Evaluation of Simultaneous Measurement of Lutropin and Follitropin with the SimulTROPIN™ Radioimmunoassay Kit

Frank H. Wians, Jr., Jal Dev, Margaret M. Powell, and James I. Heald

We evaluated the analytical performance of the "SimulTROPIN" (Becton-Dickinson Inc.) radioimmunoassay for the simultaneous measurement of lutropin (LH) and follitropin (FSH) in human serum. Dose response, linearity, analytical recovery, sensitivity, and reagent stability were all acceptable. Cross reactivity with other structurally related hormones [choriogonadotropin (CG) and thyrotropin (TSH)] was minimal. A normal reference interval was established for non-midcycle, ovulatory women. We confirm the low degree of CG and TSH cross reactivity with LH and FSH reported by Becton Dickinson and conclude that this assay is an acceptable method for laboratory use in the simultaneous quantification of these analytes.

Additional Keyphrases: hormones • variation, source of precision • reference interval • ovulation

Lutropin [human luteinizing hormone (LH)] and follitropin [follicle stimulating hormone (FSH)] are glycoprotein hormones, each with a relative molecular mass of approximately 30 000. Each hormone consists of two polypeptide chains, referred to as the alpha and beta subunits. LH, FSH, choriogonadotropin (CG), and thyrotropin (TSH) share essentially the same alpha-subunit structure. The beta-subunit structure differs among these hormones and determines their characteristic biologic and immunologic specificity. LH and FSH also differ in the position and composition of their carbohydrate residues.

The beta subunits of CG and LH share significant amino acid sequence homologies; however, CG possesses a unique C-terminal sequence of 30 amino acids. Beta-subunit sequence homology between CG and the gonadotropins accounts for the cross reactivity of these hormones in all commonly used immunoassays. Thus, increased concentrations of CG may result in spuriously high values for LH and FSH determined by radioimmunoassays that rely upon the immunological specificity of beta-subunit antisera. In addition, some of the cross reactivity with both CG and TSH may also be attributed to common antigenic determinants among alpha subunits. Owing to technological advances in techniques of antibody production over the past decade, and the availability of highly purified LH/FSH preparations, it has become possible to reduce CG cross reactivity with LH from 100% to 10–15%.

Determination of LH and FSH in serum is useful in the clinical evaluation of infertility, amenorrhea, male hypogonadism, and ovulation induction (3, 4). Several radioimmunoassay kits are currently available for the separate determination of LH and FSH in serum. Recently, kits with which LH and FSH can be simultaneously quantified have been introduced. These dual assays exploit the difference in scintillation energies produced by 57Co- and 125I-labeled tracers and the ability of gamma-counters to discriminate between radioactivity (counts) due only to 57Co or 125I in tubes containing both ligands.

The technical advantages of simultaneously measuring these analytes in a single assay are apparent. We have examined the performance of the "SimulTROPIN" kit for the simultaneous measurement of LH/FSH and have established the normal reference interval with it for non-midcycle, ovulatory women, ages 17 to 26 years.

Materials and Methods

Kits. We purchased luteinizing hormone (57Co)/follicle stimulating hormone (125I) SimulTROPIN radioimmunoassay kits and used three different lot numbers of kits for this study between November 1984 and February 1985. LH (0 to 160 int. units/L) and FSH (0 to 80 int. units/L) standards were calibrated against the WHO 1st International Reference Preparation (IRP) (LH; 68/40) and the WHO 2nd IRP (FSH; 71/333). Free ligand is separated from bound by use of goat anti-rabbit antiserum and a precipitation accelerator.

Samples and controls. Two patients' serum specimens containing an assayed amount of CG (754 and 6475 int. units/L) and TSH (64 and 300 milli-int. units/L) were analyzed in addition to CG standards (0 to 400 int. units/L; Hybritech Inc., San Diego, CA). CG and TSH in patients' sera were determined with the "Tandem-R CG" (Hybritech) and "B-D TSH" (Becton Dickinson) assays, respectively. Purified LH and FSH preparations were obtained from Cambridge Diagnostics, Boston, MA. Controls ("Level I, II, III"), each containing LH and FSH, were obtained from Environmental Chemical Specialties (ECS), Anaheim, CA.

Evaluation procedures. All assays were done in duplicate, according to the manufacturer's instructions, with freshly reconstituted tracer and up to 56 tubes in each run. We measured bound ligand by counting gamma-radiation and analyzed the results with automatic data reduction (Micromedic 4/600 with MACC; Micromedic Inc., Horsham, PA), using the logit-log transformation.

Results

Calibration curve. Standard curves for LH and FSH were obtained to assess dose–response parameters and other indicators of assay performance (Table 1).

Cross reactivity. Using the calibration curves for LH and FSH, we were able to determine the cross reactivities of CG, TSH, and LHJFSH. The mean values were 2% for CG, 14% for TSH, and 18% for LHJFSH. These values are in agreement with the values reported by Becton Dickinson for LHJFSH in the SimulTROPIN kit.

Received September 16, 1985; accepted February 21, 1986.

---

1 The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Air Force or the Department of Defense.

2 Nonstandard abbreviations: LH, lutropin; FSH, follitropin; CG, choriogonadotropin; TSH, thyrotropin; IRP, International Reference Preparation.

---

CLINICAL CHEMISTRY, Vol. 32, No. 5, 1986
FSH, we determined the percentage of cross reactivity of CG, TSH, LH, and FSH (Table 2). The percent cross reactivity of CG with LH was determined at ID$_{50}$ according to the formula:

\[
\text{cross reactivity, } \% = \frac{[A - B]}{C} \times 100
\]

where A = LH standard concentration at 50% B/B$_{0}$, calculated from least-squares analysis of the LH standard curve, B = LH concentration of matrix blank (zero CG standard), and C = CG concentration at 50% B$_{0}$, calculated from least-squares analysis of the LG curve obtained in the presence of increasing amounts of CG.

Cross reactivity of TSH and FSH with LH and of CG, TSH, and FSH with LH were similarly determined with use of this equation. TSH cross reactivity was evaluated by using serum from two male patients (TSH content 64 and 300 mili-int. units/L).

**Linearity and analytical recovery.** Linearity was evaluated by a parallelism study in which serum from a female donor, containing 160 int. units of LH and 80 int. units of FSH per liter, was serially diluted two-, four-, and 20-fold with zero standard; 10, 50, and 100 μL, respectively, of diluted sample was assayed for LH and FSH. Agreement between results for diluted samples vs expected values ranged from 81 to 104% for LH and 90 to 106% for FSH (Table 3). Analytical recovery was evaluated by measuring LH and FSH in three aliquots of a pooled serum from men that previously had been assayed for endogenous LH and FSH concentration, to which known concentrations of LH and FSH were added (Table 3). Recovery ranged from 94 to 108% for LH and 86 to 125% for FSH.

**Sensitivity.** Analytical sensitivity, defined as the detection limit at B$_{0}$ = 2 SD, was determined in a single assay with 20 replicates of zero standard. LH and FSH concentration at B$_{0}$ = 2 SD binding was obtained from the logit-log plot of the appropriate calibration curve. The detection limits for LH and FSH were 3.5 and 1.2 int. units/L, respectively.

**Intra-assay and interassay variation.** We evaluated precision by analysis of reproducibility, and sample results within a single assay for LH and FSH concentrations, including those occurring at clinical decision levels. Intra-assay variation ranged from 4.3 to 12.2% for LH and 5.2 to 10.8% for FSH, and the respective interassay variations from 6.9 to 12.3% and 6.8 to 8.3% (Table 4). In addition, interassay percent binding (B$_{0}$) varied by <3%.

**Reagent stability.** We established this by analyzing a single lot, tri-concentration controls (ECS I, II, and III) during five weeks. Control sera were tested once each week by using tracer with the same lot number. All values were within the 2 SD limits established in our laboratory.

**Normal reference interval.** We determined this by assaying with kits with the same lot number 119 serum specimens collected from women not at midcycle. The donors, screened for use of oral contraceptives and date of last menstrual period, ranged in age from 17 to 26 years and represented a diverse population from various geographic areas of the United States. We analyzed data by non-parametric statistical methods (5) and selected the 95% confidence limits as the normal reference interval. Histogram plots of these data are shown in Figure 1. The range of LH values was 0.5 to 19.6 int. units/L (mean, 4.6 int. units/L). The range of FSH values was 0.4 to 7.3 int. units/L (mean, 2.6 int. units/L). LH values were skewed toward concentrations <5 int. units/L. We established a non-parametric reference interval of 0–14 int. units/L according to percentile (2.5 to 97.5) estimation (5). For FSH a non-parametric reference interval was determined as 0–5.5 int. units/L.

**Discussion**

Our principal objective here was to examine the performance of this commercially available kit. Owing to the similarity in primary structure of CG with FSH, and especially LH, no LH or FSH antiserum completely eliminates the problem of cross reactivity with CG and TSH.

CG cross reacted with LH (15.4%) more than with FSH (2.9%); in both cases, these were close to the manufacturer's claim. Cross reactivity with CG is usually of limited clinical importance; however, it is not uncommon in our laboratory to receive specimens containing substantial CG concentration, which would falsely increase the LH/FSH value obtained with the SimulTROPIN assay. Education of the clinical staff concerning CG cross reactivity with LH/FSH can minimize this problem. Cross reactivity of TSH with LH and with FSH was low (<0.1% (LH) and 1.4% (FSH)) and would not be expected to compromise the clinical utility of LH/FSH measurement in the presence of high concentrations of TSH. Note, however, that small amounts of TSH (milli-int. units/L) are compared with much greater concentrations (int. units/L) of LH and FSH. Interestingly, we observed greater TSH cross reactivity with FSH than with LH, whereas the opposite effect is stated in the manufacturer's procedure insert. This discrepancy may be due to differences in the TSH preparation (i.e., purified standard vs patients' serum matrix) used to study cross reactivity with LH/FSH.
Table 3. Linearity and Analytical Recovery

<table>
<thead>
<tr>
<th></th>
<th>LH, int. units/L</th>
<th>FSH, int. units/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Found]</td>
<td>[Expected]</td>
</tr>
<tr>
<td>Linearity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum 1, Undiluted</td>
<td>160</td>
<td>—</td>
</tr>
<tr>
<td>Diluted 1:2</td>
<td>83.6</td>
<td>80</td>
</tr>
<tr>
<td>Diluted 1:4</td>
<td>38.8</td>
<td>40</td>
</tr>
<tr>
<td>Diluted 1:20</td>
<td>6.5</td>
<td>8</td>
</tr>
<tr>
<td>Recovery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pool 1 (0/0)</td>
<td>—</td>
<td>13.5</td>
</tr>
<tr>
<td>2 (8/4)</td>
<td>22.1</td>
<td>—</td>
</tr>
<tr>
<td>3 (40/20)</td>
<td>51.1</td>
<td>—</td>
</tr>
<tr>
<td>4 (80/40)</td>
<td>87.5</td>
<td>—</td>
</tr>
</tbody>
</table>

* [F]/[E] = [Found]/[Expected]*

Reference:

References

Table 4. Data on Precision

<table>
<thead>
<tr>
<th></th>
<th>[LH], int. units/L</th>
<th>[FSH], int. units/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Intra-assay (n = 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>15.8</td>
<td>1.9</td>
</tr>
<tr>
<td>B</td>
<td>39.0</td>
<td>1.9</td>
</tr>
<tr>
<td>C</td>
<td>72.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Inter-assay (n = 12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>8.4</td>
<td>1.0</td>
</tr>
<tr>
<td>E</td>
<td>31.0</td>
<td>2.5</td>
</tr>
<tr>
<td>F</td>
<td>52.3</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Both linearity and analytical recovery approached 100%, with deviations from 100% for quantifying low concentrations of LH or FSH. Sensitivity and reagent stability were excellent. Precision was acceptable, the maximum CV, 12%, being observed in the measurement of the lowest concentration (15.8 int. units/L) of LH tested. Others (6) have reported less precision of the SimulTROPIN assay for LH than for FSH, but we did not observe this in our study, perhaps because we used a larger sample size (n = 12) than that (n = 6) in the study by Beinlich et al. (6). In our case, our findings compare favorably with other dual-label assay kits for these analytes (6).

Reference interval data for non-midcycle, ovulatory women from our study (n = 119) were slightly different from the data reported by Becton Dickinson (n = 39), perhaps because we tested a larger sample population. These small differences should not be clinically significant.

Overall, the SimulTROPIN kit demonstrated low cross-reactivity of CG and TSH with LH and FSH and performed efficiently in terms of dose-response, linearity, analytical recovery, sensitivity, precision, and stability. We conclude that this kit is suitable for laboratory use to support the diagnosis of primary vs secondary amenorrhea and other ovarian dysfunctions associated with alterations in serum concentrations of LH and FSH.

Note: After this study was completed, Becton Dickinson changed the stated cross-reactivity of CG with LH printed in their procedure insert. The initial value of 10.5% was changed to 15.1%, a value more consistent with the result obtained in our study (15.4%). In addition, they revised their normal reference intervals, changing the values for non-midcycle, ovulatory women from 0–10 to 0–30 int. units/L for LH and from 0.7–7 to 0–8 int. units/L for FSH.
We describe a rapid, simplified isocratic "high-performance" liquid-chromatographic method for simultaneous measurement of the antiarrhythmic drug amiodarone and its major metabolite, desethylamiodarone, in small volumes of sera (100 μL). Compared with liquid-liquid extraction, the solid-phase method of extraction saves time and glassware and improves reproducibility for small sample volumes. Amiodarone and desethylamiodarone could be measured at concentrations as low as 250 μg/L. Standard curves for the drug and metabolite are linear over the range of concentrations found in our patients. Within-run CVs (n = 6) ranged from 2.7% to 4.5% for amiodarone and from 4.0% to 5.7% for desethylamiodarone over the range of 250 to 4000 μg/L. Between-run CVs (n = 12) were 8.3% and 5.7% for amiodarone and desethylamiodarone, respectively. Commonly used cardiovascular medications do not interfere with the assay.

Additional Keyphrases: chromatography, reversed-phase antiarrhythmic drugs

Amiodarone, a relatively new antiarrhythmic agent being considered for release in North America, offers three important advantages over most currently available antiarrhythmics: it has a broad spectrum of activity against both atrial and ventricular arrhythmias; it has a long duration of action, permitting once-daily dosing; and it does not appear to depress left ventricular function (7). Although a broad clinical experience with the drug in Europe has established its efficacy (2-4), there is some concern about its toxicity (5-7). One study has shown a relationship between efficacy and serum concentrations of amiodarone greater than 1 mg/L, with toxicity occurring more frequently at concentrations exceeding 2.5 mg/L (8). A better understanding of these relationships would undoubtedly make the use of amiodarone more effective and as a consequence decrease the incidence and severity of toxicity. From our experience the major barrier preventing the collection of such data is the lack of a resource analytical procedure that is accessible to the clinician.

Several liquid-chromatographic assays of amiodarone and desethylamiodarone have been reported. In most, cumbersome solvent extraction techniques are used (9-13), but some involve crude protein-precipitation methods (14-17). Our need to measure amiodarone and desethylamiodarone in small capillary-blood samples from laboratory animals, and the interest shown in therapeutic monitoring of the drug at this medical center, prompted us to develop a liquid-chromatographic method for 100 μL of serum extracted by a convenient solid-phase technique.

Materials and Methods

We developed the assay on a manual chromatographic system consisting of a Model 110A pump (Beckman Instruments Inc., Toronto, Canada M5Z 2G6), a 15 × 0.32 cm stainless-steel column packed (in our laboratory) with Spherisorb-Octyl, a 5-μm particle size reversed-phase packing (PhaseSep, Hauppauge, NY 11787); an Altex fixed-loop injector (Beckman); and a BAS LC6 fixed-wavelength (254 nm) detector (Bioanalytical Systems Inc., W. Lafayette, IN 47906). Peaks of interest were recorded and quantified with an HP 3390a recording integrator (Hewlett-Packard, Mississauga, Canada L4V 1M8). To facilitate automated sample analysis, we later transferred the chromatographic step to a Hewlett-Packard 1081B system with automatic injection.

The solid-phase extraction columns employed were cyanobonded silica (CN) minicolumns of 1-mL capacity (J.T. Baker Chemical Co., Phillipsburg, NJ 08865).

"HPLC"-grade acetonitrile, methanol, and triethylamine (TEA) were obtained from local suppliers. "HPLC"-grade water was prepared by passing distilled water through a Milli-Q system (Millipore Waters Ltd., Mississauga, Canada L4V 1M5). The mobile phase and reconstitution solvent were buffered with an aqueous sodium dihydrogen phosphate solution (10 mmol/L, pH 3.5).

The internal standard used is L8040 [2-ethyl-3-(3,5-di-bromo-4-dimethylaminopropoxybenzyl)benzothiophene]. The primary (100 mg/L) stock solutions of amiodarone, desethylamiodarone, and internal standard were prepared in methanol and kept refrigerated. All drugs were obtained from Sanofi Recherche, Paris, France. Serum standards containing both amiodarone and desethylamiodarone were prepared by serial dilution of the stock solution over the concentration range of 8000 to 250 μg/L. The working internal standard solution was diluted to 2 mg/L in methanol/water (20/80 by vol).

Chromatographic conditions. The mobile phase consisted...