The expression of the urinary forms of human luteinizing hormone beta fragment in various populations as assessed by a specific immunoradiometric assay

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Human gonadotrophins undergo metabolic transformations which result in the presence of several smaller, structurally and immunologically related forms of gonadotrophins in the urine. For luteinizing hormone (LH), a beta core fragment (LHβcf) has been isolated from the pituitary and characterized. The corresponding urinary fragment is inferred from mass spectral and immunochemical analysis of chromatographically separated urinary forms. Physicochemical characteristics, primarily mass spectral and chromatographic, indicate that the pituitary and urinary forms of LHβcf have a different structure, probably in the carbohydrate moieties. This communication characterizes the expression of LHβcf in the urine of both reproductive and post-reproductive age women and in men, employing assays highly specific for the pituitary form of the fragment. It was found that LHβcf is the predominant LH associated molecular form in the urine during peri-ovulatory period, peaking 1–3 days later than intact LH and reaching a concentration of ~600 fmol/mg creatinine, 7-fold higher than either LH or LH free beta subunit. Corresponding concentrations of human chorionic gonadotrophin (HCG) βcf were <1% that of LHβcf. LHβcf cross-reaction with some LH or LHβ monoclonal antibodies may well interfere with the accurate estimation of the day of the LH surge when urinary tests are utilized.

Key words: gonadotrophins/LH/LH beta core fragment/urinary molecular fragments

Introduction

Metabolic processing of circulating gonadotrophins includes renal excretion, presumably preceded by some form of partial hormone degradation within the kidney as well as in other tissue compartments. A major form of urinary human chorionic gonadotrophin (HCG)-associated immunoreactivity is an epitope on a molecule smaller than heterodimeric HCG (Schroeder and Halter, 1983; O’Connor et al., 1994; Birken et al., 1996a).

This molecule has been identified as an HCG beta core fragment (HCGβcf) (Birken et al., 1988; Blithe et al., 1988). It has been shown that in normal pregnancy, the core fragment constitutes a major mole fraction of urinary HCG excretion (Kato and Braunstein, 1988). Accumulating evidence has suggested that a similar luteinizing hormone (LH) fragment appears in the urine. Iles et al. (1992) and Neven et al. (1993) demonstrated that, using polyclonal antisera raised against HCGβcf, immunoreactive beta core like activity could be detected in both post-menopausal women and in the periovulatory period of the normal menstrual cycle. Both of these investigative teams ascribed this immunoreactivity to an LHβcf, which their polyclonal HCGβcf antibodies were detecting as a consequence of cross-reaction. Recently Birken et al. (1993) described the isolation and structural determination of an LHβcf from human pituitaries. Employing this material as an immunogen, Kovalevskaya et al. (1995) developed a panel of monoclonal antibodies which were used to develop specific immunometric assays for this molecule.

There is a considerable literature documenting the difficulty in obtaining accurate measurements, or even detecting at all, circulating LH by immunoassay, although normal concentrations may be detected by bioassay in the same subjects (Pettersson and Soderholm, 1991; Pettersson et al., 1991; Martin-Du-Pan et al., 1994; Barbe et al., 1995). The causes of this phenomenon are thought to include genetic variants of the LH molecule, leading to loss of expression of an epitope, or to the well documented existence of multiple circulating isoforms of LH, which may have differential recognition by the monoclonal antibodies employed in the assay, as is the case with nicking of the HCG beta subunit, which results in nearly complete ablation of binding to many monoclonal antibodies specific for the intact HCG molecule.

An analogous situation can exist in urine, in cases in which antibodies that are used for LH detection in urine cross-react with free beta subunit and/or LHβcf. This cross-reactivity results in obscuring the real LH surge, even when urinary steroid measurements indicate that ovulation has occurred (Wilcox et al., 1987). Although LHβcf, a stable LH related molecule, is not capable of accurately segmenting the menstrual cycle because of its variable day of peak occurrence, nevertheless it appears capable of confirming that the mid-cycle surge of LH has in fact occurred.

This communication describes the behaviour of LHβcf, a molecule presumably derived from the LH free beta subunit, which we show to be present in both men and women, sometimes at high concentrations, and describes how it can confound the interpretation of LH measurements in urine.

In our first publication concerning pituitary LHβcf anti-
bodies, we reported a large peak of immunoreactivity in urine which followed the LH surge by 1–3 days (Kovalevskaya et al., 1995). We observed that monoclonal antibodies to pituitary LHβcf shared at least two epitopes with the analogous protein in urine. This immunologically similar urinary molecule has been called urinary LHβcf.

Although the urinary LHβcf has not yet been isolated, and appears to have a somewhat different structure to its pituitary counterpart, its essential identity with pituitary LHβcf is based on the observations that the urinary molecule shares at least two epitopes with the pituitary form: it has a similar size on gel chromatography and it appears in urine 1–3 days subsequent to the intact LH surge, suggesting that it originated from the intact LH molecule or its free beta subunit.

This is the first communication in which a specific immunometric assay is employed to report the levels of expression of this new molecular form of LH in men and women at different stages of their reproductive history. Since urinary hormone stability, particularly for LH and follicle stimulating hormone (FSH), has been reported to be a problem (Livesey et al., 1980, 1983, Saketos et al., 1994), we also report on our studies concerning the thermal and freeze/thaw cycle stability of urinary LHβcf.

Materials and methods

Hormones

LH (AFP-4261-A), LHβ (AFP-377A), anti-human LH-2 antiserum and anti-human LH beta-1 antiserum for radioimmunoassay were kindly provided by the National Hormone and Pituitary Program, NIDDKD. Standards used in the immunoradiometric assays were LH (AFP-8270B), LHβ (AFP-3282) (all from the same source). HCGβcf and LHβcf were prepared as described by Birken et al. (1988, 1993).

Iodination of LHβcf, LH, LHβ, and purification and iodination of monoclonal antibodies

Iodination and separation of monoclonal antibodies and hormones were performed as previously described (Kovalevskaya et al., 1995).

Liquid phase radioimmunoassay with 125I-LHβcf

The liquid phase radioimmunoassay procedure was conducted as follows: 0.1 ml serial dilutions of rabbit antiserum to LH or LHβ in phosphate buffered saline (PBS) containing 1% normal rabbit serum (Sigma, St Louis, MO, USA) and 0.1% sodium azide were added to 0.2 ml 125I-LHβcf (30 000 c.p.m.) in PBS with 0.1% bovine serum albumin (BSA) (Sigma). The mixture was incubated overnight at 4°C. After 0.2 ml sheep anti-rabbit serum was added and this solution was again incubated overnight at 4°C. The precipitate containing bound radioactive LHβcf was separated by centrifugation and 125I-content determined by gamma counting (Packard Cobra; Meriden, CT, USA).

Liquid phase radioimmunoassay for LH and LHβ

Liquid phase radioimmunoassays were conducted as recommended in National Hormone and Pituitary Program instructions. In brief, the binding buffer (buffer A) consisted of PBS supplemented with 0.1% BSA and 0.1% sodium azide. A 0.1 ml solution containing standards or unknown sample was added to 0.1 ml buffer A as well as 0.1 ml LH- or LHβ-antiserum (both antisera were prepared in rabbits) in PBS containing 1% normal rabbit serum. This solution was mixed

with 0.1 ml of radiolabelled LH or LHβ (30 000–40 000 c.p.m.) in buffer A and incubated overnight at 4°C. Then 0.2 ml of sheep anti-rabbit serum was added and the mixture was incubated overnight at 4°C. The precipitate containing bound radioactive LHβcf or LH was separated by centrifugation and counted in a gamma counter.

Antibody characteristics and assay construction

The development and validation of immunometric assays for intact LH, LH free beta subunit (Krichevsky et al., 1994), HCGβcf (Krichevsky et al., 1991) and LHβcf (Kovalevskaya et al., 1995) have been described previously.

Briefly, microtitre wells (Immulon II, Dynatech, Chantilly, VA, USA) were coated (200 µl/well) with the appropriate, pretitred solution of the capture antibody in sodium bicarbonate buffer (pH 9.5, 0.2 M) by overnight incubation at 4°C. The coating antibody solution was then aspirated, and after blocking the plates with 1% BSA in PBS (overnight at 4°C) the plates were washed five times with deionized water. Urine specimens, after pH adjustment to ~7.5 (1.0 M Tris HCl, pH 9. 50 µl/ml), or standards in PBS/0.1% sodium azide/0.1% bovine IgG buffer (buffer B) and urine controls were then applied to the wells (200 µl/well) and incubated overnight at 4°C. The wells were aspirated, washed five times with deionized water and the appropriate radiodinated detection antibody (tracer) was added to the wells (200 µl/well). After an additional overnight incubation at 4°C, the tracer was aspirated, the plates were washed with deionized water five times and the wells were separated and counted in a gamma counter (Packard Cobra). Values for the samples and controls were interpolated from a smoothed spline transformation of the standard curve.

LH was measured by A407 (capture)–B207 (tracer) (LH-1 assay) and B406–A201 (LH-2) (Krichevsky et al., 1994). LHβ was measured by the B408–B409 assay (Krichevsky et al., 1994), LHβcf was detected by the B505–B503 assay (Kovalevskaya et al., 1995) and HCGβcf by the B210–B108 assay (Krichevsky et al., 1991). The sensitivity and specificity of these assays are detailed in Table I. The sensitivities of assays (least detectable dose, LDD) were calculated as plus two standard deviations (SD) of the standard ‘zero’, i.e. non-specific bound tracer (NSB).

For LHβcf, HCGβcf, hLHβ, LH-1 and LH-2, intra-assay coefficients of variation were 9, 10, 15, 21 and 10% for LHβcf, HCGβcf, hLHβ, LH-1 and LH-2 respectively.

Sample collection

First morning void urine

These specimens were collected from 15 normally cycling women, ranging in age from 20 to 42 years. The specimens were stored in the subject’s home freezer for 3–7 days until delivered to the laboratory. They were stored at ~80°C for 1–3 months in the laboratory.

Table I. Assay specificity and sensitivity

<table>
<thead>
<tr>
<th>Antigen</th>
<th>LH-1</th>
<th>LH-2</th>
<th>LHβcf</th>
<th>hLHβcf</th>
<th>HCGβcf</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH, %</td>
<td>100</td>
<td>100</td>
<td>29</td>
<td>1</td>
<td>&lt;1</td>
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<tr>
<td>LHβ, %</td>
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<td>&lt;1</td>
<td>100</td>
<td>1</td>
<td>&lt;1</td>
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<td>LHβcf, %</td>
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<td>&lt;1</td>
<td>&lt;1</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>HCGβcf, %</td>
<td>2</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>100</td>
</tr>
<tr>
<td>HCG, %</td>
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<td>&lt;1</td>
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<tr>
<td>HCGβ, %</td>
<td>31</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>1</td>
<td>&lt;1</td>
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<tr>
<td>LDD* (fmol/ml)</td>
<td>1.5</td>
<td>1.4</td>
<td>0.6</td>
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</tr>
</tbody>
</table>

*LDD = least detectable dose.
before assay. No substantial changes in hormone profiles have been observed based upon duration of storage.

Large scale peri-ovulatory urine collection
Five subjects were provided with a home ovulation detection kit (‘First Response’, Carter Wallace, Inc.; New York, NY, USA). Starting with the first day of a positive LH test signal, 24 h urine collections were made for the succeeding 7 days.

Cycles without a detectable urinary intact LH signal
Four subjects were selected from a population of women who were recruited as normal controls for an investigation of hormone metabolism in premenstrual syndrome subjects. They were between the ages of 18–40 years, and were not pregnant or planning pregnancy. They had regular menstrual cycles and were not using any medication, drug or vitamin known to perturb the menstrual cycle.

Male urine
First morning void male urine was collected from 11 subjects between the ages of 18–60 years.

Post-menopausal urine, large volume collection
Post-menopausal urine was collected from one subject (age 66 years) by pooling urine for 40 daily samples. 500 ml of this pool was processed in the same manner as the peri-ovulatory urine pool.

Post-menopausal urine random collection
Post-menopausal urine was collected from 107 subjects enrolled in a study of baseline CA-125 concentrations in post-menopausal women (Westhoff et al., 1992). The women were recruited from patients at a general medical clinic or a screening mammography appointment and ranged in age from 43 to 74 years. No woman receiving treatment for any gynaecological condition was enrolled.

Matched blood and urine collection
Matched blood and urine were obtained at the same time from a single person on two occasions, starting with the first day of a positive LH test signal in urine according to ‘First Response’ kit and continuing for a total of 4 days.

All collection protocols were approved by the Columbia Presbyterian Medical Center Institutional Review Board.

Characterization of urinary LHβcf
Aliquots of the morning urine from ovulating women were assayed for LHβcf and collections of the sequential 24 h urine samples for days which tested positive were pooled, the pH adjusted to 7.5 using 1.0 M Tris–HCl and sodium azide (0.1%) now added. A 500 ml aliquot of this pool was filtered through a 0.45 µm membrane (Nalgene, Rochester, NY, USA) and concentrated in an Amicon Cell using a YM-3 membrane (Amicon, Danvers, MA, USA). Salts and lipids were removed from the concentrate on a Sephadex G-15 column (40×2.5 cm, Pharmacia, Piscataway, NJ, USA). The eluate was lyophilized and dissolved in 0.1 M ammonium bicarbonate buffer, and half was gel filtered on double tandem columns of Superose 12 (30×1 cm, Pharmacia). The entire amount was used in the case of post-menopausal urine. Those column fractions containing LHβcf immunoreactivity were pooled, lyophilized and then dissolved in 4 M guanidine HCl containing 0.1% trifluoroacetic acid (TFA) (pH 4). This solution (1.2 ml) was applied to a Vydac C-4 Column (22×4.6 cm). A binary linear gradient was run. Solution A was 0.1% TFA in water, Solution B was 0.1% TFA in acetonitrile. Flow rate was 1.0 ml/min and gradient 10 min 10% B to 70 min 40% B.

Pituitary LHβcf was chromatographed under the same conditions as the urinary concentrates.

Urinary steroid metabolite assays
The solid phase microtitre plate-based enzyme-linked immunosorbent assay (ELISA) for oestrone-3-glucuronide (E1·3-G) and pregnanediol-3-gluconide (Pd-3-G) were performed with monoclonal antibodies kindly provided by Carter Wallace, Inc. The enzyme-conjugated steroids were provided by Dr Bill Lasley, and the assays performed according to the procedure of Munro et al. (1991).

LHβcf stability
Mid-cycle urine, encompassing the LH urinary metabolite peaks, was collected from five subjects, and pooled. The pH was adjusted to 7.5 using 1.0 M Tris–HCl and sodium azide (0.1%) now added. Aliquots of the urinary mid-cycle peak (endogenous urinary LHβcf) and blank urine (B105 immuno-extracted to remove HCG- and LH-associated urinary metabolites from the urine and thus reduce the background) were stored at −80°C (control samples). Replicate samples (plus blank) were stored at 4, 22 and 37°C for extended time periods. After each time period, the samples were returned to the −80°C freezer. The freeze/thaw specimens were removed from the −80°C freezer from one to five times/day and thawed either at room temperature or in a water bath at ambient temperature. After the indicated number of freeze/thaw cycles the samples were returned to the −80°C freezer.

At the completion of the stability study, all of the specimens were analysed in the same assay, in order to avoid interassay variation.

The B105 immuno-extracted urine exhibited the same blank value as buffer B.

Statistical analysis
Data were analysed using the SigmaStat Program, version 1.01 (Jandel Corporation, San Rafael, CA, USA). One-way analysis of variance with Bonferroni adjustment was used to evaluate stability studies. P < 0.05 was considered significant.

Creatinine
Creatinine determinations were performed in a 96-well microtiter plate format by a procedure adapted from Taussky (1954).

Mass spectrometry
Mass spectrometry was performed on a Perceptive Biosystems Voyager DE RF instrument run in linear mode using a matrix of sinapinic acid or 2,5-dihydroxybenzoic acid (DHB).

Sialic acid and sulphate analysis
Sialic acid and sulphate analysis were performed using a Dionex PAD as described (Birken et al., 1996b).

Results
LHβcf and HCGβcf in peri-ovulatory urine
In the cohort of women studied (n = 15), a peak of LHβcf was observed to occur over a 3–4 day period, commencing on the day of LH surge (day 0) and reaching a maximum value of 560 (SE119) fmol/mg creatinine at 1–3 days post-ovulatory LH peak (Figure 1). A peak of LH free beta subunit (LHβ) was observed to occur simultaneously with that of the intact molecule. Although the concentrations of LHβ approximated those of the intact hormone, the concentrations of LHβcf were severalfold higher (Figure 1).

A surge of HCGβcf immunoreactivity peaked 2 days post-intact LH, generally coincident with the peak of LHβcf but at concentrations which were 1:100 of those for LHβcf. Since the cross-reaction of the HCGβcf immunoassay with the pituitary LHβcf was determined to be 1–2%, and since the true cross-reactivity with the urinary form is unknown, it may
Figure 1. Hormone profiles in the urine of normally cycling women \((n = 15)\). Concentrations are presented as mean ± SE, fmol/mg creatinine (fmol/mg C). LH concentration has been measured using two different IRMA \((n = 8\) for LH-2 assay). Steroid hormone ratio is calculated using oestrone-3-glucuronide \((E_1-3-G)\) and pregnandiol-3-glucuronide \((Pd-3-G)\) \(× 10^3\). Day 0 is the day of LH surge.

be that the total signal detected in the HCGβcf assay is in fact due to cross-reaction with LHβcf (Birken et al., 1996a).

The urinary LH surge was detected by A407-B207 (LH-1) antibody configuration. Additionally, eight of the 15 cycles were rerun in a different antibody configuration assay B406–A201 (LH-2). These assays were constructed using monoclonal antibodies to different LH epitopes (Table I). Both LH-1 and LH-2 assays gave the same day of LH surge, but the concentration of LH in the two assays differed significantly (paired t-test, \(P = 0.0005\)).

All cycles were characterized by irregular pulsations of LHβcf. The basal concentration of LHβcf in 10 patients during first 10 days of the follicular phase (100 samples) was 32 (SE 4) fmol/mg creatinine, with a wide range of concentrations, reflecting the peaks of LHβcf occurring before the peri-ovulatory surge of LHβcf (Figure 2).

**HLHβcf in subjects without a detectable rise in peri-ovulatory intact LH**

Examination of daily first morning urine samples from four women in which the LH peri-ovulatory surge was minimal or undetectable, as measured by either of our intact LH assays, indicated that ovulation occurred as judged by the inversion of the urinary oestrogen/progesterone ratio (Baird et al., 1991). Data from two of the four women are presented in Figure 3.

**HLHβcf expression in the urine of post-menopausal women**

The concentrations of intact LH, LHβ, LHβcf, and HCGβcf were evaluated in a total of 107 healthy post-menopausal women (Figure 4). The mean concentration of LHβcf for the 107 post-menopausal women was 236 (SE 35) fmol/mg creatinine.

**HLHβcf expression in the urine of males**

Urine samples collected from 11 normal males (age 20–60 years) yielded a value of 41 (SE 13) fmol/mg creatinine (Table II).

**Comparison of LH or LHβ measurement in urine using IRMA and radioimmunoassay**

HLH and LHβ were measured in urine using IRMA incorporating specific monoclonal antibodies (Figure 5A) and by radioimmunoassay (Figure 5B), using polyclonal antisera directed against either intact LH or LHβ, supplied by the National Hormone and Pituitary Program, NIDDKD. The radioimmunoassay reagents were designed for serum assays and usually clearly provide a single day pre-ovulatory elevation of both LH and occasionally LHβ in blood.

When these same reagents are employed for LH or LHβ measurement in urine however, a broad peak for either hormone
Figure 3. The urinary LH molecular forms profile in two subjects (patient 75-3, patient 67-5) who did not express measurable intact LH in either of our LH two assays (A). Note that both LH free beta subunit and LHβcf surges are clearly apparent. (B) Corresponding urinary steroid metabolite patterns for the cycles. It can be inferred from the steroid profiles that the subjects experienced normal ovulatory cycles, even in the absence of detectable intact LH. Concentrations were normalized to creatinine. Day 1 is the first day of menses.

was obtained. These observations can be explained by the presence of LHβcf in the urine (Figure 5A). If Figures 5A and B are compared, it is apparent that the day of maximum LHβ by IRMA is different from the radioimmunoassay value, probably due to the greater cross-reactivity of the LHβ polyclonal antiserum to LHβcf.

The cross-reactivities of the polyclonal antisera to LHβ and to intact LH with LHβcf were further evaluated in a radioimmunoassay using LHβcf labelled with 125I (Figure 6). Both polyclonal antisera clearly recognized LHβcf. The pituitary form of LHβcf was used in this experiment, but a similar reactivity pattern should also be observed with the urinary variant of this molecule, since the monoclonal antibodies developed to the pituitary material all appear to share epitopes present on the urinary molecule.

**HLHβcf in blood and urine**

A comparison of the concentration of LHβcf in blood and urine was undertaken by collecting paired samples beginning on the first day of the LH surge in urine (detected by ‘First Response’ kit) and continuing for 3 days in a single subject. The collection was repeated during a subsequent cycle. Figure 7 illustrates corresponding values in blood and urine for LH, LHβ, and LHβcf. The LH-1 assay provided a significantly stronger signal in serum than did the LH-2 assay. The LHβ signal appears synchronously with the intact peak in this subject in urine. However, the LHβcf surge commences a day later and is detected only in the urine.

**HPLC analysis of LHβcf of pituitary and urinary origins**

Analysis of urine fractions after gel filtration on the Superose 12 column using the B505-B503 assay indicated that all LHβcf activity appeared in one low molecular weight peak (10 000 Da). Upon reverse phase chromatography of the gel filtered LHβcf-containing pooled fractions on a Vydac C-4 column, the elution position of both peri-ovulatory and post-menopausal LHβcf immunoreactivity were identical, while that of the pituitary-derived material appeared 4 fractions later (Figure 8). This indicates that the structure of the pituitary-derived LHβcf is likely to be different from the material.
Table II. Concentration of LH\(\beta\)cf in urine

<table>
<thead>
<tr>
<th></th>
<th>Peri-ovulatory urine, basal level</th>
<th>Peri-ovulatory urine, surge</th>
<th>Post-menopausal urine</th>
<th>Male urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SEM fmol/mg C</td>
<td>32 ± 4</td>
<td>560 ± 119</td>
<td>236 ± 35</td>
<td>41 ± 13</td>
</tr>
<tr>
<td>Size</td>
<td>100*</td>
<td>15</td>
<td>107</td>
<td>11</td>
</tr>
</tbody>
</table>

*Days 1–10 from 10 women.
C = concentration of LH\(\beta\)cf normalized per mg creatinine.

present in the urine of both pre- and post-menopausal women and that the urinary form of the LH\(\beta\)cf is substantially the same in women of any age.

**Mass spectrometric analysis of the pituitary LH\(\beta\)cf**

The pituitary form of LH\(\beta\)cf was the immunogen for the antibodies used in these studies and was our reference standard. Understanding how its structure may differ from the urinary form being measured is important. The difference in reverse phase elution profile of the pituitary and urinary forms of the LH\(\beta\)cf led us to explore such differences in structure between the pituitary and urinary forms of this molecule. We have not yet isolated the urinary form, and therefore we concentrated on the pituitary isoform. We subjected the pituitary material to reduction and carboxymethylation (RCM) and separated the constituent polypeptide chains by reverse phase high performance liquid chromatography (HPLC) as described earlier for the HCG\(\beta\)cf (Birken et al., 1988). A combination of Edman amino terminal sequence analysis and mass spectrometry permitted unequivocal assignment of the constituent non-glycosylated peptide chains of the pituitary LH\(\beta\)cf: one RCM chain began at residue 55, as reported earlier (Birken et al., 1993), but was measured as 4546 Da by mass spectrometry indicating that it terminates at residue 93, CYS. This polypeptide has a theoretical mass of 4544.83 Da for the RCM form measured. The second non-glycosylated RCM peptide exhibited an amino terminus starting at residue 49 and a size of 5188 Da which corresponds again with a COOH-terminus of CYS 93 with a theoretical mass of 5190 Da. This is in sharp contrast to the structure of the HCG\(\beta\)cf which does not include CYS 93. The glycosylated peptide, previously determined to be residues 6–40, was not subjected to mass spectrometry because of its carbohydrate heterogeneity and difficulty in isolation of its RCM form. Mass spectrometry was also performed on the native pituitary form of the LH\(\beta\)cf as compared to the HCG\(\beta\)cf and both displayed similar sizes in the range of 9000–10 000 Da. Theoretical calculation of the mass of each fragment indicates that both should be ~10 000 Da only if the LH\(\beta\)cf single carbohydrate chain is essentially intact while the two carbohydrate groups of the HCG\(\beta\)cf are known to be trimmed down to the mannose cores (Blithe et al., 1988): LH\(\beta\)cf is composed of 6–40 (3927 Da) + 49–93 (4896 Da) or + 55–93 (4249 Da) + full carbohydrate group with sulphate (2093 Da). The theoretical sizes of the LH\(\beta\)cf are 10916 Da for the form with the longer non-glycosylated peptide and 10 269 Da for the form with the shorter peptide. The HCG\(\beta\)cf is theoretically 10 347 Da. The actual mass

![Figure 5](https://via.placeholder.com/150)

**Figure 5.** Urinary hormone profile of one patient which was obtained using monoclonal antibody based IRMA (A) and radioimmunoassay on the base of polyclonal antibodies (provided by NIDDKD) to LH and LH\(\beta\) (B). Concentrations were normalized to creatinine. Day 0 is the day of LH surge.

![Figure 6](https://via.placeholder.com/150)

**Figure 6.** Binding of LH and HLH\(\beta\) specific antisera (provided by NIDDKD) with LH\(\beta\)cf in radioimmunoassay format.
Figure 7. HLH and LHβcf in serum and urine of the same patient. The blood levels of intact LH (opened circles) and LHβcf (closed circles) are illustrated in the upper panel. Note that there is an insignificant amount of the LHβcf detected in the blood. The lower panel illustrates the urinary values for LH and LHβcf in the urine for the same days of collection. The surge of LH (day 0) and the surge of LHβcf (1–2 days later) are detected in urine, but the peak of LHβcf lags that of the intact LH by 2–3 days, suggesting that the origin of urinary LHβcf is a consequence of the peripheral or renal metabolic processing of intact LH.

Figure 8. Reverse phase chromatography of peri-ovulatory and post-menopausal urine fractions which contain LHβcf activity and pituitary LHβcf. All fractions were assayed in B505-B503 assay. The open circles denote the elution position of LHβcf derived from the pituitary. The closed circles and squares denote the elution positions of LHβcf partially purified from urine. The difference in elution position denotes a structural difference (probably carbohydrate differences) between the urinary and pituitary forms.

Stability of urinary LHβcf

The results obtained from the repeated freeze/thaw cycles indicated no statistically significant change from control for up to 40 freeze/thaw cycles (P = 0.214). There was no statistically significant change from control in immunodetectable LHβcf at either 4°C or room temperature for up to 29 days. At 37°C, the molecule was stable for 14 days but showed a statistically reliable change after 29 days (P < 0.05).

Discussion

In the present study, we have investigated 15 cycles of normally ovulating women. Data presented in Figure 1 show the position and concentration of urinary LHβcf as compared with the appearance of LH and LHβ in the urine. This observation further illustrates that the concentrations of hormone detected immunologically in urine reflect the differential conservation (or stability) of LH epitopes excreted into urine and confirms the cautionary observations of others that monoclonal antibodies may in fact be too specific to provide an accurate estimation of the level of all forms of LH in either blood or urine (Pettersson and Soderholm, 1991; Pettersson et al., 1991; Pettersson, 1992; Costagliola et al., 1994; Martin-Du-Pan et al., 1994; Barbe et al., 1995; Mitchell et al., 1995). Additionally, we measured HCGβcf, because the first evidence...
of LHβcf had been obtained using a polyclonal antibody with primary specificity to HCGβcf (Iles et al., 1992; Neven et al., 1993). Using monoclonal antibodies specific to HCGβcf, we found that there is very low peak of HCGβcf exactly coincident with LHβcf (Figure 1). This signal may be accounted for by the 1–2% LHβcf cross-reactivity in the assay for HCGβcf (Birken et al., 1996a). Antibodies to HCGβcf which were used by Neven et al. (1993) and Iles et al. (1992) cross-react with LHβcf to an extent which allowed these investigators to detect LHβcf. The lag time between the appearance of intact LH or LHβ and LHβcf suggests that, analogously with HCGβcf, LHβcf is a degradation product of the intact hormone or of its free beta subunit.

Irregular pulsations of urine LHβcf outside of the main periovulatory surge (Figure 2) probably reflect peaks of LH in blood, which are a consequence of the pulsatile release of gonadotrophin-releasing hormone (GnRH) (Knobil, 1988; Shoahm et al., 1995; Van Dieten and De Koning, 1995). Peaks of LH are also observed in urine, but at a much lower amplitude than those of LHβcf.

Problems associated with the detection of a discrete LH surge in urine have been reported by Edwards et al. (1980). They found an intact LH surge in 68/79 patients employing a haemagglutination assay and confirming in some cases with an LH radioimmunoassay. In 11 cases, a satisfactory LH surge was not obtained. These investigators also noted a diurnal variation in the timing of the LH surge, demonstrating that an inadequately timed collection protocol might be implicated in this difficulty. In the course of the present investigation, we also observed several cycles which did not produce a detectable intact LH signal even though urinary steroid profiles indicated that ovulation had occurred. Two representative cycles are presented in Figure 3. They illustrate the same pattern of LHβcf as seen in cycles with a measurable intact LH value. This surge commenced on the day following ovulation as judged by urinary steroid metabolites (Baird et al., 1991), and it peaked over the succeeding 1–3 days, indicating that a normal mid-cycle surge of intact LH can be confirmed by urinary LHβcf measurements. Our results suggest that an assay incorporating the detection of all three urinary substances analysed would provide the most sensitive detection of periovulatory LH. However, although LHβ is most often observed to peak coincident with the intact molecule (Figure 1), it appears that it can occasionally occur 1 day earlier (Figure 4 in Kovalevskaya et al., 1995). On the other hand, LHβcf usually peaked over 1–3 days later than the intact molecule (Figure 1) and this mid-cycle peak of LHβcf has been detected in all four cycles in which there was undetectable intact LH in the urine (Figure 3). The intact hormone may have been completely cleared by an alternative pathway. The alternative pathway would be clearance through the liver, which has receptors for asialglycoproteins and sulphated glycoproteins (Kawasaki and Ashwell, 1976; Steer and Ashwell, 1986; Weiss and Ashwell, 1989; Flete et al., 1991). The intact hormone may have dissociated completely into subunits or have been totally degraded into fragments prior to excretion, as is the case with HCG, i.e. administration of the intact HCG molecule to either men or non-pregnant women results in the appearance of HCGβcf in the urine (Nisula et al., 1989). Finally, the antibodies used in these measurements, which were raised to the pituitary form of LH, may have failed to recognize the urinary isoform of LH present in the sample.

Some further insight into this issue is provided by the comparison of hormone profiles in blood and urine for two cycles from the same patient (one cycle is presented in Figure 7). Although our lack of an adequate number of serum samples did not permit us to confirm the synchrony of serum and urine LH secretion as reported by Cano and Aliaga (1995), our data are nevertheless supportive of their observation that intact LH in blood and urine very nearly peak simultaneously.

The basal concentration (i.e. follicular phase concentration) of LHβcf in normally cycling women was similar to the concentration which we obtained for male urine (Table II). Both of these groups differ markedly from the values obtained for post-menopausal subjects which were characterized both by much higher concentrations and a wider range of values (Figure 4). Concentrations of intact LH were low in these subjects in both assays for LH, but there was a substantial quantity of LHβ, perhaps reflecting dissociation of the intact molecule. Only low values of HCGβcf were detected.

There was no significant LHβcf surge in blood but a substantial LHβcf surge in urine, supporting the hypothesis that urinary LHβcf is a product of LH metabolic processing. The lag time in the appearance of the fragment suggests that it may be a consequence of metabolic processing by the kidney or in some other compartment.

We chose to use two assays for intact LH measurements (LH-1 and LH-2) because, although the LH-2 assay was highly specific for the intact LH molecule, it occasionally produced a weak signal in urinary assays. The LH-1 assay, although less specific for LH (some cross-reactivity with HCG, Table I), had a tendency to detect signals of greater amplitude, with a better incidence of detection when applied to urine specimens. Incidentally, the LH-2 assay barely detected LH in the serum of this subject but detected the urinary form as well as the LH-1 assay, which performed equally well in both serum and urine. The above observations probably reflect metabolic processing of the LH which affects epitope presentation upon passage from blood to urine.

Another issue is the structure of the urinary and pituitary forms of LHβcf. Are they identical or does the urinary variant, presumably arising from the intact hormone, differ in structure? The ultimate answer avoids the isolation and complete sequence and carbohydrate analysis of the urinary form. Although both the pituitary and urinary forms appear to have the same molecular size by gel filtration, on reverse phase HPLC analysis the isoforms differ in their hydrophobicity, with the urinary fragment being more hydrophilic. In contrast, urinary forms of LHβcf molecules from either pre- or post-menopausal urine concentrates are closely related in structure, as evidenced by identical elution times from the Vydac C-4 column (Figure 8). Additional structural studies of the pituitary form using a combination of mass spectrometry and ion chromatography indicate that the pituitary LHβcf resembles its parent LHβ in its carbohydrate moiety. This contrasts to the structure of the HCGβcf which has carbohydrate moieties trimmed to their
that cross-reactivity can lead to an incorrect assignment of the day of ovulation. This problem would not be encountered with these assays as long as their use was restricted to measurements in blood, since there is little or no LHβcf present in this medium.

The data illustrate a potential risk associated with the use of ovulation test kits designed for personal use. Should the antibodies used in their construction detect hormone fragments in addition to the intact molecule the test results could be ambiguous and misleading.

Acknowledgements

The work was supported by NIH grants ES07589 from NIEHS, HD15454 from NICHD and M01RR00645 from NCRR. Reagents for radioimmunoassay of LH and hLHβ were kindly provided by the National Hormone and Pituitary Program, NIDDKD. We acknowledge the generous donation of enzyme conjugated steroids from Drs B.Lasley and G.Stabenfeldt (University of California, Davis) and postmenopausal random urine samples from Dr C.Westhoff (Columbia University, New York). We thank Albert Nazareth of Carter Wallace, Inc. for the gift of 'First Response' ovulation detection kit and monoclonal antibodies for the solid phase microtiter-plate-based ELISA for oestrone-3-glucuronide (E1-3-G) and pregnanediol-3-glucuronide (Pd-3-G). We acknowledge Mr R.Apap and Mrs R.Mizuho (Columbia University) for performing some of the immunoassays and Mr Y.Maydelman (Columbia University) for post-menopausal urine fractionation. Dr T.Kakuma (Cornell University) for helpful evaluation of this manuscript.

References


Beta core LH


Received on July 4, 1997; accepted on December 17, 1997