Establishment, Value Assignment, and Characterization of New WHO Reference Reagents for Six Molecular Forms of Human Chorionic Gonadotropin

ADRIAN BRISTOW,1 PETER BERGER,2 JEAN-MICHEL BIDART,3 STEVEN BIRKEN,4 ROB NORMAN,5 Ulf-Håkan Stenman,6 and Catharine Sturgeon7* on behalf of the IFCC Working Group on hCG

Background: The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) established a Working Group to investigate means of improving the comparability of immunoassays for human chorionic gonadotropin (hCG), which was selected as a prototype glycoprotein analyte. The Working Group identified development of unambiguous nomenclature and production of new highly purified International Reference Reagents calibrated in substance concentrations as its primary objectives.

Methods: Preparations of intact hCG, nicked hCG, hCG β-subunit, nicked hCG β-subunit, hCG α-subunit, and hCG β-core fragment were purified from a crude urinary hCG preparation, ampouled, lyophilized, and assigned values in substance concentrations (mol/L). Value assignment and accelerated degradation studies were carried out in accordance with WHO protocols for International Reference Reagents.

Results: The ampouled standards were assigned final values based on the recovery of immunoreactive material after reconstitution. The degradation studies showed that the standards were highly stable.

Conclusions: The nomenclature of hCG-related molecules and immunoassays has been adopted by the IFCC, and the standards prepared and characterized by the Working Group have been formally adopted by the WHO as the First International Reference Reagents for six hCG-related molecules. These developments will enable better understanding of what assays for hCG measure and should ultimately help to improve the clinical application of these assays.

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Participants at an International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Bergmeyer Conference in 1992 considered the means by which comparability of immunoassays could be improved (1). Recognizing that different approaches are required for chemically well-defined analytes (e.g., steroids) and for larger and more heterogeneous molecules (e.g., glycoproteins), participants selected two “model” analytes—cortisol and human chorionic gonadotropin—as prototypes for study, and two IFCC Working Groups were subsequently established with the purpose of developing methods to improve comparability of immunoassay results for cortisol or human chorionic gonadotropin (hCG).8

The difficulties of standardizing assays that measure heterogeneous analytes such as hCG, which are generally

1 National Institute for Biological Standards and Control, Potters Bar, Herts, United Kingdom.
2 Institute for Biomedical Aging Research, Austrian Academy of Sciences, Innsbruck, Austria.
3 Department of Clinical Biology, Institut Gustave-Roussy, Villejuif, France.
4 Department of Obstetrics and Gynecology, College of Physicians and Surgeons of Columbia University, New York, NY.
5 Research Centre for Reproductive Health, Department of Obstetrics and Gynaecology, University of Adelaide, The Queen Elizabeth Hospital, Woodville, Australia.
6 Department of Clinical Chemistry, Helsinki University Central Hospital, Finland.
7 Department of Clinical Biochemistry, Royal Infirmary, Edinburgh, United Kingdom.
*Address correspondence to this author at: Department of Clinical Biochemistry, Royal Infirmary of Edinburgh, Edinburgh EH16 4SA, United Kingdom. Fax 44-131-242-6882; e-mail C.Sturgeon@ed.ac.uk.
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Nonstandard abbreviations: hCG, human chorionic gonadotropin; IS, International Standard; hCGβ, hCG β-subunit; IRP, International Reference Preparation; hCGα, hCG α-subunit; hCGβn, nicked hCG β-subunit; and hCGβcf, hCG β-core fragment.
more appropriately regarded as families of closely related molecules than as single species, have recently been well described (2, 3). Improvements in standardization, or assay comparability, inevitably tend to be gradual, often depending on increased knowledge of the relevant analytes, e.g., the crystal structure of hCG (3, 4). Ultimately, the ideal metrologic solution for heterogeneous analytes such as hCG would be to determine each of the principal components of the mixtures they comprise. Technologic advances may well permit this within the next decade, although the clinical utility of this approach is not yet clear. Much progress can nevertheless be made toward improved comparability of results by ensuring correct calibration of methods by use of well-characterized International Standards (1) and by specifying antibody specificities most appropriate for immunoassays designed for particular clinical applications (2). In oncology, for example, hCG results should ideally be obtained with separate immunoassays for hCG and its subunits, but if this is not practicable, it is important to ensure that the immunoassay used has appropriate specificity, recognizing the clinically relevant hCG-related molecules (2, 5). This requires that laboratories offering these tests have a clear understanding of the characteristics of the hCG method used and that manufacturers use clear and unambiguous nomenclature to describe these.

The Working Group identified the lack of universally accepted nomenclature for hCG and hCG-related molecules, together with the difficulty in comparing the extent of recognition of these molecules in different immunoassays (i.e., knowing what assays measure) as the two major areas of current concern. Because improved nomenclature to address the former and new International Reference Reagents to facilitate the latter will enable clearer and more unequivocal description of hCG immunoassays, the hCG Working Group has to date directed its resources toward achieving these objectives (1, 6). Understanding the precise cross-reaction of measuring systems with the different immunoreactive forms of hCG may help explain variations in measurements by each particular assay system (2).

**NOMENCLATURE FOR hCG-RELATED MOLECULES**

The term “β-hCG assay” is used colloquially to describe immunoassays that use antibodies specific for the β-subunit of hCG, to describe assays measuring intact hCG + β-subunit, or to describe assays measuring the β-subunit alone (1, 6). This lack of clear nomenclature still causes confusion, even among those familiar with the assays. The nomenclature proposed by the hCG Working Group is shown in Fig. 1 and Table 1, which also summarize some of the characteristics of the most important hCG-related molecules. The IFCC nomenclature is “user-friendly”, as similar as possible to that in current use, and closely resembles the nomenclature subsequently proposed by the NCCLS in the United States (7). Its adoption should decrease confusion of the kind outlined above and will also facilitate clear and unequivocal description of which hCG-related molecules different assays recognize (2).

**INTERNATIONAL REFERENCE REAGENTS FOR hCG-RELATED MOLECULES**

Internationally recognized standards for hCG [3rd WHO International Standards (IS) 75/537 and 4th WHO IS 75/589], hCG β-subunit (hCGβ) [1st International Reference Preparation (IRP) 75/551] and hCG α-subunit (hCGα; 1st IRP 75/569) have been available for more than 20 years (8). Although these standards have served the laboratory community well, they have several important drawbacks. The original assignment of units to the hCG standard, which was originally intended for use with bioassays rather than immunoassays, was based on bioactivity. The subunits, which have no bioactivity, were assigned arbitrary units based on mass (1 μg corresponds to 1 IU). This means that comparing concentrations of the three molecules is difficult, requiring several assumptions and relatively complex calculations to obtain the values in substance concentrations (9). Additionally, assessment of
cross-reactivity data is hampered by contamination of hCG IS 75/537 with nicked hCG (hCGn), a partially degraded form of hCG that is recognized to different degrees by different immunoassays (1, 6).

Preparation of new standards for six important molecular forms of hCG (Fig. 1 and Table 1) was therefore a major goal of the IFCC Working Group. Recent advances in protein purification techniques were exploited to produce new standards for hCG and its subunits and new standards for three hCG-related molecule: hCGn, nicked hCGβ (hCGβn), and hCG β-core fragment (hCGβcf). For the first time for glycoprotein hormones, these standards were assigned values in substance concentrations (mol/L) based on amino acid analysis. The purification and characterization of these were described recently (10). Here we report ampouling, value assignment, and stability studies of these preparations and their subsequent adoption by the WHO as the First International Reference Reagents for hCG and hCG-related molecules (Table 1).

### Materials and Methods

**AMPOULING OF THE CANDIDATE REFERENCE MATERIALS**

The structure of the study is outlined in Fig. 2. The purified preparations were transferred lyophilized from Columbia University (New York, NY to the National Institute for Biological Standards and Control (http://www.nibsc.ac.uk/catalog/standards/preps/sub_endo.html)). Full data on the immunoassays are available from the National Institute for Biological Standards and Control (http://www.nibsc.ac.uk/catalog/standards/preps/sub_endo.html).

**Preparation of new standards for six important hCG-related molecules**

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human chorionic gonadotropin, intact</td>
<td>hCG</td>
<td>hCG, purified to remove nicked forms and free subunits</td>
</tr>
<tr>
<td>Human chorionic gonadotropin, nicked</td>
<td>hCGn</td>
<td>Partially degraded hCG, missing peptide bonds in the hCGβ-40 to -50 region</td>
</tr>
<tr>
<td>Human chorionic gonadotropin, α-subunit</td>
<td>hCGα</td>
<td>Purified hCGα, dissociated from hCG</td>
</tr>
<tr>
<td>Human chorionic gonadotropin, β-subunit</td>
<td>hCGβ</td>
<td>Dissociated hCGβ, purified to remove intact dimeric hCG, hCG α, and hCGβn</td>
</tr>
<tr>
<td>Human chorionic gonadotropin, nicked β-subunit</td>
<td>hCGβn</td>
<td>Partially degraded hCGβ, missing peptide bonds in the hCGβ-40 to -50 region</td>
</tr>
<tr>
<td>Human chorionic gonadotropin, β-core fragment</td>
<td>hCGβcf</td>
<td>Residues hCGβ-6 to -40, joined by disulfide bonds to hCGβ-55 to -92</td>
</tr>
</tbody>
</table>

### VALUE ASSIGNMENT

Participants in the International Collaborative Study (Appendix I) received duplicate samples of the lyophilized ampouled preparations, ampouled preparations that had not been lyophilized, frozen samples of the original solutions, and ampoules of the existing International Standards for hCG (IS 75/537) and IRPs for hCGα (IRP 75/569) and hCGβ (IRP 75/351; see Table 1 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue1/). Full data on the immunoassays were requested to carry out independent analyses on separate aliquots of the original solutions, correcting for loss of recovery by use of appropriate internal standards Table 2 of the online Data Supplement).

**Amino acid analyses.** Participants performing amino acid analyses were requested to assay all preparations supplied and to provide data obtained in two independent assays. Using their usual method of data analysis, participants were requested to provide recoveries for the lyophilized and nonlyophilized ampoules expressed in terms of the nominal concentrations of the original solutions (Table 3 of the online Data Supplement).
no assay reactivity of the preparations will be the subject of a separate report.

Accelerated stability studies. Participants carrying out additional testing on ampoules subjected to thermal degradation (12) were required to assay all specimens from the stability test, i.e., six preparations, each stored at four temperatures (−20, 20, 37, and 45 °C). Results for all preparations were expressed relative to those obtained for preparations stored at −20 °C.

Results and Discussion
The 10 amino acid analyses were in reasonably good agreement for all six analytes, with CVs in the range 5–10% (Table 2 of the online Data Supplement). The measured amino acid contents of the hCG, hCGβ, and hCGβcf preparations agreed well with those estimated on the basis of $A_{280}$ measurements, whereas the hCGn and hCGα preparations varied from the predicted values by ~10% and the hCGβn preparation for varied from the predicted value by 20%. It should be noted that estimates based on ultraviolet absorbance depend on the extinction coefficients used, which in some cases have not been verified. Value assignment has therefore been based on amino acid analysis. Advantageously, this is the technique of choice for value assignment, being independent of carbohydrate content, which is likely to vary in glycoproteins such as the hCG-related molecules.

After reconstitution of lyophilized material, recovery of immunoreactivity ranged from 79% to 104% (Table 3 of the online Data Supplement). The 10–20% loss observed is typical of that observed when lyophilizing protein hormones. Preparation of WHO International Standards is a multistep process involving dilution into carrier solutions, automated distribution into ampoules, and lyophilization and sealing by heat fusion of the ampoules. Similar losses of activity have been observed during preparations of other International Standards (13, 14). Such losses of activity are difficult to ascribe to any single step in the process and, more importantly, have not been shown to compromise the stability of the remaining activity or its suitability to serve as an International Standard (13, 14).

The values assigned to each of the six hCG-related preparations based on recoveries determined by immunoassays designed to measure the ampouled substances are shown Table 3 of the online Data Supplement. Recoveries for hCG and hCGβ were determined by use of specific assays as well as assays measuring both together. On the basis of existing standards for hCG, hCGα, or
hCGβ, the specificities of the assays used were consistent with the claimed specificities. Thus, assays for “intact” hCG detected hCG + hCGn but detected <1% hCGα, hCGβ, or hCGβcf. Similar results were observed for hCGα- and hCGβ-specific assays. Assays systems for hCG + hCGβ, however, showed differences in specificity.

Loss of immunoreactivity after storage at increased temperatures forms the basis of the accelerated degradation test, which is used to predict the stability of WHO International Standards. The rate of loss is related to temperature by the Arrhenius equation, which can then be used to predict the long-term stability on storage at lower temperatures (12). No loss of immunoreactivity was seen after storage for 4 months even at temperatures of 37 or 45 °C.

On the basis of the data presented in this report, the WHO Expert Committee on Biological Standardization in November 2001 approved the six new Candidate Preparations as the First WHO Reference Reagents for Immunoassay (Table 1) (15). The Committee further recommended that the existing International Standard for hCG (4th IS 75/589) should not be disestablished and should for the present remain the primary standard for calibration of diagnostic immunoassays for hCG. The WHO Committee also stated that the new WHO Reference Reagents should be used, in the first instance, to investigate and characterize the specificity of currently available immunoassays for hCG-related molecules (15). These new Reference Reagents will enable manufacturers to characterize what their “hCG” methods are measuring and to compare the relative recognition of different hCG-related materials in different methods. The next generation of assays is likely to be calibrated in terms of the new Reference Reagents. The first report of hyperglycosylated hCG appeared 1 year after purification of the six standards described here was started (16). Its clinical utility in Down syndrome and pregnancy testing are now apparent, with other applications evolving. Problems reported in the detection of hyperglycosylated hCG in pregnancy testing and choriocarcinoma screening emphasize the clear need for establishment of an International Standard for hyperglycosylated hCG. The hCG Working Group is currently actively working toward developing such a standard as soon as is feasible.

Together with the epitope-mapping project carried out under the auspices of the International Society of Oncodevelopmental Biology and Medicine (ISOBM) (2), results of the present project may ultimately lead to development of reference methods for hCG and hCG-related molecules. The implementation of such reference systems in immunoassay, which is an important tenet of current thinking on standardization within the IFCC (3, 17), requires both well-characterized antibodies and highly purified standards such as those described here (18). These developments should lead to substantial improvement in standardization and clinical utility of the next generation of assays for hCG-related molecules.

In conclusion, we report the preparation and value assignment of the 1st WHO Reference Reagents for immunoassay standardization of six clinically important hCG-related molecules: hCG (RR99/688), hCGn (RR 99/642), hCGβ (RR 99/650), hCGβn (RR 99/692), hCGβcf (RR 99/708), and hCGα (RR 99/720; Table 1). These are the first immunoassay standardization reagents for human glycoprotein analytes with values assigned in substance concentrations or molar units (nmol/ampoule) rather than units based on bioactivity or arbitrary units. Their availability will facilitate characterization of current immunoassays for hCG-related molecules and should ultimately lead to improvements in standardization and between-method comparability. The immediate challenges are to ensure that these reference reagents are used to gain a better understanding of what assays for hCG are really measuring and, ultimately, to improve the clinical utility of those assays. To this end, results of proficiency testing surveys using the new International Reference Reagents will be the subject of a separate report.

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References


Appendix I. Participants Who Contributed Data to the Collaborative Study

Dr. Henrik Alfthan, Central Laboratory, Helsinki University, Helsinki, Finland.

Dr. Glenn Armstrong, Bayer Diagnostics, Tarrytown, NY.

Dr. Peter Berger, Austrian Academy of Sciences, Innsbruck, Austria.

Dr. Steven Birken, Columbia University College of Physicians and Surgeons, New York, NY.

Dr. Sally Byrnes, Beckman-Coulter, Chaska, MN.

Dr. Albert Chianello, Abbott Laboratories, Chicago, IL.

Dr. Tuija Halonen, Perkin Elmer Life Sciences, Turku, Finland.

Dr. Kathy Maugh, Diagnostics Products Corporation, Los Angeles, CA.

Dr. James Piersi-Perry, Dade-Behring Inc., Newark, DE.

Dr. Michael Rottmann, Roche Laboratory Systems, Penzberg, Germany.

Dr. J. Sharratt, University of Cambridge, Cambridge, United Kingdom.

Throughout this report, participating laboratories are referred to by code number. The assignation of code numbers is random and does not reflect the order of listing above.