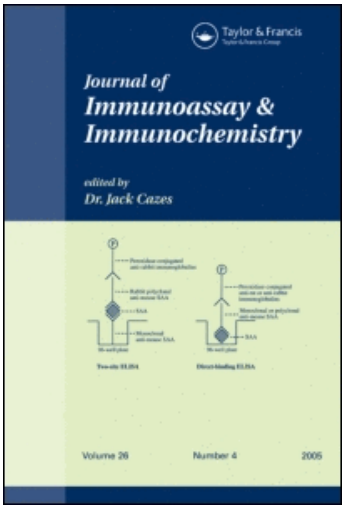

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AN ENHANCED CHEMILUMINESCENT ENZYME IMMUNOASSAY FOR FOLLICLE STIMULATING HORMONE

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ABSTRACT

An enhanced chemiluminescent enzyme immunoassay for serum follicle stimulating hormone is described which involves sequential reaction of anti-follicle stimulating hormone antibody immobilised to the inside surface of an opaque microtitre plate with sample, monoclonal anti-alpha thyroid stimulating hormone antibody, and an anti-mouse IgG - horseradish peroxidase conjungate. Bound peroxidase activity was measured using a p-hydroxycinnamic acid enhanced chemiluminescent luminol-hydrogen peroxide reaction. The assay was sensitive (detection limit 0.01 mU/well) precise (intra-assay precision 2.5-8.1%, inter-assay precision 6.7-11.9%) and results obtained with this assay and a competitive radioimmunoassay were in good agreement (correlation coefficient 0.98).

INTRODUCTION

Measurement of follicle stimulating hormone (FSH) is useful in the investigation of disorders of

reproduction. This hormone is usually measured by radioimmunoassay but an enzyme immunoassay, based on a glucose oxidase label, has also been reported (1). Previously we have described a rapid and sensitive enhanced chemiluminescent assay for horseradish peroxidase (HRP) (2) and its application in various enzyme immunoassays based on HRP labels, e.g. thyroxine (3), carcinoembryonic antigen (4), cytomegalovirus IgG antibody (5), digoxin (6). We now describe the exploitation of an enhanced chemiluminescence assay of an HRP label in an enzyme immunoassay for the glycoprotein hormone FSH.

MATERIALS AND METHODS

Instrumentation

Light emission from individual wells of an opaque black microtitre plate was measured using a chemiluminescence microtitre plate reader developed at the Wolfson Research Laboratories (2).

Reagents

FSH 78/549 and hCG 69/46 were obtained from the MRC, NIBSC, London. hCG H87B was a gift from Organon, Ltd. TSH (3-5) asf, prolactin SKF 1B, and rabbit antiserum to FSH M244/2 were obtained from the Department of Clinical Endocrinology, Birmingham.

Monoclonal antibody alpha-TSH was obtained from the Molecular Endocrinology Department, Middlesex Hospital London, UK. Antibody to mouse IgG conjugated to HRP was obtained from Dakopatts a/s (Copenhagen, Denmark). Luminol, Trizma base and BSA were purchased from Sigma Chemicals (Poole, UK). The luminol was purified by recrystallisation from aqueous sodium hydroxide solution. p-Hydroxycinnamic acid was purchased from Aldrich Chemicals (Gillingham, UK). Black polystyrene 96 well microtitre plates were obtained from Dynatech (Sussex, UK). All other chemicals used were of analytical grade.

Buffers

Coating buffer 50 mmol/L sodium carbonate-bicarbonate pH 9.

PBS 100 mmol/L sodium phosphate buffer containing 150 mmol/L sodium chloride, 0.1 g/L thimerosal.

PBS-Tween PBS containing Tween 20 1mL/L

PBS-Tween-BSA PBS-Tween containing BSA 5g/L

Tris buffer 100 mmol/L Trizma base pH 8.6.

FSH standards in the range 0.05 to 6.40 U/L were prepared in PBS-Tween-BSA.

Clinical specimens

A series of 37 serum specimens previously analysed for FSH by radioimmunoassay were obtained

from the Department of Clinical Endocrinology, Birmingham and Midland Hospital for Women, Birmingham.

Purification of antibody

The immunoglobulin (IgG) fraction of FSH antiserum was obtained by precipitation with ammonium sulphate (33% saturation). The IgG fraction was dialysed against 150 mmol/L sodium chloride. Protein concentration was determined by the method of Pesce and Strande (7).

Coating of microtitre plates

The anti-FSH IgG was diluted to 5 mg/L in coating buffer and 200 uL was pipetted into the wells of the microtitre plate. The plate was incubated overnight at 4^0C and then washed with PBS-Tween.

Immunoassay procedure (Figure 1)

First Incubation. Add 200 uL of FSH standard or 200 uL of sample (1:8 dilution in PBS-BSA) to the microtitre plate well. Cover the plate with plastic film and incubate it for 2 hours at 37^0C. Wash the plate with PBS-Tween.

Second Incubation. Add 200 uL of monoclonal anti-alpha TSH (1:2000 dilution in PBS-BSA) to each well, cover the microtitre plate with plastic film and incubate for 45 minutes at 37^0C. Wash the plate with PBS-Tween.

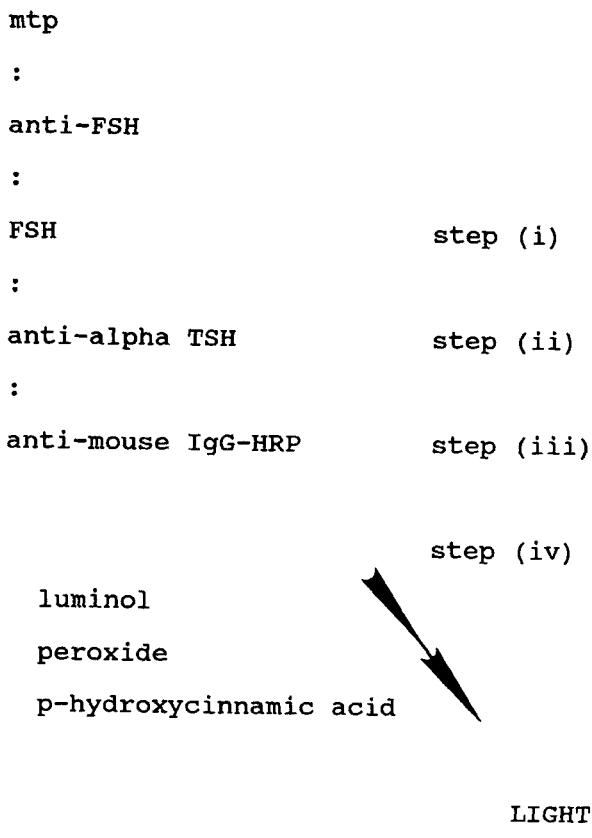

Figure 1. Enhanced chemiluminescent assay for FSH. (mtp, microtitre plate; anti-FSH, antibody to follicle stimulating hormone; anti-mouse IgG-HRP, horseradish peroxidase anti-mouse immunoglobulin conjugate)

Third Incubation. Add 200 uL anti-mouse IgG HRP conjugate (1:2000 dilution in PBS-BSA) to each well, cover the microtitre plate with plastic film and incubate for 45 minutes at 37^0C. Wash plate with PBS-Tween.

Enhanced Chemiluminescent End-point. Add 200 uL of substrate solution (1.25 mmol/L luminol, 30 umol/L p-hydroxycinnamic acid, and 2.7 mmol/L hydrogen peroxide in 100 mmol/L Tris buffer, pH 8.6) to each of the wells (complete this step within 5 minutes). Measure the intensity of the chemiluninescent emission within 5 minutes using a chemiluinescence microtitre plate reader.

Precision studies

Inter-assay precision was determined by repeated analysis (n=10) of four pools of serum (representing a range of FSH concentrations) on the same microtitre plate. Intra-assay precision was determined by analysis of the same four pools of serum on 5 separate occasions.

Determination of accuracy

Samples of a specimen with a low FSH concentration (1.36 U/L) were supplemented with FSH (0,1,2,8, and 32 U/L) and the FSH concentration determined before and after the addition of FSH.

Sample interference studies

This was assessed by assaying different volumes of a sample (6.25-100 uL diluted to a final volume of 200 ul with PBS-BSA) with a high (22.8 U/L) and a low (5.6 U/L) concentration of FSH.

Cross-reactivity

A series of hCG, hGH, LH, TSH and prolactin standards were assayed using the enhanced chemiluminescence enzyme immunoassay for FSH and in a radioimmunoassay using the same anti-FSH antiserum.

Competitive Radioimmunoassay for FSH

This was an in-house method utilizing rabbit M93/2 anti-FSH in a four hour preincubation, followed by an overnight incubation with ^{125}I labelled FSH CPDS 30 in a total volume of 500 ul. Separation of free from bound was achieved with second antibody (Scottish Antibody Production Unit, Carluke, Lanarkshire, Scotland) magnetizable particles. The assay was standardized using the International FSH Standard MRC 78/549.

RESULTS

Dose-response curve

A typical curve is shown in Figure 2. The detection limit of the assay (i.e. amount of FSH giving twice background light emission) was 0.01 mU/well.

Precision

The intra-assay precision was 8.1% at 1.37 U/L, 2.5% at 5.82 U/L, 5.6% at 13.5 U/L and 7.5% at 22.9 U/L. Inter-assay precision was 11.9% at 1.4 U/L, 8.4% at 5.5 U/L, 6.7% at 14.6 U/L, and 10.5% at 22.7 U/L.

Accuracy

Recovery of added FSH ranged from 90 to 105%.

Sample interference

Increasing the sample volume from 6.25 to 100 uL did not cause any significant interference for a sample with a low FSH concentration (5.6 U/L). However, for samples with an high FSH concentration (22.8 U/L), an increase in sample size was accompanied by a reduction in the apparent FSH concentration e.g. FSH concentration estimated in a 6.25 uL sample was 13.8% higher than the FSH concentration estimated when a 100 uL sample was used in the assay.

Cross-reactivity

The specificity of the anti-FSH antiserum as determined by the enhanced chemiluminescent enzyme immunoassay is shown in Table 1.

Comparison with radioimmunoassay

FSH values obtained on a series of 37 serum samples using the enhanced chemiluminescent enzyme

Table 1. Specificity of anti-FSH IgG as determined by an enhanced chemiluminescent enzyme immunoassay (ECLEIA) and a radioimmunoassay (RIA).

Hormone	% Cross-reactivity	
	ECLEIA	RIA
FSH	100	100
LH	8.75	0.7
TSH	0.11	0.6
HCG	0.9	0.9
HGH	<0.01	<0.3
Prolactin	<0.01	<0.3

immunoassay and a competitive radio immunoassay (y) were in good agreement (correlation coefficient = 0.98; $y = 0.973 x + 0.679$)

DISCUSSION

Various immunoassay designs, including a competitive assay using an anti-FSH-HRP conjugate

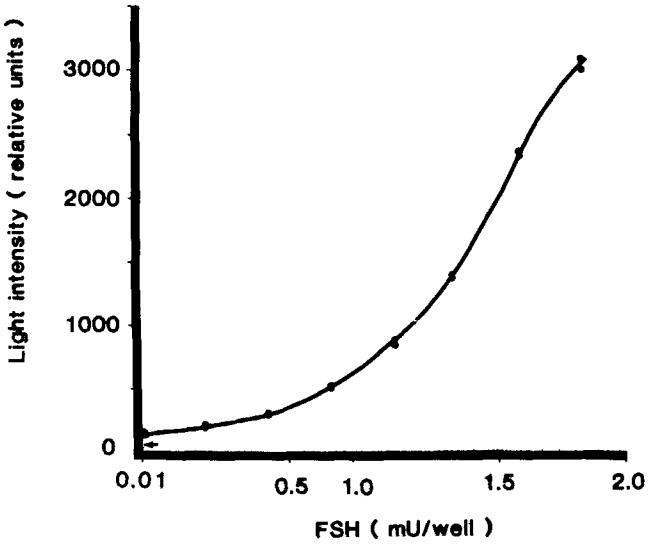

Figure 2. Dose-response curve for serum FSH assay.

(detection limit 0.16 mU), were investigated in this study. An immunoextraction assay (sandwich) was finally chosen because of its superior sensitivity (0.01 mU). In the assay (Figure 1) the anti-FSH antibody immobilised to the plastic surface provides specificity and extracted FSH is recognized via its alpha-subunit by a monoclonal anti-alpha TSH (the alpha-subunit is common to FSH, LH, hCG and TSH). Bound anti-alpha TSH is in turn recognized by an HRP conjugated anti-species antibody. Sensitivity of the assay is superior to the previously reported enzyme immunoassay for FSH (0.01 versus 4 mU) and can be

completed in a much shorter time (< 4 h versus ~ 48 h). The sensitivity of the assay also compares favorably with radioimmunoassays for FSH (e.g. detection limit of 0.04 mU). The anomaly in cross-reactivity with LH for the anti-FSH antibody in the two assays (Table 1) is possibly due to the presence of a sub-population of cross-reacting antibodies which behave differently in the competitive and immunoextraction assays. This degree of cross-reactivity would not influence FSH determinations in the normal population but would lead to an overestimation in conditions such as polycystic ovarian disease where LH is significantly increased. p-Hydroxycinnamic acid was chosen as the enhancer for the chemiluminescent HRP catalysed luminol-peroxide reaction. This enhancer has been used previously in assays for estradiol-HRP (8) and avidin-HRP (9). The influence of enhancer, luminol and peroxide concentraction on light intensity, and the kinetics of light emission have been studied in the context of an enzyme immunoassay for estradiol based on an estradiol-HRP conjungate. The assay conditions chosen for measuring estradiol-HRP were also effective for the anti-mouse IgG-HRP conjugate used here.

ACKNOWLEDGEMENTS

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