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Between-Method Variation in Human Chorionic Gonadotropin Test Results

Laurence A. Cole,[1*] Jaime M. Sutton,[1] Trefor N. Higgins,[2] and George S. Cembrowski[3]

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Background: **Results on sera and calibrators vary 1.4- to 2.3-fold among commercial human chorionic gonadotropin (hCG) assays. The relative contributions of calibrators, standards, hCG charge isoforms, and major structural variants to this variation have not been quantified.**
Methods: **Purified hCG was separated by isoelectric focusing into four fractions with pI ranges of 3–4, 4–5, 5–6, and 6–7. These four fractions together with pure hCG, hyperglycosylated hCG, hCG β-subunit (hCGb), nicked hCG, and hCGb core fragment (hCGbcf) were tested in nine commonly used commercial serum assays for hCG. The compositions of pure hCG preparations, standards, and commercial hCG preparations were determined by immunoassay.**
Results: **The three pure hCG preparations and the four hCG charge isoforms each showed parallel responses in the nine commercial hCG assays. Although wide variations were found in the detection of hCG structural variants by the nine assays (range for hyperglycosylated hCG, 468-1544 IU/L; for hCGb, 3187-5535 IU/L; for nicked hCG, 2736-4240 IU/L; and for hCGbcf, <2–130 IU/L), this did not correlate with the between-method variation observed in results for the three pure hCG preparations. Commercial preparations of hCG and calibrators showed great variation in their content of hCG structural variants (from 34% to 100% intact hCG).**
Conclusions: **Intermethod differences in hCG results were not explained by changes in responses attributable to hCG charge isoforms or to hCG structural variants, but wide variation was observed in concentrations of hCG structural variants in calibrators and in detection of these structural variants. Differences in assay specificity and in composition of the calibrators are the most likely sources of between-method variation.**
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More than 40 methods to measure serum human chorionic gonadotropin (hCG),[4] 24 urine point-of-care, and 23 home pregnancy test methods for urine are sold in the United States. All use calibrators traceable to WHO hCG standards. The first WHO International Standards (in 1938 and 1964) were both crude hCG preparations. In 1978, they were replaced by the 1st International Reference Preparation (IRP; hCG preparation 75/735), prepared from highly purified hCG batch CR119. This same preparation later became the 3rd International Standard (IS; hCG preparation 75/537), released in 1986, and then the 4th IS (hCG preparation 75/589), released in 1999. Currently, all tests use calibrators traceable to either the 3rd IS or the 4th IS. These standards (3rd IS-like standards), like all other hCG standards, are purified from a commercial crude extract of urine from pregnant women *(1, 2)*. The urine was collected and stored at ambient temperature, which allowed for cleavage or nicking of hCG. All hCG assays use antibodies generated against hCG or its subunits, purified from similar urine preparations *(3)*. Approximately 9% of the hCG molecules in the 3rd IS standard preparation are nicked or cleaved in the region of β-subunit residues 43–48 *(1)*.

A new hCG standard, the 1st Reference Reagent (RR; hCG preparation 99/688), which was isolated and prepared from a new urine extract, is in the process of being released. To improve assay recognition and between-method variation, this new standard was further purified to remove enzyme impurities and the nicked or cleaved hCG molecules that were present in the 3rd IS-like standards *(4)*.

[1] USA hCG Reference Service, Department of Obstetrics and Gynecology, University of New Mexico, Albuquerque, NM 87131.
[2] Dynacare Kasper Medical Laboratories, Edmonton, Alberta, Canada.
[3] Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Alberta, Canada.
*Address correspondence to this author at: Department of Obstetrics and Gynecology, University of New Mexico, 2211 Lomas Blvd., N.E., Albuquerque, NM 87131. Fax 505-272-6385; e-mail larry@hcglab.com.
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[4] Nonstandard abbreviations: hCG, human chorionic gonadotropin; IRP, International Reference Preparation; IS, International Standard; RR, Reference Reagent; hCGa and -b, unassociated hCG α- and β-subunit, respectively; hCGbcf, hCG β-subunit core fragment; and CAP, College of American Pathologists.

hCG is an unusual glycoprotein with eight oligosaccharide side chains. Sugar residues account for ~30% of the molecular weight, and variation in oligosaccharide sequence is a key factor in the hCG structure *(5)*. Each of the antennae of the oligosaccharide side chains can terminate in a sialic acid residue. There are two N-linked and four O-linked oligosaccharides on the β-subunit and two N-linked oligosaccharides on the α-subunit. Wide variation in the sialic acid content of hCG exists, with 8–15 sialic acid residues attached to hCG, leading to charge heterogeneity (isoforms) in all hCG clinical samples and standards *(4–7)*.

The sialic acid content and charge of hCG vary in normal and abnormal pregnancies *(5–7)*. A hyperglycosylated form of hCG, with additional antennae on the N- and O-linked oligosaccharide side chains, is the predominant form of hCG produced in early pregnancy, around the time of implantation and in the 3 weeks that follow *(8–11)*. It is also a major component of all pregnancy serum and urine samples and of hCG standards *(9, 11)*. The majority of commercially available hCG assays, whether for serum testing in a laboratory or for point-of-care use, either under- or overdetect hyperglycosylated hCG *(9, 11, 12)*. This is a potential source of hCG test disparity *(9, 11, 12)*. Hyperglycosylated hCG can vary greatly in sialic acid content, with molecules having between 0 and 19 residues *(5–7)*. The variation in sialic acid is the cause of charge variation *(6, 7)*. Hyperglycosylated hCG is generally more deficient in this acidic sugar than is regular hCG *(5)*. Compounding this dissimilarity between regular and hyperglycosylated hCG, serum and urine preparations can vary substantially in sialic acid content, as reflected in the isoelectric point or charge of hCG.

In addition to hCG and hyperglycosylated hCG, nicked or cleaved variants of these molecules are detected in serum and urine from pregnant women and in hCG standards *(1, 3, 4, 12, 13)*. Nicked and nonnicked hCG β-subunits (hCGb) are also detected in serum and urine samples *(3, 8, 12)*, and hCGb core fragment (hCGbcf), the terminal degradation product of hCGb, is detected in urine samples *(3, 8, 12)*. The hCGbcf is the principal form of hCGb present in urine samples through most of gestation *(3)*.

Taken together, pregnancy hCG immunoreactivity comes from very heterogeneous molecules that vary in sialic acid or charge content, carbohydrate structure, nicking or cleavage, and content of hCGb and hCGbcf. All of these charge, carbohydrate, and peptide structure variants of hCGb dramatically affect the recognition of samples by different total hCG assays *(3, 12)*. Most total hCG assays detect hCG and hCGb. Some also detect hyperglycosylated hCG, some detect nicked molecules, and a few detect nicked hCG or hCGb missing the C-terminal peptide, or hCGbcf *(3, 12)* (see Table 1).

Wide variation in hCG results obtained with different immunoassays has long been a problem *(14)*. Unfortunately there has been no improvement in between-method variation, and it remains a problem *(12, 15)*. In interlaboratory surveys of the College of American Pathologists (CAP), hCG quality-control preparations vary. Some may be pure hCG (e.g., C15), others made be cruder preparations (e.g., C8) contaminated with urine hCGb, nicked hCG, and hCGbcf (L.A. Cole, S.A. Khanlian, and J.M. Sutton, unpublished data). Seemingly, a mixture of pure and crude quality controls were provided by CAP for the K series surveys. Depending on the sample, the methods may show differences of 1.4- to 2.3-fold in results, and different assays may give the highest mean hCG result for different samples.

In patients with trophoblastic disease, even greater between-method variation has been observed. As much as a 58-fold variation has been reported in different assay results with single serum samples *(16)*.

Here we investigate between-method variation using structurally defined hCG preparations.

Table 1. Detection limits, cross-reactivities, and characteristics of commonly used automated hCG assays.

Commercial assay	Measuring range, IU/L	Cross-reacting molecules	LH[b] cross-reactivity. %	Targets of capture/tracer antibodies	Detection method
Abbott AxSYM	2.0–1000	hCG + hCGb	0.09	hCGb/hCGbCTP	Enzyme fluorimetry
Bayer ACS-180	2.0–1000	hCG + hCGb	<10	hCGb + hCG/hCGbCTP	Chemiluminescence
Bayer ADVIA Centaur	2.0–1000	hCG + hCGb	<10	hCGb + hCG/hCGbCTP	Chemiluminescence
Dade Dimension RXL	1.0–1000	hCG	<2.0	hCGa/hCGb	Enzyme spectrometry
Dade Stratus CS	1.0–1250	hCG + hCGb	0.7	hCGb/hCGbCTP	Enzyme fluorimetry
DPC Immulite	1.1–5000	All major variants[c]	0.7	hCGb/hCGb	Chemiluminescence
Ortho Vitros ECi	0.5–1000	hCG + hCGb	2.5	hCGb/hCGbCTP	Enzyme luminescence
Roche Elecsys	0.5–1000	hCG + hCGb	0.07	hCGb/hCGbCTP	Chemiluminescence
Tosoh AIA600	0.4–400	hCG + hCGb	NA	hCGb/hCGbCTP	Enzyme fluorimetry

[a] Data were obtained from manufacturer's assay literature (Abbott, DPC, and Dade), publications (Abbott and Bayer) *(3, 23)*, and manufacturer's technical support representatives (Roche, Tosoh, Ortho, and Bayer). The accuracies of the descriptions of epitopes of antibodies or luteinizing hormone cross-reactivities are the best interpretation of the manufacturers' technical support representatives.

[b] LH, luteinizing hormone; hCGbCTP, C-terminal peptide of hCGb; NA, not available.

[c] Refers to hCG + nicked hCG + hyperglycosylated hCG + (hCGb+ hCG).

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Materials and Methods

hCG and related molecule preparations

hCG batch CR119 is the source hCG of the WHO 1st IRP, 3rd IS (also known as 75/537), and 4th IS (also known as 75/589). CR119, hCG batch 99/688 (also known as the WHO 1st RR), and hCGb preparation CR129 were all kindly provided by Dr. Steven Birken (Columbia University). Quality-control preparation C15 was provided to our clinical laboratory by the CAP for calibration of our clinical hCG immunoassays. Pure nicked hCG, preparation M4, was prepared in our laboratory. The purification of these hCG preparations and their peptide and N-and O-linked oligosaccharide structures have been described *(5)*. Nonnicked hyperglycosylated hCG was purified from JEG-3 cells, and the structure examined as published *(9)*. hCGbcf was purified from normal first-trimester pregnancy urine, and the structure was confirmed as published *(17)*.

We isolated intact hCG from urine from women in the 8th week of pregnancy. Batch P9 hCG contains no nicked hCG *(5)*, <0.5% hCGb (DPC Immulite free β-subunit assay on the DPC Immulite Chemiluminescence Automated Immunoassay Platform), no detectable hCGbcf, and 9% hyperglycosylated hCG, the expected proportion of hyperglycosylated hCG for this time of pregnancy *(11)*. hCG batch P9, 5 mg (~55 000 IU), was separated by preparative isoelectric focusing on a Rotofor apparatus (Bio-Rad) with a miniRotofor cell, a Bio-Rad Power-Pac 3000 power supply, and a circulating refrigeration bath (Neslab). The Rotofor cell was prefocused with 1.5% ampholytes, pI range 3–7, at constant power (15 W) for 1 h at 4 °C (refrigerated circulating bath). hCG batch P9 was then focused at constant power (15 W) at 4 °C until voltage stabilization was achieved. After an additional 30 min, power was removed, and twenty 1-mL fractions were collected. The pH of each of the 20 fractions was measured with a microelectrode to determine the corresponding pI. Fractions were pooled into pI ranges 3–4, 4–5, 5–6, and 6–7. A blank run was made under similar conditions with no added hCG or other protein. The concentration of hCG in each of the four pools was measured by absorbance at 278 nm as proposed by Birken et al. *(4)* for calibrating hCG standards. To calculate mass, the absorbances of the P9 fractions were measured against corresponding pI fractions from the blank run; the concentrations were 1.331, 2.023, 4.577, and 2.235 g/L, respectively. Each of the four isoforms was diluted 10 000-fold in normal male serum (Sigma Chemical Co.), aliquoted, and distributed for immunoassay.

immunoassays

Preparations of hCG and hCG-related molecules were added to normal male serum. Portions of these preparations and the P9 preparations were blindly coded and tested in nine different total hCG assays at 10 separate clinical laboratories. Included were the assays used most frequently in North America *(14, 15)*. Samples were tested with the Abbott AxSYM platform test (used at three sites: Dynacare Kasper Medical Laboratories, Edmonton; Calgary Laboratory Services, Calgary; and Royal Alexandra Hospital, Edmonton), Bayer ACS-180 (Northern Alberta Institute of Technology, Edmonton), Bayer ADVIA Centaur (University of Alberta Hospital, Edmonton), Dade Dimension RXL (Chinook Health Region Laboratory, Lethbridge), Dade Stratus CS (Eagle Ridge Hospital, Port Moody), DPC Immulite (University of New Mexico Medical Center, Albuquerque), Roche Elecsys E170 run on the 1010 platform (University of Alberta Hospital), Tosoh AIA600 (Somagen Diagnostics, Edmonton), and the Vitros ECi (Lloydminster Regional Hospital, Lloydminster). The analytical characteristics of these nine commonly used assays *(15, 18)*, are outlined in Table 1.

analysis of hCG preparations and secondary standards

hCG batch CR119 (WHO 1st IRP, 3rd IS, and 4th IS), hCG preparation 99/688, hCG preparation P9, and CAP quality-control preparation C15 were analyzed in multiple immunoassays to determine the content of intact hCG and hCG-related molecules. The commercial hCG preparations Sigma C6322 (CHO-cell recombinant hCG) and C5297 (Sigma Diagnostics), Serono Profasi (Serono), Ferring Choragon (Ferring), Organon Pregnyl (Organon), and Scripps C0713 and C0714 (Scripps Laboratories) were purchased or provided as gifts (all but the Sigma preparations) and analyzed similarly in specific immunoassays to determine the content of intact hCG and hCG-related molecules. Total hCG and related molecule immunoreactivity was determined in the DPC Immulite hCG assay, hCGb was measured in the DPC Immulite hCGb test, and hyperglycosylated hCG was measured using the Nichols Institute Diagnostics ITA hyperglycosylated hCG-specific test on the Advantage chemiluminescence automated platform (Nichols Institute Diagnostics) *(19)*. Nicked hCG was determined in the microtiter plate immunometric assay using antibody B151, as described previously *(20)*, and 4001-peroxidase tracer (Medix Corp., Genzyme) *(12)*. hCG α-subunit (hCGa) was measured in the RIA produced by ARUP Laboratories. hCGbcf was measured using the previously described specific hCGbcf microtiter plate immunometric assay with antibody B210 (gift from S. Birken and colleagues, Columbia University) and the 4001-peroxidase *(16, 21)*. All assays included pure hCG, nicked hCG, hCGb, and hCGbcf, calibrated by mass spectrometry as described previously *(5, 17)*. To calculate the contribution of hCG structural variants to total hCG immunoreactivity, all results were calculated on a molar basis using the following molecular masses *(1, 5, 16, 17, 22)*: hCG, nicked hCG, and hyperglycosylated hCG, 36 700 Da; hCGb, 22 200 Da; hCGa, 14 500 Da; and hCGbcf, 10 000 Da.

data management

Rank correlation was determined by the Institute for Phonetic Sciences statistical analysis service. Regression

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Table 2. Composition and homogeneity of purified individual hCG preparations and hCG standards.

Preparation	Intact hCG,[a] %	Contaminant,[b] %				
		Hyperglycosylated hCG	Nicked hCG	hCGb	hCGa	hCGbcf
WHO 3rd IS/4th IS	100	15	9 *(1)*; 10 *(4)*	0.8 *(4)*	1.4 *(4)*	0.8 *(4)*
99/688 (WHO 1st RR)	100	3	<0.1 *(4)*	0.9 *(4)*	1.0 *(4)*	0.2 *(4)*
hCG preparation P9	100	9 *(5)*	<0.1 *(5)*	<0.5	<0.1 *(5)*	2.3
CAP C15 (2003)[c]	100	0.9	<0.1	0.2	ND[d]	<0.1

[a] All samples assume intact hCG as 100%.
[b] Concentrations of contaminants are either from published references or were determined in our laboratory by immunoassay for this study. Contents of free subunits and fragments are compared with intact hCG on a molar basis. Percentages are molar percentages of intact hCG concentration.
[c] Not all CAP quality-control hCG preparations are the same. Some may be pure hCG (e.g., C15); others (e.g., C6) made be cruder preparations significantly contaminated with urine hCGb, nicked hCG, and hCGbcf (Cole et al., unpublished data).
[d] ND, not determined.

equations and r^2 were determined in Microsoft Excel 2000 or in Stat-Sak (Stattools).

Results

We tested four hCG preparations added to normal male serum. We first tested hCG batch CR119 (source of three WHO standards: 1st IRP; 3rd IS, also known as 75/537; and 4th IS, also known as 75/589). We then tested hCG batch 99/688 (source of the new WHO 1st RR standard). We next tested individual pure hCG preparation P9. Finally, we tested the hCG provided by CAP for validating hCG tests run by individual laboratories [CAP quality-control preparation C15 (2003)]. As shown in Table 2, studies done in our laboratory, using immunoassays, and studies reported elsewhere, using structural methods and immunoassays, showed that these four hCG preparations contain minimal proportions of hCG-related variants. CR119, the older hCG standard, contained 15% hyperglycosylated hCG and 9–10% nicked hCG as major contaminants. The 99/688 hCG preparation was largely free of these contaminants. Major contamination of individual hCG preparation P9 was limited to the inherent 9% hyperglycosylated hCG. The CAP quality-control preparation was largely free of variants (Table 2). All of the hCG preparations had purities >11 000 IU/mg of protein (immunoreactivity per milligram of protein, not known for CAP quality-control preparation).

In contrast to these findings with pure preparations, seven commercial hCG preparations, used by manufacturers to prepare calibrators (calibrated against WHO 3rd IS or 4th IS), varied greatly in purity from 3000 to 11 900 IU/mg of protein (Table 3). These range from Sigma C6322, which is free of all hCG structural variants (Table 3); to Scripps C0713, which is rich in nicked hCG (15% of total immunoreactivity), hCGb (5%), and urine hCGbcf (16%); to Ferring Choragon; Organon Pregnyl; and Sigma C5297, which are all rich in nicked hCG (12–14% of total immunoreactivity), hCGb (5–14%), and particularly rich in urinary hCGbcf (39–52%).

Aliquots of individual hCG preparation P9 added to serum (850 μg/L), 99/688 added to serum (85 IU/L), the four preparative isoelectric focusing variants of individual hCG preparation P9 added to serum, and the common hCG structural variants added to serum [pure hCGb (230 μg/L by amino acid analysis), pure nicked hCG (313 μg/L), purified hyperglycosylated hCG (84 μg/L), and pure hCGbcf (4.0 μg/L)] were coded and blindly tested in nine commonly used commercial total hCG assays per-

Table 3. Examples of commercial hCG preparations.

Preparation[a]	Contaminant,[b] %						Calibration with 3rd IS,[c] IU/mg
	Intact hCG, %	Hyperglycosylated hCG	Nicked hCG	hCGb	hCGa	hCGbcf	
Sigma C6322	100	<0.9 *(19)*	<0.1	<0.1	<0.1	<0.1	11 900
Serono Profasi	98	6	ND[d]	1.6	ND	0.6	10 000
Scripps C0714	98	ND	14	0.8	ND	0.8	11 000
Scripps C0713	79	ND	15	5	ND	16	9000
Ferring Choragon	68	4	12	5	ND	39	~5000
Organon Pregnyl	38	7	10	12	ND	40	3000
Sigma C5297	34	ND	14	14	ND	52	3000

[a] Products are sorted in order of descending proportions of intact hCG. All were calibrated against the WHO 3rd IS.
[b] Concentrations of contaminants were determined in our laboratory by immunoassay for this study. Contents of free subunits and fragments are compared with intact hCG on a molar basis. Percentages are molar percentages of total hCG immunoreactivity (intact hCG + hCGb + nicked hCG + hCGbcf).
[c] International units are an arbitrary measurement relating to biological activity in rat models. With the introduction of immunoassays they are also used for measuring and comparing immunoreactivity. Sigma 6322 is recombinant hCG prepared in CHO cells. This is the purest and most homogeneous preparation of hCG available *(9)*. As such, we consider the purity of this preparation (11 900 IU/mg; immunoreactivity per milligram) as the closest approximation to 100% pure hCG.
[d] ND, not determined.

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formed in 10 different clinical laboratories. All assays were calibrated against the WHO 3rd IS as primary standard. The use of different commercial hCG preparations by specific manufacturers remains proprietary information; we therefore cannot relate the use of specific pure or crude hCG commercial hCG preparations to specific hCG tests.

The results (or mean results when run by multiple laboratories) obtained for hCG preparation P9 and 99/688 are shown in Table 4, which also shows the results posted by CAP [CAP 2003 Excel program *(15)*] for quality-control preparation C15 (2003; mean results from 2466 laboratories). Between-method variation was noted for all three hCG preparations: 1.44-, 1.59-, and 1.47-fold differences (highest test value divided by lowest), or a mean (SD) 1.50 (0.046)-fold difference among the nine assays. The order of the nine assays in detecting the three different hCG preparations, from the assay giving the lowest result to that giving the highest result, was significantly similar or parallel (rank correlation, each hCG preparation compared separately with each of the other two, $P < 0.006$). With all three hCG preparations, the Abbott AxSYM, Bayer Centaur, and Bayer ACS-180 gave the highest hCG results; the Dade Dimension, DPC Immulite, and Tosoh AIA600 gave midrange values; and the Roche Elecsys, Dade Stratus, and Ortho Vitros ECi gave the lowest hCG results (Table 4).

We used hCG preparation 99/699 (the new WHO standard; calibrated at 85 IU/L) to adjust, or calibrate, the nine assays. We then estimated P9 hCG and CAP quality-control preparation C15 (2003) results as if the nine tests were calibrated against a single pure hCG preparation (Table 4). This significantly reduced between-method variation for P9 hCG and the CAP results, from 1.44- and 1.47-fold differences at the extremes to 1.27- and 1.31-fold at the extremes (*t*-test, $P = 0.02$).

As shown in Fig. 1, the profiles for the four charge isoforms of hCG preparation P9, for each assay, varied overall in magnitude as would be predicted by the between-method variation (Table 4), with the Abbott AxSYM test giving the highest results for all four charge isoforms (yielded the highest result with P9 hCG; Table 4) and the Roche Elecsys giving the lowest results for all four charge isoforms (Table 4). Regardless of the changes in magnitude, the relative recognition of the four charge isoforms by each assay remained similar. Linear regression analysis was used to compare the hCG results for the four isoforms in each of the nine assays with the mean results for the four charge isoforms in the eight other assays. The results ($r^2 > 0.985$) indicated parallel recognition of the four hCG isoforms in the nine hCG assays.

We investigated the detection of hCG structural variants by the nine assays (Table 5). Wide variation was observed in the detection of hyperglycosylated hCG (at the extremes, 3.3-fold difference in the results among the nine assays), hCGb (at the extremes, 1.7-fold difference among the assays), nicked hCG (at the extremes, 1.5-fold

Table 4. Between-method variation of results for three hCG preparations.[a]

hCG preparation	Abbott AxSYM	Bayer		Dade		DPC Immulite	Roche Elecsys	Tosoh AIA600	Ortho Vitros ECi
		ACS-180	Centaur	Dimension	Stratus				
hCG preparation P9[b]	10 411 (12%)	9842 (6%)	9906 (7%)	9396 (1%)	9224 (−1%)	9815 (6%)	7241 (−22%)	9824 (6%)	7920 (−15%)
With assays calibrated against 1st RR value[c]	9513 (1%)	8283 (−12%)	8255 (−12%)	10 509 (12%)	10 454 (11%)	9701 (3%)	9617 (2%)	9382 (0%)	8976 (−5%)
99/688 (WHO 1st RR standard)[d]	93 (10%)	101 (19%)	102 (21%)	76 (−10%)	75 (−11%)	86 (2%)	64 (−24%)	89 (5%)	75 (−11%)
CAP quality-control preparation C15 (2003)[e]	94 (1%)	113 (22%)	115 (24%)	90 (−3%)	78 (−16%)	91 (−2%)	83 (−11%)	88 (−5%)	84 (−10%)
With assays calibrated against 1st RR value[f]	86 (−8%)	95 (1%)	96 (2%)	101 (8%)	88 (−6%)	90 (−4%)	110 (17%)	84 (−11%)	95 (1%)

[a] Results are in IU/L and percentage variation from the mean result for the nine commercial hCG assays (in parentheses).

[b] Concentration, 895 μg/L by amino acid analysis; average immunoreactivity, 9335 IU/L (~11× mass). At extremes a 1.44-fold difference was observed among the nine assays. A significant relationship was observed when P9 results were compared with 1st RR results (rank correlation test, $P = 0.0004$) and compared with the CAP results ($P = 0.0061$).

[c] When hCG P9 results were adjusted to the 99/688 (WHO 1st RR) pure hCG preparation, at extremes a 1.27-fold difference was observed among the nine assays.

[d] Concentration, 7.80 μg/L by amino acid analysis; average immunoreactivity, 84 IU/L (~11× mass). At extremes a 1.59-fold difference was observed among the nine assays.

[e] At extremes a 1.47-fold difference was observed among the nine assays. A significant relationship was observed when CAP quality-control C15 (2003) results were compared with 1st RR results (rank correlation, $P = 0.0013$).

[f] Concentration not determined; average immunoreactivity, 93 IU/L. When CAP quality-control preparation C15 results were adjusted to the 99/688 (WHO 1st RR) pure hCG preparation, at extremes a 1.31-fold difference was observed among the nine assays.

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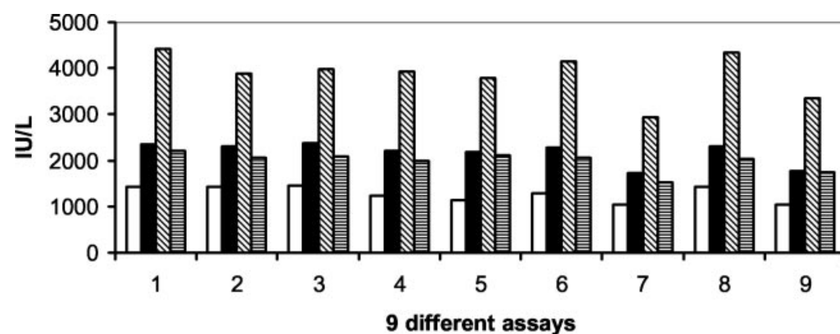

Fig. 1. Results obtained with nine common hCG assays for the four hCG charge isoforms (pI 3–4, 4–5, 5–6, and 6–7).

The assays used were the Abbott AxSYM (*group 1*), Bayer ACS-180 (*group 2*), Bayer Centaur (*group 3*), Dade Dimension (*group 4*), Dade Stratus (*group 5*), DPC Immulite (*group 6*), Roche Elecsys (*group 7*), Tosoh AIA600 (*group 8*), and the Ortho Vitros ECi (*group 9*). In each group of columns, the *first column* (□) corresponds to the pI 3–4 component, the *second column* (■) to pI 4–5, the *third column* (▧) to pI 5–6, and the *fourth column* (▤) to pI 6–7. In a regression analysis (linear regression) comparing the hCG results for the four isoforms in each of the nine assays with the mean results for the four charge isoforms for the eight other assays, the r^2 values were 0.998, 1.000, 0.999, 1.000, 0.985, 1.000, 0.999, 0.994, and 0.994, respectively.

difference among the assays), and hCGbcf (detected by only one of the assays). We looked for correlation between poor or exaggerated detection of these variants and the between-method variation of the three hCG preparations (Tables 4 and 5); we found no correlation between the order of detection of any of the hCG structural variants and the order of detection of the three preparations of hCG ($P > 0.05$ in all cases, rank correlation). Furthermore, we found no correlation between the assays giving the three highest results for any of the hCG structural variants and the three highest results for the three hCG preparations (Tables 4 and 5; $P > 0.05$ in all cases, rank correlation). Likewise, we found no correlation between the assays giving the three lowest results for any of the hCG structural variants and those giving the three lowest results for the three hCG preparations (Tables 4 and 5; $P > 0.05$ in all cases, rank correlation).

We examined the commercial hCG calibrators provided with five different commercial tests sold 5 years ago. (Calibrators are not usually provided with reagent packs today.) All five commercial tests were claimed to be calibrated against the WHO 3rd IS (CR119-based). The Hybritech Tandem test was provided with a 100 IU/L commercial hCG preparation. On a molar basis, 13% of the immunoreactivity was attributable to hCGbcf and 10% to hCGb. The Hybritech Tandem hCG test did not actually detect hCGb or hCGbcf *(16)*. In the Abbott calibrator, >55% of the immunoreactivity was attributable to hCGbcf (neither the Abbott 15/15 nor the Abbott IMX available at that time detected hCGbcf). The DPC test was provided with a 100 IU/L hCG preparation, of which >40% of the immunoreactivity was attributable to hCGbcf and 6% to hCGb. The DPC RIA available at that time detected all of these variants *(16)*. The Biomerica test was provided with a 100 IU/L commercial hCG preparation in which >32% of the immunoreactivity was attributable to hCGbcf. The Biomerica hCG immunoradiometric assay available at this time did not detect hCGbcf *(16)*. In contrast, the Serono MAIAclone test was provided with a very pure intact hCG preparation as calibrator, with no

Table 5. Detection of free hCGb, nicked hCG, and hCGbcf by nine commercial total-hCG tests, all calibrated using the 3rd IS.[a]

		Bayer		Dade					
hCG variant	Abbott AxSYM	ACS-180	Centaur	Dimension	Stratus	DPC Immulite	Roche Elecsys	Tosoh AIA600	Ortho Vitros ECi
Hyperglycosylated hCG[b]	801	947	999	468	786	917	1544	898	740
hCGb[c]	3278	5235	5535	3300[d]	3744	3843	3220	3187	4445
Nicked hCG[e]	4071	3097	3048	3658	3691	4240	2736	3827	2780
hCGbcf[f]	<2	<2	<2	<2	<2	130	<2	<2	<2

[a] All results are in IU/L.

[b] Assays were tested with purified hyperglycosylated hCG (84 μg/L). At extremes a 3.3-fold difference was observed among the nine assays. No significant correlation was observed between detection of hyperglycosylated hCG and detection of P9 hCG, 1st RR hCG, or the CAP hCG (rank correlation, $P > 0.05$; see Table 3), or between detection of the assays giving the three highest or three lowest results for hyperglycosylated hCG and the three assays giving the three highest or lowest results for P9 hCG, 1st RR hCG, or the CAP hCG (rank correlation, $P > 0.05$).

[c] Assays were tested with pure hCGb (230 μg/L). At extremes a 1.7-fold difference was observed among the nine assays. No significant correlation was observed between detection of hCGb and detection of P9 hCG, 1st RR hCG, or the CAP hCG (rank correlation, $P > 0.05$; see Table 3), or between detection of the assays giving the three highest or three lowest results for hCGb and the three assays giving the three highest or lowest results for P9 hCG, 1st RR hCG, or the CAP quality-control hCG quality control (rank correlation, $P > 0.05$).

[d] This is an intact hCG only test. Although we confirmed the source of the data, it is surprising, based on manufacturer's claims, that it detects hCGb.

[e] Assays were tested with pure nicked hCG (330 μg/L). At extremes a 1.5-fold difference was observed among the nine assays. No significant correlation was observed between detection of nicked hCG and detection of P9 hCG, 1st RR hCG, or the CAP hCG (rank correlation, $P > 0.05$; see Table 3), or between detection of the assays giving the three highest or three lowest results for nicked hCG and the three assays giving the three highest or lowest results for P9 hCG, 1st RR hCG, or the CAP quality-control hCG (rank correlation, $P > 0.05$).

[f] Assays were tested with pure hCGbcf (4.0 μg/L). Only the DPC Immulite detected urine hCGbcf. No significant correlation was observed between detection of hCGbcf and detection of P9 hCG, 1st RR hCG, or the CAP quality-control hCG (rank correlation, $P > 0.05$; see Table 1).

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detectable variants. In this case the commercial hCG preparation was more homogeneous than the primary standard, the WHO 3rd IS.

Discussion

This study shows that the WHO standards all contain minimal amounts of the major hCG structural variants. We found parallel between-method variation when we used the three virtually homogeneous pure hCG preparations with minimal contamination by variants [results at extremes varied by 1.44-, 1.47-, and 1.59-fold, respectively, or by a mean (SD) 1.50 (0.046)-fold]. The three highest and three lowest results were obtained with the same groups of assays with each of these three hCG preparations. Because the between-method variation still existed and existed in a parallel manner with all three pure hCG preparations, the between-method variation was clearly not attributable to the use of a specific pure commercial hCG preparation or standard but rather to differences in assay design.

Only the DPC Immulite detected hCGbcf, and, on a mass basis (assuming that 1 μg/L hyperglycosylated hCG or nicked hCG is equivalent to ~11 IU/L hCG, as indicted for pure hCG in Table 4), five of nine tests underdetected hyperglycosylated hCG (result <90% of mass calibration, 84 μg/L), and one test (Roche Elecsys) gave a distinctly exaggerated hyperglycosylated hCG results (140 μg/L). Furthermore, four of nine tests underdetected nicked hCG (result <90% of mass calibration, 330 μg/L), and two tests exaggerated nicked hCG results (result >110% of mass calibration). We also observed wide variability in the detection of hCGb (230 μg/L) with results ranging from 189 μg/L (Tosoh AIA600) to 323 μg/L (Bayer Centaur).

All nine assays similarly recognized multiple hCG charge variants, excluding charge or sialic acid variants as the cause of the between-method variation. Furthermore, we found no correlation between assays yielding unduly high results or unduly low results for any of the major hCG structural variants and the higher or lower results obtained with the nine assays for the three pure hCG preparations. We conclude that neither differential detection of charge variants nor differential detection of their major hCG structural variants explains the between-method variation observed with the three pure hCG preparations.

In 2001 we tested 30 serum samples with seven commercial immunoassays (Bayer Centaur and ACS-180, Beckman Access, Abbott AxSYM, DPC Immulite, Dade Stratus, and Serono MAIAclone), assays similar to those used in these studies. We observed a mean (SE) 1.51 (0.068)-fold difference in individual results at the extremes (*12*). This between-method variation is similar to that observed with the three pure hCG preparations [mean (SD) difference, 1.50 (0.046)-fold]. This between-assay variation is similar whether testing three pure hCG preparations or 30 individual serum samples (*t*-test, $P = 0.97$). It is inferred that similar causes are responsible for between-method variation whether testing pure hCG preparations or standards or individual serum samples. Furthermore, detection of structural or charge variants does not appear to be the cause of this variation. Other explanations, and remedies for them, need to be considered for the observed between-method variation in hCG tests.

WHO provides limited quantities of standards to each company. To accommodate all of the different standardizations, lot checks, instrument calibrations, and other procedures required for a hCG assay, a manufacturer needs large quantities of a hCG preparation. Manufacturers purchase a bulk hCG preparation for this task and calibrate this material based on the WHO primary standards. Many commercial hCG preparations are available. They vary from ultrapure hCG, free of any hCG structural variants, to preparations that are primarily nicked hCG, hCGb, or hCGbcf. We considered the extreme variability in structural variants in these secondary standard preparations as a source of the between-method variation observed with the pure and homogeneous hCG preparations. Calibration of one heterogeneous secondary standard by one particular company's assay may be very different from calibration by another company's test. Five years ago we were able to examine the hCG calibrators provided with five other commercial immunoassays (all claimed to be calibrated against the WHO 3rd IS). The commercial hCG preparation ranged from homogeneous hCG to hCG preparations that were primarily hCGbcf. Calibrators are not usually provided with reagent packs today, but rather are purchased separately by registered owners of automated immunoassay platforms and tests and are used approximately one time each month. This makes corroboration difficult with today's tests, especially when manufacturers restrict sale of calibrators to owners of dedicated assay platforms and users of the manufacturer's reagents.

We looked at examples of commercial hCG preparations and problems with matching commercial hCG preparations with the specificity of an individual assay (see Table 1). If, for example, the Scripps C0713 secondary standard preparation (nicked hCG is 15%, hCGb is 5%, and urine hCGbcf is 16% of the total hCG immunoreactivity) were used with the DPC Immulite test (which detects all of these hCG structural variants), the results would be very different from the results obtained for C0713 in the Ortho Vitros ECi test (which does not detect hCGbcf and poorly detects hCGb and nicked hCG). The total immunoreactivity of the secondary standard (IU/mL) could be as much as twofold different when used with these two assays. We also considered other examples of secondary standard problems. If, for example, the Roche Elecsys test, which gives exaggerated hyperglycosylated hCG results, were used with the Organon Pregnyl hCG (7% hyperglycosylated hCG), the calibration would be notably different from the calibration with the Sigma C6322 (CHO cell recombinant hCG).

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These studies raise important questions. Which calibration is correct? Which commercial hCG preparations and which contaminants are appropriate to include? None of this has been established, and there are no guidelines set by WHO or the Food and Drug Administration. On the basis of the data presented here, we deduce that the choice of secondary standard and the appropriateness of the secondary standard considering the design or specificity of the assay (see Table 1) are at the root of the between-method variation for individual patient sera and pure hCG preparations.

Calibration of a secondary standard, containing or not containing different hCG structural variants, matching or not matching the specificity of the assay (i.e., if the secondary standard contains hCGbcf, does the assay have to detect hCGbcf), is seemingly the major source of between-method variation. Similarly, some companies purchase commercial hCG preparations already calibrated against the 3rd IS (as stated by the manufacturers). Unless the company's assay has a specificity identical to the assay used to calibrate the secondary standard, it will erroneously calibrate the company's assay. Either way, these are sources of variation in assay calibration and immunoassay results. We infer that the use of different commercial hCG preparations by different manufacturers, containing various proportions of different hCG structural variants, together with the widely differing recognition of the hCG structural variants leads to miscalibration of assays.

In support of this inference, when the nine assays tested here were directly calibrated with a single pure preparation of hCG (WHO 1st RR hCG), bypassing the calibration with commercial hCG preparations, the between-method variation observed with the P9 hCG and the CAP quality-control preparation C15 (2003) were significantly reduced (*t*-test, $P = 0.02$). This shows that use of a single pure calibrant, rather than a secondary standard, improves between-method variation, indicating that the use of secondary standards is a major source of between-method variation.

Between-method variation can probably be readily reduced if manufacturers limit products to a common, pure, virtually homogeneous hCG secondary standard, such as Sigma C6322 (CHO cell recombinant hCG) or a similar preparation. This may add an additional cost to the production of the test, but it could greatly help in resolving between-method variation, permitting all laboratories to obtain comparable results with different tests and physicians to compare hCG results from different hospitals, laboratories, or assay sources.

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