Development and Validation of an Improved Chemiluminescent Assay for Inhibin B

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Abstract

Background: Inhibins are protein hormones that are secreted by the granulosa cells of the ovary and the Sertoli cells of the testes. These hormones selectively suppress the secretion of pituitary follicle-stimulating hormone (FSH); they also exert local paracrine actions in the gonads. Elevated inhibin B levels have been associated with Sertoli cell function (potential marker for spermatogenesis and testicular function), ovarian reserve, and granulosa cell tumors. Inhibin B is a 32 kDa dimeric hormone composed of 2 distinct subunits, alpha (α) and beta B (βB), which are linked by disulfide bonds. The free α subunit is usually physiologically inactive; the α-β dimer is the biologically active form.

Objective: To develop and validate a quantitative chemiluminescent assay for serum inhibin B that conforms to WHO standards.

Methods: We have developed a sandwich-type, enzymatic microplate assay. This assay uses a well-characterized monoclonal antibody pair that is specific for inhibin B (captures β subunit and detects βB subunit of inhibin B). The antibody pair does not detect inhibin A, activin A, activin B, AMH, FSH, LH, and TGF-β1, even at twice their normal physiological concentrations. The assay calibrators range from 10 pg/mL to 1300 pg/mL. In this three-step procedure, calibrators, controls, and unknown samples are added to microplate wells coated with an anti-inhibin B antibody and incubated. Inhibin B in the samples is detected using a biotinylated anti-inhibin B antibody, a streptavidin horseradish peroxidase conjugate (SHRP), and a luminogenic substrate. The emitted luminesence, measured in relative light output units (RLU) using a microplate luminometer, is directly proportional to the concentration of inhibin B.

Results: This Inhibin B assay is traceable to the WHO 96/784 IRP Standard and the assay had excellent correlation with a commercially available inhibin B assay. Comparison using 71 de-identified serum samples showed a correlation coefficient of >0.99, a slope of 1.13, and an intercept of 4.23 pg/mL. Total imprecision was 2.68% at 340 pg/mL and 10.59% at 116 pg/mL. No significant interference was observed with hemoglobin, triglycerides, or bilirubin. The LOD was <3 pg/mL and LOQ was 10 pg/mL, and the new assay had an improved dynamic range compared with that of the current commercial assay. No high-dose hook effect was observed with inhibin B concentrations up to 13,000 pg/mL. The assay was also linear for concentrations up to 1300 pg/mL (highest calibrator).

Conclusions: A highly sensitive and reproducible microplate inhibin B chemiluminescent assay has been developed. The favorable performance of this laboratory developed test makes it a useful tool for monitoring inhibin B changes in physiological and pathophysiological conditions.

Introduction

Inhibins are glycoprotein hormones produced by the Sertoli cells (testis) and granulosa cells (ovary). Inhibin B, the only inhibin found in men, is made up of an alpha (α) and beta B (βB) subunit linked by disulfide bonds. Only the dimeric α- β form is biologically active. Inhibin B negatively regulates follicle stimulating hormone (FSH) secretion and has been used as a marker of testicular function, toxicity, and spermatogenesis.

We developed and validated a quantitative chemiluminescent enzyme immunoassay for serum inhibin B that conforms to WHO standards.

Methods

The inhibin B assay is a quantitative 3-step sandwich immunoassay (each step followed by incubation and washing): 1) addition of calibrators, controls, and unknown samples to microtitration wells coated with anti-inhibin B antibody (Ansh Labs, Webster, TX); 2) addition of biotinylated second anti-inhibin B antibody; and 3) addition of streptavidin horseradish peroxidase conjugate.

A luminogenic substrate solution is then added to the wells and enzymatic turnover is assessed using luminescence measurement with a microplate luminometer. Sample inhibin values are determined by comparison against a calibration curve plotted using a set of inhibin B calibrators.

Data reported are based on experiments using serum samples. However, similar results were obtained using Li-heparin plasma (not shown).

Results

Linearity

A high-concentration sample was diluted to 75%, 50%, and 25% using a 1 pg/mL diluent and run in duplicate as an unknown. The mean of the duplicates of observed data was compared against the target values. In this first study, sample concentrations were chosen to encompass the analytical measurement range. The assay demonstrated good linearity, with an R^2 value of 0.9995 (**Figure1**).

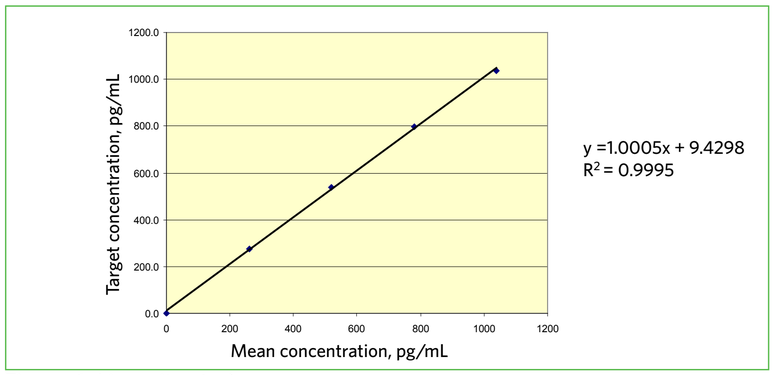

Figure 1. Linearity study.

Analytical Sensitivity: Lower Limit of Detection (LOD) and Quantitation (LOQ)

Quintuplicates of blank, 10 pg/mL, 14 pg/mL, and 27 pg/mL inhibin B were assayed in 5 different runs to determine the assay LOD (3 pg/mL) and LOQ (10 pg/mL) (**Table 1**). Based on the linearity and sensitivity studies, this assay has an analytical measurement range of 3 to 1300 pg/mL and a clinical reportable range of 10 to 1300 pg/mL.

Table 1. Analytical Sensitivity Study

Run	Inhibin B Concentration			
	10 pg/mL	14 pg/mL	27 pg/mL	0 (Blank)
1	15,146	23,385	36,585	3,141
2	16,574	23,099	40,029	2,174
3	15,215	20,972	36,644	2,247
4	17,044	24,218	40,936	2,986
5	15,775	22,899	38,360	2,787

Inhibin B concentrations represented in RLU values.

Analytical Specificity

To assess the analytical specificity of the assay, we tested sera after addition of the following potential cross-reactants to at least at 2x physiological concentration : inhibin A, activin A, activin B, activin AB, AMH, FSH, LH, and follistatin 315. None of the cross-reactants tested affected inhibin B measurements (**Tables 2, 3**).

Table 2. Inhibin B Concentrations Used for Cross-reactivity Study

	Inhibin B, pg/mL			Average	% of Neat 100%	75%	50%
Inhibin B (Neat)	175.60	175.20	180.30	177.03	177.03	132.78	88.52

Detected concentrations for neat (100%) inhibin B, and calculated concentration for 75% and 50% inhibin B used for the cross-reactivity study.

Table 3. Inhibin B Specificity: Cross-reactivity Study

	Inhibin B, pg/mL			Average	% Recovery
FSH, 50% (110.5 mIU)	81.2	80.4	85.4	82.3	93.0
FSH, 25% (55.25 mIU)	132.0	136.0	131.8	133.3	100.4
LH, 50% (121 mIU)	86.1	86.3	84.2	85.5	96.6
LH, 25% (60.5 mIU)	138.3	138.5	139.9	138.9	104.6
AMH, 50% (10000 pg/mL)	82.4	75.8	86.4	81.5	92.1
AMH, 25% (5000 pg/mL)	137.2	123.6	134.7	131.8	99.3
Inhibin A, 50% (766 pg/mL)	79.2	72.3	77.9	76.5	86.4
Inhibin A , 25% (383 pg/mL)	131.6	121.2	124.2	125.7	94.7
TGF Beta 1, 2000 pg (DS)	163.5	157.0	157.9	159.5	90.1
TGF Beta 1, 1000 pg (DS)	166.1	164.6	163.4	164.7	93.0

DS = Direct spike.

Results (cont)

Interference

Interfering substances studies included hemoglobin, bilirubin, and triglycerides at high, moderate, and low concentrations. The average of 2 assay runs was used to assess recovery. Moderate or gross contamination with bilirubin, or any amount of hemoglobin, interfered with assay performance; triglycerides had negligible impact (**Table 4**).

Table 4. Interference Study

Interfering Substance	Inhibin B Concentration, pg/mL			Recovery
	First Run	Second Run	Average	
Neat	175.6	175.2	175.4	
Triglycerides				
Gross	183	193.4	188.2	107.3%
Moderate	174.4	189.4	181.9	103.7%
Light	165.5	170.5	168	95.8%
Bilirubin				
Gross	72.9	66.7	69.8	39.8%
Moderate	111.7	118.9	115.3	65.7%
Light	164.5	153.6	159.05	90.7%
Hemoglobin				
Gross	27.8	27.4	27.6	15.7%
Moderate	42.8	41.8	42.3	24.1%
Light	119.2	123.8	121.5	69.3%

Stability

Stability was assessed with a sample pool, aliquoted and stored for various times (0, 3, 5, 7, 17, or 35 days) and temperatures (2-8 °C, -20 °C, ambient). Each combination was tested in triplicate. The samples remained stable at refrigerated and frozen temperatures for 35 days (17 days at ambient); recoveries ranged from 85% to 108% (data not shown). For operational purposes we would use more conservative stability estimates: ambient, 72 hours; refrigerated (2-8 °C), 7 days; and frozen (-20 °C), 28 days.

Comparison of Clinically Defined Samples

De-identified samples (n=71) tested on the Inhibin B Gen II ELISA (Beckman Coulter, Brea, CA) were also tested with this laboratory-developed assay. Correlation was good throughout the range tested (Figure 2).

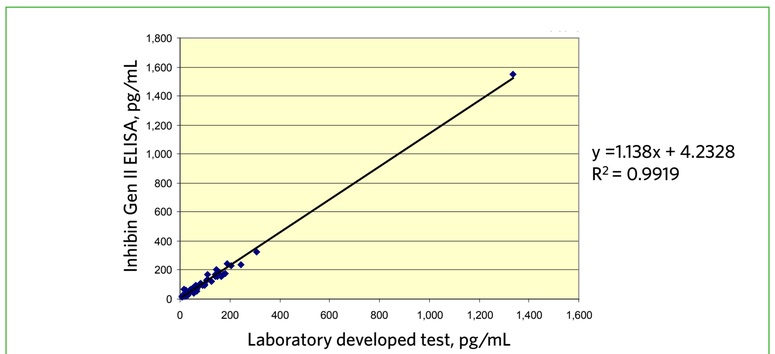

Figure 2. Correlation of laboratory-developed inhibin B assay with Beckman Coulter Inhibin B Gen II ELISA in clinical samples (n = 71).

Conclusions

- We developed a sensitive quantitative assay for measurement of inhibin B.
- This assay has excellent linearity and is well suited for automation and/or manual determination of inhibin B in serum or Li-heparin samples.
- The antibody pair in the assay is highly specific and is standardized to WHO96/784.
- This assay should be amenable for evaluating inhibin B changes and correlating these patterns with physiological and pathophysiological conditions.