A rapid and sensitive ELISA for rainbow trout maturational gonadotropin (rGtHII): validation on biological samples, in vivo and in vitro responses to GnRH

G. Salbert, T. Bailhache, Yonathan Zohar, Bernard Breton, P. Jegou

► To cite this version:

G. Salbert, T. Bailhache, Yonathan Zohar, Bernard Breton, P. Jegou. A rapid and sensitive ELISA for rainbow trout maturational gonadotropin (rGtHII): validation on biological samples, in vivo and in vitro responses to GnRH. *General and Comparative Endocrinology*, 1990, 78, pp.110-122. 10.1016/0016-6480(90)90052-N . hal-02714965

HAL Id: hal-02714965

<https://hal.inrae.fr/hal-02714965>

Submitted on 1 Jun 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

A Rapid and Sensitive ELISA for Rainbow Trout Maturation Gonadotropin (tGtH II): Validation on Biological Samples; *in Vivo* and *in Vitro* Responses to GnRH

G. SALBERT,^{*,1} T. BAILHACHE,^{*} Y. ZOHAR,[†] B. BRETON,[‡] AND P. JEGO^{*}

^{*}Laboratoire de Physiologie des Régulations, U.A. CNRS 256, Université de Rennes I, 35042 Rennes Cédex, France; [†]National Center for Mariculture, Israel Oceanographic and Limnological Research Institute, P.O. Box 1212, Eilat, Israel; and [‡]Laboratoire de Physiologie des Poissons, INRA, Complexe Scientifique de Beaulieu, 35042 Rennes Cédex, France

Accepted June 15, 1989

A rapid and sensitive heterologous enzyme-linked immunosorbent assay (ELISA) was developed to measure rainbow trout maturational gonadotropin. Purified salmon maturational gonadotropin (sGtH II) was used as reference hormone. Optimization of the procedure was performed by using an anti- β sGtH serum. Two procedures were developed: an equilibrium assay (which did not involve a preincubation step) which lasted for 8 hr and a nonequilibrium assay (which involved a preincubation step) which lasted for 26 hr. The nonequilibrium assay gave the best sensitivity (70 pg/ml sample). GtH II measurements on *in vivo* and *in vitro* samples from GnRH analogs or sGnRH experiments showed that the ELISA procedure could be used over a wide range of concentrations. The method was validated by comparing GtH II concentrations measured by both RIA and ELISA. © 1990

Academic Press, Inc.

The development of more precise and sensitive techniques has generated very important changes in some of the ideas and strategies in the field of endocrinology. Reliable hormone measurements can now be made on a few microliters of plasma: in this way, the animal physiology is not disturbed by successive samplings which are required, for example, to detect episodic hormone secretion (Atkinson *et al.*, 1970; Dierschke *et al.*, 1970; Knobil, 1980; Garnier *et al.*, 1978; Ortavant *et al.*, 1982; Zohar *et al.*, 1986). Radioimmunoassays (RIAs) for gonadotropins (GtHs) have become progressively more sensitive over the years. In some physiological situations, however, (e.g., juvenile fish, beginning of gametogenesis) and *in vitro* (e.g., whole pituitary perfusions), the GtH levels are especially

low and the classical RIA techniques are still not sensitive enough to measure them (Bailhache *et al.*, 1989). Recently, enzyme-linked immunosorbent assays (ELISAs) for hormones have been developed (Farrington and Hymer, 1987; Manquez *et al.*, 1987; Signorella and Hymer, 1984; Spearow and Trost, 1987; Yamamoto and Kato, 1986). Since these assays do not use radioactive molecules, require less time than RIAs, and, in some cases, are more sensitive than RIAs (specially with the use of the peroxidase-anti-peroxidase technique), they prove a good alternative to RIAs.

The aim of our study was to develop a rapid and sensitive ELISA for rainbow trout maturational gonadotropin (tGtH II, according to Suzuki *et al.*, 1988). After optimization of the procedure, tGtH II levels were measured in plasma samples from gonadotropin-releasing hormone analog (GnRHa)-treated female trout and in perfusion

¹ To whom correspondence should be addressed.

samples from a trout brain/pituitary preparation perfused with salmon GnRH (sGnRH). A comparison between ELISA and RIA tGtH II measurements was made.

MATERIALS AND METHODS

Salmon (*Oncorhynchus tshawytscha*) maturational gonadotropin, which has been found to correspond to the sGtH II of Suzuki *et al.* (1988), was prepared according to Breton *et al.* (1978). Purified sGtH II was used as reference gonadotropin for RIA and ELISA procedures. sGtH II subunits were prepared from the same material. Antibodies against the subunits were raised in rabbits and have already been validated for radioimmunoassay (Breton *et al.*, 1984).

Animals

For *in vivo* experiments, the rainbow trout (*Salmo gairdneri*) were obtained from an experimental fish farm. They were 2 years old and from an autumn-spawning strain. The experiment was performed in late October in nonovulated females taken from a population in which 30% of the animals had already spawned. For *in vitro* experiments, rainbow trout were kept in the laboratory in 500-liter aquaria with fresh water at 15° and under a natural photoperiod. These trout were 18 months old and at the early stage of exogenous vitellogenesis (oocyte diameter <0.5 mm).

In Vivo GnRH Treatments

Four groups of six animals received an intramuscular injection under the dorsal fin, with a saline solution (control group); 20 µg/kg body weight of [D-tryptophan⁶]luteinizing hormone-releasing hormone ([D-Trp⁶]LH-RH) purchased from Sigma Chemical Co.; 200 µg/kg body weight of a slow-release form microencapsulated [D-Trp⁶]LH-RH (Triptoreline from Ferring); or microencapsulated [D-arginine⁶, proline⁹ N-ethylamide]sGnRH ([D-Arg⁶,Pro⁹ NHet]sGnRH) releasing 20 µg/kg body weight and per day, for 2 weeks.

Biological Samples

(a) *Plasma samples.* Animals were bled from a caudal vessel using a heparinized syringe. Blood samples were taken just before the injection and 1, 3, 6, 9, 24, 48, 72, and 96 hr after the injection and then twice a week for 2 weeks. After centrifugation at 1500g for 20 min at 4°, the blood plasma were separated and frozen at -30° until assayed.

(b) *Perfusions.* Perfusions were run according to Bailhache *et al.* (1989). Briefly, the brain/pituitary was removed from the trout. A midsagittal section was

made, passing through the third ventricle, leaving thus intact most hypothalamic structures. The half fore-brain (including half a pituitary still connected) was then perfused (14°) with Hepes buffer (pH 7.4) in an appropriate chamber. After 3 hr of perfusion, sGnRH (10^{-7} M) was added to the medium for 15 min. Fractions were collected every 2 min (flow rate: 1 ml per 5 min) and then frozen at -20° until assayed.

RIA Procedure

Radioimmunological assays were run as previously described by Breton *et al.* (1978) using guinea pig IgG directed against the native sGtH II and rabbit IgG directed against guinea pig IgG. This double antibody RIA procedure has intraassay and interassay coefficients of variation (at 50% label binding) of 6.6 and 11%, respectively. The sensitivity of this RIA was 300 pg/ml sample.

ELISA Procedure

An enzymeimmunoassay was used (Ternynck and Avrameas, 1987). Briefly, hormone (reference sGtH II) coated onto the walls of microtiter plates competes for primary antibodies (anti-βsGtH II) with hormone which is free in solution. Appropriate steps reveal the immobilized sGtH II-antibody complexes. The "standard conditions" of our ELISA procedure, as used in this study, were:

Step 1. Two hundred microliters of reference sGtH II solution (2 ng/ml) in 0.05 M carbonate buffer (pH 9.6) was added to each well of a microtiter plate (NUNC IMMUNO II 96 F) and incubated for 2 hr at 37°.

Step 2. The low concentration of sGtH used in step 1 leaves many unbound sites which are saturated by unreactive proteins (nonimmunized sheep serum): 200 µl of PBS "S.T.G." (0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl, 2% sheep serum, 0.05%, Tween 20, 25 mg/liter gentamicin sulfate) was added to each well. The plates were then incubated for 30 min at 37°.

Step 3a. Without preincubation: 20 to 100 µl of sample and 180 to 100 µl of primary antibody (final dilution: 1/175,000) were added to each well. The plates were then incubated for 4 hr at 37°.

Step 3b. With preincubation: 25 to 125 µl of sample and 225 to 125 µl of primary antibody (final dilution 1/175,000) were mixed in a tube and incubated at room temperature for 24 hr. Two hundred microliters of this solution was then added to the microplate wells and incubated for 2 hr at 37°.

Step 4. Two hundred microliters of goat anti-rabbit IgG from Jackson Immunoresearch (U.S.A.) (GAR 1/2000 in PBS S.T.G.) was added to each well. Plates were incubated at 37° for 30 min.

Step 5. Two hundred microliters of rabbit peroxidase-antiperoxidase from Nordic Immunology (The Netherlands) (rPAP 1/5000 in PBS S.T.G.) was added to each well. Plates were incubated at 37° for 30 min.

Step 6. Two hundred microliters of citrate phosphate buffer (0.15 M, pH 5.1) with orthophenylenediamine (OPD) (Sigma) (0.5 mg/ml) and 0.25 μ l H₂O₂ (30%)/ml were added to each well and incubated at room temperature, in the dark for 1 hr.

Step 7. The enzymatic reaction was stopped by the addition of 50 μ l of H₂SO₄ (2 M) to each well. After 20 min, the optical density was read with an automatic microplate reader MR 700 (Dynatech, Great Britain).

In each assay, the following was determined: Blanks = optical density (OD) of wells without coated GtH; B_0 = OD of wells without free GtH (maximum binding); B = OD of wells with both coated and free GtH. All the wells received primary antibodies in equal quantity. The value of the blanks never exceeded 6% of the mean B_0 value. After steps 2, 3, 4, and 5, the wells were washed three times with 200 μ l of phosphate buffer saline containing 0.05% of Tween 20.

A first protocol is described in the legend of Fig. 1. This protocol has been used to search for optimum conditions of the assay (experimental conditions of the seven steps have been studied one by one to elaborate the standard conditions).

Linear Transformation and Statistical Analysis

After subtraction of the mean blank value, B/B_0 values were submitted to the logit transformation: $\text{Logit}(B/B_0) = \log[B/B_0/(1 - B/B_0)]$. Linear regressions were then calculated by plotting the logit (B/B_0) versus the $\ln(\text{dose})$ of free hormone added to wells. Each biological sample was assayed in duplicate. Parallelism of each plasma dilution curve with the standard curve was tested by analysis of covariance. We also calculated the intraassay and interassay coefficients of variation of the ELISA procedure. Results of the *in vivo* experiments were statistically analyzed by Student's *t* test.

RESULTS

Optimization of the ELISA Procedure

In order to give a good precision to the assay, we searched for the optimum conditions of each step (i.e., optimum time and concentrations).

(a) *Kinetics* (Fig. 1). Time course studies were run for each step. sGtH binding to the wells was more rapid at 37° than at 4°. The amount of binding was also greater at 37°

(Fig. 1a). Step 3 was relatively slow (Fig. 1b). In step 4, goat anti-rabbit (GAR) binding to the primary antibodies was extremely rapid: after 10 min, the reaction reached its maximum regardless of dilution rate (Fig. 1c). In step 7, we observed a great variability of optical densities between the B_0 wells when the plate was read immediately after H₂SO₄ addition (Fig. 1d). This variability decreased as optical densities reached their maximum value (Fig. 1d). To check if the acid concentration was suitable, we tried a 4 M H₂SO₄ solution which gave identical results.

For other time course steps, maximum binding levels of rPAP were observed after 20 min of incubation (step 5), optimal saturation after 30 min (step 2), and the highest level of substrate transformation by the peroxidase after 1 hr (data not shown).

(b) *Concentrations* (Fig. 2). Concentrations of coated GtH and primary antibody solution (steps 1 and 3) were investigated together. Primary antibody dilutions from 1/100,000 to 1/200,000 seemed to give good results (Fig. 2a). Therefore, when other parameters were optimized (see Materials and Methods: standard conditions), primary antibody dilutions were investigated in the range of 1/100,000 to 1/250,000. It appeared (Fig. 2b) that optical densities were not high enough with the 1 ng/ml coating solution, whereas 2 ng/ml gave high values (Fig. 2b).

Other antibodies were diluted to 1/2000 for GAR serum and to 1/5000 for rPAP complex. These concentrations gave good optical densities for B_0 and minimized nonspecific binding to the well. For saturation, the best results were obtained with 2% of sheep serum. In step 6, the highest observable value of B_0 was obtained by using 10 mg OPD and 5 μ l H₂O₂ (30%) in 20 ml citrate phosphate buffer.

Standard Curves

Standard curves were obtained by adding increasing doses of reference sGtH II in step 3.

FIG. 1. Time course studies of different ELISA steps for optical density (OD) B_0 values. Experiments were done using a primary protocol which differed from the standard conditions: coating sGtH II 1.6 ng/ml vs 2 ng/ml; anti- β sGtH 1/80,000 vs 1/175,000; rPAP 1/2000 vs 1/5000; incubation time in step 6: 30 min vs 1 hr. (a) Step 1, at 37° (□) or at 4° (Δ), each point is the mean $\pm$ SD of six values. (b) Step 3. (c) Step 4, with different GAR dilutions: 1/1000 (□), 1/2000 (Δ), 1/5000 (■), and 1/10,000 (▲). (d) Step 7, each point is the mean $\pm$ SD of six values.

FIG. 2. Determination of the concentrations of coated sGtH II and primary antibodies. (a) B_0 evolution for increasing doses of coating with different anti- β sGtH serum dilutions: 1/10,000 (1), 1/20,000 (2), 1/40,000 (3), 1/80,000 (4), 1/100,000 (5), and 1/200,000 (6). For the other steps, the procedure was run as described in the legend of Fig. 1. (b) B_0 evolution for increasing anti- β sGtH serum concentrations with two different doses of coating: (1) 2 ng/ml and (2) 1 ng/ml. For other steps, the procedure was run as described under Materials and Methods.

The preincubation enhanced the sensitivity of the assay, as 90% bound corresponded to 7.28 pg/well of free hormone in the procedure involving a preincubation, whereas it was 24.3 pg/well without preincubation (Fig. 3) when incubation time in step 3 was the same for the two procedures.

Then, we studied the effect of different

times of preincubation and incubation in step 3. Without preincubation, Fig. 4a shows that the equilibrium is reached after 4 hr of incubation. Thus, this procedure constitutes an equilibrium assay.

With a preincubation step, we can see that more or less than 2 hr of incubation induced a loss of sensitivity (Fig. 4b). It

FIG. 3. Standard curves obtained without or with a preincubation step. (a) B/B_0 displacement curve obtained by serial dilutions of reference sGtH from 4000 to 7.8 pg/well without preincubation step (see step 3a under Materials and Methods, except for incubation time: 2 hr vs 4 hr). (b) Logit transformation of the (a) curve. Equation of the regression is: $\text{Logit } B/B_0 = -0.964 \ln(\text{dose}) + 5.27$ with $r^2 = 0.998$ and SD slope = 0.011. (c) B/B_0 displacement curve obtained by serial dilutions of reference sGtH from 1000 to 7.8 pg/well with a preincubation step (see step 3b under Materials and Methods). (d) Logit transformation of the (c) curve. Equation of the regression is: $\text{Logit } B/B_0 = -1.047 \ln(\text{dose}) + 4.28$ with $r^2 = 0.991$ and SD slope = 0.028. The two values of each duplicate are plotted on the figure.

appears in Fig. 4c that the equilibrium is reached after 24 hr of preincubation in the tube. This procedure, with only 2 hr of incubation in step 3, constitutes a nonequilibrium assay when compared to the equilibrium one described above.

GtH Determination in Biological Samples

Serial dilutions of two plasma samples from mature female trout were assayed under both the equilibrium (Fig. 5a) and the nonequilibrium (Fig. 5b) procedures. In both cases, after logit transformation, the slopes of the regressions were not statistically different ($P > 0.25$); but GtH II contents of these plasmas were very different when assayed with the two procedures. This discrepancy also appeared when a

constant amount of reference sGtH II was added in several dilutions of a plasma from an immature female trout. Measured doses were very close to the theoretical doses, only with a preincubation (Table 1). We did not notice any nonspecific binding in perfusion samples (results not shown).

Statistical Analysis

Intraassay and interassay coefficients of variation have been calculated for biological samples ($n = 6$) around 50% bound, and they were 4.28 and 6.36%, respectively. We must also mention that the 36 outside wells were not found to be suitable for the assay when assayed samples gave about 90 or 10% bound values.

FIG. 4. Effect of incubation times in step 3 on the free sGtH doses necessary to obtain displacements of 50% (■) and 90% (▲). (a) Effect of incubation time in step 3a (Materials and Methods). (b) Effects of incubation time in step 3b, in a procedure with 24 hr of preincubation. (c) Effect of preincubation time in step 3b.

In Vivo Experiments

No fish were found to ovulate between Day 0 and Day 4 after the beginning of the treatments. In the rainbow trout, it is known that plasma GtH levels increase after ovulation (Jalabert and Breton, 1980). After Day 4, in each group, there were both ovulated and unovulated females. For this reason, Fig. 6 only shows the evolutions of the plasma GtH levels during the time for which all the fish were at a similar physiological state.

In all groups, the GtH levels were similar at the beginning of the experiment and not statistically different (3.4 to 5.9 ng/ml). In

the control group, they remained constant over the 4 days. But in all GnRHa-injected groups, the GtH levels increased significantly ($P < 0.005$), although the response varied according to the treatment.

In both native and slow-release forms, [D-Trp⁶]LH-RH induced a similar stimulation of the GtH II secretion, peaking around 30 ng/ml as early as 1 hr after the injection. With both forms, the blood plasma GtH II levels similarly decreased 24 hr after treatment but they did not return to basal values in the group receiving the LH-RH slow-release form, in which they were maintained above 12 ng/ml. There was also the same phenomenon in animals treated

with the microencapsulated [D-Arg⁶,Pro⁹NHEt]sGnRH, although the initial stimulation was not as important.

In all treated groups, the mean blood plasma GtH levels increased again after Day 4, mainly correlated with the presence of ovulated females which ovulated for 80% of the population between Day 4 and Day 7; on the contrary, in the control group, the

occurrence of ovulated females was spread over a longer period.

*Perfused Half Brain/Pituitary
Responsiveness to sGnRH*

Figure 7 represents the evolution of GtH levels (mean $\pm$ SEM, $n = 5$) in the perfusion medium of five experiments. As deter-

FIG. 5. Parallelism between standard curves and plasma sample dilution curves. (a) Displacement curves obtained with reference (Δ) sGtH II, (■) plasma from a rainbow trout at the oocyte maturation stage (mVG+), and (▲) plasma from a rainbow trout at the end of vitellogenesis (VG+), assayed under an equilibrium procedure. (b) Displacement curves obtained with reference (Δ) sGtH II and the same plasma samples as in (a), (■) plasma mVG+, and (▲) plasma VG+, assayed under a nonequilibrium procedure. Plasma dilutions were 1, 1/64; 2, 1/32; 3, 1/16; 4, 1/8; 5, 1/4, and 6, 1/2.

TABLE 1
COMPARISON BETWEEN THE ELISA GtH
MEASUREMENTS AND THE THEORETICAL CONTENT
OF DIFFERENT DILUTIONS OF A PLASMA SAMPLE
FROM AN IMMATURE RAINBOW TROUT

Dilution	Measured dose (pg/well)	Theoretical dose (pg/well)
Equilibrium procedure ^a		
1/85.3	326	334
1/42.6	383	351
1/21.3	467	385
1/10.6	595	453
1/05.3	842	590
1/02.6	1482	862
Nonequilibrium procedure ^b		
1/85.3	241	252
1/42.6	243	254
1/21.3	231	258
1/10.6	282	267
1/05.3	273	284
1/02.6	298	317

Note. A first determination gave 1.45 ng/ml under the equilibrium procedure and 0.18 ng/ml under the nonequilibrium conditions.

^a sGtH (317.5 pg/well) was added for each dilution of the plasma, when assayed under the nonequilibrium procedure. The theoretical dose (TD) is calculated as

$$TD \text{ (pg/well)} = \frac{1.45 \cdot 10^3}{\text{dilution}} + 317.5$$

^b sGtH (250 pg/well) was added for each dilution of the plasma, when assayed under the nonequilibrium procedure. TD is calculated as

$$TD \text{ (pg/well)} = \frac{0.18 \cdot 10^3}{\text{dilution}} + 250.$$

mined by a two-way analysis of variance, GnRH application induced a significant transient increase of the GtH II release ($P < 0.05$). The nonequilibrium procedure allowed us to detect this rapid response to the sGnRH application, despite the low GtH II contents of these samples.

ELISA/RIA Correlation

Plasma GtH concentrations were determined by both ELISA and RIA on 139 samples from animals treated with different analogs of the GnRH (Materials and Methods). A linear regression was calculated

with the RIA dose vs the ELISA dose. We obtained the following equation:

$$RIA \text{ dose} = 1.149 (\text{ELISA dose}) - 0.09 \\ (r^2 = 0.942).$$

We made the same comparison with 27 samples from several perfusions, spreading over a wide range (from 0.5 to 100 ng/ml). Both RIA and ELISA (equilibrium procedure) were performed on those 27 samples. The linear regression, with RIA dose vs ELISA dose, gave the following equation:

$$RIA \text{ dose} = 1.087 (\text{ELISA dose}) + 0.52 \\ r^2 = 0.884).$$

DISCUSSION

sGnRH and analogs of GnRH stimulate *in vivo* and *in vitro* GtH II secretion in salmonids. Microencapsulated or implanted analogs induce a more prolonged stimulation of the GtH II blood plasma levels. All of these observations are in agreement with those of Van der Kraak *et al.* (1983), Crim *et al.* (1983), Weil and Crim (1983), and Weil *et al.* (1986). The main purpose of this study, however, was to compare RIA and ELISA techniques for measuring GtH II levels in biological samples.

Some steps in our ELISA procedure (rPAP, primary antibody, OPD/H₂O₂) showed classical kinetics previously described by Signorella and Hymer (1984) and by Farrington and Hymer (1987), but other steps gave unusual results.

Our data show that it is necessary to wait for 20 min before reading the plate in step 7. As far as we are aware, there have been no papers recommending a delay between H₂SO₄ addition and the plate reading. Acid addition is usually admitted to stop reactions immediately. The modifications in the chromogen properties, appearing after acidification, are slower than normally thought.

The sensitivity and the range of our

ELISA procedure show that biological samples can be assayed under low dilution conditions ($1/2$). In this case (i.e., when using 100- μ l samples), sensitivity (90% bound) is 70 pg/ml sample in the nonequilibrium procedure and 125 pg/ml sample in the equilibrium procedure. Even if the sensitivity of this equilibrium assay is not as high as the one of the nonequilibrium assay, it allows us to measure hormonal concentrations over a wider range and to carry out the entire procedure in 8 hr.

Our study demonstrates another important result: displacement curve parallelism is not an absolute criterion of validity for immunoassays. Indeed, analysis of covariance showed that the dilution curves paral-

leled the standard curves, whatever the procedure was (i.e., equilibrium and nonequilibrium procedures). The other experiment (addition of sGtH in plasma) revealed the equilibrium procedure to be unable to detect the right quantity of hormone. Only the nonequilibrium procedure gave good results. Why did the dilution curves parallel the standard curve in the equilibrium procedure whereas this procedure detected too high values of GtH II? One hypothesis could be that primary antibodies recognized another plasma molecule in the equilibrium procedure and that this molecule can displace antibody binding in a specific-like manner (i.e., with the same slope than for the standard curve). On the other hand,

FIG. 6. Variations of the plasma tGtH II levels with different injections: (■) saline solution (control group); (◆) 20 μ g/kg body weight of [D-Trp⁶]LH-RH; (●) 200 μ g/kg body weight of slow-release form of [D-Trp⁶]LH-RH; and (▲) microencapsulated [D-Arg⁶,Pro⁹NH²Et]sGnRH. J0 is the day of the treatment. Fish were sampled at 0, 1, 3, 6, and 9 hr at J0 and 24 hr (J + 1), 48 hr (J + 2), 72 hr (J + 3), and 96 hr (J + 4) after. Each point is the mean $\pm$ SEM of six values.

in the nonequilibrium procedure, since anti- β GtH affinity may be greater for GtH II than for this other molecule, dissociation kinetics during incubation in step 3 may decrease cross-reactivity and increase specific detection of the gonadotropin.

In vitro experiments (with application of sGnRH to the preparation) as well as *in vivo* experiments (injections or pellet implantation of GnRH-a) allowed us to check the capacity of our procedure to detect a physiological response to a GnRH treatment. These results sustain the assumption that our assay really measures trout gonadotropin. [D-Trp⁶]LH-RH (via native or slow-

release form) evoked an immediate and more important release of GtH II than microencapsulated [D-Arg⁶,Pro⁹ NHet] sGnRH, but these two analogs (except for native [D-Trp⁶]LH-RH) induced similar GtH II levels in the days following the treatment. It is interesting that levels of GtH remained high in trout with the analog containing pellets. This could indicate that a constant high level of GnRH-a does not induce any "desensitization" at the pituitary level as reported by Zohar (1988) for another teleost, *Sparus aurata*. In the same way, continuous perfusion with [D-Ala⁶,Pro⁹ NHet]LHRH induced high

FIG. 7. tGtH II variations in the perfusion medium of a half brain/pituitary preparation. The preparation was stimulated by a 15-mn perfusion of sGnRH (10^{-7} M). The half brain/pituitary was dissected from female rainbow trout at the early stage of vitellogenesis (oocyte diameter <0.5 mm). Minimum detectable doses of both RIA and ELISA are shown on the graph. Each point is the mean $\pm$ SEM of five values.

and constant levels of GtH release from Tilapia (Levavi-Zermansky and Yaron, 1988) and from African catfish (De Leeuw *et al.*, 1986) pituitary fragments.

Our perfusion chamber is specially adapted to the brain/pituitary preparation and allows both local administrations of bioactive molecules in specific hypothalamic nuclei and electrophysiological recordings (Bailhache *et al.*, 1989). The GtH levels reported here are lower than can be seen in literature and must be due to the integrity of the half pituitaries used in these studies. However, the results obtained with our perfusion technique showed that sGnRH is able to induce a twofold transient increase of GtH II release in early vitellogenic trout. This weak pituitary responsiveness is in agreement with previous studies of Weil *et al.* (1978). The minimum detectable dose of each method (RIA and ELISA) is shown in Fig. 7 and clearly shows the value of our method. The optimal RIA sensitivity is 300 pg/ml but, in a majority of cases, this assay allows only about 1 ng/ml of sensitivity. Thus, when perfusion experiments are performed with immature trout pituitaries, an unobserved response to a GnRH treatment may be misunderstood by using the RIA technique. On the other hand, our ELISA procedure is of great interest in view of pulsatility detection and experiments on juvenile fish.

In conclusion, we have developed an ELISA procedure that allows a specific heterologous assay of *S. gairdneri* GtH II from purified salmon gonadotropin. This new reliable assay meets the general quality criteria required for an immunological assay. The good agreement between the GtH II concentrations determined by ELISA and those determined by RIA indicates that this new assay can replace RIA in other ongoing studies since sensitivity is improved (70 pg/ml against 300 pg/ml in RIA), assay characteristics are more repeatable (e.g., no loss of specific activity as in RIA),

and it is performed quicker than RIA (8 to 26 hr against 3 to 5 days).

ACKNOWLEDGMENTS

This work was supported in part by a grant of Ministère de l'Education Nationale, Direction de la Recherche, on the following topic: Control of Pituitary Hormones in Salmonids.

REFERENCES

- Atkinson, L. E., Bhattacharya, A. N., Monroe, S. E., Dierschke, D. J., and Knobil, E. (1970). Effect of gonadectomy on plasma LH concentration in the rhesus monkey. *Endocrinology* 87, 847-849.
- Bailhache, T., Salbert, G., Guillet, J. C., Saligaut, C., Breton, B., and Jegou, P. (1989). A new perfusion method adapted to rainbow trout (*Salmo gairdneri*) brain pituitary preparation for simultaneous electrophysiological recording and hormone secretion determination. *Comp. Biochem. Physiol.* 94A, 305-313.
- Breton, B. (1981). A study of salmon (*Oncorhynchus tshawytscha*) pituitary gonadotropin dissociation. *Gen. Comp. Endocrinol.* 45, 147-152.
- Breton, B., Prunet, P., and Reinaud, P. (1978). Sexual differences in salmon gonadotropin. *Ann. Biol. Anim. Biochim. Biophys.* 18(4), 759-765.
- Breton, B., Zohar, Y., and Sambroni, E. (1984). Dosages radioimmunologiques hétérologues de gonadotropines de type glycoprotéiques maturantes de poissons téléostéens. *Aquaculture* 40, 307-323.
- Crim, L. W., Evans, D. M., and Vichery, B. H. (1983). Manipulation of the seasonal reproductive cycle of the landlocked atlantic salmon (*Salmo salar*) by LHRH analogues administered at various stages of gonadal development. *Canad. J. Fish. Aquat. Sci.* 40, 61-67.
- De Leeuw, R., Goos, H. J. TH., and Van Oordt, P. G. W. J. (1986). The dopaminergic inhibition of the gonadotropin-releasing hormone induced gonadotropin release: An *in vitro* study with fragments and cell suspensions from pituitaries of the African catfish, *Clarias gariepinus* (Burchell). *Gen. Comp. Endocrinol.* 63, 171-177.
- Dierschke, D. J., Bhattacharya, A. N., Atkinson, L. E., and Knobil, E. (1970). Circoral oscillations of plasma levels in the ovariectomized rhesus monkey. *Endocrinology* 87, 850-853.
- Farrington, M. A., and Hymer, W. C. (1987). An enzyme immunoassay for rat growth hormone: Application to the study of growth hormone variants. *Life Sci.* 40, 2479-2488.
- Garnier, D. H., Cotta, Y., and Terqui, M. (1978). Androgen radioimmunoassay in the ram: Results of

- direct plasma testosterone and dehydroepiandrosterone measurement and physiological evaluation. *Ann. Biol. Anim. Biochim. Biophys.* 18(2A), 265-281.
- Jalabert, B., and Breton, B. (1980). Evolution de la gonadotropine plasmatique t-GtH après l'ovulation chez la truite arc-en-ciel (*Salmo gairdneri* R.) et influence de la rétention des ovules. *C.R. Acad. Sci. (Paris) Ser. D* 290, 799-801.
- Knobil, E. (1980). The role of signal pattern in the hypothalamic control of gonadotropin secretion. In "Rhythms and Reproduction" (R. Ortavant and A. Reinberg, Eds.), pp. 73-79. Masson, Paris.
- Levavi-Zermansky, B., and Yaron, Z. (1986). Gonadotropic secretion from tilapia pituitary exposed *in vitro* to GnRH. In "Reproduction in Fish: Basic and Applied Aspects in Endocrinology and Genetics" (INRA, Ed.), Tel-Aviv, Israel, November 10-12, Les Colloques de l'INRA, No. 44, pp. 47-62. Paris.
- Manquez, C., Urios, P., Desfosses, B., Samake, H., Lince, E., Rajdowski, K. M., and Cittonova, N. (1987). Enzyme-linked immunosorbent assay (ELISA) for steroid hormones with polyclonal and monoclonal antibodies: An assay for urinary aldosterone. *Clin. Chem. Acta* 164, 71-82.
- Ortavant, R., Daveau, A., Garnier, D. H., Pelletier, J., De Reviers, M. M., and Terqui, M. (1982). Diurnal variation in release of LH and testosterone in the ram. *J. Reprod. Fertil.* 64, 347-353.
- Signorella, A. P., and Hymer, W. C. (1984). An enzyme-linked immunosorbent assay for rat prolactin. *Anal. Biochem.* 136, 372-381.
- Spearow, J. L., and Trost, B. A. (1987). Development of a sensitive enzyme-linked immunosorbent assay for cattle, sheep, rat and mouse luteinizing hormone. *Biol. Reprod.* 37, 595-605.
- Suzuki, K., Kawauchi, H., and Nagahama, Y. (1988). Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. *Gen. Comp. Endocrinol.* 71, 292-301.
- Ternynck, Th., and Avrameas, S. (1987). Dosages immunoenzymatiques quantitatifs. In "Techniques Immunoenzymatiques" (INSERM, Ed.), Société Française d'Immunologie, pp. 41-58. Paris.
- Van Der Kraak, G., Lin, H. R., Donaldson, E. M., Dye, H. M., and Hunter, G. A. (1983). Effects of LH-RH and des-gly¹⁰[D-Ala⁶]LH-RH-ethylamide on plasma gonadotropin levels and oocyte maturation in adult female coho salmon (*Oncorhynchus kisutch*). *Gen. Comp. Endocrinol.* 49, 470-476.
- Weil, C., and Crim, L. W. (1983). Administration of LHRH analogues in various ways: Effect on the advancement of spermiation in prespawning landlocked salmon, *Salmo salar*. *Aquaculture*. 35, 103-115.
- Weil, C., Billard, R., Breton, B., and Jalabert, B. (1978). Pituitary response to LH-RH at different stages of gametogenesis in the rainbow trout (*Salmo gairdneri*). *Ann. Biol. Anim. Biochim. Biophys.* 4, 863-869.
- Weil, C., Hansen, P., Hyam, D., Le Gac, F., Breton, B., and Crim, L. W. (1986). Use of pituitary cells in primary culture to study the regulation of gonadotropin hormone (GtH) secretion in rainbow trout: Setting up and validating the system as assessed by its responsiveness to mammalian and salmon gonadotropin releasing hormone. *Gen. Comp. Endocrinol.* 62, 202-209.
- Yamamoto, H., and Kato, T. (1986). Enzyme immunoassay for cholecystokinin octapeptide sulfate and its application. *J. Neurochem.* 46, 702-707.
- Zohar, Y. (1986). Gonadotropin releasing hormone in spawning induction in teleosts: Basic and applied considerations. In "Reproduction in Fish: Basic and Applied Aspects in Endocrinology and Genetics" (INRA, Ed.), Tel-Aviv, Israel, November 10-12, Les Colloques de l'INRA, No. 44, pp. 75-79. Paris.
- Zohar, Y., Breton, B., and Fostier, A., (1986). Short-term profiles of plasma gonadotropin and 17 α -hydroxy, 20 β -dihydroprogesterone levels in the female rainbow trout at the periovulatory period. *Gen. Comp. Endocrinol.* 64, 189-198.