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Preparation and Characterization of New WHO Reference Reagents for Human Chorionic Gonadotropin and Metabolites

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Background: **The currently used standards for human chorionic gonadotropin (hCG) and its α and β subunits (hCGα and hCGβ) contain substantial amounts of contaminating variants of hCG and other impurities. Furthermore, some partially degraded forms of hCG and its subunits have become of potential clinical importance, e.g., "nicked" forms of hCG (hCGn) and hCGβ (hCGβn), which contain cuts in the peptide backbone between amino acids 44–45 or 47–48 in hCGβ, and a fragment of hCGβ (hCGβcf) consisting of amino acids 6–40 and 55–92 bound together by disulfide bridges. The IFCC appointed a working group with the aim of preparing new standards for hCG and related substances to improve standardization of their immunoassays.**

Methods: **Large amounts of hCG and its subunits as well as of hCGn, hCGβn, and hCGβcf were prepared by previously developed purification methods in combination with hydrophobic interaction chromatography and reversed-phase HPLC. Each preparation was characterized on the basis of amino acid and sequence analyses, carbohydrate composition, and electrophoretic patterns. Immunoassays for relevant contaminating proteins were also performed.**

Results: **The major preparations were homogeneous and free of contaminating proteins. Concentrations of the final preparations were determined by amino acid analysis.**

Conclusions: **Calibrated in substance concentrations (mol/L) based on amino acid analyses, these preparations will facilitate improved standardization of immunoassays for hCG and its metabolites. The six preparations have now been established by the WHO as new 1st Reference Reagents for immunoassays with the following codes: hCG 99/688, hCGβ 99/650, hCGα 99/720, hCGn 99/642, hCGβn 99/692, and hCGβcf 99/708. In contrast to the 3rd International Standard (75/537), the clinically most important Reference Reagent for hCG (99/688) contains no hCGn and negligible amounts of free subunits.**

At the Bergmeyer Conferences on Immunoassay Standardization in 1990 and 1992, participants were asked to suggest model projects. The IFCC accepted the proposition of Ulf-Håkan Stenman to establish a Working Group (WG)[9] for standardization of immunoassays for human chorionic gonadotropin (hCG) *(1)*. The main aim of the WG was to prepare new reference preparations. The previous International Reference Preparations (IRPs) for hCG and subunits were purified in 1972, later becoming

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[9] Nonstandard abbreviations: WG, Working Group; hCG, human chorionic gonadotropin; IRP, International Reference Preparation; IS, International Standard; hCGα and hCGβ, α and β subunits of hCG; hCGβcf, β-core fragment of hCGβ; hCGn, nicked hCG; hCGβn, nicked hCGβ; RR, Reference Reagent; mAb, monoclonal antibody; RP-HPLC; reversed-phase HPLC; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IEF, isoelectric focusing; IFMA, immunofluorometric assay; hFSH, human follicle-stimulating hormone; CR, Canfield–Ross preparation; and CTP, hCGβ COOH-terminal peptide.

the 3rd International Standard (IS) for hCG (coded 75/537) and the 1st IRPs for its dissociated subunits, hCGα (75/569) and hCGβ (75/551) *(2, 3)*. The recently established 4th IS (75/589) was prepared at the same time as the 3rd IS from the same batch and is essentially identical to the 3rd IS, differing only by two separate filling procedures *(3)*.

In addition to preparing improved replacements for the existing standards, the WG undertook preparation of three new standards for urinary metabolites of hCG, namely the β-core fragment of hCGβ (hCGβcf) and nicked forms of hCG (hCGn) and hCGβ (hCGβn). These hCG metabolites, which are found mainly in urine but may also occur in serum *(4–7)*, are of interest both because of their variable reactivity in existing assays and their potential clinical utility, particularly in urine assays *(1, 5, 8–10)*. Substantial amounts of these metabolites, as well as traces of protease activity, have been shown to be present in the current standard preparations for hCG (IS 75/537 and 75/589), hCGα (IRP 75/569), and hCGβ (IRP 75/551) *(3, 11, 12)*, and these contaminants may be partly responsible for variability in the behavior of the 3rd/4th IS in various commercial hCG assay systems *(13, 14)*. Contamination with hCGn is also a major problem because some hCG assays recognize hCGn poorly, whereas others recognize hCGn and hCG equally, potentially giving rise to calibration errors. Recent advances in purification technologies provided an opportunity to prepare standards essentially devoid of these contaminants *(10, 15, 16)*. The structural characteristics of the various forms of hCG that were to be prepared, together with their approved IFCC nomenclature, are shown in Fig. 1.

This report describes the preparation and chemical characterization of the new Reference Reagents (RRs) for immunoassay standardization of hCG and hCG-related variants. Detailed data on the purification procedure and protein characterization, HPLC analyses, mass spectra, and amino acid analyses appear in a data supplement available with the online version of this article (http://www.clinchem.org/content/vol49/issue1/). A description of small-scale purification of hCG and related molecules has appeared earlier *(16)*, as has a progress report on the project *(17)*. Evaluation of the final ampouled standards, including stability studies, value assignment, and comparisons of immunoassay results, will be the subject of separate reports.

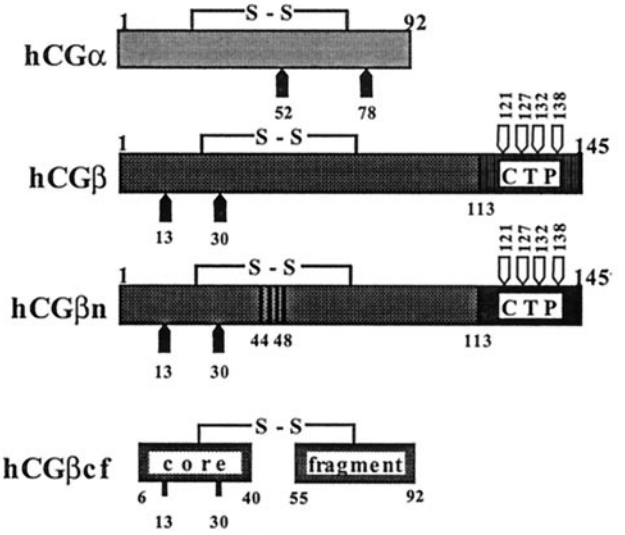

Fig. 1. Diagrams showing the structures of the subunits of hCG and its urinary metabolites.

The metabolites focus on those of the β subunit. N-glycans are indicated by *filled arrows* and O-glycans by *open arrows*. Peptide bond cleavages are indicated in nicked hCGβ by *vertical lines*. The hCG reference preparations described in this report are as follows: hCG is the intact heterodimeric hormone derived from late first trimester pregnancy urine. It consists of *hCG*α and *hCG*β with no peptide bond cleavages in either subunit. hCGn consists of intact hCGα combined with *hCG*β*n*, which has peptide bond cleavages in loop 2, generally between amino acid residues 47 and 48 but occasionally between residues 44 and 45 and at other positions. hCGβ is produced by dissociation of purified hCG and further purified by HPLC to remove nicked and oxidized material. hCGβn is a nicked form of hCGβ derived from hCGn; it is sometimes found free in urine specimens. *hCG*β*cf* is composed of hCGβ6–40 disulfide-bridged (*S-S*) to hCGβ55–92. hCGα is produced by dissociation of purified hCG and further purified by HPLC to remove nicked and oxidized material (not shown).

Materials and Methods

STARTING MATERIAL

Two lots (nos. 00010187 and 00006040) of pharmaceutical grade hCG from Diosynth (Division of Akzo/Nobel) with a biological activity of 4200 IU/mg of dry weight were used as starting material for preparation of the standards.

ANTIBODIES FOR WESTERN BLOTS

Monoclonal antibodies (mAbs; IDC) with selected specificities were chosen for Western blots: INN(sbruck)-hCG-22 (recognizes hCG and hCGβ-variants; broad specificity; epitope β_2, located on the top of β-strand loops 1 and 3 of hCGβ; amino acid regions hCGβ20–25 and hCGβ68–77); INN-hFSH-100 (hCG and hCGα; broad specificity; epitope α_2 on β-strand loop 1 of hCGα; amino acid region hCGα13–22), INN-hCG-80 (hCGα-specific; no recognition of dimer; epitope α_6 in the region of the subunit interaction site, amino acids hCGα33–42), and ISOBM-313, directed against amino acids hCGβ141–144 (epitope β_8) *(13, 18–20)*.

ANALYTICAL PROCEDURES

The methods for amino acid analysis, amino acid sequence analysis, and RP-HPLC were described previously *(16)*. Carbohydrate analyses were performed by the NIH-funded Research Center for Biomedical Complex Carbohydrates (Athens, GA). Monosaccharide composition was analyzed by gas chromatography–mass spectrometry on a HP 5890 gas chromatograph equipped with a Supelco DB1 fused-silica capillary column and interfaced to a 5970 mass spectrum detector. Trimethylsilyl derivatives of the methyl glycosides were prepared from 80 μg of each sample by methanolysis in 1 mol/L HCl in methanol at 80 °C (18–22 h), followed by re-N-acetylation with pyridine and acetic anhydride (for detection of amino sugars).

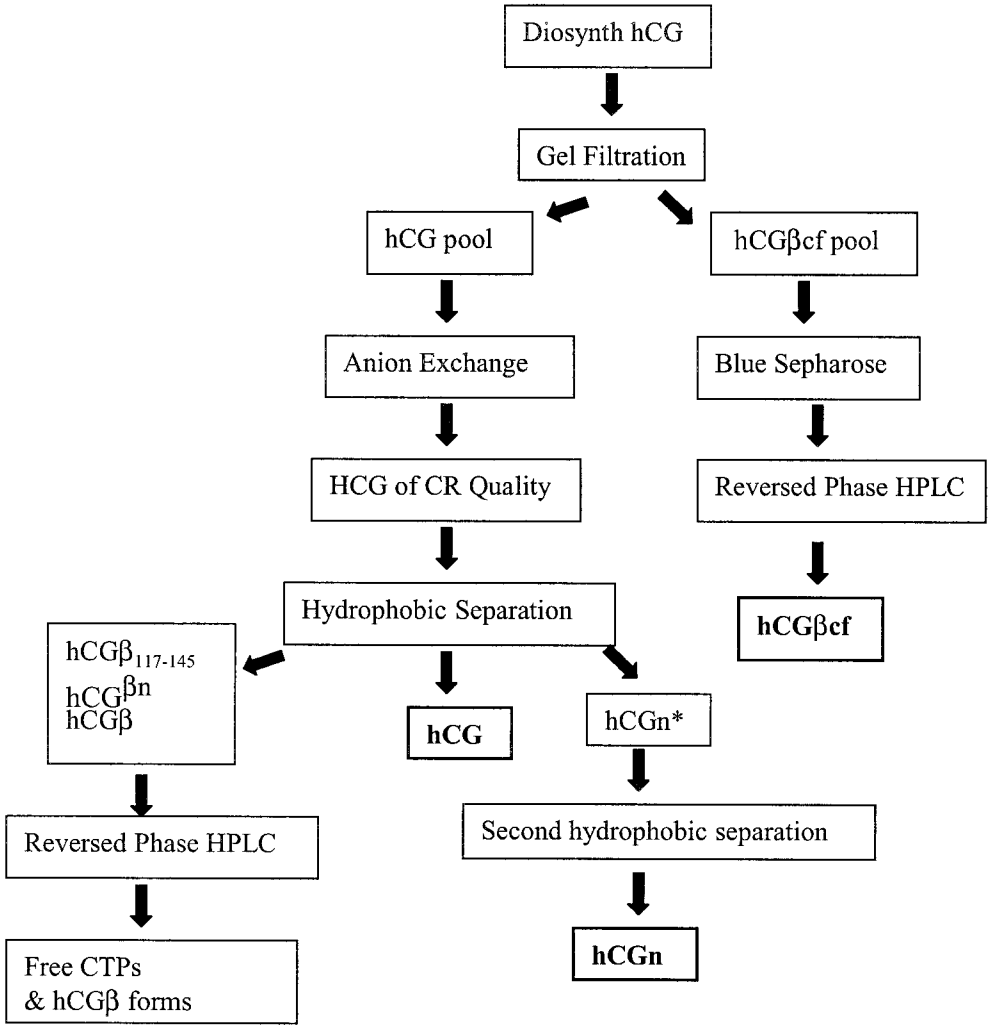

Fig. 2. Flow diagram of the purification pathway for preparation of hCG, hCGn, and hCG*β*cf from Diosynth crude urinary hCG preparation.
The final reference preparations are indicated by *bold lettering* and *boxes with thicker outlines*. *designates intermediate purity before final step.

The samples were then treated with Tri-Sil (Pierce Chemical)

SODIUM DODECYL SULFATE–POLYACRYLAMIDE GEL ELECTROPHORESIS, ISOELECTRIC FOCUSING, AND WESTERN BLOTTING

The purity and composition of the various preparations were assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting *(13, 21)*. Briefly, proteins were loaded on precast polyacrylamide gradient gels (4–20%; Novex) at equimolar concentrations and separated by SDS-PAGE (Mighty Small II; Hoefer Scientific Instruments) at 200 V for 50 min under nonreducing conditions. For isoelectric focusing (IEF), dry acrylamide gels (6% T) were reswollen to their original thickness in 3% ampholyte solution (Ampholine 3.5–9.5; Amersham Biosciences) with or without the addition of 9 mol/L urea. Protein solutions were diluted with 1 g/L bovine serum albumin in distilled water, and 5-μL aliquots were loaded (300 V, 5 mA, 5 W for 30 min) and separated (3000 V, 5 mA, 5 W for 120 min) at 20 °C.

Proteins were electrophoretically transferred from SDS-PAGE (3.5 h, 400 mA; Trans Blot® Cell; Bio-Rad) or IEF gels (60 min) onto PVDF membranes (Immobiline®; Millipore) by semidry Western blotting *(22)*. Membranes were blocked for 30 min in Tris-buffered saline-Tween buffer (0.02 mol/L Tris-HCl, 0.15 mol/L NaCl, 20 mL/L Tween 20, 0.1 g/L sodium azide, 30 g/L skim milk powder) and then incubated overnight in appropriate dilutions of reference mAbs [diluted in phosphate-buffered saline-Tween (0.05 mol/L sodium phosphate, 0.15 mol/L NaCl, 20 mL/L Tween 20, pH 7)] *(13, 18, 20)*. After extensive washing with phosphate-buffered saline, membranes were incubated for 1 h with goat anti-mouse IgG-horseradish peroxidase (Immunopure; Pierce) diluted 1:1000 in 10 g/L milk powder in phosphate-buffered saline (0.15 mol/L NaCl, 20 mmol/L sodium phosphate, pH 7.4). Chemiluminescent substrate conversion (Super

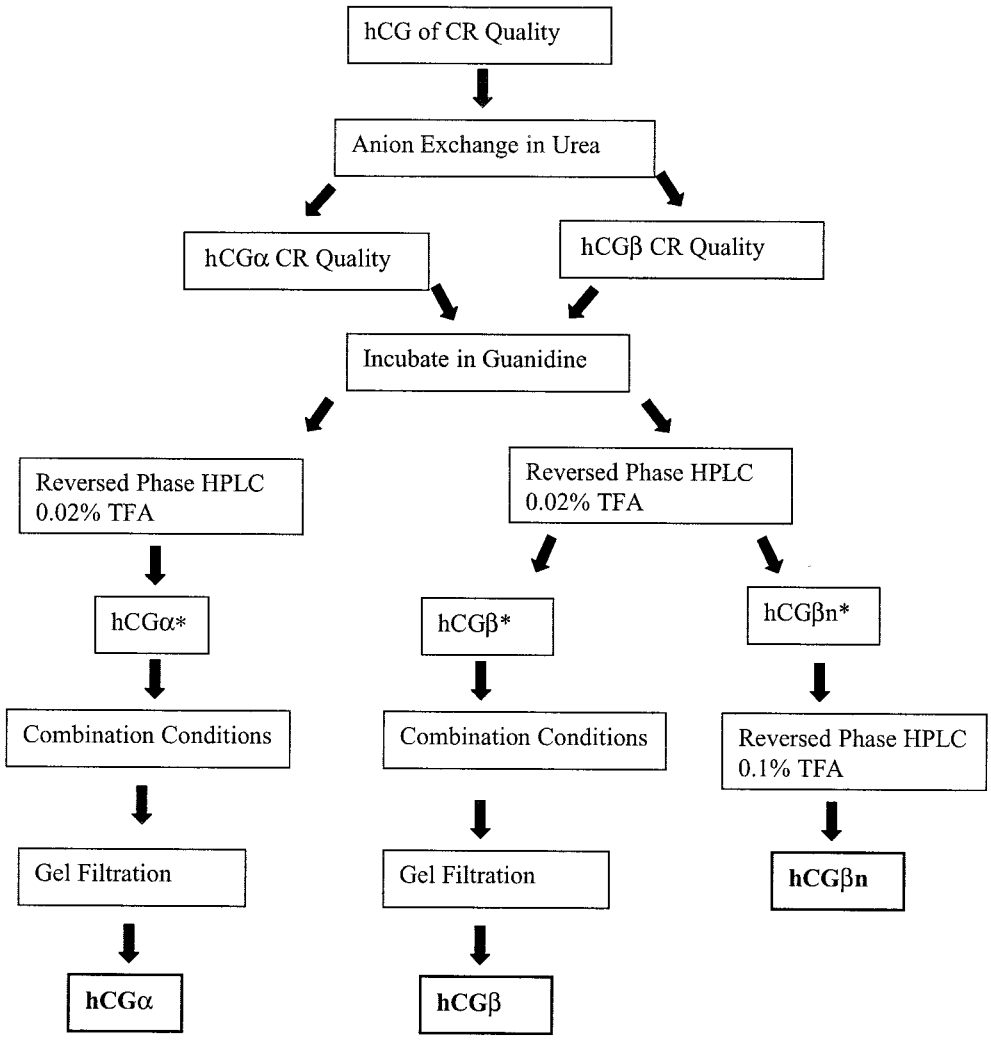

Fig. 3. Flow diagram of the purification of hCGα, hCGβ, and hCGβn from CR-quality hCG.

* designates intermediate subunit purity before the final step. The final reference preparations are indicated by *bold lettering* and *boxes with thicker outlines*. *0.02% TFA* and *0.1% TFA*, 0.2 and 1 mL/L trifluoroacetic acid, respectively.

Signal West Dura; Pierce) was detected with Hyperfilm ECL (Amersham International).

TIME-RESOLVED IMMUNOFLUOROMETRIC ASSAYS

The coating, blocking, and incubation steps of the immunofluorometric assays (IFMAs) for hCG, hCGβ, hCGβcf, and hCGα were performed as described for human follicle-stimulating hormone (hFSH) *(23)*. The characteristics of the mAbs against hCG, hCGβ, hCGβcf, hCGα, and hFSH have been described *(13, 18)*. These mAbs (IDC) served as RRs in the international TD-7 Workshop on antibodies to hCG and hCG-related molecules *(13)*. The coating mAbs were coded INN(sbruck)-45 (hCG+hCGn assay), -68 (hCGβ assay), -106 (hCGβcf assay), and INN-hCG-72 (hCGα assay). The detection mAbs (INN-hFSH-158 for the hCGα-assay; INN-hCG-22, a pan-β mAb), for all four other assays *(18)* were labeled with isothiocyanatophenylene triaminotetraacetic acid-europium (Wallac) according to the manufacturer's recommendations. Hormone standards (hCG 3rd IS 75/537, hCGβ 1st IRP 75/551, hCGα 1st IRP 75/569) used to determine the sensitivities and specificities of the IFMAs for hCG, hCGβ, and hCGα were kindly provided by the National Institute for Biological Standards and Control. hCGβcf was a gift by Drs. Klaus Mann and Rudy Hoermann (University of Essen, Essen, Germany).

Inter- and intraassay CVs for each IFMA were <10% over the entire assay ranges *(18)*. The new RRs were diluted in 0.01 mol/L $NaHCO_3$ containing 1 g/L bovine serum albumin, aliquoted, snap-frozen in liquid nitrogen, and stored at −80 °C until assayed.

SUMMARY OF PURIFICATION PROTOCOLS

Flow charts for the purification procedures are shown in Fig. 2 (hCG, hCGn, and hCGβcf) and Fig. 3 (hCGα, hCGβ,

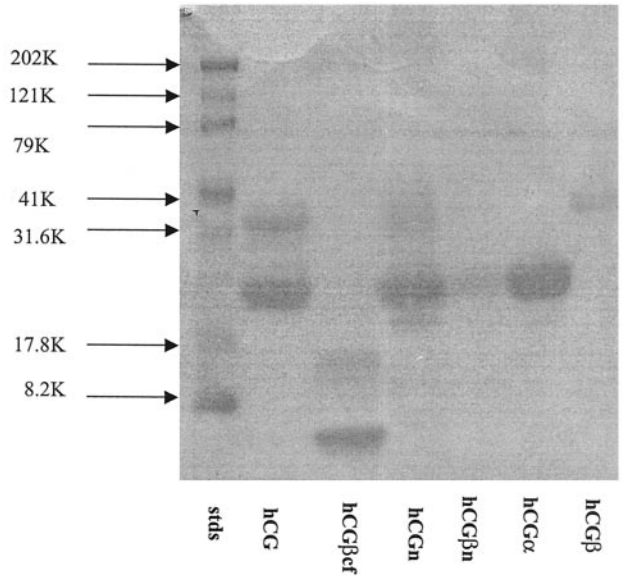

Fig. 4. SDS gel (reducing conditions) showing each of the starting materials for the RRs, stained with Coomassie Blue.

stds, molecular weight standards.

and hCGβn). Details of the purification procedures appear in the online data supplement (http://www.clinchem.org/content/vol49/issue1/).

BIOLOGICAL ACTIVITY

The biological activities of the hCG preparations were estimated in vivo in male rats by the seminal vesicle weight method *(24)* and in vitro by the Leydig cell method with testosterone production as the endpoint *(25, 26)*. The 3rd IS (75/537) was used as a standard. The in vivo assays were performed in two laboratories and the in vitro determinations in one.

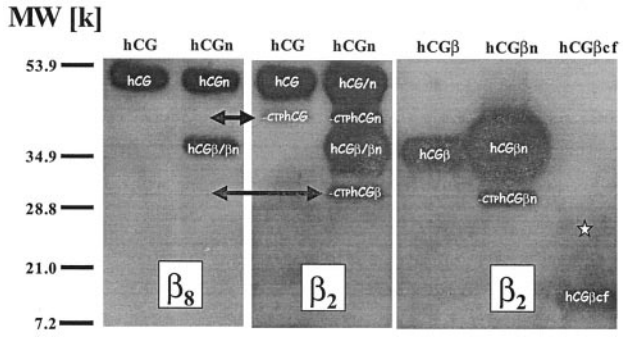

Fig. 5. Qualitative analysis of hCG and hCG-related variants by SDS-PAGE and Western blotting.

The five hCG/hCGβ variants were analyzed with use of mAbs against two different epitopes on free and assembled hCGβ. It should be emphasized that the results of this approach were not quantitative in nature. Although equimolar concentrations of hCGβ and hCGβn were loaded in each lane, the same mAb (INN-hCG-22; β_2) stained the latter preparation much more intensely. hCG, hCGβ, hCGβn, and hCGβcf appeared to be highly pure. The nature of additional bands became obvious when we compared the results of the mAb directed against the hCGβCTP (epitope β_8) with that recognizing an epitope in the core region of hCGβ (epitope β_2): These minute amounts of cross-contaminating molecules in hCG and hCGβn (*-CTPhCG*, *-CTPhCGn*, and *-CTPhCGβ/βn*; *arrows*) lack the end of hCGβCTP. hCGn obviously contains more of these contaminants, but again hCGβn might be overestimated, as mentioned above. hCGα was constantly negative with both anti-β mAbs; thus no significant hCG/hCGβ-associated contamination could be detected (data not shown).

Results

PURIFICATION OF hCG, hCGn, AND hCGβcf

Purification was started with molecular size fractionation to separate hCGβcf from the hCG components present in the starting material. Somewhat unexpectedly, the starting lot of Diosynth hCG contained only ~10% as much hCGβcf as that in lots of 10 or 20 years earlier. The method developed in 1972 and used to produce the 3rd IS [CR119; Canfield–Ross preparations of hCG made of Columbia University (CR)-quality hCG] *(2)* was improved by use of modern chromatographic media and the addition of hydrophobic interaction chromatography (Fig. 2). This permitted separation of hCGn and other minor contaminants from hCG. Some free fragments of hCGβCTP (β117–145 and other similar fragments) were also separated by this step. It is not clear whether these peptides had not been removed by previous steps or whether they were introduced by cleavage by some contaminating protease. After gel filtration, hCGβcf was further purified with use of Blue Sepharose and RP-HPLC.

PURIFICATION OF SUBUNITS

Subunit purification was accomplished starting with the CR-quality hCG preparation (Fig. 3). Although RP-HPLC can dissociate and separate subunits in a single step, oxidized forms and metabolites cause cross-contamination. Thus, oxidized forms of hCGβ coeluted with hCGα and oxidized forms of hCGα with hCGβn. Consequently, the subunits were first separated by anion-exchange chromatography *(2)* and were further purified by RP-HPLC in 0.2 mL/L trifluoroacetic acid. The fractions were immediately brought to a higher pH, lyophilized, incubated to permit recombination with any complementary subunit, and gel-filtered to remove any reformed hCG as well as to reduce salt content. Exposure to low pH was minimized to reduce loss of sialic acid residues.

hCGβn was prepared directly from the RP-HPLC purification step used to prepare hCGβ. An adequate quantity of hCGβn was isolated as a byproduct of the purification of hCGβ. A small quantity of urinary ribonuclease remained in the hCGβn preparation and was not easily removed. Other investigators have also reported copurification of this protein with hCGβcf *(27)*.

CHARACTERIZATION OF THE PREPARATIONS

The purity of each preparation was assessed by SDS-PAGE under reducing conditions (Fig. 4), immunoblotting (Figs. 5 and 6), IFMAs (Table 1), and by N-terminal sequence analysis (Table 2). The protein content of each preparation was quantified by amino acid analysis [Table S1 in the online data supplement (http://www.clinchem.org/content/vol49/issue1/)]. Comparison of the known

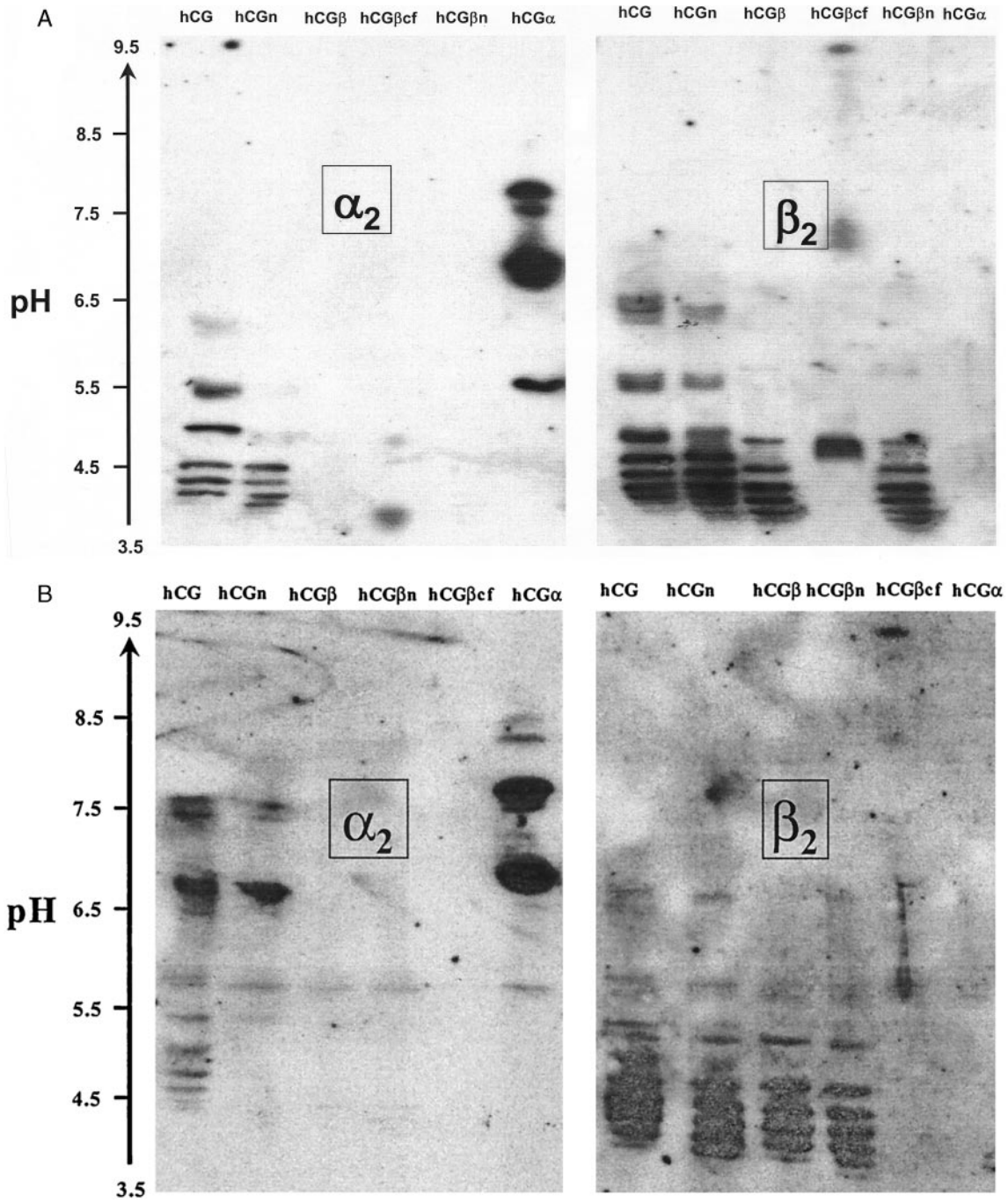

Fig. 6. Results of IEF and subsequent Western blot analysis of the six 1st WHO RRs.

Isoelectric points (pI) were determined under nondissociating (*A*) and dissociating (9 mol/L urea) conditions (*B*) with use of two reference mAbs [pan anti-α-subunit, INN-hFSH-100, epitope α_2 (*left panel*); and pan anti-β-subunit, INN-hCG-22, epitope β_2 (*right panel*)]. As shown previously *(21)*, because the carbohydrate antennae of assembled and nonassembled hCGβ(n) contained many more terminal sialic acids, all preparations except for hCGβcf and hCGα were highly acidic in nature. An array of at least six major bands appeared around pI 4.5. hCGα consisted of variants with neutral pI. hCGβcf was highly basic (pI >9.5), lacking terminal sialic acids on the carbohydrate units of the core region and the hyperglycosylated hCGβCTP. Thus, most of it has eluted from the gel. On overexposure of the film, only a contaminating acidic band can be seen, which disappears under dissociating conditions. Interestingly, some hCG variants are resistant to dissociation by urea. The *y axis* represents the approximate pH values in the gels.

primary structure with the calculated amino acid content showed good agreement.

SDS-PAGE (Fig. 4) confirmed the expected band patterns of purified hCGα, hCGβ, and hCG as well as the two bands expected for hCGβcf. Analyses of hCGn by RP-HPLC indicated the presence of ~10–15% of nonnicked hCG.

N-Terminal sequence analyses indicated that each preparation contained <2–3% contaminants; the limit of detection of the method (Table 2). The single exception

Table 1. Cross-contamination of hCG and hCG-related molecules in the frozen concentrates of the starting materials for the WHO 1st RRs hCG, hCGn, hCGβ, hCGβn, hCGβcf, and hCGα and the 3rd IS for hCG (75/537).

	Cross-contamination in assays specific for, % mol/mol[b]			
Preparation[a]	hCG, hCGn, - CTPhCG[c]	hCGβ/βn, - CTPhCGβ/βn, hCGβcf[c]	hCGβcf	hCGα
hCG 3rd IS (75/537)	100.0	3.9	0.8	1.4
hCG (99/688)	100.0	1.1	0.2	1.0
hCGn (99/642)	100.0	1.3	0.5	1.3
hCGβ (99/650)	0.3	100.0	0.2	0.02
hCGβn (99/692)	0.017	100.0	1.0	0.3
hCGβcf (99/708)	0.004	NA[d]	100.0	<0.001
hCGα (99/720)	0.031	0.015	0.04	100.0
Assay design/epitopes *(15, 16)*	c_3—β_2^{Eu}	β_7—β_2^{Eu}	β_{11}—β_2^{Eu}	α_6—α_5^{Eu}

[a] Frozen concentrates of the starting material for the WHO 1st RR except for the 3rd IS for hCG (75/537).
[b] Percentages correlated to the frozen concentrates of the new preparations and not to existing IS and IRPs.
[c] Recognition of hCG variants truncated at the CTP of hCGβ (-CTP) is suspected but not confirmed. All cross-contaminations were determined by highly specific time-resolved IFMAs (see *Materials and Methods*).
[d] NA, not applicable.

was the hCGβn preparation, which was contaminated with some urinary ribonuclease *(27)* as described above.

Characterization of the preparations by IEF and subsequent Western blotting (Figs. 5 and 6) with pan anti-α-subunit and pan anti-β-subunit antibodies showed that hCGα consists of three major bands with an approximately neutral pI. The various forms of hCG- and hCGβ-derived variants displayed at least six major bands around a pI of 4.5. hCGn and hCGβn appear to be more acidic than hCG and hCGβ. hCGβcf, with a pI >9.5, could not be analyzed by this approach. When hCG and hCGn were analyzed by IEF in 9 mol/L urea, the expected subunit patterns were observed (Fig. 6).

Table 2. N-Terminal sequence analysis of each preparation.

Ratio[a]	Molecule[b]	Sequence
hCG		
1.0	β	SKEPLRPR RPI
0.73	α1	APDVQD PE TL
0.28	α4	VQD
0.11	α3	DVQ
hCGn		
0.85	α1	APDVQD PE TL
0.28	α2	DVQD PE TLAQE
0.45	α4	VQD PE TLQED
1.0	β1	SKEPLRPR RPI
0.37	β45	LQGVLPALPQVV
0.28	β48	VLPALPQVV NY
hCGα		
1.0	α1	APDVQD PE TL
0.20	α4	VQD PE TLQED
0.07	α3	DVQ
hCGβ		
1.0	β1	SKEPLRPR RPI
hCGβn		
1.0	β1	SKEPLRPR RPI
0.60	β48	VLPALPQVV
0.54	β45	LQGVLPALP
0.10	Rnase[c]	KESR
hCGβf		
1.0	β6	RPR RPI ATLA
1.0	β55	VV NYRDVRFES

[a] Ratio is set to 1.0 for predominant molecule, and other sequences appear as a fraction of this ratio.
[b] Molecule defines the polypeptide chain and starting position of the sequence.
[c] A trace of urinary RNase could not be removed from this preparation.

The specific absorbance values for the preparations of hCG and its subunits differed slightly from published values, which were also based on protein content determined by amino acid analysis (Table 3) *(2, 16, 28)*. The extinction values for the 3rd IS were determined by absorbance at 280 nm, whereas for the current preparation, 276 nm was used, possibly contributing to this difference. However, the greater purity of the new preparation could also be responsible for the observed difference.

Results of carbohydrate analysis, RP-HPLC profiles, and mass spectra of the preparations are given in the online data supplement. The mean molecular weights were as follows: hCG, 37 500; hCGβ, 23 500; hCGα, 14 000; and hCGβcf, 10 500. Carbohydrate composition [Table S2 in the online data supplement (http://www.clinchem.org/content/vol49/issue1/)] agreed reasonably well with the expected content of each of the sugars. The sialic acid content (mol/mol) and the expected ranges were as follows: 9.8 (12–16) for hCG; 2.0 (2–4) for hCGα; 13.3 (8–12) for hCGβ; 8.5 (12–16) for hCGn, and 8.8 (8–12) for hCGβn. The reason for the higher than expected sialic acid content for hCGβ is not known and may be an experimental artifact. Complete compositional carbohydrate analysis was not performed for CR119 hCG, the 3rd IS, but the sialic acid content of CR119 hCG (10.6% by weight) was very similar to that of the new preparation (11.1% by weight). The percentage of carbohydrate by

Table 3. Extinction values of new reference preparations in water at 276 nm.[a]

Reference preparation	μmol/L = 1.0_{276nm}	g/L = 1.0_{276nm}
hCG	71.30	2.67
hCGn	63.75	2.39
hCGβ	175.90	4.13
hCGα	165.19	2.31
hCGβcf	197.10	2.07
hCGβn	138.71	3.26

[a] Concentrations in molarity and mass/volume for absorbance of 1.0 at 276 nm.

weight compared with protein weight for the purified lyophilized proteins was as follows: hCG, 29%; hCGα, 29%; hCGβ, 43%; hCGn, 35%; hCGn, 35%; hCGβn, 83%; and hCGβcf, 14%. The reason for the high sugar content of hCGβn is not known. This is obviously not protein-bound sugar but other sugar material mixed with the purified protein. Some glucose was also found, perhaps originating from the dextran columns used during purification.

BIOLOGICAL ACTIVITY

The median in vivo biopotency (with the 95% confidence intervals) measured by one laboratory was 15 400 (14 200–16 800) IU/mg, whereas in the other laboratory, it was 10 300 (8360–12 600) IU/mg. This laboratory also determined the in vitro biological potency to be 14 100 IU/mg (12 500–16 000).

PROPERTIES OF THE NEW 1st RR FOR IMMUNOASSAY OF hCG

The improved overall properties of the new hCG preparation 99/688 are compared with those of the 3rd/4th IS in Table 4. The free subunits were also improved to the same extent in terms of peptide bond intactness and freedom from contaminants, as described in the analysis above.

Table 4. Comparison of the hCG preparation (CR 119) used to create the 3rd and 4th IS and the 1st RR for immunoassay of hCG (99/688).

Property	3rd (75/537) or 4th IS (75/589)	1st RR (99/688)
Biological activity	9286 IU/mg	1.1–1.5 times more potent/mg
Polypeptide chain structures	10% cleaved hCGβ chains	Intact peptide chains only
Amino acid analysis	Agrees with structure	Agrees with structure
Sialic acid content, % by weight	10.6	11.1
hCG-related contaminants	Significant: hCGn, some hCGβ + hCGβcf (see Table 1)	No significant contaminants
Protease activity	Present	Negligible

Discussion

The major goal of this project was to develop new RRs for hCG and its subunits and major urinary metabolites to facilitate improved standardization and characterization of immunoassays for these analytes. Another goal of the WG was to evaluate the feasibility of assigning values to the standards in substance concentrations (mol/L) rather than in arbitrary units or units based on biological activity. These goals have been achieved: new RRs of high purity have been prepared and assigned values based on amino acid composition. Value assignment of the final RRs issued by WHO will be reported separately.

Preparation of the new RRs was achieved by use of an initial purification scheme similar to that for preparation of the 3rd IS of hCG, but more modern chromatographic media provided better separation, although care was taken to retain the microheterogeneity of hCG and its subunits, as revealed by IEF. This microheterogeneity primarily reflects the differences in sialic acid content typical of all glycoprotein hormones, and retention of these natural variants in the final preparations appears desirable. Removal of contaminating forms of hCG and other proteins was achieved by addition of hydrophobic interaction chromatography and RP-HPLC to the purification scheme. Use of hydrophobic interaction chromatography also facilitated removal of any residual traces of protease activities, such as those present in the 3rd IS *(3, 12, 29)* as well as in hCGn and hCGβcf *(15, 16)*.

Preparation of subunits of superior quality to the last subunit standards, IRP 75/569 and IRP 75/551, was difficult. Direct use of RP-HPLC to separate subunits in a single step was not feasible because oxidized subunits coeluted with the other subunit or with the metabolites. Methionine residues are easily oxidized, and when the single methionine in the hCGβ subunit is oxidized, hCGβ coelutes with hCGα. Because hCGα contains three methionines, it may elute as four different peaks. Anion-exchange chromatography in urea buffer did not separate oxidized from nonoxidized subunits but did allow good separation of each of the subunits. RP-HPLC was used subsequently to remove oxidized forms from each subunit. Further removal of any complementary subunit was achieved by allowing subunit recombination followed by gel filtration.

Our procedures relied heavily on analytical screening of every column fraction by RP-HPLC to decide which fraction should become part of the pool at each step. The large-scale procedure adopted facilitated production of the large quantity of protein required for IRPs. The preparations were thoroughly characterized with respect to protein and carbohydrate content and the presence of immunoreactive cross-contaminants. Amino acid sequence analysis was essential to verify polypeptide chain integrity and the extent of peptide bond cleavages in nicked materials. Because the sensitivity of Edman sequence analysis for detection of contaminating proteins is ~3%, highly sensitive IFMAs and SDS-gel electrophoresis

in conjunction with Western blotting were used for more sensitive assessment of purity.

Compared with the 3rd/4th IS for hCG, the new 1st RR for hCG contains much less hCGn, hCGβ, hCGα, and hCGβcf (Table 1). Estimation of the content of contaminating forms of hCG by time-resolved IFMAs showed that the preparations of hCG, hCGβ, hCGβ, and hCGα were of excellent purity. Only hCGn contained substantial amounts (>1%) of hCG, (-CTP)hCGβ(n), and hCGα (Table 1 and Figs. 4 and 5). These results were confirmed by Western blotting (Fig. 6). No hCG-related contaminants were observed in hCGβ, hCGβcf, and hCGα. There are minute amounts of (*a*) -CTPhCG (hCG lacking the C-terminal portion of hCGβ) and hCGα in hCG 99/688, (*b*) -CTPhCGβ(n) in hCGβn, and (*c*) -CTPhCG and -CTPhCGβ(n) and hCGα in hCGn. Presumably, a somewhat larger amount of hCGβ/βn can be found in hCGn. Because the mAbs used for Western blotting recognize the various forms of hCG/hCGβ with different affinities, the method is not quantitative. Nevertheless, it provides a qualitative screen for those hCG-derived variants for which no purified preparations are available, i.e., hCG and hCGβ variants truncated at their COOH terminus (-CTPhCG, -CTPhCGn, -CTPhCGβ, and -CTPhCGβn).

The combination of all these methods facilitated accurate characterization of the preparations and demonstration of their very high quality. Two metabolite preparations were somewhat contaminated: hCGn contained ~10% hCG, whereas hCGβn contained ~10% urinary ribonuclease and was the only preparation with detectable foreign protein. This does not prohibit their use for characterization of the specificity of immunoassays for hCG and its subunits.

The sialic acid content of the new reference preparation of hCG is similar to that of CR 119 (the 3rd IS). The major change in protein content observed by N-terminal sequence analysis was complete removal of the peptide bond cleavages within the β-subunit at positions 44–45 and 47–48 *(9)*. This, together with the IEF patterns observed (Fig. 6) and the excellent in vivo and in vitro biological activity demonstrated, provides assurance that the hydrophobic step selected molecular species representative of the physiologically relevant active hormone.

The availability of the new reference preparations provides a sound scientific basis for improving the standardization of assays for hCG-related molecules. The 3rd IS for hCG (75/537) was calibrated by bioassay in international units (IU) against the 2nd IS. The subunits, which lack bioactivity, were assigned arbitrary units (also IU) based on mass. This common unitage falsely suggests that such "standardization" of hCG and its subunits has a common basis. Value assignment on the basis of biological activity is appropriate for hCG standards intended for therapeutic purposes. In contrast, for hCG standards used to calibrate immunoassays, value assignment in substance concentrations on the basis of amino acid analysis, as for the RRs reported here, is much more informative. As an important first step toward improving the comparability of immunoassays for hCG, the new standards will allow manufacturers to calculate the cross-reactivity of these six hCG-related molecules in the immunoassays they supply. Inclusion of such data in assay inserts will enable direct comparison of the relative recognition of these molecules in different assay systems. This should be achievable for currently available hCG immunoassays.

Realistically, the new standards are likely to be used for primary calibration of the next generation of hCG immunoassays rather than for current assays. Careful consideration will then be required as to how best to transfer values for the primary (nonnicked) hCG standard to the "working" secondary standards used by manufacturers, because the latter are likely to contain substantial amounts of nicked hCG. At the same time, convincing clinical colleagues of the advantages of adopting molar units for hCG will present a major challenge to laboratorians.

The use of molar units is particularly helpful when the ratios of various forms of hCG are compared, as in the diagnosis of Down syndrome and trophoblastic tumors. Some groups already report results for hCG, its subunits, and fragments in molar concentrations based either on locally prepared standards or on theoretical values calculated from the WHO standards *(30, 31)*. Value assignment in substance concentrations also increases the probability that subsequent standards can be calibrated identically. [Unfortunately this is not possible retrospectively for the 3rd/4th IS because these preparations contain carrier protein.] Measurement of the molar content of protein by amino acid analysis also eliminates some of the practical problems associated with glycoproteins, e.g., difficulties in gravimetric determination of glycoprotein mass because of tight binding of water molecules. Although carbohydrate residues present on glycoproteins rarely affect immunoreactivity, they may strongly affect biological activity, e.g., by modulating the half-lives of circulating glycoproteins or by influencing receptor binding and activation. Because antibodies almost exclusively recognize peptide epitopes, the molar content of proteins, rather than their bioactivity, may be expected to best reflect what is measured by immunoassays.

Although antibodies that recognize glycan-associated epitopes within hCG are very rare *(32)*, the potential utility of measurement of hyperglycosylated hCG (acid variants of hCG with high carbohydrate content) in early pregnancy and in cancer patients has been the subject of recent reports *(33 34)*. The current interest in hyperglycosylated hCG is related to the hCG isoforms recognized by antibody B152, which is directed to the glycans and peptide structure within the hCGβ COOH-terminal peptide (CTP) region *(35)*, but this antibody does not recognize all forms of hyperglycosylated hCG., e.g., those with modifications in the N-glycan region only. Preparation of a reference standard for hyperglycosylated hCG would be desirable but was not part of the focus of the current project.

It is not likely, however, that the presence of various hyperglycosylated hCG forms in serum or urine causes errors in current commercial assays for hCG. It has recently been shown by IEF, Western blotting, and sandwich immunoassays that all mAbs tested, including those against all major epitopes located in the immunodominant regions such as the top of the two adjacent β-strand loops 1 and 3 of hCGβ, recognize the entire spectrum of glycosylation variants, including deglycosylated hCG, asialo-hCG, variants of more neutral pI, and highly acidic (hyperglycosylated) variants of pregnancy hCG as well as highly purified acidic hCG variants of tumor patients *(21)*. All of these epitopes are therefore predominantly determined by the protein backbone, and the carbohydrate moieties that form a biochemical basis for hCG heterogeneity are neither of major antigenic relevance nor are they structurally related to any immunodominant antigenic region. Most manufacturers of "hCG" reagent sets use mAbs with epitope specificities identical to the reference mAbs used for these studies *(13)*. The calibration of the RRs in substance concentrations (mol/L) is undoubtedly an important advantage for the measurement of hCG that is heterogeneous in nature with respect to glycosylation.

In conclusion, we have produced highly purified preparations of hCG, hCGα, hCGβ, and three partially degraded forms of these. The protein contents of these preparations were determined by amino acid analysis. The WHO has approved these preparations as RRs, and their use should facilitate improved comparability among immunoassays for measurements of hCG, its subunits, and its metabolites. The characterization of the final WHO preparations as well as their application in external quality assessment (proficiency testing) schemes will be reported separately.

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References

1. Stenman UH, Bidart JM, Birken S, Mann K, Nisula B, O'Connor J. Standardization of protein immunoprocedures. Choriogonadotropin (CG). Scand J Clin Lab Invest Suppl 1993;216:42–78.
2. Canfield RE, Ross GT. A new reference preparation of human chorionic gonadotrophin and its subunits. Bull World Health Organ 1976;54:463–72.
3. Storring PL, Gaines-Das RE, Bangham DR. 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 α and β subunits of human chorionic gonadotrophin for immunoassay. J Endocrinol 1980;84:295–310.
4. Alfthan H, Stenman UH. Pregnancy serum contains the β-core fragment of human choriogonadotropin. J Clin Endocrinol Metab 1990;70:783–7.
5. Birken S, Kovalevskaya G, O'Connor J. Metabolism of hCG and hLH to multiple urinary forms. Mol Cell Endocrinol 1996;125:121–31.
6. Birken S, Gawinowicz MA, Maydelman Y, Milgrom Y. Metabolism of gonadotropins: comparisons of the primary structures of the human pituitary and urinary LH β cores and the chimpanzee CG β core demonstrate universality of core production. J Endocrinol 2001;171:131–41.
7. O'Connor JF, Birken S, Lustbader JW, Krichevsky A, Chen Y, Canfield RE. Recent advances in the chemistry and immunochemistry of human chorionic gonadotropin: impact on clinical measurements. Endocr Rev 1994;15:650–83.
8. Birken S, Armstrong EG, Kolks MA, Cole LA, Agosto GM, Krichevsky A, et al. Structure of the human chorionic gonadotropin β-subunit fragment from pregnancy urine. Endocrinology 1988; 123:572–83.
9. Birken S, Gawinowicz MA, Kardana A, Cole LA. The heterogeneity of human chorionic gonadotropin (hCG). II. Characteristics and origins of nicks in hCG reference standards. Endocrinology 1991; 129:1551–8.
10. Cole LA, Kardana A, Andrade-Gordon P, Gawinowicz MA, Morris JC, Bergert ER, et al. The heterogeneity of human chorionic gonadotropin (hCG). III. The occurrence and biological and immunological activities of nicked hCG. Endocrinology 1991;129:1559–67.
11. Wehmann RE, Blithe DL, Akar AH, Nisula BC. β-Core fragments are contaminants of the World Health Organization Reference Preparations of human choriogonadotrophin and its α-subunit. J Endocrinol 1988;117:147–52.
12. Daja MM, Hiyama J, Scott GK, Renwick AG. The detection and isolation of protease activity associated with purified preparations of human chorionic gonadotropin. Endocrinology 1993;132: 1766–73.
13. Berger P, Sturgeon C, Bidart JM, Paus E, Gerth R, Niang M, et al. 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 2002;23:1–38.
14. Cembroski GS, Hanna AN, Hofer TL, Blakney TN, Higgins TN, Wesenberg JC, et al. Measurement of inaccuracy and imprecision

in hCG measurements performed by Abbott AxSYM, Roche Elecsys, Bayer Centaur, Beckman Access, Dade Stratus CS, Tosoh AIA 600 II, and Dade Opus Plus using dilutions of the WHO 4th IS hCG standard and a pregnant patient's serum [Abstract]. Clin Chem 2002;48(Suppl A):138.

15. Birken S, Chen Y, Gawinowicz MA, Lustbader JW, Pollak S, Agosto G, et al. Separation of nicked human chorionic gonadotropin (hCG), intact hCG, and hCG β fragment from standard reference preparations and raw urine samples. Endocrinology 1993;133: 1390–7.
16. Birken S, Maydelman Y, Gawinowicz MA. Preparation and analysis of the common urinary forms of human chorionic gonadotropin. Methods 2000;21:3–14.
17. Sturgeon C, Stenman UH, Birken S, Berger P, Lequin RM, Norman RJ, et al. IFCC Working Group on Standardization of human chorionic gonadotropin measurements: progress report. Proc UK NEQAS Meeting 1998, 1999;3:134–9.
18. Madersbacher S, Berger P. Antibodies and immunoassays. Methods Enzymol 2000;21:41–50.
19. Berger P, Panmoung W, Khaschabi D, Mayregger B, Wick G. Antigenic features of human follicle stimulating hormone delineated by monoclonal antibodies and construction of an immunoradiomometric assay. Endocrinology 1988;123:2351–9.
20. Berger P, Klieber R, Panmoung W, Madersbacher S, Wolf H, Wick G. Monoclonal antibodies against the free subunits of human chorionic gonadotrophin. J Endocrinol 1990;125:301–9.
21. Berger P, Schwarz S, Spottl G, Wick G, Mann K. Variants of human chorionic gonadotropin from pregnant women and tumor patients recognized by monoclonal antibodies. J Clin Endocrinol Metab 1993;77:347–51.
22. Kyhse-Andersen J. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J Biochem Biophys Methods 1984;10:203–9.
23. Madersbacher S, Shu-Chen T, Schwarz S, Dirnhofer S, Wick G, Berger P. Time-resolved immunofluorometry and other frequently used immunoassay types for follicle-stimulating hormone compared by using identical monoclonal antibodies. Clin Chem 1993; 39:1435–9.
24. European Directorate. Gonadotrophin, chorionic. European Pharmacopoeia. Strasbourg: European Directorate for the Quality of Medicine, 2001.
25. Dufau ML, Pock R, Neubauer A, Catt KJ. In vitro bioassay of LH in human serum: the rat interstitial cell testosterone (RICT) assay. J Clin Endocrinol Metab 1976;42:958–69.
26. Van Damme MP, Robertson DM, Diczfalusy E. An improved in vitro bioassay method for measuring luteinizing hormone (LH) activity using mouse Leydig cell preparations. Acta Endocrinol (Copenh) 1974;77:655–71.
27. Griffiths SJ, Bramley TA, Menzies GS, Adams DJ. Co-purification of a ribonuclease and human chorionic gonadotrophin β-core protein from human urine and displacement of ^{125}I-human luteinizing hormone from *Candida albicans* binding sites by ribonucleases. Mol Cell Endocrinol 1997;134:69–76.
28. Morgan FJ, Canfield RE, Vaitukaitis JL, Ross GT. Properties of the subunits of human chorionic gonadotropin. Endocrinology 1974; 94:1601–6.
29. Hiyama J, Renwick AG. Separation of human glycoprotein hormones and their subunits by reversed-phase liquid chromatography. J Chromatogr 1990;529:33–41.
30. Alfthan H, Haglund C, Roberts P, Stenman UH. Elevation of free β subunit of human choriogonadotropin and core β fragment of human choriogonadotropin in the serum and urine of patients with malignant pancreatic and biliary disease. Cancer Res 1992;52: 4628–33.
31. Marcillac I, Cottu P, Theodore C, Terrier-Lacombe MJ, Bellet D, Droz JP. Free hCG-β subunit as tumour marker in urothelial cancer. Lancet 1993;341:1354–5.
32. Birken S, Agosto G, Amr S, Nisula B, Cole L, Lewis J, Canfield R. Characterization of antisera distinguishing carbohydrate structures in the β-carboxyl-terminal region of human chorionic gonadotropin. Endocrinology 1988;122:2054–63.
33. Cole LA, Shahabi S, Oz UA, Bahado-Singh RO, Mahoney MJ. Hyperglycosylated human chorionic gonadotropin (invasive trophoblast antigen) immunoassay: a new basis for gestational Down syndrome screening. Clin Chem 1999;45:2109–19.
34. Kovalevskaya, Birken S, Kakuma T, O'Connor JF. Early pregnancy human chorionic gonadotropin (hCG) isoforms measured by an immunometric assay for choriocarcinoma-like hCG. J Endocrinol 1999;161:99–106.
35. Kovalevskaya G, Birken S, Kakuma T, Ozaki N, Sauer M, Lindheim S, et al. Differential expression of human chorionic gonadotropin (hCG) glycosylation isoforms in failing and continuing pregnancies: preliminary characterization of the hyperglycosylated hCG epitope. J Endocrinol 2002;172:497–506.