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Quantitation of human chorionic gonadotrophin (hCG) by radioreceptor assay

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Abstract: A sensitive radioreceptor assay was developed for pharmaceutical preparations of human chorionic gonadotrophin with the use of rat testicular membranes as receptor preparation and human ^{125}I-chorionic gonadotrophin as tracer. The addition of unlabelled human chorionic gonadotrophin or luteinizing hormone inhibited the binding of ^{125}I-chorionic gonadotrophin to the receptors in a concentration dependent way. Concentrations of human chorionic gonadotrophin between 30–300 mIU ml^{-1} were normally used for a three-dose assay fulfilling pharmacopoeial statistical requirements for assay validity. The relative standard deviation for five assays was 7%. Estimates of potency of commercial preparations of human chorionic gonadotrophin obtained with the radioreceptor assay correlated well with corresponding estimates from *in vivo* assays. The proposed radioreceptor assay, however, provides a considerable saving in the number of animals required, requires less technical support, and is more precise than the *in vivo* method.

Keywords: *Chorionic gonadotrophin; rat testicular membranes; radioreceptor bioassay;* in vitro/in vivo *correlation.*

Introduction

Pharmaceutical preparations of gonadotrophins are declared in international units. To control their potencies, conventional rat *in vivo* assays are used. According to the European Pharmacopoeia [1], assays of chorionic gonadotrophins are performed using the rat seminal vesicle weight method, whereas preparations of human menopausal gonadotrophin (HMG) are assayed for follicle stimulating (FSH) and luteinizing (LH) activity measuring effects on weights of ovaries and seminal glands, respectively. These established *in vivo* assays require for each potency determination 50–90 animals which are dosed for a week, demanding the work of several technicians. Thus, *in vitro* assays for potency readings of gonadotrophins, which fulfil the pharmacopoeial requirements for validity would be of great value for control laboratories in regulatory agencies as well as industry. We have previously described such assays for cholecystokinin [2], glucagon [3], insulin [4, 5] and human growth hormone [6].

In the present study, binding of chorionic gonadotrophins to specific receptors on a crude preparation of rat testicular membranes as described by Catt *et al.* [7–9] has been characterized. A radioreceptor assay for human chorionic gonadotrophin (hCG) has been developed using a design fulfilling pharmacopoeial requirements. The *in vitro* assay has been validated against an established *in vivo* method.

Materials and Methods

Male Sprague-Dawley rats were obtained from Alab (Sollentuna, Sweden). All chemicals were of reagent grade. ^{125}I human chorionic gonadotrophin with specific activity of 3.0×10^6–3.7×10^6 Bq μg^{-1} was from New England Nuclear (Dreieich, FRG). The international standard used was the 2nd standard for hCG for bioassay containing 5300 IU per ampoule, corresponding to 2180 IU mg^{-1} bulk powder. The following international standards and reference preparations were also used. The 1st Reference Preparations of chorionic gonadotrophin, alpha and beta subunits, respectively, human, for immunoassay; 2nd Reference Preparation of pituitary FSH and LH, human for bioassay; 1st Standards of pituitary LH, alpha and beta subunits, respectively, human; 2nd Standard of serum gonadotrophin (PMSG), equine, for bioassay; 2nd Reference Preparation of thyroid stimulating hormone (TSH), human, for immunoassay and bioassay. They were kindly supplied by the

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WHO laboratory at the National Institute of Biological Standards and Control (London, UK). Commercial preparations of chorionic gonadotrophin were Gonadex from Leo (Helsingborg, Sweden), Pregnyl from Organon (Oss, Holland) and Profasi from Serono (Geneva, Switzerland). A menopausal gonadotrophin preparation, Humegon, from Organon and a preparation of urinary FSH, Fertinorm, from Serono were also used. Biosynthetic human growth hormone (HGH), Genotropin, was obtained from Kabi (Stockholm, Sweden) and human insulin, Humulin, from Eli Lilly (Indianapolis, IN).

Preparations of testicular membranes

The method described by Catt *et al.* [9] was followed with some modifications. A rat weighing around 300 g was killed by a blow to the head. The testicles were removed, decapsulated and homogenized in phosphate buffered saline (PBS) with the following composition per litre of buffer: NaCl 8 g, KCl 0.2 g, $CaCl_2$ 0.1 g, $MgCl_2$ 0.1 g, K_2HPO_4 0.2 g, and Na_2HPO_4 0.9 g, and with 0.1% bovine serum albumin (BSA) at pH 7.5. The homogenization was carried out either for 10 s by a Polytron homogenizer (System Technik, Ruschlikon, Switzerland) set at position 3.5 or by 7 strokes in a Teflon-glass homogenizer. The homogenate was centrifuged at 100 ***g*** for 20 min at 4°C in a Beckman J6 centrifuge (Beckman Instruments, Palo Alto, CA). The supernatant was aspirated and centrifuged at 20,000 ***g*** for 30 min at 4°C in a Sorvall RC-2B centrifuge (Sorvall Instruments, Wilmington, DE). In some experiments, the second centrifugation was carried out at 6000 ***g*** in a J6 centrifuge. The pellet was suspended in 14 ml of PBS. The protein concentration of the suspension was determined by the method of Lowry *et al.* [10]. Normally, membrane protein concentration in the incubation medium was 1.5–2.0 mg ml^{-1}.

Binding studies

Approximately 2×10^{-11} M of ^{125}I-hCG was incubated with testicular membranes with and without specified concentrations of unlabelled hormone (hCG or various preparations of hormones or subunits) at 22°C when not otherwise stated. Two 200 μl samples of the incubation suspension were withdrawn and placed on top of a layer of ice-cold PBS in 500 μl microfuge tubes and centrifuged at 9000 ***g*** in a Beckman microcentrifuge type 12 for 1 min. The pellets were washed four times with PBS and counted in a Packard 800 C gamma counter (Packard Instrument Co., Downers Grove, IL). The total radioactivity of the medium was determined by counting 200 μl samples of the incubation suspension.

Results from experiments with varying amounts of unlabelled hCG added to the incubation medium were analysed by a computer program for Scatchard analysis using an algorithm for least-squares curve fitting with objective measurement of goodness of fit [11]. With the computer program, binding parameters for ligand-binding site data during apparent equilibrium conditions were estimated.

In separate experiments, dissociation of membrane bound ^{125}I-hCG was studied. After an initial incubation period of 18 h at 22°C, membranes were centrifuged at 20,000 ***g*** in a Sorvall RC-2B centrifuge for 15 min, resuspended in fresh buffer and centrifuged once more at the same conditions. After resuspension, 200 μl samples were withdrawn and treated as above.

In radioreceptor assays, three serial dilutions of unlabelled hCG were chosen from the linear portion of the ^{125}I-hCG binding curve. In most experiments, the concentrations ranged from 30 to 300 mIU ml^{-1}. In three identical incubation series, 370 μl of membrane suspension was incubated with 185 μl of ^{125}I-hCG solution and 370 μl of PBS alone or with three specified concentrations of standard and test preparation of hCG for 18 h at 22°C. Two tubes with an excess of unlabelled hCG (60 IU ml^{-1}) were also included in each series in order to define unspecific binding. The specific binding was obtained by subtracting unspecific binding from total binding. Total binding was on average 17.7 ± 1.2% (SE) and unspecific binding 1.5 ± 0.2% (SE) of added radioactivity. Results from the three separate series of incubations carried out in each experiment, were used for calculation of the relative potency of the test preparation in comparison with the standard preparation, with the log concentration of unlabelled ligand as the dose and the degree of specific binding as the response.

In vivo *assay of hCG*

Three or four doses of standard and test preparation of hCG in geometric progression

were given to 8–10 groups of six or eight immature male rats, 19–28 days old. A control group was treated with solvent alone. Subcutaneous injections were administered on 4 consecutive days. About 24 h after the last injection, animals were killed and the seminal vesicles removed and weighed. The mass of the vesicles were used as responses for calculation of the relative potency of the test preparation in comparison with the standard preparation [1].

Statistical analysis of data

The potency of various test preparations in relation to a standard preparation was calculated by analysis of variance for a three-dose, or in some experiments a two-dose, assay according to pharmacopoeial rules [12]. The statistical weight is the reciprocal of the variance of the $\log_{10}$ potency estimate [12]. Index of precision is calculated by dividing the standard deviation of the responses by the slope of the log dose–response relationship [13]. The weighted log potency estimates were used for combination of results from separate assays, after testing for homogeneity [12].

Results

The time-course for ^{125}I-hCG binding to rat testicular membranes is shown in Fig. 1. Maximal binding was obtained after around 20 h of incubation at 22°C. The amount bound was related to the membrane concentration of the incubation medium (Fig. 1).

Binding of ^{125}I-hCG to testicular membranes was reversible, since bound ^{125}I-hCG, after an incubation period of 18 h dissociated to more than 50% after two washes with alternate centrifugation of membranes and resuspension in fresh buffer (data not shown).

Addition of increasing concentrations of unlabelled hCG inhibited tracer binding in a dose-related way (Fig. 2). In the concentration range from 30 to 300 mIU ml^{-1} corresponding approximately to 2–20 ng of pure hCG per ml [7, 14], there was a linear relationship between concentration of unlabelled hCG and inhibition of tracer binding. Therefore, normally concentrations of 30–300 mIU ml^{-1} of unlabelled hCG were used in the radioreceptor assay (Fig. 3).

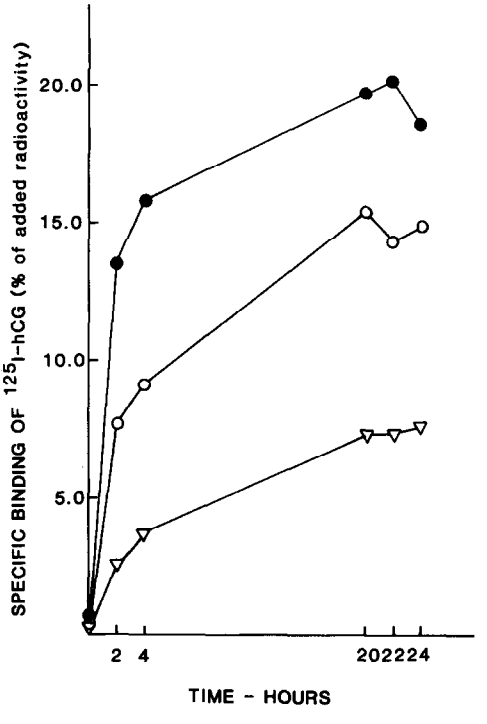

Figure 1
Time course of specific binding of ^{125}I-hCG to rat testicular membranes. Membranes [protein concentration 0.6 mg ml^{-1} (▽) 1.2 mg ml^{-1} (○) 2.4 mg ml^{-1} (●)] were incubated with approximately 2 × 10^{-11} M of ^{125}I-hCG in phosphate buffered saline with 0.1% BSA at pH 7.5 and 22°C. In separate tubes, an excess concentration of hCG (60 IU ml^{-1}) were added for determination of unspecific binding. Each point represents the mean of two separate experiments.

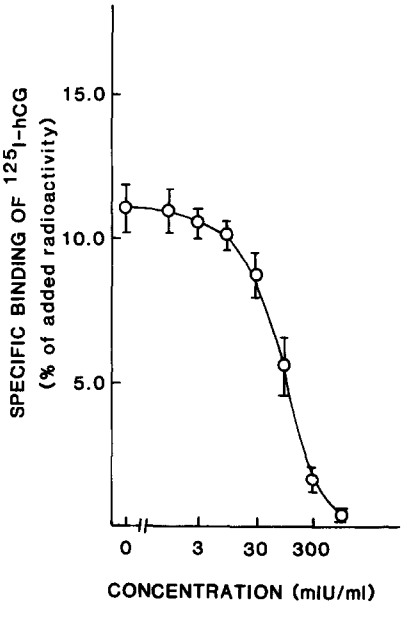

Figure 2
Inhibition of specific binding of ^{125}I-hCG (2 × 10^{-11} M) by addition of increasing concentrations of unlabelled hCG. Membranes at 1.2 mg ml^{-1} were incubated at 22°C for 18 h. Unspecific binding was determined by addition of 60 IU of hCG ml^{-1}. Binding of ^{125}I-hCG is expressed as percentage of added radioactivity bound specifically. Each point represents the mean from five separate experiments. Vertical bars denote SE.

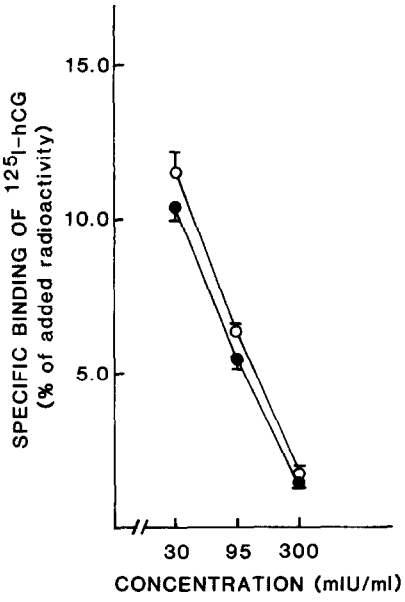

Figure 3
Radioreceptor assay of a commercial preparation of hCG (●) against the 2nd International Standard of hCG (○). Each point is the mean from three separate incubations. Assay showed commercial preparation to contain 123% of the activity of the standard. Limits of error (P = 0.95) were 88–113%. Vertical bars denote SE.

When binding data from two experiments with varying concentrations of unlabelled hCG were analysed according to Scatchard using the computer program Ligand [11], the results were compatible with a single class of binding sites with an estimated mean association constant of 1.3 ± 0.2 (SD) × 10^{10} M^{-1} and a mean binding capacity 9.8 ± 1.0 fmol mg^{-1} protein.

In specificity tests, the alpha and beta subunits of hCG reduced ^{125}I-hCG binding only at concentrations higher than 0.5 μg ml^{-1} (Table 1). The LH/FSH Reference Preparation caused dose-related inhibition of binding when added at concentrations of 67–266 mIU LH/ml corresponding approximately to 1–5 μg pituitary extract/ml. The LH subunits were devoid of binding activity. Serum gonadotrophin as well as menopausal gonadotrophin reduced ^{125}I-hCG binding in concentrations from 40 mIU to 40 IU ml^{-1} and 120–1080 mIU LH/ml, respectively, while urinary derived FSH did not cause inhibition. TSH inhibited binding at concentrations of 80–320 ng ml^{-1} suggesting contamination with LH. Human growth hormone and insulin produced by recombinant DNA-technology did not reduce binding of ^{125}I-hCG at concentrations tested (Table 1).

A number of commercial preparations of hCG have been assayed against the international standard (Table 2). The mean statistical weight of an individual assay was 1970 ± 792 (SE). Index of precision was low, on average 0.071 ± 0.012 (SE), indicating a high precision of the assay. In further experiments, the precision was even higher (Tables 3 and 4). When the assay of one batch of hCG was repeated four times the RSD was 7%. In some experiments, the results deviated slightly from linearity. Since the variation in binding results was limited, such deviations sometimes reached statistical significance.

In a series of experiments, a preparation of hCG was diluted to a certain percentage and then assayed against the undiluted preparation which was used as standard (Table 3). There was a close agreement between estimated and nominal potencies (Table 3).

In a separate experiment, a sample of a commercial hCG preparation stored at 56°C for 24 h was assayed against the same preparation stored frozen. The sample stored at 56°C was found to have 37% of the control activity (limits of error 94–107%, P = 0.95).

In a final series of experiments, a number of preparations of hCG were assayed against the international standard with both the radioreceptor assay and the *in vivo* seminal vesicle weight assay (Table 4). There was no statistical significant difference in potency determined with the *in vitro* and *in vivo* assays. However, the mean statistical weight (4393 ± 663 SE) was significantly higher with the receptor assay than with *in vivo* assay (569 ± 212 SE).

Discussion

The present study confirms earlier observations by Catt *et al.* [7–9] showing that a crude preparation of rat testicular membranes binds hCG specifically, with high affinity and in a saturable and reversible way.

The results of the Scatchard analysis suggesting one class of binding sites with an association constant of 1.3 × 10^{10} M^{-1} and a binding capacity of 0.15 × 10^{-10} mol l^{-1} are also in good agreement with findings of Catt *et al.* [7, 14]. However, since the present binding data have not been corrected for factors, such as degradation of hormone and receptors, the estimates should be interpreted with caution.

The binding sites reacted as expected not only with hCG but also with various preparations containing LH and PMSG, which also

Table 1
Inhibition of binding of ^{125}I-hCG by various preparations of hormones and subunits. 2×10^{-11} M of ^{125}I-hCG was incubated with membranes and various concentrations of unlabelled material for 18 h at 22°C. Control binding, is binding of ^{125}I-hCG in absence of unlabelled hormones or subunits

Preparation	Concentration	Specific binding, % of control
hCG, α-subunit	0.6 μg ml^{-1}	86
	0.06 μg ml^{-1}	100
hCG, β-subunit	1.0 μg ml^{-1}	41
	0.1 μg ml^{-1}	87
	0.01 μg ml^{-1}	93
FSH/LH, pituitary	266 mIU LH ml^{-1}	11
	133 mIU LH ml^{-1}	77
	67 mIU LH ml^{-1}	93
LH, α-subunit	0.27 μg ml^{-1}	97
LH, β-subunit	0.27 μg ml^{-1}	94
HMG	1080 mIU LH ml^{-1}	14
	360 mIU LH ml^{-1}	45
	120 mIU LH ml^{-1}	88
PMSG	40 IU ml^{-1}	0.2
	4 IU ml^{-1}	5
	0.4 IU ml^{-1}	53
	40 mIU ml^{-1}	89
	4 mIU ml^{-1}	102
FSH, urinary	10 IU ml^{-1}	102
TSH	0.32 μg ml^{-1}	28
	0.08 μg ml^{-1}	92
	0.02 μg ml^{-1}	96
HGH, biosynthetic	160 mIU ml^{-1}	101
Insulin, human, biosynthetic	4 IU ml^{-1}	110

Table 2
Potencies of pharmaceutical preparations of hCG determined against the 2nd International hCG Standard using radioreceptor assay

Preparation	Potency, % of standard	Limits of error ($P = 0.95$)	Statistical weight	Index of precision
1	119	87–115	1319	0.058
1	123	88–113	1726	0.051
Combination	121	92–109	3046	
2	122	80–129	424	0.102
2	112	70–143	203	0.149
Combination	115	83–121	627	
3	113	84–119	897	0.071
3	106	83–120	763	0.077
Combination	110	89–113	1659	
4	102	95–105	9398	0.022
4	117	75–133	319	0.118
Combination	102	95–105	9717	
5	124	91–109	3304	0.038
6	101	86–113	1192	0.061

exerts LH-like activity [9, 15]. The effect of the 2nd Reference Preparation of TSH on ^{125}I-hCG binding may be explained by LH-contamination. The material has been reported to contain 194 IU of LH per mg [16]. On the other hand, preparations of subunits of hCG and LH as well as the commercial preparation of FSH showed little or no binding activity.

Table 3
Potency of a hCG preparation diluted to various nominal concentrations. Potency, determined by radioreceptor assay

Potency % of undiluted preparation		Limits of error		
Nominal	Estimated	(P = 0.95)	Statistical weight	Index of precision
90	95	94–105	7000	0.025
90	90	93–107	5554	0.028
80	80	93–107	6123	0.042
80	77	94–106	8245	0.036
50	55	94–107	7535	0.040
50	42	95–105	13223	0.029

Table 4
Comparison of hCG activity in various commercial preparations as determined against the 2nd International Standard of hCG with radio-receptor assay and seminal vesicle weight *in vivo* assay

	Radioreceptor assay					Seminal vesicle weight assay				
Preparation	Potency, % of standard	Limits of error (P = 0.95)	Statistical weight	Index of precision	No. of expts	Potency, % of standard	Limits of error (P = 0.95)	Statistical weight	Index of precision	No. of expts
A	62	94–107	5855	0.027	1	68	87–115	1142	0.088	1
B	108	95–106	8406		2	113	78–129	325	0.247	1
C	124	91–110	2687	0.040	1	112	83–120	625	0.154	1
D	112	95–105	9659		2	106	71–141	183	0.255	1

This was also the case for the chemically unrelated biosynthetic hormones, HGH and insulin, demonstrating the specificity of the binding sites for hCG and LH.

The concentration of hCG found to cause maximal stimulation of rat testicular testosterone production *in vitro* is approximately 20 mIU ml^{-1} [17]. The present results where maximal inhibition of tracer binding was obtained with about 900 mIU of hCG ml^{-1} indicate that only a limited number of the binding sites have to be occupied to induce maximal stimulation of steroidogenesis, which is in accordance with previous findings [7].

Using three concentrations of hCG from the linear part of the log dose–response curve for binding of ^{125}I-hCG to testicular membranes, it was possible to obtain an assay with high sensitivity and precision, giving similar but much more precise potency readings than the established *in vivo* method [1]. The present results suggest that the described radioreceptor assay for hCG may be an advantageous alternative to established *in vivo* assays for potency readings of pharmaceutical preparations of hCG.

Further advantages of the radioreceptor assay over the established *in vivo* assays are its reduced requirements for laboratory animals and labour hours. One technician could assay several preparations in one test using testicles of one or two rats while *in vivo* methods require the presence of two or more technicians for significant parts of the working days of 1 week using 50 or more rats for one assay.

While there is, on the whole, a good correlation between receptor affinity and biological activity *in vitro* the radioreceptor assay may not always reflect the biological activity *in vivo* [17–19]. This is probably mainly due to the fact, that desialylated hormones retain their biological activity *in vitro* but are rapidly broken down *in vivo* [17, 18, 20, 21]. Thus, the present receptor assay may fail to detect an activity loss due to desialylation. However, the concordance between the present results obtained with the radioreceptor assay and the *in vivo* assay does not suggest the occurrence of significant amounts of desialylated hormones in presently available commercial formulations of chorionic gonadotrophins. Thus, the described assay may serve well for example as a screening method for chorionic gonadotrophin activity in pharmaceutical preparations.

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