Overnight Radioimmunoassay of Human Follicle-Stimulating Hormone

Loretta P. Brown, Paige K. Besch, Dean S. Kelley, and Veasy C. Buttram, Jr.

A radioimmunoassay procedure for follicle-stimulating hormone in serum has been developed in which the assay mixture is incubated at 37°C for a total of 22 h, rather than at 4°C for 4–7 days. The within-assay coefficients of variation for the 37°C and 4°C methods, respectively, are 7.7% and 5.0%, while the between-assay variations are 13.8% and 9.9%. Comparison of values for 4°C (x) were related to values for 37°C (y) according to the equation y = 1.2x + 0.8, with a correlation coefficient of 0.98.

Additional Keyphrases: normal range • double-antibody system • diagnostic aid

FSH¹ is most often measured by an RIA procedure in which a double-antibody system is used that requires four to seven days to complete (1–3). If assays are run once a week, it can be at least two weeks before the physician receives the results. In addition, the requisite time intervals at which various components are added to the typical assay may make it difficult to adapt the procedure to the usual work week. We describe an overnight RIA for FSH, which is easily adapted to use in the clinical laboratory.

Materials

Biochemicals

FSH, anti-FSH, and standard. FSH (LER-1366), anti-FSH (Batch No. 3) and standard (LER-907) were obtained from the National Pituitary Agency at NIAMD, NIH, Bethesda, Md. 20014. Both LER-1366 and LER-907 are pituitary extracts. Anti-FSH, prepared in rabbits, was pre-absorbed with HCG.² One milligram of LER-907 contains 20 international units (int. units) of FSH and 48 int. units of luteinizing hormone (bioassay: 2nd IRP HMG).

¹Nonstandard abbreviations used: RIA, radioimmunoassay; FSH, follicle-stimulating hormone; HCG, human chorionic gonadotrophin; ARGG, anti-rabbit gamma globulin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; and AB, antibody.

²This HCG was obtained from Ayerst Laboratories, Inc., New York, N. Y. 10017, through the courtesy of Dr. John B. Jewell, and was used in conjunction with the Calbiochem kit.

Received Oct. 30, 1972; accepted Nov. 22, 1972.

Second antibody (anti-rabbit gamma globulin, ARGG). ARGG, obtained from Antibodies, Inc., Davis, Calif. 95616, was prepared in goats against rabbit gamma globulin.

¹³I. Radioactive iodine for iodination was obtained from International Chemical and Nuclear Corp., Irvine, Calif. 92664.

Solutions

PBS. Phosphate-buffered saline consists of sodium phosphate buffer (10 mmol/liter, pH 7.0) incorporating, per liter, 0.14 mol of sodium chloride and 0.1 g of thimerosal ("Merthiolate") as preservative.

BSA. The various dilutions of bovine serum albumin were prepared in PBS.

EDTA. EDTA, 50 mmol/liter, was dissolved in PBS at pH 7.0, with 2.5 ml of normal rabbit serum added per liter.

Chloramine-T (N-chloro-p-toluene sulfonamide) sodium. The chloramine-T solution (10 mg/liter) was made in sodium phosphate buffer (50 mmol/liter, pH 7.5) with 0.1 g of thimerosal per liter as preservative.

Metabisulfite solution. This was sodium metabisulfite, 24 g/liter, in sodium phosphate buffer (50 mmol/liter, pH 7.5) with 0.1 g of thimerosal added per liter.

KI. The potassium iodide solution was made to a concentration of 10 g/liter, in sodium phosphate buffer (50 mmol/liter, pH 7.5) with 0.1 g of thimerosal and 160 g of sucrose per liter.

Dilutions

LER-907. The standard was diluted with BSA solution (1 g/100 ml) to give 100 units of FSH per liter. This was then serially diluted with the BSA solution (1 g/100 ml) to give 100 units of FSH per one (i.e., 100, 50, 25, etc. units/liter).

Anti-FSH. The first antibody (anti-FSH) was diluted in EDTA reagent to a working dilution of 6000-fold. The final dilution in the assay tubes was 30,000-fold.

¹³I-FSH. Labeled FSH was diluted with BSA solution (5 g/liter) so that 0.1 ml gave about 20,000 counts per minute (cpm).

Second antibody. ARGG was diluted in PBS to
to give a working dilution of 50-fold and a final dilution of 250-fold in the assay tube.

Methods

Iodination

FSH was iodinated with $^{131}$I according to a modification of Greenwood et al. (4). FSH, 3 μg, was reacted with 1 mCi of $^{131}$I for 1–2 min in 25 μl of sodium phosphate buffer (0.5 mol/liter, pH 7.5) to which 10 μl of chloramine-T reagent had been added. The reaction was stopped by adding 50 μl of sodium metabisulfite reagent. KI reagent, 100 μl, was then added as carrier.

The iodination mixture was chromatographed on Biogel P-60, 100–200 mesh, 0.8 × 21 cm column (a disposable 10-ml pipet). Before use, the Biogel column had been coated with 1.5 ml of a 50 g/liter BSA solution and then rinsed with sodium phosphate buffer (50 mmol/liter pH 7.5, incorporating 0.1 g of thimerosal per liter). This buffer was also used as the eluent. One-milliliter fractions were collected into tubes containing 1.0 ml of a BSA solution (50 g/liter).

Two radioactive peaks were observed, the first being the labeled FSH and the second the free iodide. The tube with the highest number of counts per minute in the first peak was saved for dilution for the RIA. It was quick-frozen in a mixture of solid carbon dioxide and ethanol and stored at −20°C until needed, when it was thawed under warm running water and an aliquot removed for dilution and use in the assay, the remainder being immediately quick-frozen and again stored at −20°C. With this procedure, iodination was required only at monthly intervals.

It is not advisable to use the labeled FSH from a particular iodination if the counts in the FSH peak are less than 50% of the total counts from all the fractions. The amount of labeled FSH per tube that would be required to give a count rate sufficiently high for statistical accuracy might overload the assay system. This could result in a loss of sensitivity, as well as a decrease in the percentage of labeled hormone bound to the antibody in the absence of unlabeled hormone ($B_0$).

Assay

A sample protocol for the FSH assay is presented in Table 1. The actual mechanics of the assay can be summarized as follows:

1. Add BSA (10 g/liter), EDTA reagent, standards or unknowns, and first antibody to appropriate tubes.
2. Vortex-mix for 1–2 s.
3. Incubate at 37°C for 2 h.
4. Add labeled antigen ($^{131}$I-FSH) to all tubes.
5. Mix as in step 2.
6. Incubate at 37°C for 2 h.
7. Add the second antibody (ARGG).
8. Mix as in step 2.
9. Incubate at 37°C for 18 h.
10. Centrifuge all tubes except numbers 1 and 2 (total count) at 0–4°C for 30 min at 1000 × g.

11. Decant the supernatant fluid from all centrifuged tubes and allow them to drain inverted over absorbent paper for at least 15 min.

12. Count all tubes in a well-type gamma counter—generally 1 min per tube.

Calculations

In Table 1, tubes number 1 and 2 represent the total counts added to each tube. Tubes No. 3 and 4 represent the nonspecific binding of the labeled FSH to anything in the assay mixture other than the first antibody, and this value must be subtracted from all subsequent counts. Tubes No. 5 and 6 represent the

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>BSA</th>
<th>EDTA</th>
<th>Conc of standard int. units/liter</th>
<th>Vol of standard</th>
<th>1st AB</th>
<th>Label</th>
<th>2nd AB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–2</td>
<td>0.9</td>
<td></td>
<td>...</td>
<td>...</td>
<td>0.1</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>3–4</td>
<td>0.5</td>
<td>0.2</td>
<td>...</td>
<td>...</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>5–6</td>
<td>0.5</td>
<td></td>
<td>...</td>
<td>0.78</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>7–8</td>
<td>0.25</td>
<td></td>
<td>...</td>
<td>1.56</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>9–10</td>
<td>0.25</td>
<td></td>
<td>...</td>
<td>3.13</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>11–12</td>
<td>0.25</td>
<td></td>
<td>...</td>
<td>6.25</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>13–14</td>
<td>0.25</td>
<td></td>
<td>...</td>
<td>12.50</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>15–16</td>
<td>0.25</td>
<td></td>
<td>...</td>
<td>25.00</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>17–18</td>
<td>0.25</td>
<td></td>
<td>...</td>
<td>50.00</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>19–20</td>
<td>0.25</td>
<td></td>
<td>...</td>
<td>100.00</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>21–22</td>
<td>0.25</td>
<td></td>
<td>...</td>
<td>...</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>23–24</td>
<td>0.25</td>
<td></td>
<td>...</td>
<td>...</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>25–26</td>
<td>0.25</td>
<td></td>
<td>...</td>
<td>...</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>etc.</td>
<td>etc.</td>
<td></td>
<td>...</td>
<td>...</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[Sample]</td>
<td>[Unknown A]</td>
<td>0.2</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[Serum]</td>
<td>[Unknown B]</td>
<td>0.2</td>
<td>0.1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*a* May be varied, but volume of BSA + volume of standard or unknown must = 0.5 ml.

*b* May be varied. Linear to 0.3 ml.

---

Table 1. Sample Protocol for Radioimmunoassay of Follicle-Stimulating Hormone

---

198 CLINICAL CHEMISTRY, Vol. 19, No. 2, 1973
amount of labeled antigen bound to antibody in the absence of unlabeled antigen, and, of all the precipitated tubes, these will contain the highest number of counts. This zero standard is designated $B_o$ (amount bound in the absence of unlabeled antigen), and is given the value of 100%. All subsequent tubes are expressed as $B$ and as a percent of $B_o$. For the quantitation of unknowns, percent $B/B_o$ is plotted vs. the logarithm (log) of milli-int. units of FSH. As a quality-control check on the system, logit $B/B_o$ is plotted vs. log milli-int. units of FSH, easily performed with the use of logit transformation paper (Codex Book Co., Inc., Norwood, Mass. 02062).

**Results**

**Analytical**

*Standard curve.* Figure 1 shows a plot of percent bound vs. the log of the concentration of the LER-907 standard expressed in milli-int. units (same as used for the quantification of unknowns). The sensitivity for FSH at 37°C is about 0.5 milli-int. unit, somewhat less than the 0.2 milli-int. unit sensitivity available at 4°C.

*Immunologic identity.* For comparison, standard curves for the assay at 37°C and 4°C are plotted on a logit vs. log concentration for LER-907 in Figure 2. Linearity and parallelism are good over the entire range of FSH concentrations. Figure 2 also shows the log of the amount of serum in the 37°C assay vs. logit $B/B_o$. This curve also parallels the standard curves.

*Binding.* Nonspecific binding amounts to 1–2% of the total count. $B_o$ usually binds 25 to 40% of the total count.

*Error.* The within-assay error for the 37°C method had a coefficient of variation of 7.7% ($n = 9$), and that for between-assay error was 13.8% ($n = 13$), as compared to 5.0% within-assay and 9.9% between-assay for the 4°C assay.

*Conversion.* When 32 paired samples from the 37°C (y) and 4°C (x) assays were compared statistically, they showed a $y = 1.2x + 0.8$ relationship, with a correlation coefficient of 0.98.

**Clinical Application**

Serum concentrations of FSH in some clinical conditions are shown in Figure 3.

FSH concentrations were measured in one individual with the testicular feminization syndrome. This patient had the classic karyotype for this syndrome, 46XY, with phenotypic female development. Samples were collected before surgery (on three different days of the same week) and again one year after removal of the testis (again on three different days of the same week).

FSH concentrations (on two different days of the same week) in a typical perimenopausal woman were determined before she received estrogen therapy and again after she received estrogen (“Premarin”: 1.25 mg per day) daily for three weeks.

![Fig. 1. Standard curve for FSH at 37°C](image1.png)

![Fig. 2. A comparison of the logit transformation curves for FSH standards at 37°C and 4°C, and serum dilutions at 37°C](image2.png)

FSH in Turner's and Sheehan's syndrome was measured on one patient each who exhibited classic clinical symptoms of their particular syndrome.

**Discussion**

Although the sensitivity of 0.5 milli-int. unit at 37°C is somewhat less than that observed at 4°C, the method is sufficiently sensitive to be useful for FSH determinations in patients with hypopituitarism and other conditions resulting in low FSH concentrations.

The parallelism between standards at 37°C and 4°C indicates that, immunologically, the two systems are behaving identically. Furthermore, the parallelism between the serum FSH values and the standard curve at 37°C indicates identical behavior between standard and unknown. Because the concentration in serum is linear over a wide range (50–300 μl), the assay is easily adaptable to the study of all types of patients, whether their FSH values be high or low. Sera with suspected high concentrations may be
measured by use of only 50 μl of serum, to avoid wasting standard by producing values beyond the standard curve. Conversely, sera with suspected low concentrations may be measured by use of as much as 300 μl of serum, to avoid having their values fall below the measurement range.

Although the second antibody in this report was used at a 50-fold dilution, each new batch of ARGG should be titrated before use, because the best working dilution may vary greatly (it may be 25- to 100-fold) from batch to batch.

Crossreactivity of the anti-FSH with HCG or luteinizing hormone is of no concern, because it was preabsorbed with HCG. However, if an anti-FSH that has not been preabsorbed with HCG is used, HCG must be added to the assay system; 2.0 int. unit per tube is usually sufficient.

The assay’s applicability to clinical situations has been indicated in Figure 3. We find that normal FSH concentrations in a cyclic, menstruating woman, range from a low of 5.0 int. units/liter in the follicular and luteal phases to a high of 30.0 int. units/liter in the ovulatory phase. FSH concentrations in the patient with testicular feminization syndrome, after removal of the testis that had produced enough steroids to suppress FSH concentrations, appeared very much like those of a menopausal patient by a year after surgery. A typical perimenopausal patient had elevated FSH concentrations, which were readily decreased with the aid of estrogen.

Although the assays reported here were performed with use of materials from the National Pituitary Agency, the 37°C assay was also run with FSH antigen and FSH antibody obtained from Calbiochem, San Diego, Calif. 92112, with similar results. Thus, this assay method is available for purely service-oriented measurements by the clinical laboratory.

This work was supported in part by the Roderick Duncan McDonald Fund, St. Luke’s Episcopal Hospital, Houston, Tex. 77025. We thank Dr. L. Russell Malinak for making two of his patients available for study.

References