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Immunoextraction-Tandem Mass Spectrometry Method for Measuring Intact Human Chorionic Gonadotropin, Free β -Subunit, and β -Subunit Core Fragment in Urine

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Abstract

BACKGROUND—Human chorionic gonadotropin (hCG) stimulates testosterone production by the testicles. Because of the potential for abuse, hCG is banned (males only) in most sports and has been placed on the World Anti-Doping Agency list of prohibited substances. Intact hCG, free β -subunit (hCG β), and β -subunit core fragment (hCG β cf) are the major variants or isoforms in urine. Immunoassays are used by antidoping laboratories to measure urinary hCG. Cross-reactivity with isoforms differs among immunoassays, resulting in widely varying results. We developed a sequential immunoextraction method with LC-MS/MS detection for quantification of intact hCG, hCG β , and hCG β cf in urine.

METHODS—hCG isoforms were immunoextracted with antibody-conjugated magnetic beads and digested with trypsin, and hCG β and hCG β cf unique peptides were quantified by LC-MS/MS with the corresponding heavy peptides as internal standard. hCG isoform concentrations were determined in urine after administration of hCG, and the intact hCG results were compared to immunoassay results.

RESULTS—The method was linear to 20 IU/L. Total imprecision was 6.6%–13.7% (CV), recovery ranged from 91% to 109%, and the limit of quantification was 0.2 IU/L. Intact hCG predominated in the urine after administration of 2 hCG formulations. The window of detection

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ranged from 6 to 9 days. Mean immunoassay results were 12.4-15.5 IU/L higher than LC-MS/MS results.

CONCLUSIONS—The performance characteristics of the method are acceptable for measuring hCG isoforms, and the method can quantify intact hCG and hCG β separately. The limit of quantification will allow LC-MS/MS hCG reference intervals to be established in nondoping male athletes for improved doping control.

Human chorionic gonadotropin (hCG)⁴ is a glycoprotein hormone consisting of α and β subunits with a mean molecular weight of 37.5 kDa (1,2). The α subunit of hCG is not unique and is identical in luteinizing hormone, follicle-stimulating hormone, and thyroid-stimulating hormone. The 145-amino acid β subunit of hCG confers its distinct biological function despite having approximately 80% homology with the β -chain of luteinizing hormone (2-4). hCG is produced in high concentrations by placental trophoblasts and in low concentrations by the pituitary (5-7). The majority of hCG in the circulation is metabolized by the liver, and approximately 20% is excreted by the kidneys (8). There are several different variants or isoforms of hCG, including intact hCG, nicked hCG, free β -subunit (hCG β), nicked hCG β , and degradation products including β -subunit core fragment (hCG β cf) (9, 10). Almost all hCG β cf is produced during renal excretion and accounts for 50% of the hCG found in urine (4, 11, 12).

In males, hCG stimulates testosterone production by the testicles (13) and has the potential to be abused by athletes in an attempt to enhance performance in sports. hCG may be used not only to increase testosterone production but also to normalize testosterone concentrations that have been suppressed by anabolic steroid use (4, 13). The use of hCG by males is banned in most sports, and hCG is on the World Anti-Doping Agency (WADA) list of prohibited substances (14). The WADA guideline document recommends that the initial hCG screening test used by accredited antidoping laboratories should be an immunoassay recognizing multiple isoforms of hCG (15). A urine hCG concentration >5 IU/L is considered positive and would be confirmed by a second immunoassay that detects only intact hCG (15). Depending on the antibody configuration and the cross-reactivity of the assay with various hCG isoforms, widely varying hCG results can be obtained with different commercial immunoassays (16, 17). For example, the Siemens Immulite immunoassay has 35% cross-reactivity with hCG β cf, whereas the Abbott Architect immunoassay has only 1% cross-reactivity (18). Thus, there is a critical need to standardize urinary hCG immunoassays for doping control purposes.

The high analytical sensitivity and specificity of LC-MS/MS-based methods make them an attractive alternative to antibody-based hCG immunoassays. Liu and Bowers developed a solid-phase immunoaffinity trapping technique with mass spectrometric detection of tryptic peptides to identify hCG in a urine sample fortified with 25 IU/L intact hCG (19). Several improvements resulted in a quantitative method that could measure hCG at concentrations as low as 5 IU/L with the β T5 peptide for detection, which was shown to be unique to hCG β (20). A more recent study used an antibody recognizing multiple hCG isoforms for immunoextraction and signature peptides for detecting hCG β , nicked forms of hCG β , and hCG β cf, with a limit of quantification (LOQ) of 5 IU/L (21, 22). Although this method is a

major step in the quantification of hCG isoforms in urine and blood, it does not distinguish between hCG and hCG β , since both isoforms are captured during the immunoextraction procedure and contain the same β T5 peptide used for quantification.

In this study, we developed an antibody-based immunoextraction method that would allow the differential capture of intact hCG and hCG β from urine samples. This is important because the ratio of intact hCG to hCG β in the urine of males dping with different formulations of hCG has not been established by LC-MS/MS. The method was used to measure the concentration of hCG isoforms in timed urine samples after the administration of pregnancy urine-purified and recombinant hCG to male subjects.

Materials and Methods

CHEMICALS

Tris(2-carboxyethyl)phosphine hydrochloride, Tris-HCl, bovine serum albumin, Tween-20, glycine, lysine, and iodoacetamide were obtained from Sigma-Aldrich; trypsin from Promega Corp.; and acetonitrile (LC-MS grade), formic acid (LC-MS grade), and EDTA from Fisher Scientific.

hCG REFERENCE STANDARDS AND PEPTIDES

WHO reference standards of intact hCG (99/688), hCG β (99/650), and hCG β cf (99/708) were purchased from the National Institute for Biological Standards and Control. The mean immunoassay estimate for intact hCG in terms of the international standard for hCG is 800 IU/ampoule. On this basis, 1 IU was equivalent to 2.35 pmol (1 IU/L = 2.35 pmol/L). For comparison, 2.35 pmol/L hCG β or hCG β cf was considered equivalent to 1 IU/L.

hCG β -specific T5 tryptic peptide (VLQGVLPAL-PQWCNYR; amino acids 44-60; M_r 1927.2 Da), hCG β cf-specific T5 tryptic peptide (WCNYR amino acids 55-60; M_r 810.7 Da), and the corresponding heavy peptides (mass shift of 10 Da) labeled at the C-terminal arginine residue ($^{13}\text{C}_6$, $^{15}\text{N}_4$) were synthesized by Pierce Thermo-Fisher Scientific. All peptides were >97% pure and were modified with a carbamido-methyl group at the cysteine residue.

ANTIBODIES AND COUPLING TO MAGNETIC PARTICLES

The β 7 (INN-hCG-68) monoclonal antibody recognizing hCG β and hCG β cf, but not intact hCG, was purchased from GeneTex. Interestingly, the supplier indicated that the β 7 antibody bound only hCG β . The β 1 (INN-hCG-2) monoclonal antibody recognizing all 3 hCG isoforms was purchased from Abeam. The specificity of these antibodies has been previously reported (23, 24).

The β 7 and β 1 monoclonal antibodies were conjugated to carboxylate-modified Sera-Mag Magnetic SpeedBeads (carboxyl content 0.4885 mEq/g, Thermo Scientific) according to the manufacturer's instructions. Antibody (12 μ g) was coupled to each milligram of beads. After antibody coupling, the beads were incubated in a 500-mmol/L solution of lysine at 4 °C to block active carboxyl sites. The antibody-coupled beads were stored in buffer containing 50

mmol/L Tris-HCl, 5 mmol/L EDTA, 0.1% BSA, and 0.1% Tween-20 at 4 °C and were used within 2 weeks.

IMMUNOEXTRACTION OF hCG ISOFORMS

Sample (1 mL) was incubated with 0.5 mg β 7-coupled magnetic particles for 3 h at room temperature with gentle rotation. In initial experiments, we fortified negative urine and buffer samples with hCG. After incubation, the magnetic particles were trapped by a magnet supplied by the manufacturer, and we saved the supernatant for immunoextraction of intact hCG. The magnetic particles were washed with 1 mL of 10 mmol/L Tris-HCl (pH 7.4), and antibody-bound hCG β and hCG β cf were eluted from the particles after incubation for 2 min with 15 μ L of 100 mmol/L glycine solution containing 1 mmol/L EDTA (pH 2.55). This step was repeated twice, and the pooled eluate (30 μ L) was neutralized with 1.8 μ L of 4 mol/L Tris-HCl (pH 8.6) and 2.2 μ L of 2 mol/L ammonium bicarbonate. Intact hCG failed to bind β 7 antibody-coupled beads at multiple hCG concentrations (see Supplemental Fig. 1, which accompanies the online version of this article at <http://www.clinchem.org/content/vol60/issue8>). Intact hCG was immunoextracted from the supernatant not binding β 7-coupled magnetic particles by overnight incubation with 0.5 mg β 1-coupled magnetic particles. Intact hCG was eluted from the particles as described above.

hCG DIGESTION

Immunoextracted hCG isoforms underwent reduction with Tris(2-carboxyethyl)phosphine hydrochloride, alkylation with iodoacetamide, and tryptic digestion before analysis by LC-MS/MS as described in the online Supplemental Data.

LC-MS/MS ANALYSIS

We used a Shimadzu LC-20AD HPLC system with an autosampler coupled to an AB Sciex 5500 QTRAP tandem mass spectrometer with an electrospray ionization interface for analysis. Peptides were separated by a Kinetex C18 50- $\times$ 2.1-mm HPLC column (Phenomenex) with 2.6- μ m particle size and 100-Å pore diameter. Solvent A was 0.1% formic acid in water, and solvent B was 90% acetonitrile/0.1% formic acid. A gradient of 0% to 50% solvent B over a 30-min period at a flow rate of 0.1 mL/min was used for separation. Optimization and LC-MS/MS parameters are provided in the online Supplemental Data. The mass spectrometer was operated in multiple reaction monitoring mode, and the transitions monitored were ion pairs 642.7/ 518.3, 642.7/711.4, and 963.8/610.5 for the T5 β -specific peptide and 646.3/523.2, 646.3/721.5, and 968.7/610.5 for the corresponding internal standard (IS). The transitions 405.7/711.3 and 405.7/612.3 were monitored for the T5 β cf-specific peptide, and 410.7/ 721.6 and 410.7/622.4 were monitored for the corresponding IS. Peptide identification required the presence of all monitored transitions. The chromatographic area ratios for the T5 β -specific product ions 518.3 and 523.2 (IS) and the T5 β cf-specific product ions 405.7 and 410.7 (IS) were used for quantification.

METHOD VALIDATION

To determine linearity, a urine pool was fortified with a mixture of intact hCG, hCG β , and hCG β cf, each at a concentration of 20, 10, 5, 2.5, or 0.7 IU/L. The 3 hCG isoforms were

immunoextracted from duplicate samples and analyzed by LC-MS/MS. We determined imprecision by immunoextraction and LC-MS/MS analysis of urine pools fortified with 7, 2, or 0.7 IU/L hCG isoforms in quadruplicate on 5 separate days. To determine recovery, we compared results obtained after immunoextraction and LC-MS/MS analysis of fortified urine samples to those obtained when the same urine was first immunoextracted, and then the eluted material was fortified with the same concentration of hCG isoforms (20, 10, 5, 2.5, or 0.5 IU/L). We determined the LOQ by adding known concentrations of each hCG isoform to elution buffer and analyzing quadruplicate samples on 4 different days. The LOQ was defined as the concentration with a CV <20%. To investigate matrix effects, the results obtained with urine samples fortified with hCG isoforms were compared to those obtained when the same concentration of each hCG isoform was added to buffer and analyzed in the identical manner.

IMMUNOASSAY

We performed the Roche intact hCG electrochemiluminescence immunoassay (HCG STAT) with the Elec-sys 2010 immunoanalyzer according to the manufacturer's instructions. The assay measures only intact hCG. The assay was validated to measure hCG in urine samples. Interassay CVs at mean urinary concentrations of 5.7, 16.6, and 79.4 IU/L were 5.7, 2.5, and 2.9%, respectively. The assay was determined to have a slight positive bias (mean 112% recovery) when analyzing intact hCG reference standard at concentrations ranging from 5 to 25 IU/L. The LOQ was 1 IU/L.

hCG ADMINISTRATION AND SAMPLE PREPARATION

We recruited 12 healthy men (21-30 years old) to receive a single dose of hCG in the UCLA Clinical and Translational Research Center. Exclusion criteria included the use of any prohibited substances or a random urine intact hCG concentration >2 IU/L. Intact hCG concentrations in random urine samples from the participants ranged from undetectable to 1.9 IU/L. Participants were randomized into 4 groups of 3 participants each. Two groups received Novarel® (Draxis Specialty Pharmaceuticals, intramuscular use only), a purified form of hCG derived from human pregnancy urine; 1 group received 10 000 IU and the other group received 30 000 IU intramuscularly. The other 2 groups received Ovidrel® (EMD, Serono, subcutaneous use only), a recombinant form of hCG produced in genetically modified CHO cells; 1 group received 250 µg and the other group received 750 µg subcutaneously. This study was approved by the Office of the Human Research Protection Program at UCLA. After hCG administration, urine was collected in 12-h intervals for 9 days to calculate the total amount of hCG appearing in the urine. Urine was collected in 24-h urine containers without preservative. Urine aliquots were stored at -70 °C and were thawed at room temperature before analysis. Urinary hCG concentrations are expressed in international units per liter.

The concentration of intact hCG in timed urine samples was initially determined by the Roche intact hCG immunoassay so that samples could be diluted (<20 IU/L) for complete immunoextraction with antibody-coupled magnetic particles. An appropriate volume of urine was added to PBS containing 0.1 mmol/L Tris-HCl and 0.1% BSA to a final volume

of 1 mL. Calibrators containing 2.5 and 5 IU/L of each hCG isoform were prepared in buffer solution and used for quantification.

Results

ANALYTICAL PERFORMANCE

The nonglycosylated hCG β T5 peptide was monitored for identification and quantification of hCG β because it was found in high abundance (retention time 21.7 min) after tryptic digestion and the amino acid sequence was unique to hCG β (21). The double-charged 963.8 and triple-charged 642.8 precursor ions of the T5/y peptide produced the highest signal-to-noise ratio. In contrast to the T9 β cf peptide used by another group of investigators (21), we found that the T5 β cf peptide (retention time 9.6 min) and the doubly charged 405.7 precursor ion produced the best signal-to-noise ratio and the lowest LOQ for hCG β cf.

Imprecision of the immunoextraction method and LC-MS/MS detection system was determined by analyzing urine samples fortified with different concentrations of hCG isoforms in quadruplicate on 5 separate days. Total imprecision ranged from 6.6% to 13.8%, depending on the hCG concentration and isoform being detected (Table 1). The method was linear at concentrations ranging from 0.7 to 20 IU/L for each hCG isoform on the basis of linear regression analysis (r^2 values >0.99) (Fig. 1). Linearity was also verified by the polynomial evaluation method; nonlinear β -coefficients were not found to be statistically significant by t -test (25).

To determine recovery, equivalent concentrations of each hCG isoform were added to a urine sample before and after immunoextraction (hCG was added to the eluate), and the concentrations were determined. Recovery of hCG isoforms ranged from 90% to 108% at concentrations ranging from 0.5 to 20 IU/L (Table 2). After immunoextraction of hCG isoforms (each at 10 IU/L) from a buffer solution, the supernatant (unbound material) contained <0.2 IU/L of each hCG isoform (data not shown), providing additional evidence for the effectiveness of the immunoextraction procedure.

The LOQ was determined to be 0.2 IU/L for each hCG isoform on the basis of a CV $<20\%$ (see online Supplemental Fig. 2) and was then validated in subsequent experiments at 0.7 IU/L. For a CV $<10\%$, the LOQ would be 0.2, 0.4, and 0.7 IU/L for intact hCG, hCG/y, and hCG β cf, respectively. Ion suppression due to the urine matrix was evaluated, and the percentage recovery (compared to buffer) ranged from 93% to 110% for hCG isoforms at concentrations ranging from 2.5 to 20 IU/L, indicating no significant ionization interference (see online Supplemental Table 1).

URINARY CONCENTRATIONS OF hCG ISOFORMS AFTER ADMINISTRATION

After administration of 10 000 IU of Novarel to male participants, urinary hCG β cf concentrations ranged from 316 to 485 IU/L, whereas intact hCG ranged from 47 to 158 IU/L in the first 12-h timed urine (Fig. 2, A-C). Urinary hCG β cf concentrations ranged from 10 to 78 IU/L in the second timed urine and were <7 IU/L for all participants on days 7-9. In contrast, intact hCG peaked in the first or second timed urines (range of 158-351 IU/L) and did not drop below 13 IU/L until day 7 (Fig. 2, A-C). Urinary hCG β concentrations peaked

in the first or second timed urines (range of 8-20 IU/L) and were <5 IU/L in all participants on days 4-9 (Fig. 2, A-C). Administration of 30 000 IU of Novarel resulted in a similar urinary excretion pattern, except that peak concentrations of hCG β cf and intact hCG were considerably higher, ranging from 1039 to 1562 and 883 to 1624 IU/L for hCG β cf and intact hCG, respectively (Fig. 2, D-F). The mean percentage (both groups) of the administered dose that was excreted in the urine during 9 days as intact hCG was 8.3%, and only 0.5% and 4.6% was excreted as hCG β and hCG β cf, respectively.

Administration of either 250 or 750 μ g of Ovidrel did not produce the rapid increase in hCG β cf that was observed following Novarel administration, with hCG β cf concentrations <21 IU/L in all timed urines (Fig. 3). Urinary intact hCG concentrations peaked in the second to sixth timed urines and ranged from 77 to 172 and 239 to 435 IU/L following administration of 250 and 750 μ g Ovidrel, respectively (Fig. 3). Urinary hCG β concentrations never exceeded 17 IU/L in any of the timed urines. The mean percentage (both groups) of the administered dose that was excreted in the urine during 9 days as intact hCG was 5.2%, and only 0.2% and 0.3% was excreted as hCG β and hCG β cf, respectively.

CORRELATION STUDIES

Roche intact hCG concentrations were slightly higher than those obtained following immunoextraction and LC-MS/MS analysis on the basis of Deming regression line slopes of 1.06 and 1.11 following administration of Novarel and Ovidrel, respectively (Fig. 4, A and B). The r^2 values were 0.98 for both hCG formulations; however, the Roche immunoassay produced a mean positive bias of 15.5 and 12.4 IU/L following administration of Novarel and Ovidrel, respectively (Fig. 4, C and D).

APPLICATION TO DOPING CONTROL

The window of detection for a doping violation (intact hCG >5 IU/L) with the immunoextraction and LC-MS/MS method was as follows: 6-7.5 days for 10 000 IU Novarel; 9 days for 30 000 IU Novarel; 6.5-9 days for 250 μ g Ovidrel; and 7.5-9 days for 750 μ g Ovidrel. The window of detection with the Roche intact hCG immunoassay was 8-9 days for 10 000 IU Novarel, 9 days for 30 000 IU Novarel, and 8.5-9 days for Ovidrel at both concentrations. A specific gravity adjustment was not performed as recommended by WADA (15), because timed 12-h urine samples were analyzed. Urine samples were only collected for 9 days after hCG administration, so it was possible that the detection window exceeded 9 days in some cases.

Discussion

By use of monoclonal antibodies with differential reactivity against hCG isoforms, an immunoextraction method was developed for isolating intact hCG separately from hCG β and hCG β cf. Urinary concentrations of each hCG isoform were then measured by LC-MS/MS with unique hCG β and hCG β cf tryptic peptides. The throughput of the method is approximately 60 samples per week. The method is linear up to 20 IU/L, which is well above the WADA threshold of 5 IU/L for a doping violation (15). Total imprecision is 10.4% for each hCG isoform at concentrations of 2 and 7 IU/L, making the assay suitable

for antidoping control purposes. The imprecision of the method is considerably improved compared with other LC-MS/MS based methods (21), which is important in the setting of doping control.

Our method differs from a previously published method (21), since intact hCG and hCG β can be measured separately with a 25-fold lower LOQ (0.2 vs 5 IU/L). Another difference is that our method does not require a solid-phase extraction step after tryptic digestion, which simplifies the cleanup procedure and helps minimize hCG loss during sample manipulation. The improved LOQ will be critical for establishing LC-MS/MS hCG isoform reference intervals in nondoping male athletes. Although the median concentration of hCG in urine samples from males <50 years of age has been shown to be <1 IU/L with the Delfia® time-resolved immunofluorometric assay (11), the ratio of intact hCG to hCG β has not been determined in individual male urine samples, which may be critically important for establishing an optimal testing strategy for detecting doping with hCG.

Although the $\beta 7$ antibody used to immunocapture hCG β and hCG β cf does not bind other hCG isoforms, the $\beta 1$ antibody binds nicked forms of hCG and hCG missing the C-terminal peptide (23). Nicked forms of hCG β are missing peptide linkages in hCG β molecules between amino acids 44/45 and 47/48 (26). Nicked forms of hCG β have been found in urine after hCG administration (27), but our method was not designed to detect nicked forms of hCG[3 since nicked forms would produce peptide fragments with precursor masses (amino acids 45-60, 44-47, and 48-50) that differ from the $\beta T5$ peptide and T5 β cf peptide being monitored by our method. Although our LC-MS/MS detection method could be modified to detect nicked forms, we chose to focus on the most abundant hCG isoforms in urine. Because hCG β missing the C-terminal peptide would be immunoextracted by the $\beta 1$ antibody and contains the identical $\beta T5$ tryptic peptide, this isoform would contribute to the concentration of intact hCG measured by our method. However, hCG β lacking the C-terminal peptide is found only in the urine of some cancer patients (28) and in a rare benign condition called familial hCG syndrome (29).

The intact hCG molecule contains 8 carbohydrate moieties (6 attached to the hCG β chain), and variations in the size of the carbohydrate chains occur (2). The carbohydrate heterogeneity of hCG molecules should not affect the ability of the $\beta 7$ and $\beta 1$ antibodies to recognize hCG isoforms, since these antibodies recognize an epitope around the cystine knot, which is not affected by differences in glycosylation (23). In addition, the T5 β and T5 β cf tryptic peptides being monitored by the LC-MS/MS method are not glycosylated, so detection of these peptides is not altered by carbohydrate heterogeneity.

Consistent with a previous study (30), we found that injection of purified hCG (Novarel) produced a large spike in urinary hCG β cf that rapidly decreased within a day. Purified preparations of hCG are known to be contaminated with hCG β cf (30, 31), and the hCG β cf is rapidly cleared by renal excretion (10). We determined that the purified hCG preparation used in our study contained intact 71% hCG, 27% hCG β cf, and 2% hCG β . In contrast, recombinant hCG contains >99% intact hCG (32) and, as expected, did not produce a rapid increase in urinary hCG β cf after administration (Fig. 3). Notably, the majority of hCG excreted into the urine was intact hCG regardless of the formulation or dose administered.

Approximately 91%, 3%, and 6% (mean for both doses) of the hCG found in urine after administration of recombinant hCG was intact hCG, hCG β , and hCG β cf, respectively. The percentage of intact hCG in urine after administration of purified hCG was considerably lower at 62%, whereas the percentage of hCG β cf increased to 34% owing to contamination with hCG β cf. The percentages of hCG isoforms were similar when different doses of the same hCG formulation were administered. For doping control purposes, an hCG assay that detects intact hCG, hCG β , and hCG β cf equally would be highly desirable and would maximize detection times, since all 3 isoforms are found in urine after administration of both hCG formulations.

On the basis of a cutoff of 5 IU/L for intact hCG (15), we found concentrations above the cutoff for 6-9 days after the administration of either purified or recombinant hCG. Because the study was designed to collect urine for only 9 days, it is possible that intact hCG could be detected for longer periods of time, especially after administration of 30 000 IU purified hCG. In a previous study, administration of either 5000 IU purified hCG (Pregnyl®) or 250 μ g recombinant hCG (Ovitrelle®) produced urinary total hCG β concentrations >5 IU/L for 6-14 days, when measured by LC-MS/MS (27). The increased detection times compared to those determined in our study might partially be due to the different formulations of hCG used in the 2 studies. In addition, the previous study used total concentrations of hCG β (sum of intact and hCG β) and not intact hCG, which would produce higher urinary concentrations. We were somewhat surprised that the Roche intact hCG assay produced results that were higher than our LC-MS/MS method, which resulted in slightly longer detection times. The Roche intact hCG assay is FDA approved only for blood testing and may suffer from matrix effects that result in positive bias.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Linearity of the immunoextraction and LC-MS/MS detection method. Intact hCG (A), hCG β (B), and hCG β cf (C)

Each point represents the mean from duplicate aliquots. The area ratio is the mean hCG isoform peak area divided by the mean IS peak area, y-int, y-intercept.

Fig. 2. Measurement of intact hCG, hCG β , and hCG β cf concentrations by immunoextraction and LC-MS/MS after administration of either 10 000 IU (A-C) or 30 000 IU (D-F) Novarel.

Fig. 3.
Measurement of intact hCG, hCG β , and hCG β cf concentrations by immunoextraction and LC-MS/MS after administration of either 250 μ g (A-C) or 750 μ g (D-F) Ovidrel.

Fig. 4. Comparison of immunoassay intact hCG results to those obtained by immunoextraction and LC-MS/MS analysis

Deming regression analysis of intact hCG concentrations in urine following administration of Novarel (A) or Ovidrel (B). Gray line represents unity ($x = y$). The corresponding Bland-Altman difference plots are shown for Novarel (C) and Ovidrel (D). The x axis is the mean of the immunoassay and LC-MS/MS concentrations. Gray line represents unity, and dotted lines represent ± 2 SD. y-int, y-intercept.

Table 1Imprecision of the immunoextraction and LC-MS/MS detection method.^a

hCG isoform and concentration, IU/L	Intra-assay CV, %	Interassay CV, %	Total CV, %
Intact			
0.7	9.0	7.5	11.7
2	6.8	7.9	10.4
7	4.8	4.4	6.6
hCG β			
0.7	8.4	11.0	13.8
2	4.8	7.1	8.6
7	6.1	8.0	10.2
hCG β cf			
0.7	5.5	7.7	9.5
2	4.6	6.2	7.7
7	5.5	8.0	9.7

^aOne-way ANOVA was used to calculate the various components of precision.

Table 2

Recovery of hCG isoforms after immunoextraction and LC-MS/MS detection.

Concentration (IU/L) and hCG isoform	Area ratio ^a	Area ratio CV, %	Recovery, % ^b
0.5			
Intact	0.09	12.9	105
hCG β	0.06	7.5	108
hCG β /cf	0.03	6.0	97
2.5			
Intact	0.40	7.2	96
hCG β	0.27	12.0	90
hCG β /cf	0.14	3.0	91
5			
Intact	0.83	6.3	100
hCG β	0.59	2.0	102
hCG β /cf	0.30	4.0	93
10			
Intact	1.56	6.4	98
hCG β	1.29	2.3	95
hCG β /cf	0.56	3.0	101
20			
Intact	3.17	5.4	96
hCG β	2.52	6.0	102
hCG β /cf	1.07	1.3	96

^a Mean hCG isoform peak area divided by mean IS peak area.^b Recovery is the mean peak area of the urine sample fortified before immunoextraction divided by the mean peak area of the urine sample fortified after immunoextraction, expressed as a percentage.