prepared guidelines for immunoprocedures for determination of hCG and related substances. These guidelines have been discussed at the IV Bergmeyer conference in Lenggries on November 8-10, 1992, and they will be published as a supplement to Scand. J. Clin. Lab. Invest. [6]. This presentation is a brief summary of these recommendations.

Present status of immunoprocedures for hCG

Results from the EQAS for Peptide Hormones and Related Substances in the UK reveal poor accuracy and considerable crossreactions in some methods. Most methods designed to measure "total hCG" (*i.e.* hCG and hCGß) overestimate hCGß, and some "intact" procedures also measure hCGß. In some methods, an excess of hCGß interferes with the measurement of hCG causing underestimation of the true concentration (called covert crossreaction). These results demonstrate that (a) the major methods give fairly similar

results but many minor procedures have a bias larger than 25%, (b) many procedures have inadequate specificity, (c) procedures for "intact hCG" perform better than those measuring "total hCG" and, (d) procedures for "total hCG" tend to overestimate hCGß. Data on quality control sera further suggest that the detection limit of most commercial methods is not low enough for measurement of physiological concentration of hCG in men and nonpregnant women [6].

hCG as well as hCGß, especially in urine but also in sera from some cancer patients, may have intrachain nicks, i.e. missing peptide linkages at position ß 47-48 and/or ß 44-45. These are critical for biological activity and they also may affect the reactivity with some antibodies. Therefore the concentrations measured in serum and urine may be dependent on the antibodies used [7]. However, insufficient information is available on the severity of this problem, which requires further investigation using homogeneous standards. Because the present international standards for hCG and hCGß contain some nicked material, it has been suggested that new reference materials of non-nicked and nicked forms of hCG and hCGß should be prepared [6].

Information on the performance of pregnancy test is scarce [8]. A recent study shows some differences in the detection limit and robustness of various procedures [9], but practical experience suggests that most modern pregnancy tests perform in a satisfactory manner [10].

RECOMMENDATIONS

Nomenclature and abbreviations

HCG-like molecules exist both as intact or modified hCG, free α and ß subunits and degradation products. The following nomenclature for these is suggested:

- *Human choriogonadotropin,* also called chorionic gonadotropin; abbreviation hCG.
- *Free ß subunit of hCG,* referring to the non-combined ß subunit; abbreviation hCGß.
- *Free α subunit* common to hCG, LH, FSH and TSH; abbreviation hCGα.
- *Core fragment of hCGß*; abbreviation hCGßcf.
- *Nicked hCG and nicked hCGß* are partially degraded forms of hCG and hCGß, in which the peptide chain of hCGß is nicked in the region 43-49 (usually between

amino acids 47 and 48 and less frequently between 44-45). Abbreviation; hCGn and hCGßn, respectively.

Description of measurement procedures

The nomenclature of hCG measurement procedures is confusing. Commercial hCG methods are often called ßhCG or hCGß assays to indicate that they do not react with LH and/or that hCGß has been used as immunogen. The procedure specificity should be defined to indicate the reactivity of the antibodies and whether the method is of sandwich- or inhibition type. Thus a sandwich method employing a solid phase antibody against hCGß and detector antibody against hCGα would be defined a ß−α sandwich method specific for intact hCG. An αß method of inhibition type would employ an αß dimer-specific antibody. A ß−ß method would indicate a sandwich method employing two antibodies reacting with the ß subunit. Such a method could recognize both hCGß and hCG or only hCGß. The specificity of the method should be clearly indicated on the package and in other material concerning the kit.

Units

International units (IU) based on bioactivity are generally used clinically to express the concentrations of hCG, hCGß and hCGα [11]. Mass concentrations are also used for subunits and fragments. Because antibodies recognize epitopes on the peptide chain, the concentrations measured by immunoprocedures can be expected to correlate better with substance concentrations than with bioactivity or mass. It is recommended that the concentrations of new reference preparations of hCG, its subunits and fragments are determined by amino acid analysis and expressed in mol per litre [6].

Validation of measurement procedures

Measurement procedures should be validated with respect to specificity, precision, detection limit and robustness according to the following guidelines.

Specificity should be established by determining the reactivity of the analyte with potentially crossreacting substances at concentrations corresponding to the intended use (*e.g.* pregnancy, cancer), type of sample (*e.g.* plasma, serum or urine) and expected pathophysiological concentrations of the crossreactants. Important crossreactions are (a) LH and hCGß in methods for hCG, (b) hCG in methods for hCGß and (c) hCGß in methods measuring hCGßcf. The crossreactions should be calculated on the basis of

substance concentrations expressed in mol per litre [6].

Nonspecific *matrix effects* caused by the sample may generate large differences in results obtained by various methods. Matrix effects may be due to (a) proteins, salts and pH, (b) complement factors, (c) rheumatoid factors (RF), (d) heterophilic antibodies and human antibodies to animal IgG (HAMA). These factors vary from one sample to another [12 , 13]. Increasing concentrations of protein and salt and extremes in pH tend to inhibit binding. This causes too low results in sandwich methods and too high results in inhibition methods. Because nonspecific interference is caused by substances occurring at high concentrations, their effects are mostly rapid. Therefore short incubation times tend to exacerbate the problem [13].

The influence of matrix effects on the true detection limit of the procedure should be estimated by analyzing samples from healthy males and females of age groups 20-40 and 60-80 years. The effect of potentially interfering substances is estimated by analyzing samples containing high concentrations of rheumatoid factor, heterophilic antibodies and human anti-mouse antibodies.

Recovery is analyzed by measuring samples spiked with low, medium and high concentrations of the analyte (relative to the concentrations of the calibrators). When relevant, covert crossreactions should be tested for by measuring recovery in the presence of crossreacting antigens at concentrations that may occur under pathophysiological conditions.

Comparability with an accepted procedure, preferably a reference measurement procedure, should be established using at least 30 samples representing each pathological and physiological condition for which the procedure is intended to be used.

If appropriately determined *reference values* are available, these should be provided. The concentrations of the various forms of hCG in plasma from healthy men and nonpregnant women are below the detection limit of most routinely used immunoprocedures, but with methods having very low detection limits, the upper reference limits have been established. For hCG the upper reference limit (based on the 97.5 percentile) of postmenopausal women is 5 IU/l (16 pmol/l) [1], but levels up to 9 IU/l (26 pmol/l) are occasionally observed [14]. In menstruating women the limit is 3 IU/l (9 pmol/l) and in men 0,7 IU/l

below and 2 IU/l in those above 50 yr. It is notable that the hCG concentrations are higher in women than in men and that the concentrations increase with age. By contrast, the concentrations of hCGß are similar in both sexes (2 pmol/l) and there is very little increase with age. The reference values for urine have also been determined. [1]. Until confirmed in other studies, these values are valid only for the methods used, but similar values have been reported in other studies [2, 5, 15, 16].

It should be noted that validation of the measurement procedure does not obviate the need to perform clinical validation. *E.g.* reference values to be used for prenatal screening need to be determined separately in each laboratory.

Method requirements for various clinical applications
Generally, immunoprocedures measuring specific forms of hCG are to be preferred over methods measuring mixtures of different molecular forms. The detection limit required depends on the clinical use, but generally it should be at least 5-fold lower than the discrimination limit [6].

A discrimination limit of 10 IU/l (29 pmol/l) for hCG in plasma is often used for diagnosis of pregnancy in order to avoid false positive results in postmenopausal women, who may have concentrations up to 9 IU/l (26 pmol/l) [14]. Crossreactions with LH in hCG determinations below 1% may be considered adequate.

Determination of maternal plasma concentrations of hCG or hCGß (mostly in combination with alphafetoprotein, AFP) during pregnancy weeks 10-18 is used for *prenatal diagnosis* of chromosomal aberrations and especially trisomy 21 [17, 18]. Because of a low ratio of hCGß to hCG, i.e. 0.2 - 2%, a low crossreaction (<0.1%) is essential.

Diagnosis of pregnancy by determination of hCG in urine. Many immunochemical "pregnancy tests" for detection of hCG in urine have a detection limit of 25-50 IU/l, which is adequate. The specificity of these procedures is also acceptable [8, 10]. It is desirable that pregnancy tests are characterized and evaluated according to the same criteria as other immunoprocedures [9].

HCG is an extremely sensitive marker for *gestational trophoblastic disease*. Because the ratio of hCGß to hCG is higher in patients with malignant disease, simultaneous determi-

nation of hCG and hCGß can be used to differentiate between malignant and benign trophoblastic tumors, [19, 20]. The ratio should be calculated on the basis of substance concentrations in mol/l.

Non-seminomatous testicular germ cell tumors often produce hCG or its free subunits, and hCG together with AFP are very valuable markers for these [21]. In *seminomas*, hCG in serum is seldom elevated [22] but about 30% of these tumors secrete hCGß. Since these cases would be missed by methods detecting only intact hCG, the use of methods measuring both hCG and hCGß can be recommended [23], if specific methods for each component are not available.

Non-trophoblastic tumors often secrete hCGß but only occasionally intact hCG [5, 24, 25]. HCGß is a promising marker at least for cancer of the pancreas [25] and the bladder [24]. The urinary excretion of hCGßcf is often elevated in various nontrophoblastic tumors [4, 25, 26] and it appears to be useful marker for gynecologic cancers [26]. The concentrations correlate with those of hCGß in serum, and at least i pancreatic cancer, hCGß in serum is a better marker than hCGßcf in urine [25]. Endocrine gastrointestinal malignancies (malignant apudomas) often produce hCGα and hCGß. The latter is often elevated in patients with carcinoid tumors. [27].

When hCGß methods are used for diagnosis of cancer, it is essential to use specific methods with a very low detection limit [2, 5, 25]. Methods measuring both hCG and hCGß are less useful, because the basal levels of hCG fluctuate and they are 5 to 10-fold those of hCGß [1].

CONCLUSIONS

The performance of determinations for hCG and related substances is generally adequate for diagnosis and follow-up of pregnancy and pregnancy-related disorders. However, further improvement is desirable for methods to be used in cancer diagnosis. This can be achieved by careful assay design and selection of high affinity antibodies, which have been well characterized for reactivity with various forms of hCG. This characterization requires preparation of new standards for intact and nicked forms of hCG and its subunits. Appropriately designed quality control schemes will disclose procedures with inadequate performance, and this can be used to encourage use of appropriate methods.

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