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False-Negative Results in Point-of-Care Qualitative Human Chorionic Gonadotropin (hCG) Devices Due to Excess hCGβ Core Fragment

Ann M. Gronowski,[1*] Mark Cervinski,[1] Ulf-Håkan Stenman,[2] Alison Woodworth,[3] Lori Ashby,[4] and Mitchell G. Scott[1]

BACKGROUND: During pregnancy, human chorionic gonadotropin (hCG) immunoreactivity in urine consists of intact hCG as well as a number of hCG variants including the core fragment of hCGβ (hCGβcf). We identified 3 urine specimens with apparent false-negative results using the OSOM® hCG Combo Test (Genzyme Diagnostics) qualitative hCG device and sought to determine whether an excess of 1 of the fragments or variants might be the cause of the interference.

METHODS: We measured concentrations of hCG variants in the urine from 3 patients with apparent false-negative hCG results. Purified hCG variants were added to urines positive for hCG and tested using the OSOM, ICON® 25 hCG (Beckman Coulter), and hCG Combo SP® Brand (Cardinal Health) devices.

RESULTS: Dilution of these 3 urine samples resulted in positive results on the OSOM device. Quantification of hCG variants in each of the 3 patient urine specimens demonstrated that hCGβcf occurred in molar excess of intact hCG. Addition of purified hCGβcf to hCG-positive urines caused false-negative hCG results using the OSOM and ICON qualitative urine hCG devices.

CONCLUSIONS: Increased concentrations of hCGβcf can cause false-negative results on the OSOM and ICON qualitative urine hCG devices.

Since the introduction of rapid, qualitative urine human chorionic gonadotropin (hCG) devices in the 1970s, their use has become ubiquitous: in hospital emergency departments, physicians offices, over-the-counter home use, and other point-of-care settings. The sensitivity of these devices for detecting pregnancy depends on urine concentration, number of days postfertilization, and the lower limit of quantification of the various kits on the market. Although rare, false-positive and false-negative test results are known to occur. Most commonly these are attributed to incorrect reading time (i.e., reading test results too early or too late); insufficient or dilute urine; mislabeled specimens (in hospital and physician office settings); and "biochemical pregnancy" (i.e., early pregnancy loss). The high dose "hook effect" has also been reported to cause false-negative results in qualitative hCG devices *(1)*. For this reason, hospitals and laboratories using these qualitative devices should investigate the hook effect in the device used at their institution. Because the behavior of different specimens may vary based on hCG variant ratios, investigation should be done with samples from several different patients. In the hospital setting, qualitative hCG devices are often used to rule out pregnancy in women before an intervention that could potentially harm a fetus. Therefore, false-negative results are particularly dangerous.

In pregnancy, hCG immunoreactivity in urine is a result of intact hCG (intact heterodimer comprising α and β subunits) as well as partially degraded variants detectable in serum and urine. In addition to intact hCG, hyperglycosylated hCG (hCG-h),[5] nicked hCG (hCGn), hCG missing the β-subunit C-terminal peptide, free β-subunit (hCGβ), hyperglycosylated free β-subunit (hCGβ-h), and nicked hCGβ (hCGβn) can be detected in both serum and urine; the core fragment of hCGβ (hCGβcf) is predominantly detected in urine *(2–5)*. The fraction of total hCG that each of these variants represents in urine changes during pregnancy, with hCG-h high in early pregnancy and hCGβcf high in midterm pregnancy *(4, 5)*. Although most manufac-

[1] Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO; [2] Department of Clinical Chemistry, Helsinki University Central Hospital, Helsinki, Finland; [3] Department of Pathology, Vanderbilt University, Nashville, TN; [4] Barnes-Jewish Hospital, St. Louis, MO.
* Address correspondence to this author at: Washington University School of Medicine, Department of Pathology and Immunology, Box 8118, 660 S. Euclid, St. Louis, MO 63110. Fax 314-362-1461; e-mail gronowski@wustl.edu.
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[5] Nonstandard abbreviations: hCG, human chorionic gonadotropin; hCG-h, hyperglycosylated hCG; hCGn, nicked hCG; hCGβ, free β-subunit hCG; hCGβ-h, hyperglycosylated hCGβ; hCGβn, nicked hCGβ; hCGβcf, core fragment of hCGβ; BJH, Barnes-Jewish Hospital; ED, emergency department; POC, point-of-care; WBC, white blood cell; UTI, urinary tract infection; IFMA, immunofluorometric assay; IRP, international reference preparation.

turers claim that their qualitative devices are designed to detect the intact hCG molecule, little has been done to characterize the hCG variants that these devices detect. Recent reports demonstrate that many devices do recognize some of the other hCG variants *(6)*. What is not known is whether an excess of 1 or more of these variants may cause false-negative results by binding to one of the antibodies in the devices but not the other, thus preventing formation of a complete antibody–antigen–antibody sandwich. We hypothesized that increased concentrations of 1 of the hCG variants was the cause of 3 confirmed false-negative results in a commonly used qualitative hCG device.

Materials and Methods

PATIENTS

Patient 1 was an 18-year-old woman who presented to the Barnes-Jewish Hospital (BJH) emergency department (ED) with vaginal spotting and cramping. She stated that she was 3 months pregnant. A point-of-care (POC) urine pregnancy test was performed in the ED using the OSOM® hCG Combo Test (Genzyme Diagnostics; lot 081299; hereafter referred to as OSOM) and was negative. Urine was cloudy, and specific gravity was 1.020 (reference interval 1.003–1.030), pH 5.5 (5.0–8.0). Urine was positive for white blood cells (WBCs) and bacteria. Ultrasound showed a live intrauterine pregnancy. Serum hCG (Siemens Centaur) was 419 680 IU/L. Discharge diagnosis was threatened abortion and urinary tract infection (UTI).

Patient 2 was a 23-year-old woman who presented to the BJH ED with sharp left flank pain. She stated that she was 10 weeks pregnant. A POC urine pregnancy test was performed in the ED (OSOM; lot 081299) and was negative. Urine was cloudy and specific gravity was 1.020, pH 7.5. There was no evidence of urinary tract infection. Ultrasound showed a live intrauterine pregnancy. Serum hCG (Siemens Centaur) was 133 860 IU/L. The patient had an MRI study to rule out appendicitis. Discharge diagnosis was low back pain. She had a medical history of UTI and subsequently came in 1 month later with threatened abortion and UTI.

Patient 3 was a 23-year-old woman who presented to the Vanderbilt University Medical Center ED with continual nausea with vomiting and abdominal pain for the past 4 weeks. She stated that she was 13 weeks pregnant. A POC urine pregnancy test (OSOM; lot 081334) performed in the Vanderbilt University Medical Center Core Laboratory was negative. Urine was orange and turbid and specific gravity was >1.035, pH 6.0. Urine was positive for trace ketones, WBCs, and bacteria. Ultrasound showed a live intrauterine pregnancy. Serum hCG (Siemens Immulite 1000, Siemens Centaur) was 99 118 IU/L. Discharge diagnosis was hyperemesis gravidarum, UTI, and hyperthyroidism.

DETERMINATION OF HOOK EFFECT

Urine from a patient with gestational trophoblastic disease (serum hCG 3 200 000 IU/L) was serially diluted in hCG-negative urine from a male subject. We measured the hCG concentration using the Siemens Centaur total hCG assay that we have previously validated for use with urine *(7)*. Diluted urine was tested using the OSOM device.

EFFECT OF hCG VARIANTS ON QUALITATIVE hCG TEST RESULTS

We obtained purified hCG variants from the National Institute for Biological Standards and Controls (Hertfordshire, UK) [first WHO reference reagent, 2001 (hCGn, 99/642), (hCGβ, 99/650), (hCGβn, 99/692), and (hCGβcf, 99/708)]. Cross-contamination of these materials ranged from 0.004% to 1.3% *(8)*. Each purified hCG variant was diluted in normal saline to a concentration of 3000 nmol/L, which corresponds to about 1 000 000 IU/L for intact hCG. A randomly chosen positive urine specimen with an hCG concentration of 17 800 IU/L (Siemens Centaur) was mixed with purified hCG variants at various concentrations and tested using the OSOM (lot 081299), hCG Combo (lot 8080021), and ICON 25 (lot 8029001) devices according to manufacturer instructions.

MEASUREMENT OF hCG VARIANTS

We determined urine hCG variant composition using a combination of commercial and in-house immunofluorometric assays (IFMAs). We quantified intact hCG using a time-resolved IFMA specific for intact hCG and hCGn (AutoDELFIA®, Perkin-Elmer Wallac) according to the instructions of the manufacturer. This assay uses a monoclonal capture antibody specific for the β-subunit (MAb 5008) and a detection antibody (MAb 5501) specific for the α-subunit *(9)*.

We measured the concentrations of hCGβ and hCGβcf using in-house IFMA. Both assays use the same detection antibody (MAb 1B2) that recognizes hCG, hCGβ, and hCGβcf. The capture antibody used in the hCGβ assay (MAb 9C11) specifically detects the free β-subunit of hCG (cross-reaction with intact hCG and hCGβcf is about 0.5%). The capture antibody in the hCGβcf assay (MAb 3C11) is highly specific with MAb 1B2 as tracer (cross-reaction with intact hCG and hCGβ is <0.5%). These assays were calibrated using international reference preparations (IRPs) 99/650 and 99/708, respectively. Cross-reactivities were determined as described *(3)* using IRPs for the various forms of hCG.

We measured total hCG (hCG + hCGβ) using the Siemens Centaur Total hCG assay. This 2-site sand-

Table 1. hCG variants present in the urine from 3 women with false-negative results on the OSOM device.

Patient	Total hCG (Siemens Centaur), IU/L	Intact hCG (AutoDELFIA), IU/L	Intact hCG, pmol/L (% of total)[a]	hCGβ, pmol/L (% of total)	hCGβcf, pmol/L (% of total)	Total hCG immunoreactivity, pmol/L[b]
1	176 498	242 148	709 494 (21)	54 652 (1.6)	2 674 100 (78)	3 438 246
2	>1000[c]	273 573	801 569 (26)	46 591 (1.5)	2 185 330 (72)	3 033 490
3	89 195	101 541	297 515 (34)	18 539 (2.1)	554 120 (64)	870 174

[a] As measured by the AutoDELFIA assay.
[b] Sum of the intact hCG, hCGβ, and hCGβcf immunoreactivities.
[c] Not diluted to endpoint.

wich chemiluminescent assay uses a polyclonal goat and a monoclonal mouse antibody against different epitopes of the hCG β-subunit. This assay is standardized with the WHO Third International Standard 75/537. The Bayer Centaur hCG assay does not recognize hCGβcf *(10)*.

Results

PATIENT SAMPLES

Two separate urine specimens from patient 1 were tested neat and diluted 1:5 with saline on the OSOM device. The specimens were negative when tested neat and clearly positive after dilution. The two specimens were diluted 2000 and 20 000 times and measured on the Siemens Centaur, with results within 82% of each other. The urine from patient 2 was clearly positive after 1:3 dilution with saline. Patient 2 urine was also tested with 2 alternative lot numbers of the OSOM device (071717 and 081334). These devices gave a very weak positive result when urine was tested neat. The urine from patient 3 was diluted 1:5 and 1:10 with saline, and qualitative hCG results were weakly positive and strongly positive, respectively.

HOOK EFFECT ON THE OSOM DEVICE

All 3 patient urine specimens were negative when tested neat using the OSOM device but positive after dilution, initially suggesting the possibility of a hook effect *(11)*. Parallel 10% (i.e., 90%, 80%, 70%, etc.) dilutions of urine from a patient with gestational trophoblastic disease (serum hCG 3 200 000 IU/L) demonstrated that the OSOM device produced a positive result when the hCG concentration was <1 600 000 IU/L and a negative result when the hCG was >1 900 000 IU/L. Thus, the OSOM does have a hook effect above concentrations of 1.6–1.9 million IU/L. The hCG concentration in urine from the 3 patients was far less than 1 600 000 IU/L, however, indicating that a hook effect was not the cause of the false-negative results.

RESULTS USING OTHER QUALITATIVE hCG DEVICES

The urine from patient 1 was tested on 2 other hCG devices. When tested on the ICON hCG device, the results were faintly positive using the neat sample and strongly positive after a 1:3 dilution into saline. When tested using the hCG Combo device, the results were clearly positive using both a neat specimen and after a 1:3 dilution. Because the hCG Combo device gives positive results in the presence of purified hCGβcf, but the ICON does not *(6)*, this finding led us to believe that excess hCGβcf in these urines may be causing the false-negative results in the OSOM and ICON devices.

CHARACTERIZATION OF hCG VARIANTS IN PATIENT URINE

We measured hCG variants in the urine specimens from these 3 patients using variant-specific immunoassays (Table 1). In all 3 urines, hCGβcf occurred in molar excess of intact hCG, representing 64%–78% of the immunoreactive hCG. The results for intact hCG measured by the AutoDELFIA assay were higher than those obtained by Siemens Centaur. This is likely explained by differences in calibration and/or recognition of the hCG variants occurring in urine.

hCGβcf INHIBITS TEST RESULTS ON SOME QUALITATIVE DEVICES

To further investigate the possibility that excess hCGβcf may inhibit the immunoreaction on some devices, we added purified hCGβcf to an hCG-positive urine with an hCG concentration of 17 800 IU/L as determined by the Siemens Centaur method. Results of these studies are summarized in Table 2 (and Supplemental Fig. 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue7). When 1 000 000 pmol/L hCGβcf was added to hCG-positive urine, the result changed from clearly positive to negative using the OSOM device and from clearly positive to negative/faint positive using the ICON device, but no visible effect occurred on the hCG Combo device. As the concentration of hCGβcf decreased, the positive signal returned to the OSOM and ICON devices. The positive signal also returned with a

Table 2. Effect of hCGβcf added to hCG-positive urine with an hCG concentration of 17 800 IU/L (Siemens Centaur) on the results of the OSOM (Genzyme Diagnostics), ICON 25 (Beckman Coulter), and hCG Combo (Cardinal Health) devices.

Final hCGβcf concentration, pmol/L	OSOM	ICON 25	hCG Combo
0	Positive	Positive	Positive
63 000	Positive	Positive	
125 000	Positive	Positive	
250 000	Faint positive	Positive	
500 000	Negative/faint positive	Faint positive	Positive
1 000 000	Negative	Negative/faint positive	Positive

1:5 dilution in saline, as observed with the patient samples (data not shown).

To determine if other hCG variants might have similar effects on the OSOM device, we added purified hCGβ, hCGβn, and hCGn to hCG-positive urine (Table 3 and online Supplemental Fig. 2). At 500 000 pmol/L, hCGβ inhibited the positive signal and hCGβn and hCGn diminished the positive signal.

Discussion

During our investigation of the cause of false-negative qualitative urine hCG results from 3 patients tested using the OSOM device, we reviewed the report by Sigel and Grenache *(6)* that examined the hCG variant specificity of 6 POC qualitative hCG devices. Their data showed that the OSOM device recognizes only intact and nicked hCG, and no other hCG variants. We hypothesized that perhaps there was a high concentration of 1 of the other hCG variants that was binding to 1, but not both, of the antibodies in the OSOM device and preventing the formation of a "sandwich" by saturating either the capture or detector (or tracer) antibody (both antibodies are bound to a solid phase in pregnancy test). To investigate this hypothesis, we demonstrated that (1) the OSOM device is capable of detecting urine hCG up to concentrations of 1.6 million IU/L; (2) in these 3 specimens, the concentration of hCGβcf was in molar excess of the intact hCG (64%–78% of all hCG immunoreactivity), and (3) the addition of purified hCGβcf to hCG-positive urine can cause false-negative hCG results using the OSOM and ICON qualitative urine hCG devices.

Table 3. Effect of hCG variants added to hCG-positive urine (hCG concentration 17 800 IU/L, Siemens Centaur) on the results of the OSOM (Genzyme Diagnostics) device.

hCG, pmol/L	hCGβ	hCGβn	hCGn
63 000	Positive	Positive	Positive
125 000	Positive	Positive	Positive
250 000	Faint positive	Faint positive	Faint positive
500 000	Negative	Faint positive	Faint positive

hCGβcf is a fragment of hCGβ lacking the C-terminal peptide amino acids 93–145, 41–54, and 1–5 from the N-terminus *(12)*. From approximately 5–8 weeks of gestation until term, hCGβcf is the major hCGβ-subunit–related molecule in urine and accounts for up to 90% of the immunoreactive urine hCG from midpregnancy *(5)*. Because the concentrations of hCGβcf in serum are <0.1% of those in urine *(13)*, it is likely that most of it is formed during excretion of hCG and hCGβ in the kidneys *(14)*. However, it is not known why the proportion of hCGβcf in urine from some women is higher than in others.

The hook effect is a well-documented interference that occurs when extremely high concentrations of an analyte occupy all the sites on both the capture and detection antibodies and prevent the formation of a so-called sandwich in 2-site immunoassays. As a result, few or no tracer-antibody + antigen + capture-antibody complexes are formed, yielding a false-negative result. However, the measured total hCG concentrations in these 3 urines was far less than the concentrations necessary to cause a hook effect in these devices.

In these assays, we believe that hCGβcf is recognized by 1 of the 2 antibodies in the device. When the hCGβcf occurs in high concentrations and in molar excess of the forms of hCG recognized by both antibodies, it saturates one of the antibodies and blocks the ability of intact hCG to form the double antibody–antigen sandwich. This observation confirms in clinical practice the prediction of Madersbacher and Berger *(15)*. Similar to traditional hook effects, dilution of the

sample decreases the concentration of hCGβcf to a point where sandwich formation can again occur. The epitopes of various forms of hCG have been extensively characterized. The adjacent epitopes β2 or β4 on loop 1 and 3 of hCGβ occur on both hCGβcf and hCG and are used in many hCG assays *(16)*. One of the antibodies in the OSOM and ICON assays apparently recognizes 1 of these epitopes. Other hCG variants, i.e., hCGβ, hCGβn, and hCGn, may also cause this phenomenon to varying degrees with some devices. This is probably related to the use of an antibody recognizing epitope C1 or C2. These epitopes are found only on heterodimeric intact hCG, are sensitive to nicking in hCGn, and are not present on hCGβ *(16)*. In addition to hCG and hCG-h, the variants most frequently encountered in urine during normal pregnancy have been reported to be hCGn, hCGβ, and hCGβcf *(5)*. A negative interference is likely to occur whenever 1 of these variants is present in great excess and recognized by only 1 of the 2 antibodies. The negative interference is more of an issue in hospital settings where qualitative devices are being used at later stages of pregnancy, when the concentrations of hCG and its variants are high.

Our results illustrate how physiological changes in the concentrations and variants of hCG in urine may cause problems for our routinely used hCG assays. The cases presented here illustrate the importance of considering the target population when designing immunoassays. Hospital and laboratory point-of-care hCG devices are not simply being used to detect early pregnancy in women trying to achieve pregnancy. They are being used in a wide variety of gestational ages. Our results show that in hospital settings, the immunoassay characteristics need to be different. Immunoassays in which hCGβcf causes a false-negative result may be acceptable for use in detection of early pregnancy but are unreliable later in pregnancy. The concentrations of hCGβcf in urine from different pregnant women vary considerably, and in addition there may be more than 10-fold differences between consecutive samples from single individuals. Compared with results from earlier studies, the concentrations of hCGβcf observed in our patients appear to represent relatively high values *(17, 18)*. They are not unique, however; we have observed numerous urine specimens from women at 5–8 weeks' gestation that exhibit faint positive results using the OSOM device but are clearly positive after dilution.

This is the first report, to our knowledge, that hCG variants occurring at high concentration in pregnancy urine cause a false-negative result in POC qualitative hCG devices. Caution should be used when hCG devices in which hCGβcf causes negative interference are used to test women who are pregnant beyond 5–8 weeks' gestation, as false-negative results may occur. Devices used for this purpose should be tested to identify this problem.

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