A SENSITIVE IN VITRO BIOASSAY METHOD FOR LUTEINIZING HORMONE (LH) ACTIVITY

By

M.-P. Van Damme, D. M. Robertson, P. Romani and E. Diczfalusy

ABSTRACT

A specific and sensitive in vitro bioassay method is described for the measurement of LH activity of HCG, HMG and human hypophyseal gonadotrophin (HHG) preparations. The method is based on the principle that a linear relationship exists between graded doses of HCG, HMG or HHG preparations and the amount of testosterone released into the incubation medium by decapsulated mouse testes. The sensitivity of the method is 0.4 mIU/ml incubation medium for HCG and 2 mIU/ml for HMG. The useful range of the method is 0.4–12.8 mIU/ml for HCG and 2–32 mIU/ml for HMG preparations. A 3 × 3 point design with 6 individual observations per dose is used. Under these experimental conditions the mean index of precision (λ̂) in 44 assays was 0.22.

All HCG, HMG and HHG preparations yielded parallel dose-effect lines. In the concentrations studied, the assay was not influenced by the presence of FSH, ACTH, STH, LTH, vasopressin, oxytocin and LH releasing hormone activities. The high LH contamination of the highly purified TSH preparations studied precluded an adequate assessment of a possible synergistic or antagonistic effect of TSH on the assay.


1) Ford Foundation Fellow in Reproductive Endocrinology.
There is a major need for a specific and highly sensitive bioassay method for the measurement of low levels of LH activity. Such a method is needed to establish the relationship between the immunological and biological activities of various human gonadotrophins present in body fluids and tissues and to assess the purity of other pituitary hormone preparations.

Dufau et al. (1971, 1972a) reported that there is a dose-response relationship between the amount of testosterone produced by decapsulated rat testes in vitro and the quantity of HCG or LH added to the incubation medium. In these studies ovine FSH and ovine prolactin did not stimulate testosterone production nor was there a synergistic or antagonistic effect observed in the presence of added ovine LH or HCG. It has also been applied as a bioassay (Dufau et al., 1972b, Catt et al., 1973). The present paper describes systematic studies on the specificity of the in vitro bioassay method, which gives statistically valid potency estimates of the LH activity of HCG, HMG and HHG preparations, comparable to those obtained by established in vivo bioassay methods.

MATERIALS AND METHODS

Abbreviations

CV = coefficient of variation; dibutyryl cAMP = N6O2-dibutyryl adenosine 3',5'-cyclic monophosphoric acid; HHG = human hypophyseal gonadotrophin; HHLH = human hypophyseal luteinizing hormone; HHTSH = human hypophyseal thyroid stimulating hormone; KiSs = dissociation constant; λ = index of precision; KRb = Krebs-Ringer bicarbonate; LHRH = luteinizing hormone-releasing hormone; OAAD = ovarian ascobic acid depletion assay; RIA = radioimmunoassay; testosterone = 17β-hydroxy-4-androsten-3-one; TLC = thin layer chromatography; WITARO = assay method based on the weight increase of the total accessory reproductive organs (in intact immature male rats).

Experimental animals

Sprague-Dawley rats and mice of the Naval Medical Research Institute strain (Bethesda, USA) were purchased from AB Anticimex, Stockholm, and caged for a minimum of 24 h prior to sacrifice.

Radioactive steroids. – [1,2-3H]Testosterone (45 Ci/mM) and [1,2,6,7-3H]testosterone (100 Ci/mM) were obtained from the Radiochemical Centre, Amersham, Bucks., U.K., and from NEN Chemicals GmbH, Frankfurt, Germany, and stored in freshly distilled benzene:ethanol (9:1) at 4°C at a concentration of 10–20 ng/ml. Radiochemical purity was checked by TLC on silica gel (Merck F254) in two systems (chloroform:ethyl acetate 1:1 and benzene-ethyl acetate 3:2) and by crystallization to constant specific activity.

Steroids and solvents. – The various steroids used in the cross-reaction studies were obtained from Steraloids (Pauling, USA) and crystallized three times before use.

All organic solvents were of reagent or analytical grade.

The marker dye F9 (Lacquer violet, 1,4-diamino-anthaquinone) was supplied by ICI Ltd., Dyestuff Division, U.K.

The incubation medium was KRb buffer containing 200 mg/100 ml of glucose and bubbled with carbogen (93.5:6.5/O2:CO2) for 45 min at 34°C prior to use. For RIA
of testosterone a 0.1 m sodium phosphate buffer pH 7.4 containing 0.9% sodium chloride, 0.01% gelatin and 0.1% sodium azide (w/v) was employed. The Norit A charcoal (Serva, Heidelberg, Germany) was washed three times with water to remove floating material and dried at 100°C overnight.

The testosterone antiserum was diluted with buffer (1:1000) every 2–3 months and frozen in suitable portions. The stock solution was diluted further 1:10 prior to assay.

The hormone preparations used in the bioassay (Tables 1 and 6) were dissolved in 0.9% sodium chloride containing 0.1% bovine serum albumin prior to assay.

HCG and LH potencies were determined by the method of Diczfalusy (1954) based

Table 1.
Hormone preparations used in the assays; a list of HCG preparations is included in Table 6.

<table>
<thead>
<tr>
<th>Hormone preparation</th>
<th>Identification</th>
<th>Source</th>
<th>Potency IU/amp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>FSH</td>
</tr>
<tr>
<td>Urinary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human menopausal gonadotrophin</td>
<td>WHO 2nd IRP</td>
<td>MRC Division for Biological Standards London</td>
<td>40 (a)</td>
</tr>
<tr>
<td>Homogonal V 1935</td>
<td>Leo AB (Helsingborg - Sweden)</td>
<td>75 (b)</td>
<td>30 (b)</td>
</tr>
<tr>
<td>Humegen 641 726</td>
<td>Diosynth (Oss - Holland)</td>
<td>75 (b)</td>
<td>150 (b)</td>
</tr>
<tr>
<td>70/45</td>
<td>MRC Division for Biological Standards London</td>
<td>63 (c)</td>
<td>45 (d)</td>
</tr>
<tr>
<td>Pituitary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human hypophyseal gonadotrophin</td>
<td>HHLH 68/40</td>
<td>MRC Division for Biological Standards London</td>
<td>&lt;0.9 (a)</td>
</tr>
<tr>
<td>XL E 3</td>
<td>Dr. Bettendorf (Hamburg - Germany)</td>
<td>16 (c)</td>
<td>440 (d)</td>
</tr>
<tr>
<td>B/62</td>
<td>Dr. Bettendorf (Hamburg - Germany)</td>
<td>222 (c)</td>
<td>770 (d)</td>
</tr>
<tr>
<td></td>
<td></td>
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</tbody>
</table>

(cont.)
### Table 1 (cont.).

<table>
<thead>
<tr>
<th>Hormones used for specificity studies</th>
<th>Identification</th>
<th>Source</th>
<th>Specific potency IU/mg</th>
<th>LH content IU/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (Human urinary)</td>
<td>E 161 Ter-4</td>
<td>Serono (Rome - Italy)</td>
<td>1055 (b)</td>
<td>4.2 (b)</td>
</tr>
<tr>
<td>TSH (Human pituitary)</td>
<td>68/38</td>
<td>MRC Division for Biological Standards</td>
<td>0.147 (a) (i)</td>
<td>57.2 (a) (i)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>London</td>
<td></td>
<td>63 (e) (i)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dr. Pierce (Los Angeles - USA)</td>
<td>10 (b)</td>
<td>4000 (j)</td>
</tr>
<tr>
<td></td>
<td>Kabi May 72</td>
<td>AB Kabi (Stockholm-Sweden)</td>
<td>7.5 (b)</td>
<td>700 (b)</td>
</tr>
<tr>
<td>ACTH (Synthetic)</td>
<td>Synacthen</td>
<td>Ciba (Basel - Switzerland)</td>
<td>100 (b)</td>
<td>Not indicated</td>
</tr>
<tr>
<td>STH (Human pituitary)</td>
<td>Lqc. 145</td>
<td>AB Kabi (Stockholm-Sweden)</td>
<td>3.4 (b)</td>
<td>Not indicated</td>
</tr>
<tr>
<td>LTH (Ovine pituitary)</td>
<td>4273</td>
<td>Calbiochem (Los Angeles - USA)</td>
<td>1000 (i)</td>
<td>Not indicated</td>
</tr>
<tr>
<td>LH-RH (Synthetic)</td>
<td>Op. 3</td>
<td>Hoechst (Frankfurt/M - Germany)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Oxytocin (Synthetic)</td>
<td>Syntocinon</td>
<td>Sandoz (Basel - Switzerland)</td>
<td>5 (b) (k)</td>
<td>Not indicated</td>
</tr>
<tr>
<td></td>
<td>48 K 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vasopressin (Synthetic)</td>
<td>007 B 2</td>
<td>Sandoz (Basel - Switzerland)</td>
<td>10 (b) (k)</td>
<td>Not indicated</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) Bangham et al. (1971).
b) Indicated by supplier.
c) Assay conducted in this laboratory (Steelman & Pohley 1953). Figures in parentheses indicate 95% fiducial limits and index of precision.
d) Assay conducted in this laboratory (OAAD). For details consult Table 7.
e) Assay conducted in this laboratory (WITARO). For details consult Table 7.
f) OAAD: invalid assay (Bangham et al. 1971).
g) Assay based on the ventral prostate weight in immature hypophysectomized rats (Bangham et al. 1971).
h) IU/mg.
i) IU/amp.
j) Assay conducted in this laboratory (RIA).
k) IU/ml.
on the weight increase of the total accessory reproductive organs in intact immature male rats (WITARO) and by the OAAD method of Parlow (1961), using a 3 + 3 point assay design with 5 animals per dose level. In a few experiments a 2 + 2 design was used with litter mate control. The bioassay results were statistically analysed following the methods of Gaddum (1953) and Finney (1964) using the simplified calculations of Borth et al. (1957a,b) and Borth (1960).

The methods of RIA for the measurement of HCG, HMG and HHG were based on the method of Midgley (1966) as modified by Robyn et al. (1971), using HCG 2nd IS, HMG 2nd IRP and HHLH 68/40 as standards.

RESULTS

Proposed method

Assay design. — A 3 + 3 point assay is used with 6 individual testes for each dose level and with equal spacing of log doses. Usually 5 unknown preparations are assayed against 1 standard. Validity tests are performed in each assay following standard statistical techniques as described for the in vivo bioassay methods in the section “Materials and Methods”.

Incubation of decapsulated testes. — Adult mice (3–4 months old) in groups of ten are sacrificed by cervical dislocation and the testes removed and placed in Petri dishes on ice. The testes are decapsulated using glass instruments and care is taken that the testes are not damaged. The decapsulated testes are allocated to different doses on the basis of random numbers (e.g. Fisher & Yates 1953) and are then placed in vials in ice containing 2 ml of KRB buffer pH 7.4 and the required concentration of hormone. The vials are tightly stoppered and incubated at 34°C in a moving water bath at 50–100 cycles/min and gassed with carbogen.

After incubation for 3 h the vials are placed in ice, the testes are removed and dried to constant weight (for a minimum of 24 h at 65°C). The media are diluted (1:500) with distilled water and the testosterone levels are assayed by a RIA method (see below). The results are calculated in terms of ng testosterone produced/mg dry weight of testis.

Radioimmunoassay method

The RIA procedure consists of incubating 0.1 ml of the unknown sample (1:10 000 of the incubation media) with a portion (0.2 ml) of the mixture containing tritium labelled testosterone (75 pg) and the appropriate dilution of antiserum. The mixture is incubated at 60°C for 10 min followed by 30°C for 30 min. The tubes are iced and a charcoal suspension (0.5 ml 1 % Norit A suspended in assay buffer) is added within a predetermined time interval (3–5 min), using a dispenser (Oxford Model M). The tubes are kept in ice for a further 10 min prior to centrifugation at 1000 × g for 5 min at 4°C. Portions
(0.5 ml) of the supernatant are added to counting vials containing toluene scintillation fluid (Mešter et al. 1970). The vials are then tightly stoppered, heated at 60°C for 5 min and vortexed for 15 seconds in order to ensure total dissolution of the labelled testosterone into the toluene, then cooled and counted. The counting efficiency for tritium in this system in a Nuclear Chicago Liquid Scintillation Spectrometer Mark II is 55–60%. A standard curve (0–500 pg testosterone) is processed in an identical manner with the unknown samples. The RIA standard curve is linearized by a logit transformation (Rodbard et al. 1969) and the equation of the line and its linearity determined. All individual values are calculated from the equation of the RIA line. All computations are performed on a Wang 700 Advanced Programming Calculator according to a computer programme developed in this laboratory (Cekan, Robertson & Diczfalusy, unpublished).

Variables affecting the assay

Conditions of incubation

Rat and mouse testes at various ages from weaning to old age were investigated in order to optimize assay sensitivity and to maximize the useful range of the assay.

Immature rats (21–23 days, 55 g) gave a sensitive dose response curve with a limited working range (0.1–0.8 mIU HCG/ml). Rats and mice during puberty (50 days: 210 g and 30 g, respectively) gave a less sensitive dose response curve with an enlarged working range (0.2–3.2 mIU HCG/ml). One experiment with mature rats (3 months, 450 g) gave results similar to those obtained with rats at puberty. Mature mice (3–4 months, 35–40 g) gave an increased working range with lower sensitivity (0.4–12.8 mIU HCG/ml). For all assays the index of precision ranged from 0.13 to 0.25. Testes of mature mice rather than of rats (cf. Dufau et al. 1971, 1972a,b) were chosen as the experimental system, as mice in large numbers are easy to handle and house and since no additional incubation step is necessary because the amount of preformed testosterone present is negligible.

The conditions of incubation and RIA varied with the source of testes. With immature rats, firstly, a 2 h incubation was employed, secondly, a much lower dilution of the media (final dilution 1:200) was needed for RIA and thirdly, a heating step (60°C for 15 min) was introduced prior to RIA to denature proteins which bound testosterone (Ritzén et al. 1973). In adult animals the influence of the binding protein on the assay was negligible, probably due to the high dilution of the media needed for RIA.

Mature rat testes required a high O₂/CO₂ pressure to maintain the incuba-
tion pH at 7.4, otherwise a marked increase in pH to a maximum of pH 9 was observed. This effect was not observed with mouse testes.

A considerable variability of testosterone values between individual testes at one gonadotrophin dose level was observed for all hormone preparations. This variability could not be related to animal differences (as testes from the same animal behaved differently), the decapsulation technique or to any examined aspect of the methodology.

**Choice of RIA conditions**

The testosterone antiserum used throughout the study was produced against 3-oxo-testosterone haemocyanin in rabbits (Nieschlag & Loriaux 1972) and gives by analysis of Scatchard (1949) a straight line over a large range of binding (bound to free ratios 2.0–0.05) with a \( K_{\text{dis}} \) at 30°C of \( 0.6 \times 10^{-10} \) M. For use in the in vitro bioassay the antibody dilution chosen was 1:30 000 (2.3 \( \times \) \( 10^{-10} \) M of binding sites). Under the RIA conditions employed this gave an optimal calibration curve for 0–500 pg testosterone. The first incubation step (60°C for 10 min) was introduced to facilitate a more rapid approach to equilibrium condition.

**Reliability of the testosterone assay.** – The sensitivity of the RIA method used in the in vitro bioassay as assessed by the lower 95% fiducial limit in the absence of added steroid was 6 pg. The precision of the testosterone measurements (Snedecor 1952) in the range of testosterone values (5–150 pg) found over the working range of the in vitro bioassay gave a CV of 6–7% (\( n = 90 \)). The specificity of the antiserum was established by comparing increasing amounts of different steroids with testosterone under the assay conditions described above. Specificity was defined in terms of the amount of steroid which displaced 50% of labelled testosterone. The following steroids were investigated for cross-reaction with testosterone:

3α-Hydroxy-5α-androstan-17-one, 5α-androstane-3α,17β-diol, 5-androstene-3β,17β-diol, 4-androstene-3,17-dione, 4-androstene-3,11,17-trione, 3β-hydroxy-5α-androstan-17-one, 17β-hydroxy-5α-androstan-3-one, 17β-hydroxy-4-oestren-3-one, 1,3,5(10)-oestriene-3,17β-diol, 1,3,5(10)-oestratriene-3,16α,17β-triol, 3-hydroxy-1,3,5(10)-oestratrien-17-one, 5-cholest-3β-ol, 3β-hydroxy-5-androstan-17-one, 17,21-dihydroxy-4-pregnene-3,20-dione, 18,11-haemiacetal of 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al, 5β-pregnane-3,20-dione, 3β-hydroxy-5α-pregn-20-one, 4-pregnen-3,20-dione, 20α-hydroxy-4-pregnen-3-one, 20β-hydroxy-4-pregnen-3-one, 17α-hydroxy-4-pregnen-3,20-dione, 5β-pregnane-3α,20α-diol, 11β,17α,21-trihydroxy-4-pregnen-3,20-dione, 11β,21-dihydroxy-4-pregnen-3,20-dione and 17α,21-dihydroxy-4-pregnen-3,11,20-trione. Of these steroids, 17β-hydroxy-5α-androstan-3-one (79%), 17β-hydroxy-4-oestren-3-one (34%), 4-androstene-3β,17β-diol (24%), 3α-hydroxy-5α-andro-
stan-17-one (19%) and 4-androstene-3,17-dione (1%) gave a cross-reaction equal to or greater than 1%. These results are in general agreement with those obtained by Nieschlag & Loriaux (1972).

**Conditions for using charcoal.** – The choice of charcoal as a suitable means of separating the bound and free steroids was checked by measuring the rate of dissociation of the complex in the presence of charcoal and by determining the amount of adsorption of the complex by the charcoal. In the presence of charcoal the complex dissociates at 4°C with a half-life of 255 min, so that for the addition of charcoal in large assays, an automatic dispenser is a requirement. The charcoal at a concentration of 1%, while removing 99.5% of the free steroid did not adsorb any detectable amount of the complex.

**Characterization of the antibody-competing material present in the incubation medium.** – To assess whether testosterone was the major antibody-competing material released by the mouse testis into the medium, portions of the media (with and without HCG treatment) were processed through two successive TLC steps prior to RIA. The Rs values of the various steroids in the two steps are presented in Table 2.

A portion of the incubation medium, containing labelled testosterone to assess procedural losses, was extracted with ether, partitioned between equal volumes of 90% methanol and petroleum ether and the evaporated methanol extract applied to TLC. The testosterone region in the second TLC step was extracted with ether, evaporated, diluted with buffer and assayed by RIA.

The mean levels of testosterone in the medium of 5 different incubations

**Table 2.**
The Rs values (s = dye marker F9, slowly migrating component) in two solvent systems.

<table>
<thead>
<tr>
<th>Compound</th>
<th>System A</th>
<th>System B</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-Hydroxy-4-androsten-3-one</td>
<td>0.97 (0.91-1.03)</td>
<td>0.79 (0.76-0.82)</td>
</tr>
<tr>
<td>17β-Hydroxy-4-oestren-3-one</td>
<td>0.94 (0.88-1.00)</td>
<td>0.72 (0.66-0.78)</td>
</tr>
<tr>
<td>5α-Androstone-3α,17β-diol</td>
<td>0.69 (0.65-0.73)</td>
<td>0.78 (0.66-0.90)</td>
</tr>
<tr>
<td>4-Androstene-3β,17β-diol</td>
<td>0.74 (0.70-0.78)</td>
<td>0.85 (0.79-0.91)</td>
</tr>
<tr>
<td>3α-Hydroxy-5α-androstan-17-one</td>
<td>1.17 (1.06-1.28)</td>
<td>1.09 (1.05-1.13)</td>
</tr>
<tr>
<td>17β-Hydroxy-5α-androstane-3-one</td>
<td>1.18 (1.11-1.25)</td>
<td>1.11 (1.06-1.16)</td>
</tr>
<tr>
<td>4-Androstan-3,17-dione</td>
<td>1.42 (1.35-1.49)</td>
<td>1.03 (0.98-1.08)</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate 95% fiducial limits.

System B: Isooctane/ethyl acetate 7:3.
measured with and without the TLC procedure were for the untreated testes 35.3 ± 4.7 and 33.6 ± 7.4 ng (mean ± sd) and in the presence of 1.6 mIU HCG/ml 215.7 ± 48.8 and 226.5 ± 48.1 ng, respectively. The difference between the testosterone values obtained before and after TLC purification is not significant. Hence testosterone is the principal steroid released by the decapsulated mouse testes into the medium, as postulated previously by Dufau et al. (1971, 1972a) for decapsulated rat testes.

Reliability of the in vitro bioassay method

The useful range of doses giving a linear log dose response curve for HCG and HMG was 0.4–12.8 mIU/ml and 2–32 mIU/ml, respectively. As a consequence, the doses chosen for assay were 0.4, 0.8, 1.6 or 0.8, 1.6, 3.2 mIU/ml for HCG and 2, 4, 8 or 4, 8, 16 mIU/ml for HMG (Fig. 1).

The sensitivity of the method was 0.4 mIU/ml for HCG and 2 mIU/ml for HMG as assessed by t-test analyses ($P < 0.05$).

The precision of the method was assessed from the mean λ value of the potency estimates of all preparations and by repeated assays of an HCG

![Fig. 1.](attachment:fig1.png)

Dose-response curve with mouse testes for HCG and LH (HMG). Each point is the mean of 6 incubations. The standard deviation is also indicated.
preparation. From 54 assays the mean \( \lambda \) was 0.22, of which 44 assays were 3 + 3 point design \( (\bar{\lambda} = 0.22) \) and 10 assays were 2 + 2 point design \( (\bar{\lambda} = 0.20) \). In 6 repeated assays the weighted mean relative potency of HCG (2nd IS) in terms of HMG (2nd IRP) was 3.37, with 95% fiducial limits at 2.72 and 4.02.

No significant deviation from linearity or parallelism for HCG, HMG or HHG was observed in any assay.

The practicability of the method is such that the assay of 5 unknown hormone preparations against 1 standard can be performed by a team of 3 persons in 2 days.

The specificity of the method was assessed in three ways. Firstly, the assay results presented in Table 3 indicate that with the exception of TSH and

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Batch No.a)</th>
<th>LH potency (IU/mg)</th>
<th>Index of precision (( \lambda ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH</td>
<td>E 161 Ter-4</td>
<td>4</td>
<td>3.4 (2.6-4.5)</td>
</tr>
<tr>
<td>HHTSH</td>
<td>68/38b)</td>
<td>148c)</td>
<td>103 (67-175)c)</td>
</tr>
<tr>
<td>Dr. Pierce</td>
<td>4000</td>
<td></td>
<td>4030 (2760-5750)</td>
</tr>
<tr>
<td>Kabi - May 72</td>
<td>2280</td>
<td></td>
<td>1910 (1895-2595)</td>
</tr>
<tr>
<td>ACTH</td>
<td>Synacthen</td>
<td>Not assayed</td>
<td>No response when assayed at a concentration of 300 mIU/ml</td>
</tr>
<tr>
<td>LTH</td>
<td>Calbiochem</td>
<td>..</td>
<td>No response when assayed at a concentration of 83 mIU/ml</td>
</tr>
<tr>
<td>STH</td>
<td>Lqc. 145</td>
<td>..</td>
<td>No response when assayed at a concentration of 3.4 mIU/ml</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>48 K 9</td>
<td>..</td>
<td>No response when assayed at a concentration of 500 mIU/ml</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>007 B 2</td>
<td>..</td>
<td>No response when assayed at a concentration of 500 mIU/ml</td>
</tr>
<tr>
<td>LH-RH</td>
<td>Hoechst</td>
<td>..</td>
<td>No response when assayed at a concentration of 1 µg/ml</td>
</tr>
<tr>
<td>Dibutyryl c-AMP</td>
<td>Sigma D 0627</td>
<td>..</td>
<td>d)</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate 95% fiducial limits.

a) The potencies of the hormone preparations are indicated in Table 1.
b) The LH content estimated by in vivo bioassay is indicated in Table 1.
c) IU/amp.
d) 0.1 µM dibutyryl c-AMP/ml gave a response equivalent to 0.4 mIU/ml.

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FSH, no LH activity could be demonstrated in the various hormone preparations.

The LH potency of the FSH preparation was very low (FSH/LH = 328); however, that of TSH was very high (TSH/LH = 0.0025 and 0.011).

Secondly, various hormones were added separately to HCG, HMG and HHG preparations and the combination assayed for a synergistic or antagonistic effect (Table 4).

The validity of the in vitro bioassay was not influenced by the presence of the hormones studied at levels corresponding to those reported in plasma under pathological conditions. Unless otherwise indicated, the same amount of hor-

**Table 4.**

Specificity of the in vitro bioassay method: The effect of adding to HCG, HMG and HHG preparations various hormones at concentrations corresponding to pathologically elevated levels found in plasma.

<table>
<thead>
<tr>
<th>Hormone added</th>
<th>Levels tested/ml</th>
<th>HCG 760/531</th>
<th>HMG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Relative potency against HCG</td>
<td>Index of precision (λ)</td>
</tr>
<tr>
<td>FSHa)</td>
<td>100 mIU</td>
<td>0.85 (0.49–1.26)</td>
<td>0.19</td>
</tr>
<tr>
<td>TSHa)</td>
<td>5–20 μIU</td>
<td>0.99 (0.67–1.47)</td>
<td>0.25</td>
</tr>
<tr>
<td>ACTH</td>
<td>20 μIU</td>
<td>1.08 (0.68–1.72)</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTH</td>
<td>8.3 mIU</td>
<td>1.00 (0.72–1.43)</td>
<td>0.28</td>
</tr>
<tr>
<td>STH</td>
<td>340 μIU</td>
<td>1.15 (0.80–1.82)</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxytocin</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>0.5 mIU</td>
<td>0.93 (0.74–1.17)</td>
<td>0.15</td>
</tr>
<tr>
<td>LH-RH</td>
<td>100 ng</td>
<td>0.73 (0.36–1.14)</td>
<td>0.23</td>
</tr>
<tr>
<td>HCGa)</td>
<td>0.4–1.6 mIU</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>HHGa)</td>
<td>1–4 mIU</td>
<td>0.97 (0.72–1.30)</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate 95% fiducial limits.

a) Graded doses were added to graded doses of HCG, HMG or HHG (see text).
b) Test material: HMG 2nd IRP.
c) Test material: Homogonal.
d) Test material: HHLH 68/40, which was assayed against HMG 2nd IRP (cf. Table 7).
e) Not assayed at this level as no effect was observed at very high (pharmacological) levels.
mone was added to increasing doses of HCG or HMG. However, in the assessment of hormone preparations possessing an intrinsic LH-like activity (TSH and FSH) it was necessary to combine graded doses of these hormones with graded doses of the gonadotrophins to be tested. For instance, 5, 10 and 20 µIU of TSH (preparation from Dr. Pierce estimated to correspond to the activity of 1, 2 and 4 mIU of LH) were combined with 1, 2 and 4 mIU of HMG and these three combinations were assayed against 2, 4 and 8 mIU of HMG. The addition of TSH or FSH to HCG was performed in a similar manner to that indicated above. From the data of Table 4 it can be concluded that at the levels tested, the presence of the hormones studied did not influence the validity of the in vitro bioassay of HCG, HMG or HHG. Additivity was also observed with various combinations of HCG, HMG and HHG preparations. Very high or pharmacological levels of the hormones indicated in Table 4 were also studied in a similar manner to that above. The data of Table 5 suggest that ACTH and LTH may have a small synergistic effect and STH a small antagonistic effect.

Table 5.
Specificity of the in vitro bioassay method: The effect of adding very high or pharmacological levels of hormones to HCG, HMG and HHG preparations.

<table>
<thead>
<tr>
<th>Hormone added</th>
<th>Levels tested/ml</th>
<th>HCG 760/531 Relative potency against HCG 2nd IS</th>
<th>Index of precision ((\text{I}))</th>
<th>HMG Relative potency against HMG 2nd IRP</th>
<th>Index of precision ((\text{I}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH&lt;sup&gt;a&lt;/sup&gt;</td>
<td>155–620 mIU</td>
<td>1.33 (0.85–2.22)</td>
<td>0.29</td>
<td>0.75 (0.55–1.00)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSH&lt;sup&gt;c&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ACTH</td>
<td>300 mIU</td>
<td>1.52 (1.05–2.31)</td>
<td>0.24</td>
<td>1.33 (0.92–1.98)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.24</td>
</tr>
<tr>
<td>LTH</td>
<td>83 mIU</td>
<td>1.29 (0.77–2.29)</td>
<td>0.31</td>
<td>1.36 (1.01–1.85)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.19</td>
</tr>
<tr>
<td>STH</td>
<td>3.4 mIU</td>
<td>0.83 (0.48–1.40)</td>
<td>0.31</td>
<td>0.71 (0.52–0.94)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.19</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>5 mIU</td>
<td>1.12 (0.80–1.72)</td>
<td>0.20</td>
<td>1.06 (0.80–1.42)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.18</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>5 mIU</td>
<td>0.72 (0.48–1.02)</td>
<td>0.20</td>
<td>0.69 (0.50–1.12)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate 95% fiducial limits.

<sup>a</sup> Graded doses were added to graded doses of HCG, HMG or HHG (see text).

<sup>b</sup> Test material: HMG 2nd IRP.

<sup>c</sup> Test material: Homogonal.

<sup>d</sup> Test material: HHLH 68/40, which was assayed against HMG 2nd IRP (cf. Table 7).

<sup>e</sup> The high ratio TSH/LH activities precluded the assay of this material.

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Table 6.
Potency of HCG preparations of varying purity as estimated by *in vivo* and *in vitro* bioassay methods and by radioimmunoassay.

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>Supplier</th>
<th><em>In vivo</em> estimation (WITARO)</th>
<th><em>In vitro</em> estimation</th>
<th>RIAa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IU/mg</td>
<td>Index of precision</td>
<td>IU/mg</td>
</tr>
<tr>
<td>MSKG 03</td>
<td>This laboratory</td>
<td>17,150 (14,010–21,130)c)</td>
<td>0.11</td>
<td>17,140 (10,270–30,340)c)</td>
</tr>
<tr>
<td>02b)</td>
<td></td>
<td>12,250 (8,860–16,580)c)</td>
<td>0.19</td>
<td>9,310 (5,700–13,690)c)</td>
</tr>
<tr>
<td>E 231 - 2</td>
<td>Serono - Rome</td>
<td>9,820 (7,480–12,160)d)</td>
<td>0.10</td>
<td>8,320 (5,330–12,090)</td>
</tr>
<tr>
<td>71:75:16</td>
<td>Leo - Helsingborg</td>
<td>9,240 (7,360–11,350)</td>
<td>0.12</td>
<td>7,650 (5,700–10,010)</td>
</tr>
<tr>
<td>J 26:30</td>
<td>Leo - Helsingborg</td>
<td>7,130 (5,570–9,600)d)</td>
<td>0.11</td>
<td>8,580 (5,880–13,200)</td>
</tr>
<tr>
<td>MPPD 01</td>
<td>This laboratory</td>
<td>2,900 (2,220–3,780)</td>
<td>0.15</td>
<td>2,700 (2,090–3,460)</td>
</tr>
<tr>
<td>428 ZEP</td>
<td></td>
<td>2,720 (1,640–3,890)</td>
<td>0.19</td>
<td>2,460 (1,660–3,570)</td>
</tr>
<tr>
<td>760 - 531</td>
<td>Diosynth. - Oss</td>
<td>2,660 (2,070–3,460)</td>
<td>0.13</td>
<td>2,560 (1,810–3,590)</td>
</tr>
<tr>
<td>MPPD 02</td>
<td>This laboratory</td>
<td>1,680 (1,340–2,200)</td>
<td>0.14</td>
<td>1,660 (1,140–2,510)</td>
</tr>
<tr>
<td>03</td>
<td></td>
<td>1,250 (940–1,680)</td>
<td>0.15</td>
<td>1,510 (1,170–1,950)</td>
</tr>
<tr>
<td>04</td>
<td></td>
<td>1,200 (993–1,530)</td>
<td>0.12</td>
<td>1,040 (712–1,560)</td>
</tr>
<tr>
<td>05</td>
<td></td>
<td>633 (457–862)</td>
<td>0.17</td>
<td>579 (437–753)</td>
</tr>
</tbody>
</table>

All estimates are in terms of the 2nd IS of HCG.
Figures in parentheses indicate 95% fiducial limits.

a) RIA carried out in this laboratory.
b) Purified urinary HCG prepared in this laboratory.
c) IU/mg protein.
d) 2 + 2 assay.
e) Robyn & Diczfalusy (1968).
f) Not assayed.
Table 7.
Potency of HMG and HHG preparations of varying purity as estimated by in vivo and in vitro bioassay methods and by radioimmunoassay.

<table>
<thead>
<tr>
<th>Hormone preparations</th>
<th>OAAD</th>
<th>WITARO</th>
<th>In vitro</th>
<th>RIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Urinary: HMG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogonal</td>
<td>49 (30–95)</td>
<td>0.22</td>
<td>58 (46–77)</td>
<td>0.13</td>
</tr>
<tr>
<td>Humegon</td>
<td>52 (33–92)</td>
<td>0.19</td>
<td>58 (33–90)</td>
<td>0.20</td>
</tr>
<tr>
<td>70/45</td>
<td>45 (37–54)</td>
<td>0.12</td>
<td>45 (36–57)</td>
<td>0.15</td>
</tr>
<tr>
<td>B. Pituitary: HHG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HHLH 68/40&lt;sup&gt;d&lt;/sup&gt;</td>
<td>92 (67–131)</td>
<td>0.20</td>
<td>63 (49–76)</td>
<td>0.12</td>
</tr>
<tr>
<td>XL - E&lt;sub&gt;3&lt;/sub&gt;</td>
<td>440 (270–770)</td>
<td>0.22</td>
<td>198 (133–268)</td>
<td>0.13</td>
</tr>
<tr>
<td>B/62</td>
<td>770 (550–1050)</td>
<td>0.16</td>
<td>794 (577–1080)</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate 95% fiducial limits.

- a) RIA carried out in this laboratory.
- b) Weighted mean of 4 estimations (Borth et al. 1957b).
- c) Mean value of 4 assays.
- d) IU/amp.
Thirdly, a comparison of the potency estimates obtained by the WITARO and in vitro bioassay methods for 12 HCG preparations ranging in potency from 600 to 17 000 IU/mg gave a linear correlation with a coefficient of correlation of 0.97 and a slope of 1.05 (Table 6).

Similar agreement between the in vitro and in vivo bioassay methods (WITARO, OAAD) was obtained with HMG preparations on a limited number of samples. With HHG preparations there was a marked difference in potency estimates by the three methods (Table 7).

The in vitro bioassay method was also compared with RIA for 10 of the 12 HCG preparations and the HMG and HHG preparations (Tables 6 and 7). The correlation coefficient between the in vitro bioassay method and RIA for HCG was 0.95 with a slope of 0.86. The close correlation between RIA and WITARO methods with potency estimates of highly pure HCG preparations appears to be at variance with previously reported data (Robyn et al. 1971).

**DISCUSSION**

The in vitro bioassay method fulfils the recognised criteria of sensitivity, precision and specificity. In terms of sensitivity, the assay is approximately 800 and 1000 times more sensitive for the determination of HCG and 150 and 800 times for the determination of HMG than the OAAD and WITARO methods, respectively. In terms of precision, the mean λ value of 0.22 based on 54 assays indicates that the precision of the method is satisfactory. In terms of specificity, the assay is not influenced by other pituitary or urinary hormones, including FSH and prolactin. This confirms the previous observations by Dufau for these two hormones (Dufau et al. 1972a) using decapsulated adult rat testes. Furthermore, there was strict additivity if combinations of HCG, HMG and HHG were assayed by this technique. All hormones preparations used in the specificity studies were of human origin, except LTH, which was an ovine preparation.

In estimating the potencies of HCG preparations, a good agreement was obtained with the WITARO bioassay method and the RIA method. However, in case of the few pituitary gonadotrophin preparations studied, the agreement between potency estimates by the various methods was less satisfactory. Further studies will be needed to assess adequately this point.

A number of other in vitro bioassay methods have been described for LH recently (Watson 1971; Kamiyoshi et al. 1972; Rees et al. 1973). At the present stage of development it is not yet possible to assess the relative merits of the various methods.
ACKNOWLEDGMENTS

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