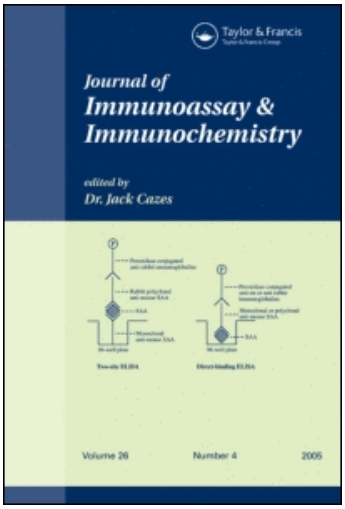

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An Enzyme Immunoassay for Rat Growth Hormone: Validation and Application to the Determination of Plasma Levels and in Vitro Release

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AN ENZYME IMMUNOASSAY FOR RAT GROWTH HORMONE : VALIDATION AND APPLICATION TO THE DETERMINATION OF PLASMA LEVELS AND IN VITRO RELEASE

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ABSTRACT

A competitive enzyme immunoassay for rat growth hormone (rGH) has been developed using polyclonal anti-rGH antibodies and an acetylcholinesterase (EC 3.1.1.7.) enzymatic tracer coupled covalently with rGH. The assay was performed in 96-well microtiter plates coated with rabbit polyclonal anti-goat immunoglobulin antibodies. Molecular sieve filtration and Western blot analysis revealed a single immunoreactive peak for rat plasma or pituitary extracts. Cross-reactivity with other rat pituitary hormones or human GH was less than 1%. Assay of samples in a concentration range of 0.7 to 69 ng/ml by enzyme immunoassay and radioimmunoassay were well correlated (r=0.87 and 0.85 respectively for plasma and culture medium samples). Intra- and inter-assay variations in plasma were 4 (n=24) and 14% (n=9) respectively. Minimal detectable amounts of rGH were 0,6 ng/ml. A two-site immunometric assay also developed with the same antibodies allowed a detection threshold of 0.25 ng/ml. (KEY WORDS : rat growth hormone, enzyme, immunoassay, immunometric assay, acetylcholinesterase, plasma).

INTRODUCTION

Growth Hormone (GH), a polypeptide hormone with a molecular weight of 23000 released from somatotropes of the anterior pituitary, is regulated by several neurotransmitters and neuropeptides (1). Among other functions it plays an essential role in regulating body growth.

Over 200 annual publications report experiments on the regulation of GH secretion in the rat. Most of them rely on rat GH (rGH) radioimmunoassays, the most common being that provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). Radioimmunoassays however present a few drawbacks : their duration usually exceeds three days, and they require frequent preparation and purification of radioiodinated hormone involving relatively high radioactivities.

In recent years, our laboratory has developed sensitive immunoassays for drugs, peptides or proteins based on enzymatic conjugates between haptens or proteins and acetylcholinesterase (AChE) from *Electophorus electricus* (2). We developed for instance a highly sensitive and easy to perform enzyme immunoassay for rat prolactin (3).

In the present paper we report development of an enzyme immunoassay (EIA) for rat growth hormone based on a competition method using rGH labeled with AChE and specific anti-rGH antibodies. Validation of the assay was achieved for both culture media and plasma samples, by checking the parallelism of curves obtained in different biological fluids and comparing the data with those of a

conventional radioimmunoassay. In addition, a non-competitive assay based on a "sandwich" or immunometric method was also developed ; in that case the same antibody served as complexant and as tracer, and a slighly higher sensitivity could be achieved.

MATERIALS AND METHODS

Reagents and Materials

rGH and pituitary hormones used for cross-reaction studies were from UCB Bioproducts (Nanterre, France). rGH was calibrated against rGH-RP2 from the NIDDK (Bethesda, USA). Rat prolactin standard RP3 was gift from the NIDDK and recombinant prolactin was obtained in our laboratory (4). Standard chemicals were from Merck (Darmstadt, Germany) or Sigma (Saint-Louis, MO, USA). Normal goat serum was from Institut Pasteur (Paris, France). Assays were performed in 96-well microtiter plates (Immuno-NUNC Maxisorp) from Nunc (Denmark). Microtitration was performed using a microtiter plate washer 120 and a Multiskan MCC from Titertek Flow (Helsinki, Finland). Acetylcholinesterase (AChE) (E.C.3.1.1.7) was extracted from the electric organ of eels (*Electrophorus electricus*) and purified by one-step affinity chromatography as described elsewhere (5). Use of the G4 form (6) of the enzyme for synthesis of enzymatic tracers used in enzyme immunoassays has been patented by our laboratory (7). The purified enzyme is available from SPI-BIO (Saclay, France). Enzyme activities were measured using Ellman's reagent, an AChE substrate containing 2.2 g acetylthiocholine and 1 g dithionitrobenzene in 200 ml 0.05 M

phosphate buffer (PB) at pH 7.4. Anti-rabbit immunoglobulins conjugated to alkaline phosphatase (AP), biotinylated molecular weight markers were purchased from Sigma (St Louis, USA) and Streptavidin AP from Amersham France.

Immunogen Preparation and Immunization

rGH used for immunization was purified from rat pituitaries at Pasteur Institute (Paris, France) by M.M. Gabellec. The immunogen (5 mg) was emulsified in 1 ml apyrogen 0.9% NaCl solution with 1 ml Freund's complete adjuvant and injected intradermally at forty sites on the back of an adult goat (n°520). One ml perthydral was injected subcutaneously in order to enhance the immune reaction. Booster injections were repeated every three months for 12 months. For each booster injection 0.5 mg immunogen suspension, in 0.5ml apyrogen 0.9% NaCl solution and 0.5 ml Freund's complete adjuvant, was administered intradermally at four sites and intramuscularly at one site. Twenty-four hours later, a second intravenous injection of 0.5 mg rGH in 3 ml of apyrogen 0.9% NaCl solution was performed. Goat was bled the first, the second and the third week after each booster injection. After centrifugation sera were stored in 0.01% sodium azide (w/v) at 4°C. Antiserum 520.3 was used to develop a competitive EIA. After purification, it is also used to coat plates and to prepare Fab for the enzymatic tracer, in a non-competitive EIA.

Synthesis of the Enzymatic Tracer rGH-AChE

The enzymatic tracer was obtained by conjugation of rGH to acetylcholinesterase (AChE). Thiol groups were first introduced into

the polypeptide by mixing rGH (125 mg) with S-succinimidyl-S-acetyl-thioacetate (1 mmol) at pH 8.5 in 0.1 M borate buffer. The mixture was allowed to react for 30 min at 30°C and thioester groups were hydrolyzed by addition of 0.2 ml 1 M hydroxylamine. Thiolated rGH was isolated from excess thiol reagents by chromatography on a Sephadex column (1x10 cm). Elution was performed with 0.1 M PB pH 6 containing 5 mM EDTA. The thiol-containing rGH (5 nmol) was mixed with AChE (1 nmol) previously activated with N-succinimidyl-4(N-maleimidomethyl) cyclohexane 1-carboxylate (SMCC), as described elsewhere (3), and incubated for 16 h at 4°C. The enzymatic tracer was purified by molecular sieve chromatography using a Biogel A (90x1.5 cm) 1.5 m column (Biorad, Paris, France) eluted with EIA buffer. Fractions (2 ml) were collected and the peak corresponding to AChE activity was stored at -20°C.

Antibody Purification for the Non-competitive EIA

Anti-rGH antiserum (3 ml) was diluted with 0.05 M acetate buffer pH 7.4 and the pH adjusted to 4.6 with 1 M HCl. Caprylic acid (7.5 M, 0.55 ml) was added and the mixture incubated for 1 h at room temperature and then centrifuged for 1 h at 2,000 g (8). The supernatant was dialyzed against 0.05 M PB pH 7.4 and the dialysate containing the immunoglobulin G (IgG) fraction of the antiserum used as the complexing antibody in the sandwich EIA. (Fab')2 anti-rGH IgGs were obtained by applying ficin (30 ml of 24 mg/ml solution) for 1 hour at 37°C (9). The reaction was stopped by addition of 0.1 ml of 0.01 M N-ethyl-maleimide. The mixture was filtered through an AcA 44 gel

(Pharmacia, St-Quentin en Yvelines) in 0.1M PB pH 6.4 containing 10^{-3} M EDTA. Fractions corresponding to the protein were lyophylized and dissolved in 2 ml of water. Disulfur bridges were reduced by addition of 0.2 ml 0.02 M ß-mercaptoethanol for 1 hour at 37°C. Fab fragments were purified by filtration on a Sephadex G25 column (1x25 cm)(Pharmacia), eluted with 0.1 M PB pH 6 containing 5 mM EDTA and reacted with AChE (0.2 nmol) previously activated with SMCC as described elsewhere (3), and incubated for 16 h at 4°C. The enzymatic tracer was purified by molecular sieve chromatography on a Biogel A 1.5 m column (90x1.5 cm) eluted with 0.01 M Tris buffer pH 7.4 containing 1 M NaCl, 0.01 M MgCl2 and 0.01% sodium azide. Fractions (2 ml) corresponding to AChE coupled to the antibody were stored at -20°C in 1 ml aliquots. The enzymatic tracer was used at a concentration of 10 Ellman units (EU). One EU is defined as the concentration of enzyme producing an absorbance increase of 1 during 1 min in 1 ml substrate medium for an optical path length of 1 cm. It corresponds to about 8 ng enzyme.

Competitive EIA

All assays were performed in EIA phosphate buffer (0.1 M at pH 7.4) containing 0.15 M NaCl, 0.001 M EDTA, 0.1% BSA, 0.01% sodium azide and 0.2% Triton X100. The 96-well microtiter plates were coated with rabbit polyclonal antibodies specific for goat IgG (Immunotech, Marseille, France) at a concentration of 10 µg/ml for one night at room temperature and saturated in the EIA buffer without Triton X-100. Before use, plates were extensively washed with 0.01 M PB pH 7.4

containing 0.1% Tween 20. The assay was performed in a total volume of 150 µl. Plasma and culture medium samples were diluted in EIA buffer. Reagents were dispensed as follows : 50 µl sample or standard (rGH from UCB Bioproduct calibrated against the NIDDK standard rGH-RP2), and 50 µl rGH antiserum (diluted 1/50.000). After a 20 hr incubation at room temperature 50 µl enzymatic tracer (0.25 EU/ml) was added for a further 20 hr incubation at room temperature. Plates were then washed again as described above and Ellman's reagent (200 µl) was distributed into each well and incubated 3 hours. Absorbance was measured with a spectrophotometer at 414 nm. Unknown concentrations were calculated from a model standard curve using a Log-logit plot (Assay Zap, Biosoft). All measurements for standards and samples were made in duplicate.

Non-competitive EIA

Microtiter plates were coated with acid caprylic-purified antibodies (10 µg/ml) in 0.05 M PB pH 7.4. After a 16 hr incubation at room temperature, the plates were washed and saturated with EIA buffer for 24 hr at room temperature. Before use, the buffer was discarded and the plates were incubated with varying concentrations of rGH standard (0 to 2500 pg/ml in EIA buffer) or with rat plasma samples. After a 16 hr incubation at 4°C, the plates were washed and 0.1 ml enzymatic tracer (10 EU/ml) diluted in EIA buffer in the presence of 10% normal goat serum. Plates were left for 18 hr at 20°C and the enzymatic activity bound to the solid phase was measured as described above for competitive EIA. Unknown concentrations were calculated from a model standard curve using a Spline function (Immunofit

software from Beckman, Gagny, France). All measurements for standards and samples were made in duplicate.

Competitive EIA Validation

The specificity of the antibodies was studied by measuring cross-reaction of various rat pituitary hormones (prolactin (PRL), recombinant PRL, follicule stimulating hormone (FSH), thyrotropin stimulating hormone (TSH) and luteinizing hormone (LH)) and of mouse and human GH at concentrations comprised between 10 and 1000 ng/ml. Cross-reactivity was calculated as the ratio (IC 50 of the rGH curve) over (IC 50 of the tested hormone), IC 50 being the mass of hormone that induces a 50% decrease of tracer binding. Possible interference of matrix components was tested by measuring the apparent immunoreactivity of hypophysectomized rat plasma or of heterospecific plasma (human, monkey, dog and rabbit). The absence of interference from higher molecular weight component was demonstrated by measuring the immunoreactivity of rat plasma (1 ml) fractionated by chromatography on an AcA 44 column (70x1cm). Elution was performed with EIA buffer and the immunoreactivity of each fraction was measured by the competitive EIA.

Rat pituitary extracts or plasma from intact or hypophysectomized rat as well as the NIDDK GH standard, were subjected to Western blot analysis as described elsewhere (10). rGH antiserum at a dilution of 1/100 was used as the probe. Alkaline phosphatase (AP) was used to visualize rGH immunoreactive bands. Molecular weight markers were biotinylated and reacted with Streptavidin AP.

Intra-assay variations were estimated by measuring the coefficient of variation of 3 rat plasma samples assayed eight times. Inter-assay variations were measured by assaying 1 sample in nine independent assays. Accuracy was tested by recovery experiments with rat plasma spiked with rGH at different concentrations. Reliability of the assay was investigated by correlating RIA and EIA data from the same samples. Culture medium conditioned by incubation for 24 hr with GC tumor derived rat pituitary cells (11), as well as female rat plasma sampled at random and containing various concentrations of endogenous hormone (between 10 and 200 ng/ml), were run in parallel by EIA and RIA using the NIDDK kit (12). In addition, episodic peaks characteristic of GH secretion were evaluated by both assays on plasma samples every 15 mn over a period of 6 hours from cannulated, free moving rats prepared as previously described (12).

Non-competitive EIA Validation

Since antibody specificity and reproducibility of the microplate technology were already assessed during validation of the competive EIA, validation of the non-competitive assay mainly focused on the presence of non-specific interferences liable to yield false positives in for rGH-free plasma.

RESULTS

Specificity of the anti-rGH antibodies used for the competitive EIA was demonstrated by Western blot analysis (Fig. 1). The specificity of the EIA was further checked by comparing standard curves obtained

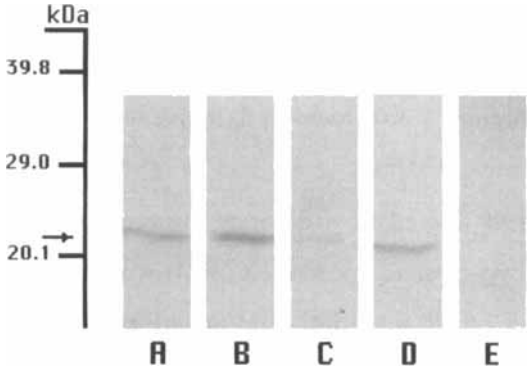

Figure 1 : Western blot analysis of rat pituitary extract (lane A, B), rGH-RP2 standard (lane C), and plasma from a normal (lane D) or an hypophysectomized rat (lane E). Molecular mass markers are indicated: 39.8 K, alcohol dehydrogenase; 29 K carbonic anhydrase; 20.1 K trypsin inhibitor.

with rat GH and other pituitary hormones (rPRL, rrPRL [recombinant rat prolactin], rLH, rFSH). Rat prolactin (standard RP3 from the NIDDK) and rFSH showed weak or very weak cross-reactivities (<1% and <0.1% respectively); this was not observed with rrPRL (<0.01%) (Fig. 2). Other rat pituitary hormones (rFSH, rTSH, rLH) or human GH did not cross react with the anti-rGH antibody. In contrast, a 91% cross reactivity of mouse GH (mGH) with respect to the rat standard was observed in the assay (Fig. 2).

Rat plasma fractionated on gel filtration revealed only one immunoreactive peak co-eluting with rGH (Fig. 3).

Preliminary experiments permitted to define optimal parameters of sensitivity and duration of the rGH competitive EIA. As indicated under Material and Methods, Figure 4 shows the standard curve obtained under these conditions. A mean IC 50 value of 3.7±0.3 ng/ml

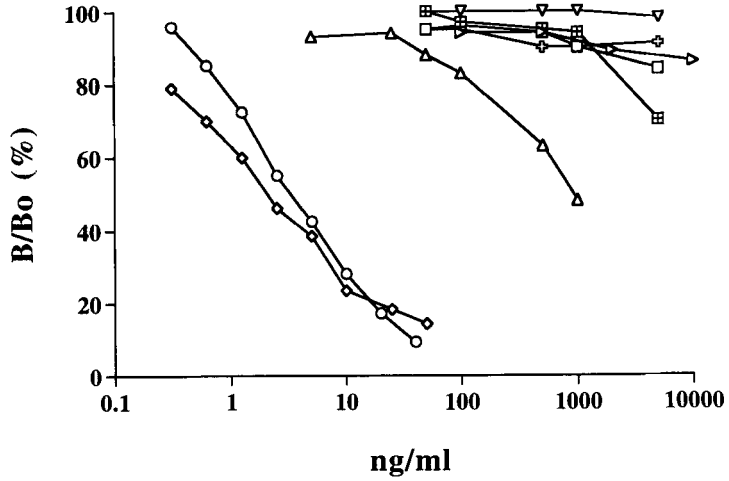

Figure 2 : Standard curve of rGH measured by EIA (o). Cross reactivities of anti rGH with other rat pituitary hormones (rPRL (Δ), rFSH (⊞), rTSH (□), rLH (∇), rrPRL (▷)), human GH (+) and mouse GH (◊).

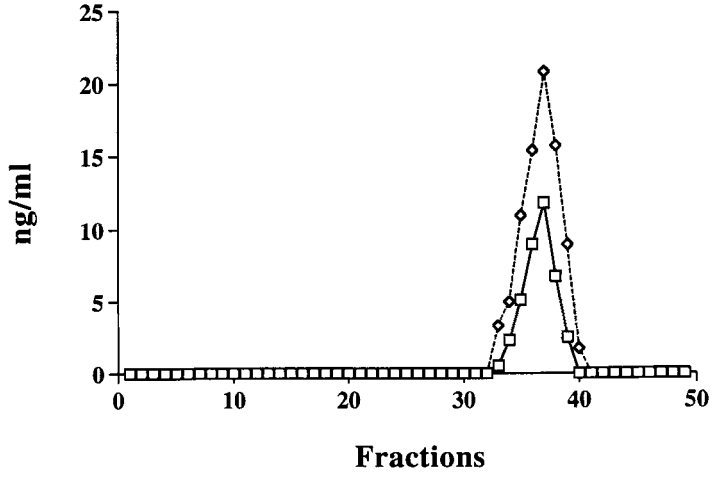

Figure 3 : Immunoreactive profile of rGH standard (□) and rat plasma (◊) after filtration on AcA 44.

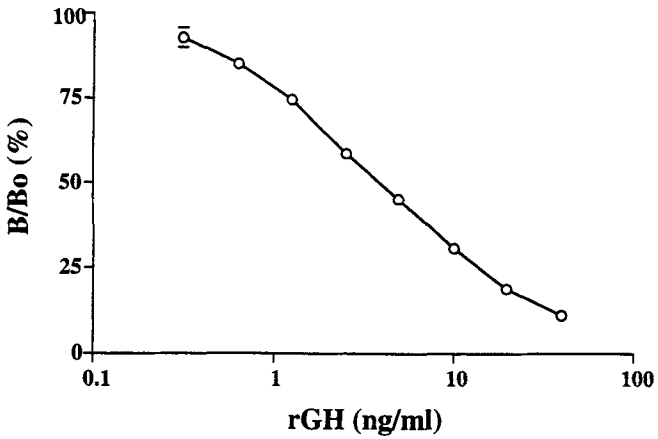

Figure 4 : Standard curve of the rGH competitive EIA. Each point represents the mean ± standard deviation of 4 independant experiments. Bars indicate standard deviation; when non visible, these bars are included in the symbol.

(n=11) was observed. The detection limit (concentration producing 15% displacement of initial tracer) was 0.6 ng/ml. Intra- and inter-assay coefficients of variation were 4 (n=24) and 14% (n=9). In presence of Triton X-100, addition of plasma from hypophysectomized rats did not modify the standard curve.

Anticoagulant agents as EDTA, lithium heparine, potassium oxalate or sodium citrate did not interfere with the assay (data not shown).

In order to validate the EIA, correlation with data obtained by RIA was investigated in two series of samples: culture medium of GC tumor derived pituitary cells and normal rat plasma collected every 15 mn over a period of 6 hr to monitor episodic bursts of the hormone. The data presented in Figs 5 and 6 point out to a fair correlation of both

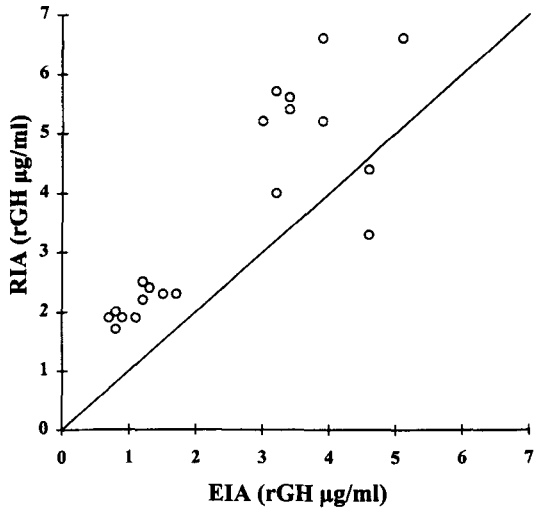

Figure 5 : Correlation between data obtained by competitive EIA and RIA. rGH was measured in culture media of GC tumor derived pituitary cells. (RIA=1.175+1.002 EIA; n=20; r=0.853, $p<0.001$); the line represents the theoritical perfect agreement between the two methods.

assay methods in all cases, with correlation coefficients ranging from 0.85 to 0.87. Comparison of ultradian rGH fluctuations indicates that profiles obtained by both methods are fairly well correlated (Fig. 6). On the other hand, addition of increasing amounts of exogenous rGH to plasma from normal and hypophysectomized rats or addition of same amounts of exogenous rGH to culture media allowed satisfactory recovery of the added hormone (Tables 1 and 2).

In order to increase assay sensitivity a sandwich (i.e. non competitive) EIA was developed. After optimizing reagent concentration and incubation conditions, a standard curve with a detection limit of 0.25 ng/ml was obtained (Fig.7). Measurements of

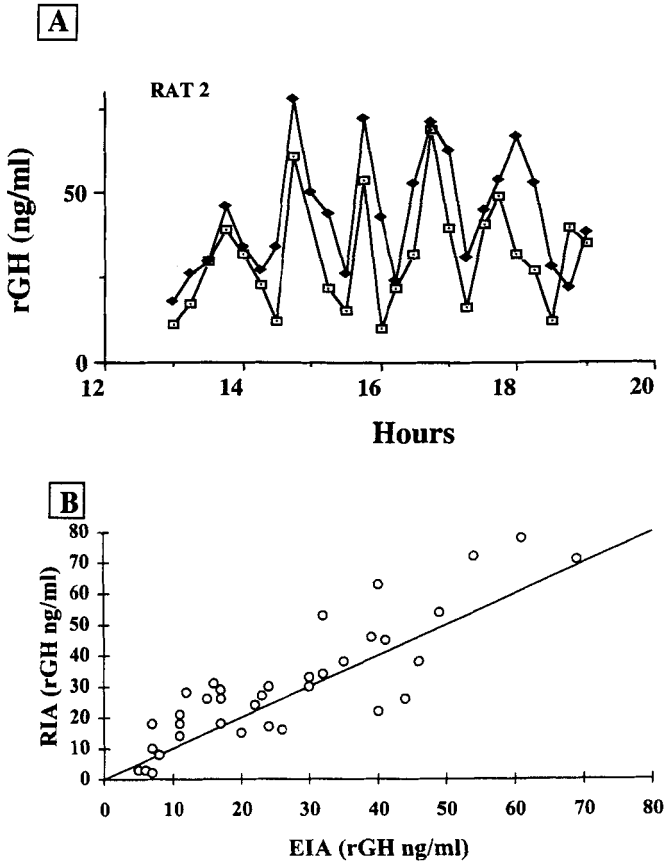

Figure 6 : Correlation between data obtained by competitive EIA and RIA. Assays were performed on sequential plasma samples collected from adult female rats every 15 min over a period of 6 hours.

A : Individual GH secretory profil. GH plasma concentrations were measured by EIA (▣) or RIA (◆).

B : Correlation between EIA and RIA data for female rat plasma samples. (RIA= 3.543 + 1.012 EIA; n=36, r=0.868, $p<0.001$); the line represents the theoritical perfect agreement between the two methods.

TABLE 1

Recovery of exogenous rGH added to rat plasma samples.

Exogenous rGH (ng/ml) added to the sample	0	0.6	1.2	2.5	5	10	20	Average recovery of exogenous hormone (%)
HRP								
Th. Conc.	0	0.6	1.2	2.5	5	10	20	102 ±12
Act. Meas.	0	0.5	1.3	2.5	5	10.1	24.4	
NRP 1/20								
Th. Conc.	1.5	2.1	2.7	4.0	6.5	11.5	21.5	91 ±11
Act. Meas.	1.4	1.9	3.1	4.0	5.9	11.0	17.8	
NRP 1/5								
Th. Conc.	6.0	6.6	7.2	8.5	11.0	16.0	26	116 ±7
Act. Meas.	5.9	7.1	8.9	10.4	13.4	17.8	28.8	

Th. Conc. : Theoretical Concentration
Act. Meas : Actual Measurement

Increasing amounts of rGH were added to 10 µl hypophysectomised rat plasma (HRP) and to 10 µl normal rat plasma (NRP) previously found by RIA to contain 30 ng/ml endogenous GH and diluted 1/5 and 1/20. Recovery was calculated as the ratio x 100 of (amount estimated by the EIA-endogenous hormone) over (theoretical amount of hormone in the sample).

TABLE 2

Recovery of exogenous rGH added to culture media samples.

Sample number	C	1	2	3	4	5	6	Average recovery of exogenous hormone (%)
Th. Conc.	8	14.1	12.9	12.7	3.8	13.3	12.5	103±4
Act. Meas.	9.6	14.8	13.7	13.5	13.3	13.9	12.7	

Th. Conc. : Theoretical Concentration
Act. Meas : Actual Measurement

Eight ng exogenous rGH of a control solution (C) and 2.5 µl of six different culture media of GC tumor derived pituitary cells were diluted to 1ml. Recovery was calculated as the ratio x 100 of (amount estimated by the EIA-endogenous hormone) over (theoretical amount of hormone in the sample).

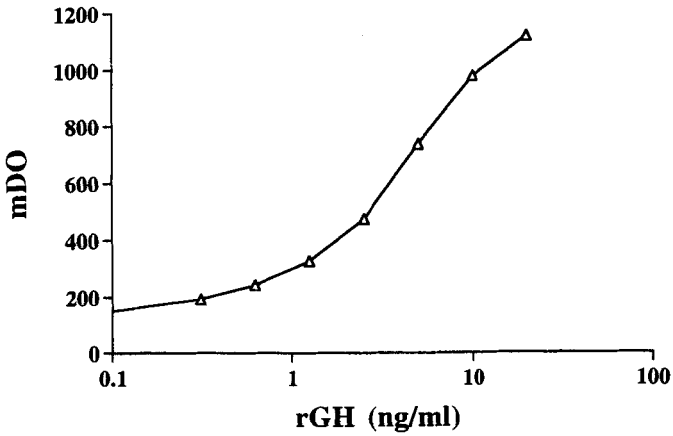

Figure 7 : Standard curve of the non-competitive rGH EIA.

rGH-free plasma from hypophysectomized rats or from other species revealed the existence of high apparent immunoreactivity unrelated to the presence of rGH. Addition of 10% normal goat serum to the tracer was effective in completely eliminating these false positive immunoreactivities.

DISCUSSION

Although radioimmunoassays are familiar to most endocrinologists, alternate techniques as enzyme immunoassays present potential advantages ; they do not require radioactive sources and are quite satisfactory in terms of tracer stability, safety, easy automation and low sample volumes. Following current trends tending to substitute radioactive by enzyme labels for immunoassays (13), the present work indicates that the novel rGH EIA satisfies criteria of sensitivity and specificity and thus qualifies as a substitute to rGH radioimmunoassays.

Assessment of non-specific interferences is a major problem in immunoassay development. Such interferences are usually accounted for by binding proteins, enzymes or lipids contained in plasma samples, and generate apparent, false positive immunoreactivities (14). Immunoassays for growth hormone are particularly sensitive to interferences, since plasma samples are known to contain important concentrations of growth hormone binding proteins (GHBP) (15), a moiety which corresponds to the extracellular domain of the growth hormone receptor released into the general circulation (16). As a consequence of this, most GH radioimmunoassay methods require either prior precipitation of larger proteins, or addition to the standard

curve of plasma from hypophysectomized animals. In the latter case bound GH is likely to precipitate along with GHBP, so that assays mainly measure free forms of the hormone.

Under our experimental conditions, recovery of exogenous rGH from plasma samples was satisfactory ; yields indicates that the assay is reliable. Under these conditions, EIA may evaluate preferentially free rGH plasma levels in the presence of detergents in immunoassays (17), in contrast to radioimmunoassays which evaluate total amount of the hormone. This may account for the slight underevaluation of high rGH plasma concentrations by the EIA with respect to the RIA (fig.6), in agreement with previous conclusions by Leung et al (15) on the influence of binding proteins. Nevertheless, profiles obtained by both methods are strictly homothetic. In addition, the good correspondence between measured EIA values and theoretical estimations, when exogenous rGH was added to plasma or medium samples (Tables 1 and 2), further subtantiates the validity of the EIA. This statement however may not be valid beyond five-fold dilutions of plasma, as suggested by

The competitive EIA was developed in agreement with specific recommendations for immunoassay validation (18). Specificity was demonstrated by the absence of cross-reactivity with other pituitary hormones or with rGH-related compounds (glycosylated and polymeric forms, data not shown). Nevertheless weak or very weak (over three orders of magnitude) cross-reactivities was observed with rat prolactin RP3 (NIDDK) and rat FSH, but not with rat recombinant prolactin, thus suggesting that cross-reactivity is due to rGH contamination of the purified hormone standards.

In contrast, recognition of mGH in the EIA-rGH assay was very satisfactory (91% cross-reaction), suggesting that the assay can be applied to interspecific evaluation of mGH, an attractive perspective in view of the increasing development of new transgenic mice models.

Values of repeatability (intra-assay precision) and reproducibility (inter-assay precision) are consistent with those reported for other immunoassays (coefficient of variation usually below 15%). Recovery of rGH added to rat plasma and correlation with the radioimmunoassay from the NIDDK fulfills accuracy requirements. In spite of slight, barely significant differences, comparison of rGH secretion profiles during a 6 hr sampling period shows that data obtained by EIA and RIA generate almost superimposable curves.

Studies on *in vitro* or *in vivo* modulation of rGH release require detection of low levels (below 1 ng/ml) of the hormone, in particular during episodic troughs, a condition under which the hormone is usually undetected by either radioimmunoassays or competitive EIA. Since the sensitivity of the competitive immunoassay is limited by the specific activity of the tracer and the affinity of the antibody (19), we also developed a non-competitive (sandwich type) immunoassay with the same antibodies. This procedure previously applied to a bovine prolactin assay permitted to obtain a detection limit of 0.1 ng/ml (20).

A frequent problem with this assay format however stems from plasma components which may bind both capture and labelled antibody, thus leading to artificially elevated concentrations. Addition of normal goat serum to the tracer preparation made it possible to eliminate such interferences, since most interfering components are

heterophilic antibodies (21). With a detection limit of 0.25 ng/ml, the resulting assay was three-fold more sensitive than the competitive assay, a property which renders it attractive for studies involving very low levels of plasma rGH. Its use is however limited by the fact that it requires large amounts of purified immunoglobulins.

In conclusion, the enzyme immunoassays presented here offer new reliable tools for rGH assessment in biological fluids. The competitive EIA is comparable to available rGH RIAs in terms of assay performance, but offers practical advantages, such as the possibility to use reduced sample volumes (10 µl) and to rely on stable tracers and easy manipulation owing to microplate technology. The non competitive assay based on related reagents makes it possible to increase the sensitivity of the competitive enzyme immunoassay if needed.

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