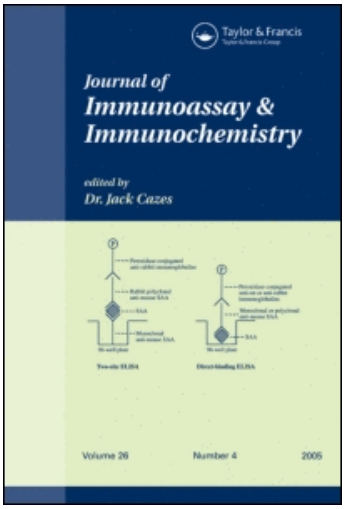

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Şerif Akman[a]; Charlie McLain[b]; John Landon[b]
[a] Department of Biochemistry, School of Medicine, Gulhane Military Medical Academy, Etlik-Ankara, TURKEY [b] Saint Bartholomews' Hospital Research Center, West Smithfield, U. K.

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THE DEVELOPMENT OF AN ENZYMEIMMUNOMETRIC ASSAY FOR LH AND THE EFFECTS OF THE METHODS ON THE IMMUNOREACTIVITY OF THE CONJUGATES

Şerif AKMAN[1], Charlie McLAIN[2], John LANDON[2]

Department of Biochemistry, School of Medicine, Gulhane Military Medical Academy, Etlik-Ankara - TURKEY[1]
Saint Bartholomews' Hospital Research Center, West Smithfield EC1A 7BE U. K.[2]

ABSTRACT

Using an affinity purified sheep anti-human luteinizing hormon IgG-horseradish peroxidase conjugate (sheep anti-hLH IgG-HRP) and rabbit anti-human luteinizing hormone antiserum (rabbit anti-hLH) directed against different antigenic determinants, a solid-phase "sandwich" enzyme immunometric assay (EIMA) for human luteinizing hormone (hLH) was developed. The assay was validated and compared with a liquid phase "two site" immunoradiometric assay (IRMA) for hLH which uses same two antibodies. The sheep anti-hLH IgG, which had been affinity purified by eluting at pH 3,5 from a hLH-sepharose 4 B column, was labelled with ^{125}I. The IRMA is based on simultaneous addition of two antibodies to standards and samples. After overnight incubation, separation was achieved by addition of Sheep anti-Rabbit Fc (SARFc) antiserum.

In EIMA; partially denaturated (at pH 2.5) Sheep anti-Rabbit FcIgG (SARFcIgG) coated polystyrene tubes or microtitre plates were employed as solid-phase second antibody. The substrate was N, N'-o-phenylene diamine (2 mg/ml) and H_2O_2 (0,02 %).

Two methods, modified $NaIO_4$ and 4-(N-maleimido methyl)-cyclohexane-1-carboxylic acid N-hydroxy succinimide ester (SMCC), were employed in the preparation of sheep anti-hLH IgG-HRP conjugate. The immunoreactivity and peroxidase activity of conjugate prepared with

$NaIO_4$ method was impared to various extends. Both EIMA and IRMA had good specificity, were not susceptible to interference from serum components and exhibited very low non-specific binding. The values determined by EIMA were independent of the serum volume employed. Standard added to serum samples was accurately determined and the results obtained from the analysis of serum samples correlated closely with those obtained by IRMA.

(KEY WORDS: EIMA, Luteinizing Hormone, protein conjugation, EIMA)

INTRODUCTION

Luteinizing hormone (LH) is a glycoprotein secreted from anterior pituitary (1). This hormone has a molecular weight of 28000 and is composed of α-and β-subunits held together by hydrophobic force. The α-subunit of this hormone is identical to that of the other glycoprotein hormones including follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH) and human chorionic gonadotropin (hCG). The β-subunit is distinct among different hormones and is responsible for their differential biological activities. LH and FSH were known to play important roles in the regulation of ovulation and the ovarian function during the control of menstrual cycle. Ovulation and luteinization only occur after a significant increase in the level of circulating LH. Since LH is rapidly excreted (short circulation half-life ≤ 1h) the rise in the concentration of LH, in the circulation and urine, within the defined limits of the occurance of ovulation (~ 30 hours), can be used as a marker for the initiation of the fertile period in women. Therefore, rapid, sensitive and specific assays of LH are required for the clinical detection of ovulation. Time consuming competitive RIAs or conventional IRMAs are used to date for LH determination which are required expensive automatic γ-counting equipment and facilities suitable for the radioiodination of proteins. In addition, the relatively short half-life of ^{125}I means that labelling must be carried out every month or so, which cause health hazards and greatly increasing costs, especially when small numbers of samples must be assayed. Furthermore most two-site systems used for determination of LH are, in reality, not truly two-site, because the same antibody is used for preparing both the labelled and the solid-phase unlabelled reactants. A true two-site assay would have antibodies to separate antigenic determinants on the

antigen. Raising antibodies to ligand in two different species may provide antibodies with different specificities. Although the major drawback of RIAs and conventional IRMAs (cross reactivity, instability of radiolabeled LH, non-specific serum effect, time-consuming assay procedures, limited range of analyte concentration) have approximately been removed in liquid phase "two-site" IRMA by using two different specific antibodies (one radiolabeled) in excess, instability of radiolabeled antibodies, high dose "hook" effect are still existing as major problems (2). Moreover, resulting in poor recoveries of antibodies with reduced immunoreactivity during the affinity chromatography of specific antibodies because of the denaturating effects of low pH solutions needed to desorb is another disadvantage of antibody labeled IRMAs and EIMAs (2, 3, 4). On the other hand, EIMAs have similar specificities and potentially higher detectabilities, wide operating range with IRMA and offer, in addition important advantages such as cheaper equipment required, reagents which are generally of long half-life (2, 3, 4, 5, 6). However, preparation of enzyme-antibody, enzyme-macromolecule or others, enzyme-hapten conjugates is a difficult part of EIMA which requires a suitable choose of enzyme and IgG or other macromolecules for conjugation. Conventional procedures of macromolecule conjugation described are usually resulted with low recovery and inactivation of the enzyme or antibody (4, 7, 8).

This study was designed to compare solid phase EIMA and IRMA of hLH. In the liquid phase "two-site" IRMA the single reagent added to the serum sample (or standard) for hLH comprising rabbit antiserum together with ^{125}I labelled specific sheep anti-hLH IgG, after equilibrium reached, an excess of sheep anti-rabbit Fc (SARFc) antiserum was added to separate bound fraction. After centrifugation radioactivity of bound fraction in precipitate was measured.

In solid-phase EIMA the same antibodies were employed. SARFc IgG was coated to polystyrene tubes or microtiter plates. After incubation with rabbit antiserum followed by washing and incubation with standards or unknown. Washing and adding sheep anti-hLH IgG-HRP conjugate into tubes or wells followed by in turn incubation and washing. Finally substrate sol was added and incubated before stopping enzyme reaction by adding 4M H_2SO_4. Intensity of chromophore was measured at 490 nm.

MATERIALS AND METHODS

Materials

Human LH, TSH, FSH and hGH were prepared in this laboratory according to procedure described by Lowry et al (9). Sheep anti-hLH, rabbit

anti-hLH, SARFc, normal sheep serum (NSS) and normal rabbit serum (NRS) were obtained through the courtesy of Polyclonal Antibodies Ltd. (14-15 Newbury Street, London ECI). Bovine serum albumin (BSA, fraction V), HRP (grade I), S-acetylmercapto succinic anhydride (AMSA), Thiomerosal, 2-Mercaptoethylamine, Hydrogen Peroxide (H_2O_2), N-N'-o-phenylene diamine (OPD), Hydroxylamine and sodium-m-periodate were purchased from Sigma Chemical Company. 5-5'-dithio-bis (2-nitro benzoic acid) (DTNB, Elman's reagent) was obtained from Aldrich. 4-4'-dithiodipyridine and 4-thiopyridone were also obtained from Sigma Chemical Company.

CNBr-Activated sepharose-4B and Sephacryl S-200, Sephadex G-25, G-50 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). 4-(N-Maleimido methyl)-cyclohexane-l-carboxylic acid, N-hydroxy succinimide ester (SMCC) and iodogen (1, 3, 4, 6-tetrachloro-3a, 6 a-diphenylglycoluril) were purchased from Pierce Chemical Co Ltd. (Cambridge UK). Chromatography columns for use of affinity chromatography and for desalting were purchased from Bio-Rad Laboratories (Watford, Hertfordshire). Columns for purifications of POase conjugated and iodine labelled IgGs were purchased from Whatman Laboratory Sales Ltd. (Maisdone, Kent, England).

Buffers

The most frequently used buffers were;

1. IRMA assay buffer; 0.05M phosphate buffer, pH 7.4, containing 0.5 % BSA (w/v), 2% PEG 6000 (w/v), 1 % NRS (v/v), 2 % NSS (v/v).
2. Buffer A; 0.05 M phosphate buffer, pH 7.4, containing 0.1 % BSA and 0.1 % NaN_3.
3. Buffer B; Buffer A containing no azid.
4. PBS-Alb; 0.1 M phosphate buffer containing 0.9 % (w/v) NaCl and 1 % BSA.
5. Substrate buffer; 0.1 Mol/L citrate buffer pH 5.0.

Methods

Preparation of Immunoglobulin Fraction

The immunoglobulin fractions of sheep antiserum (sheep anti-hLH),

SARFc and rabbit anti-hLH were prepared according to the procedure described by Akman et al (5).

Affinity Purification of Sheep Anti-hLH IgG

An immunoadsorbent for the affinity purification of sheep anti - hLH IgGs was prepared using CNBr - activated sepharose 4B following the instructions recommended by the manufacturer. Five ml solution of hLH (5 mg) was transferred into 2 ml settled CNBr-activated sepharose 4B and mixed on an end-over-end mixer overnight at 4°C. Excess ethanolamine (1 M) was added to block remaining active groups and incubated again overnight at 4°C. The non-specifically adsorbed materials were removed with successive washes of coupling buffer. Immunoadsorption of 10 ml (10 mg) sheep anti-LH IgG on hLH coated solid-phase (CNBr activated sepharose-4B) was carried out by continous mixing on an end-over-end mixer for overnight at 4°C. This immunoadsorbent (2 ml gel) was then packed into a small column and washed with 0.15 M NaCl. Small samples (200 µl) from this washing solution were kept for assesment of the progress of the fractionation procedure. To each fraction collector tube 200 µl 1M NH_4HCO_3 was added before elution of the immunoadsorbed sheep anti-hLH IgG. The selective elution of the immunoadsorbed sheep anti-hLH IgG was carried out by stepwise reduction of pH (pH6, pH5, pH4, pH 3.5, pH 3) (Figure 1). Each buffer used for the stepwise elution of IgG contained acetonitrile (20 % v/v). The eluting volume of each solution was 10 ml. The IgG content of each eluent fraction (1.5 ml including 0.2 ml NH_4HCO_3) was determined by absorbance assuming that $A^{1cm}_{280\ nm}$, of a 1 mg/ml solution is 1.4. At each of the pH values used, those fractions containing the most IgG were pooled separately.

Immunoreactivity of each pool was estimated by preparing serial dilution curves. The dilutions of IgG which bound 50 % of added hLH-I^{125} from each dilution curves were chosen for the comparision of immunoreactivity of each pool.

Iodination of Sheep Anti-hLH IgG

The immunoaffinity purified sheep anti-hLH IgG from pH 3.5 pool was iodinated using a modification of the iodogen method of Salacinski et al (10). The iodination mixture was then loaded onto a sephacryl S-300 column and IgG was pooled. The incorporation of radioiodine in the IgG was

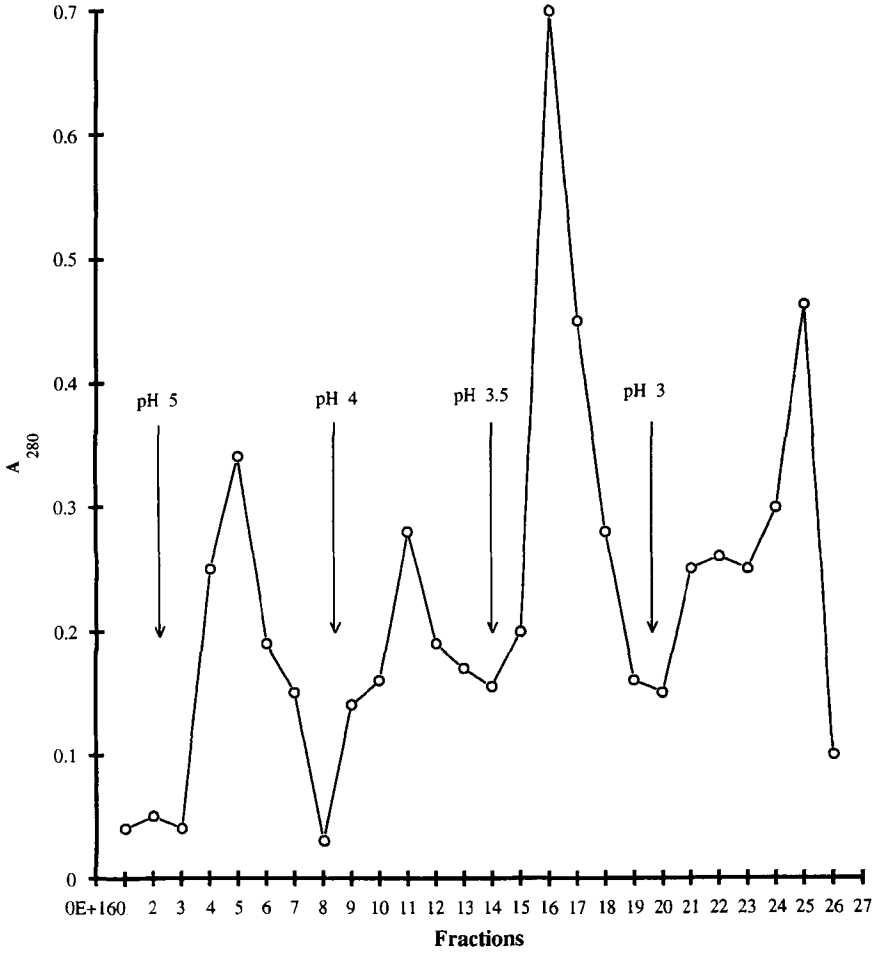

Figure 1: Selective elution of sheep anti-hLH IgG by stepwise reduction of pH.

normally between 70 and 90 % and the specific activity of the tracer ranged from 8-13 μCi/μg.

Preparation of Sheep Anti-hLH IgG-HRP Conjugate by Simplified Periodate Method

Conjugation of HRP to affinity purified sheep anti-hLH IgG was performed using a modification of the $NaIO_4$ method of Nakane et al (11).

Briefly, 1 mg HRP in 0.5 ml of freshly prepared 100 mM $NaHCO_3$ was activated with 0.5 ml of 10 mM $NaIO_4$. 5 mg IgG was then added into activated peroxidase and incubated for 3 h at room temperature. After addition of 0.1 ml $NaBH_4$ the reaction mixture was applied onto the column of Sephadex G-25 for desalting. Fractions containing protein were pooled and stored at -20 °C.

Conjugation of Affinity Purified Sheep Anti-hLH IgG with HRP by SMCC

Enzyme conjugated to IgG was prepared as follows:

a- Thiolation of IgG

Introduction of thiol groups with AMSA has been performed according to procedure modified by Imagawa et al (12). 5 mg IgG in 1 ml of 125 mM phosphate buffer pH 7.0 was incubated with 100 molar excess AMSA in 0.1 ml N, N-dimethylformamide (DMF) at room temperature for 30 min with continous stirring. Then 0.2 ml of 100 mM Tris-HCl buffer pH 7.0 containing 0.5 M hydroxylamine and 10 mM EDTA was added into reaction mixture and incubated for 4 min at 30 °C. Reaction mixture was applied to sephadex G-25 column for desalting. IgG containing fractions were pooled and deep-freezed until conjugation.

Determination of Thiol and Maleimide Groups

Thiol groups were determined using 4.4'-dithiodipyridine and maleimide groups were determined by incubation with a known amount of 2-mercaptoethylamine and then measuring remaining thiol groups using 4-4' dithiodipyridine (13, 14).

b- Introduction of Maleimide Groups into HRP Using SMCC

Maleimide groups were introduced into HRP following the methods of Yoshitake et al (8).

2 mg HRP in 0.3 ml of 0.1 M fosfat buffer pH 7.0 and 1.6 mg SMCC in 20 µl DMF were mixed and incubated at 30°C for 1h in 1 ml polypropylene vial and centrifuged. Supernatant was loaded on a sephadex G-25 column equilibrated with 10 mM phosphate buffer, pH 6.0. Fractions containing HRP were pooled.

c- Conjugation of Maleimide Peroxidase and Thiolated IgG

The thiolated IgG and maleimide introduced peroxidase were mixed so that the final concentration of both HRP and thiolated IgG is between 2-6

mg/ml. Overnight incubation at 4 °C was followed by gel filtration on a sephacryl S-200 column equilibrated with 100 mM phosphate buffer (Figure 2). Absorbance at 280 and 403 nm were measured in each fraction and fractions containing HRP conjugated IgG were pooled and stored at -20 °C as 100 µl aliqouts containing 0.1 % BSA and 0.002 % thiomerosal.

Testing immunoreactivity of HRP conjugated IgGs

To test the antigen-binding ability of sheep anti-hLH IgG and its HRP conjugates; the dilution curves prepared with native and HRP conjugated sheep anti hLH IgGs were compared. Figure 3 shows the dilution curves obtained with native and HRP conjugated sheep anti-hLH IgG prepared with periodate and SMCC methods. The same amount of IgGs were serially diluted and used in each curve.

Assay of Peroxidase Activity

Peroxidase activity was spectrophotometrically determined using OPD. The substrate solution was freshly prepared in 0.1 M citrate buffer pH 5.5, H_2O_2 0.02 %, OPD 2 g/L and protected from light. The colour intensity was measured at 490 nm with and at 445 nm without acid addition.

Calculations

The amount of IgG was calculated from absorbance at 280 nm by using its extinction coefficient at this wavelength ($A_{280}^{1mg/ml}$, 1 cm = 1.4). The amount of peroxidase was also calculated spectrophotometrically from absorbance at 403 nm using extinction coefficient ($A_{403}^{1mg/ml}$, 1 cm = 2.25).

Preparation of hLH Standards

hLH purified in this laboratory was calibrated against hLH standard preparation from the National Institute of Biological Standards and Control, Holly Hill, London NW3, and diluted from the stock in the filtered donor bovine serum to give a range of 0.7 IU/L-175 IU/L. The stock standard was stored in aliquots at 20 °C.

IRMA Procedure

100 ml of standarts or samples were incubated overnight at room temperature, with 200 µl of hLH IRMA reagent mixture. This reagent mixture consisted of 100.000 cpm ^{125}I labelled sheep anti-hLH I_gG and rabbit anti-hLH antiserum (1:50.000) in IRMA assay buffer. Separation of hLH bound from free labeled antibody was achieved by precipitation of bound

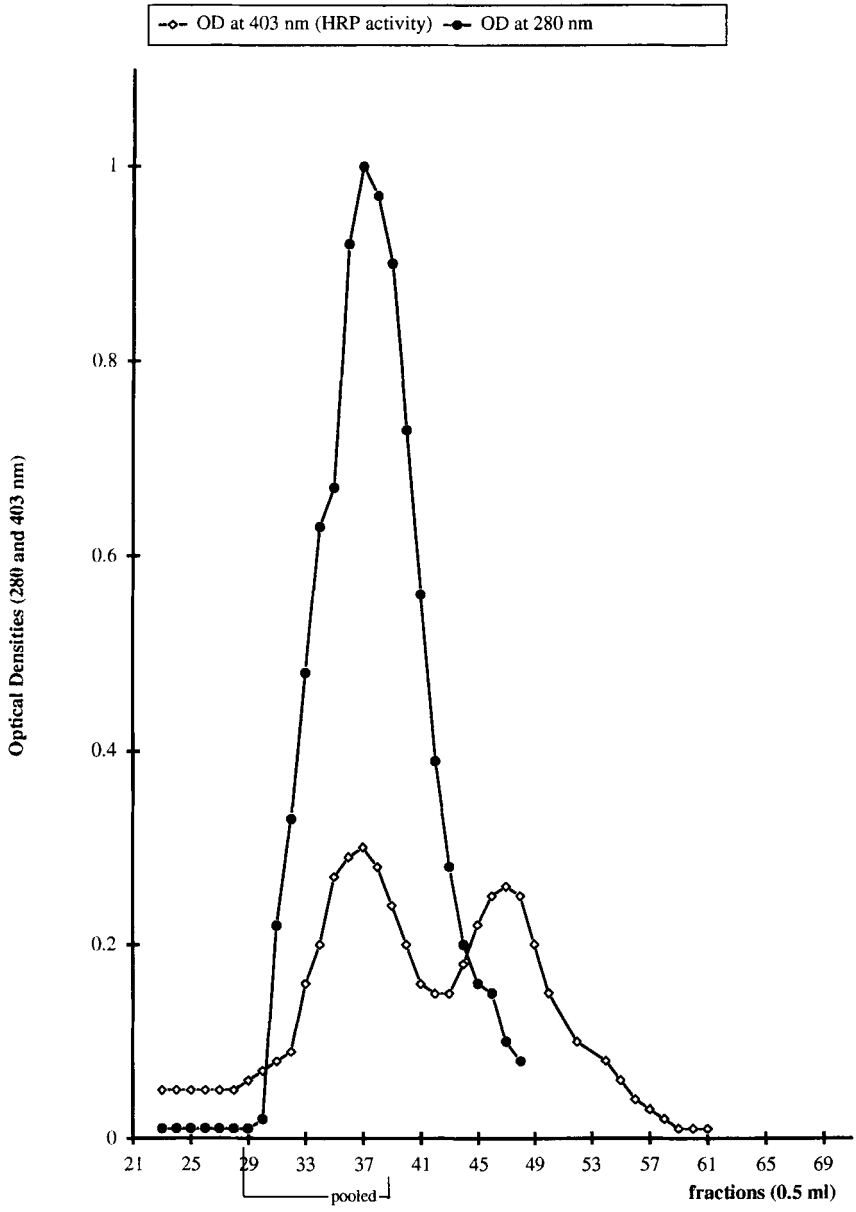

Figure 2: Purification of sheep anti-hLH IgG-HRP conjugate prepared with SMCC on sephacryl S-200 column. Eluted with o.1 M phosphate buffer, pH 6.0.

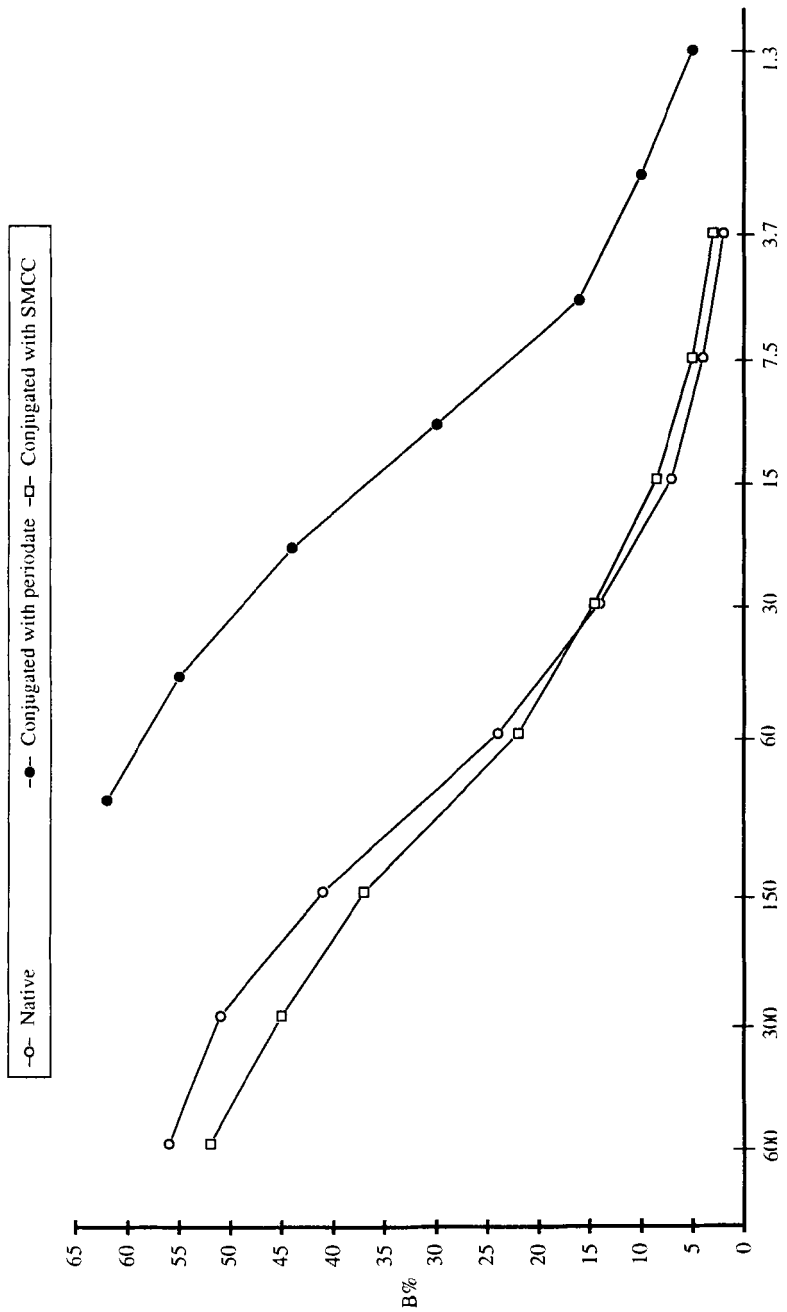

Figure 3: Dilution curves of native and conjugated sheep anti-hLH IgG

complex using SARFc antiserum. Precipitating reagent was prepared as a single reagent in 0.05 M phosphate buffer, pH7.4, containing 1/20 dilution of SARFc antiserum, 2% (w/v) PEG 6000, and 1% v/v normal rabbit serum as carrier. This reagent was left standing at 4°C to pre-precipitate before use in the assay as 200 µl. After 1/2h incubation at room temperature 1ml of saline was added into each tube and centrifuged at 2300g for 20 minutes at 4°C. The soluble free fraction was aspirated to waste and radioactivitiy was determined in the precipititate in an innotron hydrogamma - 16 counter.

EIMA PROCEDURE

The wells of micro titer plates (Nunc, DK 4000 Roskilde, Denmark) were coated with SARFcIgG, washed three times with PBS-Alb and 100µl of rabbit anti-hLH antiserum (1:20.000 dilution in buffer B) was added. After incubation for 1 hour at 37°C all wells were washed thrice with PBS-Alb. Addition of 100µl hLH standards or samples followed by 1 hour incubation at 37°C and washing again three times with PBS-Alb. Finally 100 µl of sheep anti-hLH I_gG-HRP (50ng/100µl) in buffer B was added to all wells and incubated for 1 hour at 37°C followed by washing as described above. The bound enzyme activity was assayed by addition of 100µl substrate solution (0.02%H_2O_2, 2mg/ml OPD in substrate buffer) and incubation at room temperature for 30min. The reactions were stopped using 100 µl 4M-Sulphuric acid and the colour was measured at 490nm.

RESULTS AND DISCUSSION

Preparation of Conjugate with Periodate and SMCC Methods

From 50 to 200 molar excess AMSA over I_gG were tested for mercaptosuccinylation of I_gG 200 molar AMSA at pH 7.0 was more effective than other concentrations and introduction of average number of thiol groups at this ratio were 1.2- 9.6 per I_gG molecule.

Figure 2 indicates the elution profile of conjugate prepared with SMCC, from sephacryl S-200 column. Conjugate formed were efficiently separated from unreacted HRP by gel filtration with sephacryl S-200. Unconjugated I_gG has not been separated since molecular weight of conjugated and unconjugated I_gG was not sufficiently different.

The recoveries of HRP and I_gG in conjugate slightly varied in the SMCC method increasing with increasing number of thiol groups introduced per antibody molecule. In the periodate method, although recovery could be increased by prolonged oxidation of HRP, highly

polymerized formation of conjugate was increased. Immunoreactivity of affinity purified sheep anti-hLH I_gG on the basis of dilution curve didn't significantly change by the conjugation in the SMCC method but tended to decrease in periodate method, Figure 3. Loss of immunoreactivity of conjugated I_gG prepared by periodate method was nearly more than 50%, whereas conjugate done by SMCC has an increased immunoreactivity which has been evaluated by the fact that conjugation could have effected hinge structure of I_gG with an increase of immunoreactivity. This result was consistent with the previous results that antibody activity was impaired in periodate methods (4,7). In addition, another problem in the periodate method is the sensitivitiy of the carbohydrate moiety of HRP to oxidation. Although this varied from lot to lot of HRP, the enzyme is over-oxidated in all current methods and consequently stronger oxidation produces the formation of polymers. On the other hand, we obtained the best results with the heterobifunctional reagent, SMCC, which was particularly stable and has been adopted successfully for the conjugation of HRP with mainly monoconjugates, resulting in lower non specific binding (results not shown). In this sdudy, I_gG-HRP conjugates prepared by SMCC contained more than 70% HRP incorporated into I_gG with highly much recovery of peroxidate activity and fully retained I_gG immunoreactivity.

Optimization and characteristics of EIMA

The sandwich solid-phase EIMA for hLH with affinity purified sheep anti-hLH I_gG-HRP conjugate prepared by SMCC method described above is more sensitive than the previously reported enzyme immunoassays in which monoclonal antibodies were employed. (The detection limit calculated from the standard deviation of absorbances for 20 replicates of the zero concentration standard at BO-(2xSD) was 0.15). There are several reasons for this high sensitivity. The first was the use of partially denaturated SARFcIgG to coat small surface of wells to lower the non-specific binding which was seen in the test systems using polystyrene balls as solid phase which provide large surface. The second reason for this high sensitivity was use of three antibodies in our EIMA. The third reason was that affinity purified sheep anti-hLH I_gG-HRP conjugate and one of the most sensitive substrate OPD were employed in our test. Standard curve of hLH-EIMA (Figure 4) is characterized by a 35 fold change in absorbances across the range of standards. In the same way a 40-fold change in bound radioactivity was found in our in-house hLH-IRMA in which same antibodies have been used as in EIMA.

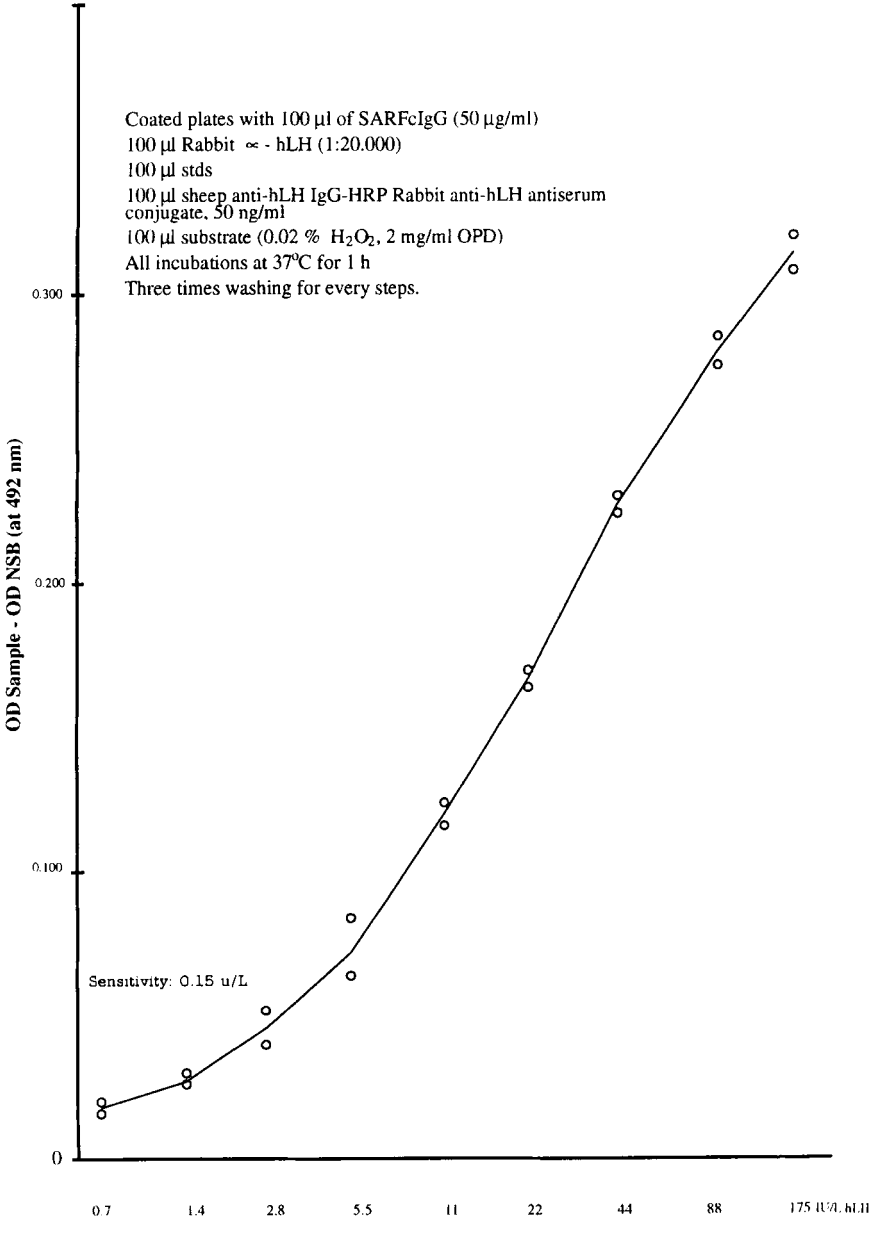

Figure 4: Standard curve of hLH-EIMA

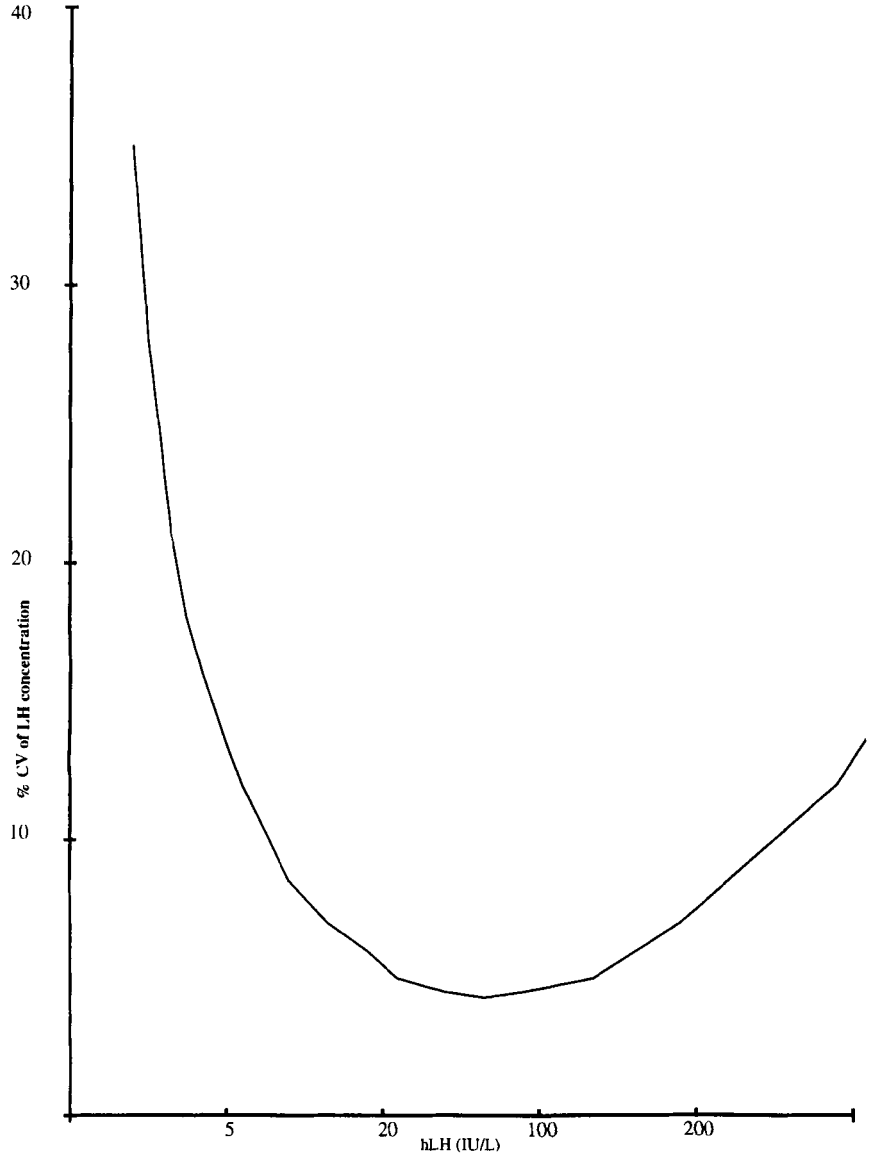

Figure 5: Precision profile of the hLH-EIMA employing the affinity purified sheep anti hLH IgG-HRP conjugate

The standard curves, precision profiles (Figure 5) of solid phase EIMA and liquid-phase "two-side" IRMA for hLH indicate that the sensitivities and precisions of the assays were satisfactory. A good correlation was found between these two methods (regression equation. y(IRMA)=0.95 (EIMA)+3.2 and coefficient for correlation 0.97). Furthermore, EIMA, which can be run in 3 hours, offers all the conveniences associated with the use of microtiter plates and associated equipment, was valid alternative to the IRMAs currently in use, which often need gamma counter for the iodine-label to be counted.

On the negative side, EIMAs described above are characterized by inconvenient successive washes to reduce non-specific binding and the enzyme-antibody conjugate, while it has a much longer half life than radioiodinated labels, was more complex to prepare.

The results of the application of the assays in a wide range of clinical experiments and determinations of ovulation are consistent with the assay being not just analytically valid but also clinically valid.

The EIMA presented here permits specific and accurate determination of hLH using standard equipment.

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