Screening Method to Evaluate Point-of-Care Human Chorionic Gonadotropin (hCG) Devices for Susceptibility to the Hook Effect by hCG β Core Fragment: Evaluation of 11 Devices

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BACKGROUND: The predominant hCG variant in urine, hCG β core fragment (hCGβcf), has been demonstrated to cause false-negative results in qualitative point-of-care (POC) hCG devices. This is a major concern for healthcare professionals using POC pregnancy tests. We developed a screening method to evaluate qualitative POC hCG devices for their susceptibility to inhibition by hCGβcf. Using this method, we evaluated the performance of 11 commonly used devices.

METHODS: A wide range of purified hCG and hCGβcf concentrations were mixed and tested on 2 POC devices. By use of those results, a screening method was defined and 9 additional POC devices were evaluated. Two solutions containing (a) 500 pmol/L (171 IU/L) intact hCG with 0 pmol/L hCGβcf and (b) 500 pmol/L intact hCG with 500 000 pmol/L hCGβcf were used to screen all POC devices.

RESULTS: The OSOM and Cen-Med Elite devices were found to be most susceptible to false-negative results due to hCGβcf. The BC Icon 20 and the Alere were the least susceptible. The remaining 7 were moderately affected. Devices that gave the strongest signal with hCGβcf alone were those that were least likely to show a hook effect.

CONCLUSIONS: The screening method put forth here can be used by device users and manufacturers to evaluate POC devices for inhibition by hCGβcf. Of 11 devices evaluated, only 2 have been identified that exhibit minimal to no susceptibility to hCGβcf.

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1 Nonstandard abbreviations: hCG-h, hyperglycosylated hCG; hCGn, nicked hCG; hCGcf, core fragment of hCGββ; POC, point-of-care.
traditional hook effect. When the variant is present in high enough concentrations, a false-negative result can occur. Maderbacher and Berger referred to this as due to "an overhang in antigen recognition of the coating monoclonal antibody as compared with the detection monoclonal antibody." A positive signal following sample dilution can be observed in both the traditional and variant hook effects. In contrast to the traditional hook effect, which is now observed rarely at extremely high hCG concentrations associated with gestational trophoblastic disease, the variant hook effect has been shown to cause false-negative results in qualitative POC hCG devices due to the presence of hCG variants at concentrations regularly observed during healthy pregnancy (7). Clearly, this is a concern for the medical profession.

For researchers and manufacturers to investigate the effect of hCGBcf and improve hCG devices, a standardized validation method needs to be established. The original variant hook effect study and subsequent studies were performed by adding increasing concentrations of purified hCGBcf to a healthy pregnant urine sample. The problem with this approach is that each healthy pregnant urine sample contains a different mixture of hCG, hCGBcf, and other hCG variants that may affect device performance. A standardized method, by use of defined concentrations of hCG, could be used to evaluate the performance of currently available devices, providing valuable information to the device users. Additionally, manufacturers could use this method as a model to facilitate improvements to existing devices and the development of new devices. We describe here a screening method that can be easily used to examine the effect of hCGBcf and have used this method to interrogate 11 POC hospital hCG devices.

**Materials and Methods**

**hCG PURIFICATION AND IMMUNOREACTIVITY**

Institutional review board approval was obtained for this study. Purified hCGBcf was generously provided by Church and Dwight Co., Inc., and was prepared from crude urinary hCG purchased from a commercial source (Shangdong Top Science Biotech Co.). Material was purified by using an affinity column (Bio-Rad Econo-column) that was prepared by packing N-hydroxysuccinimide-activated agarose (Pierce Biotechnology) conjugated to 11D6 anti-hCG monoclonal antibody (Scantibodies Laboratory), which recognizes all hCG isoforms. The column was subjected to 20 mL quenching buffer (1 mol/L ethanalamine, pH 7.4) at room temperature for 30 min over a rotary mixer. After removal of the quenching buffer, the column was washed with 50 mL PBS buffer followed by 20 mL of 0.1 mol/L glycine buffer, pH 2.0. We repeated this washing process 3 times before sample purification.

The column was equilibrated with 3 times bed volume of 0.01 mol/L PBS buffer, pH 7.4, before purification. Crude hCG was loaded on the column at a flow rate of 0.5 mL/min; then the column was washed with 20 mL PBS buffer at the same flow rate. The bound hCG was eluted with 0.1 mol/L glycine buffer, pH 2.5. The eluted material was neutralized by adding 1/25 volume of 2 mol/L Trizma (Tris base). Affinity-purified material was further fractionated with a Sephadex G75 column (1.5 × 95 cm) at a flow rate of 0.24 mL/min in PBS buffer. Fractions were collected and pooled on the basis of the results of SDS-PAGE analysis.

We characterized the fraction containing purified hCGBcf by SDS-PAGE electrophoresis and ELISA assay. Homogeneity and purity of the final product was confirmed by use of SDS-PAGE electrophoresis performed under nonreducing conditions. A single band with a molecular weight of approximately 17 KDa was observed. The SDS-PAGE result was comparable to that of pre-reduced hCGBcf purified with the Sephadex G-75 column, as previously reported (10). We also assessed purity by MALDI/TOF mass spectrometry analysis. MALDI/TOF mass spectra were acquired with a Voyager DE STR instrument (Applied Biosystems). A solution (1 mg/100 μL) of 3,5-dimethoxy-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid was used as matrix. The mass spectrometer was calibrated with insulin, cytochrome c, myoglobin, and BSA in linear mode. The spectrum of hCGBcf was acquired in positive linear mode with a scan range from 5 to 40 KDa. The peaks in the spectrum represent the protonated molecular ion of different forms of hCGBcf proteins. We observed a major peak with molecular weight of approximately 9.5 KDa. The MALDI/TOF result was consistent with reduced purified hCGBcf with molecular weights of approximately 7.5 and 8.9 KDa observed on SDS-PAGE by de Medeiros et al. (10) (see Supplemental Fig. 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol60/issue4). Immunoactivity of the final product was examined by comparing the purified hCGBcf with the hCGBcf standard (IRR 99/708) by use of an in-house ELISA. The NUNC MaxiSorp plates were coated with 4 μg/mL 11D6 monoclonal antibody overnight followed by BSA blocking. Purified and IRR hCG-Bcf were assayed with the coated plates and detected with 0.2 μg/mL biotinylated monoclonal antibody B210 (Scantibodies Laboratory) that is specific to hCGB cf. The plates were developed with Strep-AKP (Jackson Immunologicals; 1:2500 dilution) for colorimetric measurement. Equivalent immunoreactivity between the purified hCGBcf and the hCGBcf standard (IRR 99/708) was confirmed on an equal mass basis.
The concentration of the purified hCGβcf was determined by use of the Immulite 1000. A calibration curve was generated by use of the hCGβcf standard (IRR 99/708) diluted with negative urine pool at 300, 610, and 1210 pmol/L. The purified hCGβcf was diluted with negative urine pool to the linear range; its concentration was determined by use of the regression equation.

Intact hCG was obtained from Scripps Laboratories (cat. C0714, lot 2436602, 11584 IU/vial; purity >99% by SDS-PAGE) and resuspended in 100 mmol/L Tris, 1 g/L BSA, pH 8.0, according to manufacturer instructions.

Stock hCG and hCGβcf were stored at −80 °C for 3 days and thawed at ambient temperature, and working solutions were prepared by use of male urine confirmed to generate negative hCG qualitative test results. Final working solutions containing 0, 5 × 10^2, 5 × 10^3, 5 × 10^4, 5 × 10^5, or 1 × 10^6 pmol/L intact hCG (2.93 pmol/L hCG = 1 IU/L hCG) as well as 0, 5 × 10^3, 5 × 10^4, 1 × 10^5, or 2 × 10^5 pmol/L hCGβcf were stored overnight at 4 °C before device testing. Three solutions from the original 30 were selected for testing with additional POC devices: 5 × 10^2 pmol/L intact hCG + 0 pmol/L hCGβcf, 5 × 10^4 pmol/L intact hCG + 5 × 10^5 pmol/L hCGβcf, and 0 pmol/L intact hCG + 5 × 10^4 pmol/L hCGβcf. These 3 selected solutions (3 mL) were prepared by use of stock hCG, hCGβcf, and hCG-negative male urine and stored at 4 °C for 5 days before use.

QUALITATIVE POC hCG DEVICES

The study devices were as follows: SP hCG Combo Rapid Test, Cardinal Health, cat. B1077–23, lot hCG3050059, exp. 03/2015; OSOM hCG Combo Test, Genzyme Diagnostics, lot 131243, exp. 05/2015. The validation devices were as follows: hCG Combo, Alere, cat. 92215, lot hCG1100167, exp. 09/2013; ICON 20 hCG, Beckman Coulter, cat. 395097, lot 032M11, exp. 08/2014; ICON 25 hCG, Beckman Coulter, cat. 43025, lot hCG3040237, exp. 04/2015; Elite Plus hCG, Cen-Med, lot hCG2100115, exp. 09/2014; Clinitest hCG Pregnancy Test, Siemens, cat. 1760, lot 038280, exp. 08/2014; hCG Urine Test, McKesson, cat. 2–102, lot hCG3040234, exp. 03/2015; QuickVue+ One-Step hCG Combo Test, Quidel Corp., cat. 0507202 hCG, lot 219330, exp. 02/2015; QuPID One-Step Pregnancy Test, Stanbio Laboratory, cat. 1221–001, lot hCG3020085, exp. 01/2015; and Sure-Vue Serum/Urine hCG-Stat, Fisher Healthcare, cat. 23–900–528, lot hCG3030187, exp. 01/2015. Devices were used according to package inserts. We shipped aliquots at 4 °C overnight for testing on the Siemens Clinitest immediately on receipt. Instrument-generated readouts were obtained. Devices were immediately shipped overnight back to the authors for visual interpretation with the other devices.

DEVICE INTERPRETATION AND SCORING

The hCG devices were tested in duplicate and interpreted by 10 untrained personnel who were not involved in any other way in the study and were unaware of the study purpose. One representative test result was reported for each pair of devices. We used a 4-point scoring system: 0 = negative; 1 = weak positive; 2 = clear positive; 3 = highest possible positive signal (i.e., greater than the control band). Mean test scores were calculated and displayed graphically plus or minus SE. We calculated statistically significant differences (P < 0.05) in device interpretation by use of Student paired t-test with a 2-tailed distribution. We calculated the magnitude of the false-negative effect by subtracting the mean score for the solution containing 500 pmol/L intact hCG and 0 pmol/L hCGβcf from the mean score for the solution containing 500 pmol/L intact hCG and 500 000 pmol/L hCGβcf.

Results

Varying concentrations of intact hCG and hCGβcf were combined in an hCG-negative urine matrix. Each solution was tested in duplicate by use of the SP hCG Combo Rapid Test (Combo) and the OSOM hCG Combo Test (OSOM) as described in Materials and Methods. These devices were chosen because the OSOM device has previously been shown to demonstrate the variant hook effect, and the Combo device, while affected, was the least affected device previously tested (7, 11).

Fig. 1A and B illustrates that with increasing concentrations of hCGβcf, both the OSOM and the Combo devices produce diminished signal in a dose-dependent manner; the OSOM device was more profoundly affected than the Combo. In the absence of intact hCG, the Combo device (Fig. 1A, blue line) gave a clearly positive signal at 50 000 pmol/L hCGβcf, and this signal decreased with increasing concentrations of hCGβcf. The Combo device was also able to retain detectable positive signal in the presence of 2 000 000 pmol/L hCGβcf. In contrast, the OSOM device (Fig. 1B, blue line) gave a very faint positive signal at 50 000 pmol/L hCGβcf that disappeared completely at hCGβcf concentrations ≥500 000 pmol/L. Importantly, Fig. 1A and B demonstrate that as the concentration of intact hCG increased, more hCGβcf was required to diminish the signal (additional concentrations of intact hCG are shown in online Supplemental Fig. 2).

Fig. 1C and D demonstrates that, in the absence of hCGβcf (blue line), both the OSOM and Combo devices were also subject to some degree of the traditional hook effect induced by increased concentrations of intact hCG. The Combo device (Fig. 1C) showed a slight
decrease in band intensity at hCG concentrations >500 000 pmol/L (170 648 IU/L). This hook effect was still apparent in the presence of 50 000 pmol/L hCGβcf (red line). As the concentration of hCGβcf increased, the signal was diminished across all the hCG concentrations. The OSOM device (Fig. 1D) was most affected by the traditional hook effect and, in the absence of hCGβcf (blue line), began to decrease its signal intensity at concentrations >50 000 pmol/L (17 065 IU/L) intact hCG. These findings were similar to those seen in the presence of 50 000 pmol/L hCGβcf (red line). Fig. 1C and D also illustrates that in the absence of intact hCG, the Combo device gave a clearly positive signal with 50 000 and 500 000 pmol/L hCGβcf. The OSOM device gave a very faint signal at 50 000 pmol/L hCGβcf, but this disappeared at higher concentrations.

The data were analyzed to identify conditions that could reliably detect devices susceptible to the hook effect due to hCGβcf. Ideally, such conditions would require modest hCG and hCGβcf concentrations to facilitate device evaluation in a cost-effective manner. On the basis of our findings, solutions containing 500 pmol/L (171 IU/L) intact hCG with 0 pmol/L hCGβcf and 500 pmol/L intact hCG with 500 000 pmol/L hCGβcf were selected (for reference, see Fig. 1A and B, red lines, first and third points). These 2 solutions were selected because they contained the lowest concentration of hCG and hCGβcf that could reliably distinguish a decrease in signal in both index devices. For the purposes of this study, we tested a third solution containing 50 000 pmol/L hCGβcf and 0 pmol/L intact hCG to evaluate the devices’ ability to detect hCGβcf alone.

This screening method was then used to evaluate the performance of 9 additional qualitative hCG POC devices (Fig. 2). Visible differences in device performance were evident, resulting in the classification of the devices into 3 distinct groups: best performance, moderate performance, and poor performance. The 2
Fig. 2. Hook effect due to hCG β core fragment in 11 POC devices.

Devices were tested in duplicate and given a single score by each reader. Bars represent the mean result from 10 untrained readers ± SEM. Representative device images are included as well as the magnitude of the hook effect observed for each device [(intact hCG + hCGβBcf) – intact hCG only (i.e., black bar – white bar)]. The Clinitest was also read in duplicate by the instrument and results were as follows: hCG, positive/positive; hCGβBcf, positive/positive; hCG + hCGβBcf, negative/borderline.

*Statistically significant differences in device interpretation between the zero point and all subsequent points.
best-performing devices were the Beckman Coulter Icon 20 and the Alere. The Icon 20 was the only device to exhibit an increased signal when hCGβcf was added to intact hCG (+0.3). The Alere device was very modestly affected by the addition of hCGβcf (−0.3). The group of devices with intermediate performance consisted of the Sure-Vue, Combo, McKesson, Clinitest, QuPID, Beckman Coulter Icon 25, and QuickVue devices. All of these devices demonstrated noticeable susceptibility to the addition of hCGβcf, with scores from −1.0 to −1.5. Devices in this group generated faint positive signals when tested with the solution containing both intact hCG and hCGβcf. The group of devices with the poorest performance consisted of the OSOM and Cen-Med Elite devices. These devices were read as negative by all but 2 readers when used to test the solution containing intact hCG and hCGβcf.

As expected, the ability of each device to withstand inhibition due to increased concentrations of hCGβcf correlated with the ability to detect hCGβcf (Fig. 3). The best-performing devices generated strong positive signal when tested with the solution containing 50 000 pmol/L hCGβcf alone, whereas the poorest-performing devices generated weakly positive or negative signal when tested with the solution containing hCGβcf alone.

Having identified the BC Icon 20 and the Alere as the 2 devices least affected by hCGβcf at 500 000 pmol/L, we evaluated the performance of these 2 devices, as well as the Combo, with a solution containing 500 pmol/L intact hCG and \(3 \times 10^6\) pmol/L hCGβcf, which was greater than the highest hCGβcf concentration reported previously (7). As shown in Fig. 4, the BC Icon 20 generated faint to clearly detectable positive signal, whereas the Alere and Combo devices generated negative to faint positive signals, confirming the BC Icon 20 as the device least affected by hCGβcf in our study.

**Discussion**

False-negative point-of-care hCG results, because of the presence of high concentrations of hCGβcf, in women with hCG concentrations regularly observed during healthy pregnancy are a clinically significant concern for the healthcare profession. Here we sought to develop an easy-to-use, affordable screening method that could be used by device users to select devices and select specific device lots (since interferences have been shown to vary with both device and lot number (12)) and also be used by manufacturers to develop better hCG devices and potentially modify existing devices.

The phenomenon referred to as the variant hook effect (9) occurs when, in addition to intact hCG, 1 of the antibodies used in the assay recognizes an hCG variant (such as hCGβcf) and the other antibody does not recognize it at all, distinguishing the variant hook effect from the traditional variant hook effect. Here, we show that the majority of devices examined had some recognition of hCGβcf alone. Therefore the inhibition seen due to increasing amounts of hCGβcf cannot be...
called a variant hook effect and is more consistent with a traditional hook effect. Hence we have tried to avoid the term variant hook effect.

Both the Combo and OSOM had biphasic responses to intact hCG (Fig. 1). In other words, the devices showed an initial signal with hCG alone that began to diminish when the concentration got too high. This is the traditional hook effect and would be predicted in a 1-step sandwich assay. The OSOM device was much more affected than the Combo device. In fact, the OSOM device signal was diminished to a faint positive at 1 000 000 pmol/L (341 297 IU/L) intact hCG, a concentration that can be achieved in the first trimester. The Combo device also had a biphasic response to hCGβcf (Fig. 1A). In other words, the device showed a signal with 50 000 pmol/L hCGβcf alone that began to diminish as the concentration increased. This is also consistent with the traditional hook effect.

It is unclear if the OSOM device recognizes hCGβcf. Previously, Sigel and Grenache (13) reported that the OSOM device did not recognize hCGβcf (up to a concentration of 10 200 pmol/L). We observed a very faint signal (4 of 10 readers rated the device as faint positive) at concentrations of 50 000 pmol/L (Fig. 1B). It is unclear if the OSOM device has some ability to recognize hCGβcf or if this positive signal represents a very small contamination with intact hCG. At a concentration of 50 000 pmol/L, even a 0.1% contamination could theoretically cause a positive result. Thus it is unclear if the OSOM device demonstrates a true variant hook effect or traditional hook effect. Epitope mapping of both antibodies, such as has been done recently (14), would be needed to clarify this question.

McChesney et al. examined urine concentrations of total hCG, hCGβcf, hCGβ, and intact hCG during the first 6 weeks postconception and demonstrated that, in the first several weeks, intact hCG concentrations are greater than hCGβcf (5). However, hCGβcf concentrations become greater than intact hCG with time. By about 7 weeks of gestation, concentrations of hCGβcf are approximately 10-fold higher than intact hCG (5). Stenman et al. also measured the concentrations of hCG and hCGβcf in urine across pregnancy in a single patient (6). These authors also reported that hCGβcf concentrations are in general about 10-fold greater than intact hCG. We observed that both the OSOM and Combo devices were markedly inhibited at concentrations consistent with approximately 7 weeks of pregnancy [50 000 pmol/L (17 000 IU/L) intact hCG and 300 000 pmol/L hCGβcf; purple line].

We sought to select a pair of intact hCG and hCGβcf concentrations that could be used to screen POC hCG devices. Because we observed the general trend that more hCGβcf was required to cause inhibition as the concentration of intact hCG increased, and to minimize the cost of the screening method, minimum intact hCG and hCGβcf concentrations were preferred: (a) 500 pmol/L (171 IU/L) intact hCG with 0 pmol/L hCGβcf; and (b) 500 pmol/L intact hCG with 500 000 pmol/L hCGβcf. These were the minimal concentrations of hCG and hCGβcf with which we could observe a visible difference between solutions with and without hCGβcf. This combination of intact hCG and hCGβcf was in no way meant to mimic hCG concentrations at any single point in pregnancy. Rather, it was meant to illustrate that a device was in fact susceptible to the hook effect due to hCGβcf.

Because the OSOM and Cen-Med Elite devices were unacceptable due to their inhibition in the presence of hCGβcf, these devices present a clinically significant risk of false-negative results in patients after approximately 5–7 weeks of pregnancy. The OSOM has in fact been shown previously to give false-negative results in pregnant patients (7, 11).

The BC Icon 20 and the Alere were not affected by increased concentrations of hCGβcf. The BC Icon 20 was the only device to actually exhibit increased signal when hCGβcf was added to intact hCG. Only the BC Icon 20 retained a clearly positive signal at 3 000 000 pmol/L hCGβcf. These 2 devices are manufactured by Beckman Coulter but include different antibodies and have different analytical sensitivities. As might be expected, we found that the devices that gave the strongest signal with hCGβcf alone were those least affected by the addition of hCGβcf.

Our findings raise the long-debated question of whether monoclonal antibodies are too specific. A lack of understanding of the exact analytical specificity of antibodies can lead to problems. For analytes such as hCG, which are a heterogeneous mix of variant forms, one has to ask if polyclonal antibodies or a mix of monoclonal antibodies might actually be better, especially in 1-step sandwich assays. Detailed knowledge of the epitopes recognized by monoclonal antibodies will facilitate the development of assays for proper clinical use (14).

There are many unanswered questions about hCGβcf. What causes some women to have higher hCGβcf concentrations than others? How often do women achieve hCGβcf concentrations that could cause a false-negative result in the majority of devices that are subject to the hook effect? Clearly more studies are needed. Until these questions are answered, we suggest that healthcare professionals choose qualitative hCG devices that are least subject to inhibition caused by increased hCGβcf concentrations to avoid the possibility of false-negative results. The screening method put forth here should help users choose devices and select device lot numbers. We hope that manufacturers will use this information to develop devices that gener-
ate clearly positive signal even in the presence of high hCGβcf concentrations.

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