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Analytical and Clinical Validation of the Immulite 1000 hCG Assay for Quantitative Analysis in Urine

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Abstract

Background—The Siemens Immulite hCG assay detects all major hCG variants in serum. Currently, this assay is only FDA approved for qualitative measurement of hCG in urine.

Methods—Complete validation of the hCG assay in urine was performed on the Siemens Immulite 1000 immunoassay platform. Reference intervals were established for females <55 y, females 55 y, and males 20–70 y.

Results—The limit of quantitation was 2.0 IU/l. The Immulite hCG assay was precise for measuring hCG in urine from pregnant patients with intra- and inter-assay imprecision of <11% CV. The assay was linear over a dynamic range of 2–2600 IU/l and 2–3500 IU/l for hCG and hCGβ respectively. The assay was non-linear for hCGβcf. No hook effect was observed at concentrations up to 1,200,000 pmol/l, for hCGβ or hCGβcf. The reference intervals were <2.0 IU/l for males, <2.2 IU/l for females <55 y, and <12.2 IU/l for females 55 y.

Conclusion—The Immulite 1000 hCG assay can accurately quantify hCG in urine.

Keywords

human chorionic gonadotropin; hCG; urine; quantitative measurement

1.0 Introduction

Human chorionic gonadotropin (hCG), a member of the glycoprotein hormone family, is produced by placental trophoblasts and acts on the corpus luteum to maintain progesterone production during early pregnancy. During the first trimester, hCG doubles every 40–48 h and peaks at ~8–11 weeks [1]. Serum hCG concentrations vary widely among women of the same gestational age; therefore doubling times are commonly measured to assess normal

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progression of pregnancy. Distinct hCG isoforms are present in serum and urine of women at different times during pregnancy [2,3]. Intact hCG, composed of an α and β subunit, and free beta hCG (hCG β) are both found in serum and urine. Nicked forms of hCG and hCG β , feature a break between amino acids 47 and 48 on the beta chain. Hyperglycosylated hCG (hCG-H) is the predominant isoform in serum and urine in early pregnancy [3]. Intact hCG is the predominant form in serum after 7 weeks gestational age. hCG β core fragment (hCG β cf), is formed in the kidney during renal clearance, and is the predominant hCG isoform in urine after 5–7 weeks gestational age [4].

Current laboratory testing for hCG includes qualitative and quantitative testing. Qualitative testing, primarily performed on urine specimens at the point of care, has a manufacture-claimed analytical sensitivity of ~20 IU/l depending on device [5]. Most qualitative assays are chromatogenic sandwich type immunoassays with antibodies targeted to distinct epitopes on the alpha and beta subunits designed to detect intact hCG heterodimer. Qualitative tests are subject to false-negative results due to dilute urine, operator error, high-dose hook effect, and hCG variant effect [6–8].

Quantitative testing is performed primarily using automated immunometric assays that target different epitopes of the hCG molecule and many are designed to detect both intact hCG heterodimer and hCG β . Quantitative testing is currently only FDA approved for serum or plasma specimens but one assay (Siemens Immulite hCG) is FDA approved for qualitative, not quantitative, measurement of hCG in urine.

Analytical variability exists among different quantitative serum hCG assays in serum due to different antibody specificities for the various hCG variants and a lack of assay harmonization [9,10]. hCG may not be detected if samples are collected in very early pregnancy and results may be falsely decreased due to the high-dose hook effect [11,12]. False positives results can occur when interfering antibodies react with the assay reagents. hCG may also be detected in peri- and post-menopausal women due to hCG production from the pituitary gland [13].

For many of the reasons listed above, it is not uncommon to have inconsistent results between urine qualitative and serum quantitative hCG tests. This can cause clinical confusion and may result in delay of necessary treatment or initiation of unnecessary treatment. In these cases, a sensitive, quantitative urine hCG assay that recognizes all hCG isoforms would be useful, particularly to rule out inherent differences between typical qualitative and quantitative hCG assays.

The Siemens Immulite hCG assay detects all major recognized hCG variants in urine [9,10]. Here we report the analytical performance of this assay to quantify hCG in early pregnancy urine and purified preparations of hCG, hCG β , and hCG β cf. In addition, we establish valuable urine-specific reference intervals.

2.0 Materials and Methods

2.1 Study Samples

The hCG-negative urine pool was created from 30 residual drug screen negative urine specimens from males age 18–40 y sent to the Vanderbilt University Medical Center toxicology laboratory for physician ordered drug screens. Samples were pooled, aliquotted, and stored at –80°C until use. Specimens were thawed at 4°C, to ensure stability. The pool was determined to be hCG free by measuring aliquots on the Immulite hCG assay.

Pregnancy urine and serum specimens were obtained by collecting residual urine and serum specimens sent to the Vanderbilt University Core Laboratory for physician ordered

qualitative and quantitative hCG testing. Specimens were stored at -80°C until use. Specimens were thawed at 4°C . A review of medical record was performed to determine gestational ages (3–5 weeks by last menstrual period).

Non-pregnant urine samples were collected from three cohorts: 120 non-pregnant females <55 y, 120 females 55 y, and 120 males 20–70 y. Healthy patient volunteers were recruited and consented at ARUP Laboratories (Salt Lake City, UT). Basic information was recorded for female volunteers including: age, current medications, last menstrual period, and current pregnancy status (self-declared by subject). Only age and current medications were documented for male subjects. No patients were excluded based on medications. Specimens were shipped to Vanderbilt and stored frozen at -80°C until analysis. Specimens were thawed at 4°C . Institutional review board approval was received for this study.

2.2 hCG Standard Preparations

The fourth WHO International Standard (4th IS) preparation of hCG (75/589) and first WHO International Reference Reagent (IRR) preparations of hCG β (99/650) and hCG β cf (99/708) were obtained from the national Institute for Biological Standards and Controls (Hertfordshire, UK). For linearity and low-end linearity/analytical sensitivity studies, each ampule of lyophilized standard was reconstituted in 1 ml of hCG free urine pool equivalent to 1880 nmol/l hCG, 880 nmol/l hCG β , and 1020 nmol/l hCG β cf. Concentrated stock solutions were subsequently diluted in hCG-free urine to create working stocks of 18, 17 and 35 nmol/l for hCG, hCG β , and hCG β cf respectively. These working stocks were subsequently diluted to tested concentrations in hCG-free urine. For the high-dose hook effect experiments, hCG β and hCG β cf IRRs were reconstituted in hCG-free urine pool to a working concentration of 3,000 nmol/l.

2.3 hCG Assay

Quantitative analysis of hCG was performed on the Immulite hCG assay on an Immulite 1000 instrument according to the manufacturer's instructions. The manufacturer's package insert states that the lower and upper limits of detection of hCG in serum are 0.4 and 5000 IU/l respectively, while the urine assay is qualitative with a positive cut off of 30 IU/l. The precision of the assay in serum is < 10% at all concentrations tested from 30–3500 IU/l. No high-dose hook effect is observed with hCG concentrations up to 2,000,000 IU/l in serum and the assay is not affected by the hCG variant effect.[11]

2.4 Validation of Analytical Sensitivity, Linearity, and Recovery

The limit of the blank (LOB) was defined as the highest concentration expected when a sample containing no analyte is tested. The limit of detection (LOD) was defined as the lowest concentration that can be distinguished from the LOB. LOB and LOD were determined by diluting pregnancy urine to an expected concentration of 2 IU/l in hCG-free urine. Ten replicates each of the hCG-free pool and the 2 IU/l specimen were measured and means and standard deviations were calculated. The following calculations were performed: $\text{LOB} = \text{mean}_{\text{blank}} + 2\text{SD}_{\text{blank}}$; $\text{LOD} = \text{LOB} + 2\text{SD}_{\text{low concentration sample}}$. The limit of quantitation (LOQ) was defined as the lowest concentration which could be measured with an imprecision of <20% CV. This was determined by diluting pregnancy urine into hCG-free urine to mean concentrations of 1.6 and 2.0 IU/l and measured 10 times.

Low end linearity was tested by serially diluting WHO standards or pregnancy urine in hCG free urine to the expected concentrations of 0.5, 1, 2, 4, 8, 10, 20, 30, 40, and 80 IU/l and assayed in duplicate.

For linearity studies, WHO 4th IS hCG or hCG β and hCG β cf IRRs were diluted to working concentrations of 18, 17 and 35 nmol/l respectively. These were subsequently diluted 1:2.2, 1:3.3, 1:5, 1:10, 1:20, 1:50, 1:100, 1:200, 1:400, 1:1000, and 1:5000 in hCG free urine and each assayed in duplicate.

Recovery studies were performed by diluting serum from a pregnant patient (Gestational age of 4 weeks by last menstrual period) into hCG-free urine to 3 expected concentrations of 2.5, 10 and 80 IU/l. The dilutions were 1:320, 1:80, 1:10, serum to urine respectively, in order to minimize alterations to the urine matrix. Each concentration was assayed in duplicate.

2.5 Validation of Imprecision

Pregnancy urine was diluted into the hCG free urine pool to 3 different concentrations for inter-assay and intra-assay precision studies. Samples were aliquotted and frozen at -80°C until use. Samples were thawed at 4°C prior to experiments. For intra-assay precision, aliquots for each concentration were assayed 10 consecutive times. For inter-assay precision, an aliquot of each concentration was assayed in duplicate for 5 consecutive days for a total of 10 replicates.

2.6 High-dose Hook Effect

WHO IRRs for hCG β and hCG β cf, at working concentrations of 3000 nmol/l, were diluted in hCG free urine to the following concentrations 1200, 1080, 960, 840, 720, 600, 480, 360, 240, and 120 nmol/l and assayed in duplicate. All samples were subsequently further diluted with hCG-free urine to check for accuracy of dilutions.

2.7 Reference Interval Validaiton

Reference intervals were determined by measuring hCG in 120 urine samples from each of three cohorts: non-pregnant females <55 y, females 55 y, and males 20–70 y. Reference intervals were determined by the non-parametric method using EP evaluator 9 software (Data Innovations, South Burlington, VT).

2.8 Statistical Analysis

Precision, linearity, analytical sensitivity, recovery, and reference intervals were evaluated using EP evaluator 9 software (Data Innovations).

3. Results

3.1 Analytical Sensitivity, Linearity, and Recovery

The LOB and LOD were determined to be 0.5 IU/l and 1.3 IU/l respectively. The LOQ was determined to be 2.0 IU/l (CV 5.7%). Linearity specimens were prepared by mixing various concentrations of pregnancy urine and/or WHO hCG preparations into hCG free urine and measuring hCG in each concentration in duplicate. The Immulite hCG assay was linear over a dynamic range of 2–2600 IU/ml (4–8100 pmol/l) and 2–3500 IU/ml (3.4–7600 pmol/l) with the WHO 4th IS for total hCG (Fig. 1A), and the WHO IRR for hCG β (Figure 1B). Measurement of hCG β cf was not linear throughout the dynamic range, but was linear from 100–5000 IU/l (331–10,635 pmol/l) (Fig. 1C) and <2.0–100 IU/l (7–331 pmol/l) (Figure 1C inset). The low-end linearity was determined by measuring serial dilutions the WHO 4th IS for hCG, WHO IRRs for hCG β and hCG β cf or early pregnancy urine. Low-end linearity was between 1–2 IU/l for pregnancy urine (Figure 1D) and all hCG isoforms tested (data not shown).

Each WHO standard was reconstituted in hCG free urine to specified molar concentrations (nmol/l). Then various dilutions of these standard preparations were measured on the

Immulite 1000 hCG assay and results reported in IU/l. The estimated conversion factors in IU/nmol (activity ratios) for the hCG isoforms in urine were calculated based upon slopes in the linearity studies (Fig. 1A–C). The urine activity ratios were 324 IU/nmol, 442 IU/nmol, and 376 IU/nmol for WHO hCG 4th IS (75/589), WHO hCG β IRR (99/650), and WHO hCG β cf (99/708) respectively. Recovery was ~108% when serum from a patient approximately 4 weeks pregnant was diluted into hCG free urine at three expected concentrations (2.5, 10, and 80 IU/l) (Table 1).

3.2 Imprecision

Intra-assay repeatability and inter-assay imprecision were determined using three concentrations of hCG in urine. For intra-assay precision, three concentrations of early pregnancy urine were diluted into hCG-free urine and measured 10 consecutive times on the Immulite 1000 hCG assay (Table 2). For inter-assay precision, three concentrations of urine pregnancy were diluted into hCG free urine and measured two times per day for five consecutive days. Intra-assay imprecision ranged from 6.1–6.5% CV for concentrations ranging from 6.9–973 IU/l. The within laboratory CVs in 10 samples were all <11% at mean concentrations of 6.6, 68, and 908 IU/l (Table 2).

3.3 High-dose Hook Effect

Table 3 shows expected concentrations in pmol/l and equivalent concentrations in IU/l based on activity ratios calculated in this study and average observed concentrations measured in duplicate on the Immulite 1000 hCG assay. No hook effect was observed at concentrations as high as 1,200,000 pmol/l or up to 700,000 IU/l of free β hCG and 428,000 IU/l hCG β cf. (Table 3)

3.4 Reference Intervals for hCG in Urine

Reference intervals for hCG in urine were derived for each of three healthy patient populations (n=120 for each). The reference intervals were found to be <2.0 IU/l for males, <2.2 IU/l for females <55 y, and <12.2 IU/l for females age $\geq$ 55 y (Fig. 2).

4. Discussion

In this study we validate the measurement of intact hCG as well as the major urine hCG isoforms, hCG β and hCG β cf, in urine using the Immulite hCG assay. Importantly, we have established reference intervals for urine hCG in healthy males, pre-menopausal woman and post-menopausal women.

The analytical measuring range for WHO preparations of total hCG and hCG β was validated from 2–2600 and 2–3500 IU/l respectively, while hCG β cf was nonlinear. Imprecision, in urine from pregnant women, was less than 20% even at an hCG concentration <5 IU/l. Recovery of hCG from early pregnancy serum was ~108% at all concentrations tested. Calculated activity ratios for WHO preparations of hCG, hCG β , and hCG β cf were 324, 442, and 376 IU/nmol respectively. These ratios are different from those previously calculated in serum for the Immulite hCG assay [10], likely due to a matrix differences between serum and urine.

In order to utilize the Immulite 1000 hCG assay to detect early pregnancy it must provide excellent analytical sensitivity. We determined the limit of quantification was 2 IU/l. This degree of sensitivity would allow for detection of hCG in urine above our established normal reference interval (< 2.2 IU/l) as early as cycle day 23 (~9 days following ovulation; i.e. ~6 days before expected day of menses) [14].

Some hCG assays are subject to the high-dose hook effect with extremely high concentrations of total hCG [11,12]. High concentrations of hCG β cf and hCG β are seen in urine in the first trimester of pregnancy or in cancer patients [7,15]. Therefore it is critical to determine whether the Immulite 1000 hCG assay show a hook effect with extremely high concentrations of hCG β and hCG β cf in urine. No high-dose hook effect was observed with concentrations up to 1,200,000 pmol/l of either isoform, which is consistent with previous studies of Immulite 2000 assay in serum [11].

Quantitative urine hCG tests may be of use when investigating discrepant results obtained from qualitative urine and quantitative serum hCG tests. Thus it is critical to understand background urine hCG concentrations in various non-pregnant patient populations. Studies have demonstrated variable concentrations of hCG in urine for non-pregnant females and males [16–18]. In this study we determined the reference intervals for hCG in men was lowest at <2.0 IU/l, followed by females < 55 y <2.2 IU/l, and then females $\geq$ 55 y <12.2 IU/l. The higher concentrations in post-menopausal women is likely due to the presence of pituitary hCG [13].

A validated quantitative urine hCG assay with age and sex specific reference intervals will be most useful for investigating discrepant serum hCG results. A direct comparison of results between similar quantitative serum and urine immunoassays allows laboratories to rule out assay differences as the cause of discordances. Further studies are needed to determine the utility of a quantitative urine hCG assay in diagnosis and monitoring of pregnancy.

One drawback to measuring hCG in urine is that concentrations vary depending upon fluid intake. Despite this, we intended to establish reference intervals from a random set of patients most similar to patients who would have hCG testing in urine. Therefore our results reflect a practical reference intervals for urine hCG, and were not normalized to creatinine concentration.

5. Conclusion

In conclusion, the Immulite 1000 hCG assay can be used to accurately quantify hCG in urine which may aid in monitoring pregnancy and troubleshooting discrepant serum hCG results.

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Abbreviations

hCGβ	human chorionic gonadotropin free β subunit
hCG-H	hyperglycosylated human chorionic gonadotropin
hCGβcf	human chorionic gonadotropin beta core fragment
IRR	International Reference Reagent

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Highlights

- We validated the Immulite 1000 hCG assay for quantitative measurement in urine using urine from pregnant patients and WHO International Reference Reagents
- We determined that there was no high dose hook effect seen on the Immulite hCG assay with two common hCG variants (hCG β and hCG β cf)
- We established reference intervals for hCG in urine for three populations: Males 20–70 years, Females < 55 years and Women $\geq$ 55 years

Fig. 1a

Fig. 1b

Fig. 1c

Fig. 1c_inset

Fig. 1d

Figure 1.

Linearity of standard reference reagents for hCG and pregnancy urine diluted into hCG free urine. **(A)** Total hCG measured using WHO hCG 4th IS (75/589) diluted into hCG-free urine, linear regression: $y = 0.617x + 15.5$. **(B)** WHO hCG β IRR (99/650) reconstituted in hCG free urine was diluted at various concentrations into hCG-free urine, linear regression: $y = 0.755x - 38.3$ **(C)** WHO hCG β cf (99/708) diluted into hCG-free urine, Observed and Expected concentrations, $y = 1.054x - 57.7$; **Inset** Low-end linearity of hCG β cf, $y = 0.378x + 0.47$ **(D)** Urine from a pregnant patient was serially diluted into hCG-free urine pool.

Reference Interval Males 20 - 70 years

Fig. 2a

Reference Interval Women < 55

Fig. 2b

Fig. 2c

Figure 2. Nonparametric reference intervals for hCG in urine in three cohorts: Males 20–70 y (**A**), Females <55 y (**B**) and Females age ≥ 55 y (**C**). The reference range for each cohort was derived from 120 healthy non-pregnant control subjects.

Table 1

Recovery of hCG in urine using a pregnant patient's serum.

	Expected IU/l	Observed IU/l	Recovery (%)
	2.5	2.7	108
Pregnancy Serum	10	10.8	108
	80	87.0	109%

Table 2

Intra- and inter-assay precision studies using urine from a pregnant patient.

	Mean (IU/l)	SD (IU/l)	CV (%)
	6.9	0.45	6.5
Intra-assay (n=10) ^a	74.0	4.56	6.2
	973	59.8	6.1
	6.6	0.67	10.2
Inter-assay (n=10) ^b	68.1	2.36	3.5
	908	48.9	5.4

^a Each concentration was assayed 10 consecutive times.^b Each concentration was assayed in duplicate for 5 consecutive days for a total of 10 replicates.

Table 3Expected and observed concentrations of hCG β and hCB β cf in high-dose hook effect experiments

Concentration (pmol/l)	Expected hCG β^a (IU/l)	Average hCG β (IU/l)	Expected hCG β cf ^b (IU/l)	Average hCG β cf (IU/l)
1,200,000	530,400	>5000	451,200	>5000
1,080,000	477,360	>5000	406,080	>5000
960,000	424,320	>5000	360,960	>5000
840,000	371,280	>5000	315,840	>5000
720,000	318,240	>5000	270,720	>5000
600,000	265,200	>5000	225,600	>5000
480,000	212,160	>5000	180,480	>5000
5,333	2,357	2975	2,005	4460
2,133	943	789	802	566
<2.0	<2.0	<2.0	<2.0	<2.0

^aWHO hCG β IRR (99/650) reconstituted to 3000 nmol/l (3×10^6 pmol/l) in hCG free urine was further diluted in hCG free urine. IU/l estimates were calculated based upon an activity ratio of 442 IU/nmol (calculated in this study).

^bWHO hCG β cf (99/708) reconstituted to 3000 nmol/l (3×10^6 pmol/l) in hCG free urine was further diluted in hCG free urine. The calculated Activity ratio was 376 IU/nmol (calculated in this study).