Studies on Purification and Coatation of Polyclonal Antibody for Prolactin Solid Phase Radioimmunoassay in Human Serum


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ABSTRACT

The objective of the present study was oriented to produce purified polyclonal antibody to prepare a prolactin solid phase coated tubes radioimmunoassay system. In the present study, production of polyclonal antibodies was carried out through immunization of three healthy white male mature New Zealand rabbits with a highly purified sheep prolactin antigen. The obtained anti-sera was purified using an anion exchange reactive group, diethylaminoethyle (DEAE) covalently linked to Sepharose. The purified polyclonal antibody was used for coating polystyrene tubes. The preparation of $^{125}$I-prolactin tracer was carried out using chloramine-T method. The preparation of standards was performed using assay buffer to cover the range from 2 to 200 ng/ml. The optimization and validation tests of the assay were performed to evaluate the validity of the prepared system. In conclusion, this low cost assay would be used in diagnosis of pituitary dysfunction and diagnosis of infertility in males and females.

Key Words: Prolactin, Ab Purification, RIA.

INTRODUCTION

Prolactin (PRL) is a protein hormone of the anterior pituitary gland (adenohypophysis). The human PRL is composed of 199 amino acids with molecular mass of 23000 Dalton$^{(1)}$. PRL circulates in blood in different forms, monomeric PRL 32 K Da dimeric PRL 48-56 KDa and polymeric forms $> 100$KDa$^{(2)}$. The monomeric form is considered the most bioactive of the different forms found in the circulation and demonstrates the greatest response to thyrotropin releasing hormone (TRH), the hypothalamic releasing factor that stimulates the pituitary to release PRL.

The measurement of prolactin in human sera is widely being used all over the world as a significant tool in diagnosis of pituitary dysfunction and possible reproductive disability$^{(3)}$.

IgG may be purified from serum by a simple one-step ion exchange chromatography procedure. This method is widely used and works on the principle that IgG has a high basic isoelectric point than most serum protein. Therefore, if the pH is kept below isoelectric point of most antibodies, the immunoglobulins do not bind to an anion exchanger and are separated from the majority of serum proteins bound to the column matrix. The high capacity of anion exchange columns allows for large scale purification of IgG from serum. The anion exchange reactive group, diethylaminoethyle (DEAE) covalently linked to Sepharose is useful for this purpose.

Solid phase separation methods in immunoassays can be classified into two groups: (1) Mobile solid phase reagents (particles free to move in solution) using centrifugation at low temperature for separation such as cellulose particles or magnetic rack for separation such as magnetic particles. (2) Stationary solid phase reagents (surface on which receptors or ligands have been adsorbed or covalently coupled)
which doesn't need centrifugation for separation such as coated tubes or coated beads (4). When selecting a solid phase for an immunoassay the assay should select a material that permits the covalent coupling of ligands onto its surfaces. This will avoid problems associated with poor adsorption onto surfaces and maximize the binding capacity of the sorbent. A system of finely divided solid phase coated tube has low-non specific binding with no need for centrifugation (5, 6).

**MATERIALS AND METHODS**

For production and establishment of RIA for prolactin, a number of different reagents were used. Highly purified prolactin antigen (Lactogenic hormone) L-6520, Sigma Chemical Co, Na-^{125}I (5 mCi/50 μl), pH 7-11 (IZOTOP Co, Budapest, Hungary), no carrier added and reducant free solution. Complete and incomplete Freund's adjuvants, Bovine Serum Albumin, Chloramine-T, Sodium Metabisulphite, sodium tetraborate (Borax) and D-Mannitol anhydrous were purchased from Sigma Chemical Co., USA. DEAE-Sepharose CL-6B Pharmacia, Uppsala, Sweden. All other chemical reagents were analytical (AR) grade obtained from reputed manufacturers.

Our research plan was achieved through the local preparations of the following:

**a- Polyclonal Antibody Production:**

Production of polyclonal anti-prolactin was carried out according to Shafik et. al. (7) through immunization of three male mature white New-Zealand rabbits, (2-2.5 Kg body weight). They were kept under the same hygienic conditions, well balanced diet and water was supplied ad-libitum. This production was undertaken by immunizing the three rabbits with highly purified sheep prolactin antigen (25 μg prolactin per rabbit). The immunogen preparation was performed by dissolving 0.1 mg highly purified prolactin antigen in a small volume of alkaline solution (0.01N NaOH and 0.05M Na₂CO₃-NaHCO₃, pH 9.5) and diluted up to 1 ml with saline (0.1 mg/mL). Then the solution was emulsified with 3 ml complete Freund's adjuvant. The immunization was carried out by injecting each rabbit with 1 ml emulsion at six sites subcutaneously over the shoulder region and four sites intramuscularly. The first booster immunization was given after 2 weeks interval from the primary immunization. The booster doses were giving in the same way except that the complete Freund's adjuvant was replaced by incomplete Freund's adjuvant. The titre of collected anti-sera for prolactin was assessed.

**b- Purification of Produced Polyclonal Antibody:**

A selected pool of anti-prolactin with highest titre was subjected to purification using DEAE-Sepharose method according to Majeei et al. (8). The purification step was started by adding 50% ammonium sulphate to participate the collected rabbit antisemum. The solution was stirred continuously for 30 min at room temperature, then centrifuged at 10,000 rpm for another 30 min. The collected clear supernatant contained IgG was dialyzed thoroughly against 0.07M phosphate buffer, pH 6.3 followed by ion exchange chromatography using DAEA-Sepharose. The IgG content was measured using A₂₈₀ UV spectrophotometer.

**c- Preparation of solid phase coated tubes:**

The coating process was carried out using the method described recently with some modifications (9). 300μl of diluted purified polyclonal antibody (IgG) 1:600 in 0.05 M borate buffer, pH8.0 were dispersed into polystyrene tubes. The tubes were incubated at 25°C overnight. After incubation the tubes were aspirated completely and washed. Then 300μl of 1% Bovine Serum Albumin (BSA) were pipetted into the tubes. The tubes were incubated at 25°C overnight. At the end of the incubation time, the content of the tubes were aspirated and washed. Then 300μl of 2% Mannitol (in distilled water)
were added to all tubes. The tubes were incubated for two hours at 25°C. Then the tubes were aspirated and washed well. The efficiency of the coated polystyrene tubes was tested by radioimmunoassay technique.

d- Iodination of prolactin:

The preparation of radiolabeled prolactin was performed using chloramine-T oxidation method as described by Hunter and Greenwood (10). To an eppendorf tube containing 5 μg of highly purified sheep prolactin in 10 μl phosphate buffer (0.05M, pH 7.4), 10 μl 0.5M phosphate buffer (pH 7.4) and 5 μl of Na$^{125}$I (500 μCi, 18.5 MBq) were added, respectively. The reaction was started by addition of 10 μl of 0.05M phosphate buffer (pH 7.4) containing 10 μg chloramine-T. The reaction was allowed to proceed for two minutes and stopped by addition of 10 μl 0.05M phosphate buffer (pH 7.4) containing 10 μg sodium metabisulphite. 100 μl 0.05M phosphate buffer (pH 7.4) containing 20 μg KI were added as a carrier. The product was purified by gel filtration using Sephadex G-100 (11).

e- Prolactin standards:

The preparation of standards for prolactin was done by dilution of highly purified sheep prolactin antigen using an assay buffer. The assay buffer, contained: 5 ml phosphate buffer (0.5 M, pH 7.4), 5 ml NaCl (3 M), 0.1 ml of triton (10% ), 0.1 g sodium azid and 10 ml protein solution (BSA) per 100 ml distilled water. The prolactin standard ranged from 2 - 200 ng/ml (7).

RESULTS AND DISCUSSION

The results and observations of the present work are summarized to prepare a valid assay to detect prolactin concentration in human serum.

With respect to the production of polyclonal anti-prolactin, the data obtained revealed that rabbit No. 2 gave the highest titre and highest displacement (Fig 1, 2). The data obtained for rabbit 2 showed that the Ab production increased up to 2 weeks after 1st booster followed by a slight decrease thereafter (Fig. 1).

![Fig. 1. The response obtained with booster doses of rabbit 2. (Where K=10¹).](image-url)
To obtain the best dilution for selected antibody, an experiment was designed to get the highest displacement between zero and high standard (200 ng/ml) as indicated in Fig. (2). Serum aliquots were taken from the individual bulk antiserum for testing in terms of specific binding percent and displacement percent. Different dilutions of antisera obtained were tested using liquid phase RIA system and the results show a high displacement was observed at dilution titre (1: 8000) of antibody produced from rabbit (R₂). Rabbits (R₁, R₃) gave poor immune response. So the polyclonal antibodies produced from rabbit R₂ were selected for coating after purification process.

Immunized rabbit sera were collected and precipitated by 50% ammonium sulphate. After dialysis against phosphate buffer, ion exchange chromatography was carried out using DEAE-Sepharose. The purity of eluted protein was checked using U.V. The results show a high peak was obtained after elution volume 45 ml eluent buffer but the top of the peak obtained after 30 ml elution buffer with OD= 2.5 at 280 nm. The concentration of IgG was calculated using $E_{1%}=13.6$ (i.e 1 mg/ml solution has 1.36
so that the IgG concentration equal to 1.838 (2.5 divided by 1.36). The purified PRL antibody was introduced directly for the preparation of this RIA technique.

Radioiodination of prolactin was carried out using chloramine-T as oxidizing agent and the elution profile was constructed by drawing activity (µCi) fraction No. as indicated in Fig.(3). The results show two peaks corresponding to \(^{125}\text{I}\)-prolactin (80.5%) and free \(^{125}\text{I}\) (16.4%). The specific activity was 60 µCi/µg.

3- Optimization of solid phase coated tube RIA system:

For achieving reliable assays, the general principals of the assay optimization must be followed. Optimization of RIA system for prolactin was carried out by studying the effect of incubation time, sample volume, incubation temperature, radioactivity of the tracer, washing methods and stability of coated tubes.

a- Incubation time:

The influence of the incubation time on this system using the locally prepared basic reagents including the prepared tracer was carried out throughout 24 hours, ranged from one hour to 24 hours, all at room temperature. The results showed that the displacement percent between the three concentrations (5, 50 and 100 ng/ml) used increases with increasing incubation time and reach the optimum at 3 hrs incubation time. This can be explained that 3 hrs incubation times were sufficient to reach the equilibrium between antigen-antibody complex and free antigen. So that the optimum incubation time proved to be 3 hours to get a reliable and valid determination for prolactin.

Table (1): Effect of incubation time on PRL- RIA solid phase.

<table>
<thead>
<tr>
<th>Incubation Time→</th>
<th>PRL Standard (ng/ml)↓</th>
<th>1h</th>
<th>2h</th>
<th>3h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.0</td>
<td>80.2</td>
<td>82.1</td>
<td><strong>84.6</strong></td>
<td>85.0</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>60.1</td>
<td>56.5</td>
<td><strong>52.8</strong></td>
<td>53.4</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>42.4</td>
<td>28.8</td>
<td><strong>21.3</strong></td>
<td>21.3</td>
</tr>
</tbody>
</table>

The data are presented as percent bound (B/Bo %).

b- Incubation temperature:

The assay procedure was performed as previously mentioned, but at variable temperatures. The data obtained reveal that the highest differences in binding between the three concentrations of prolactin were demonstrated using 37°C incubation temperature. This may be due to the thermodynamic reaction between Ag and Ab. This finding is in good agreement with Abdel-Fattah et al.(13).

Table (2): Effect of incubation temperature on solid phase RIA for PRL.

<table>
<thead>
<tr>
<th>Incubation Temp.→</th>
<th>PRL (ng/ml)↓</th>
<th>4 °C</th>
<th>25 °C</th>
<th>37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>80.4</td>
<td>82.9</td>
<td><strong>85.4</strong></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>71.2</td>
<td>52.4</td>
<td><strong>51.5</strong></td>
<td></td>
</tr>
<tr>
<td>100.0</td>
<td>51.2</td>
<td>23.7</td>
<td><strong>20.0</strong></td>
<td></td>
</tr>
</tbody>
</table>

The data are presented as percent bound (B/Bo%).

c- Radioactivity of the tracer:

Numerous counts of radiotracer ranged from 5000 to 20000 cpm in 200 µl were investigated. The results obtained reveal very interesting and consistent findings with the prepared tracer. The data
obtained shows that, the highest differences in percent binding between the three concentrations studied (5.0, 50.0 and 100.0 ng/ml) were proved using 15,000 cpm/200µl.

Table (3): Effect of tracer mass on the solid phase RIA for PRL.

<table>
<thead>
<tr>
<th>Tracer activity (cpm)→ PRL Standard (ng/ml)</th>
<th>5 000</th>
<th>10 000</th>
<th>15 000</th>
<th>20 000</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>72.2</td>
<td>80.3</td>
<td>84.6</td>
<td>82.1</td>
</tr>
<tr>
<td>50.0</td>
<td>62.3</td>
<td>56.5</td>
<td>52.2</td>
<td>50.0</td>
</tr>
<tr>
<td>100.0</td>
<td>30.4</td>
<td>28.0</td>
<td>20.9</td>
<td>21.0</td>
</tr>
</tbody>
</table>

d- Sample volume:

The assay procedures were performed using different volumes of PRL standards. The results obtained showed that the highest differences in binding between variable levels of PRL were found using 100 µl of standards. So, it can be concluded that the optimum volume of the PRL standards and that of the samples was 100 µl.

e- Washing procedure:

Our motivation to proceed with washing step in the local solid phase RIA system of the present study was to eliminate the problem related to inadequate separation between the free and the bound fractions of the tracer. With the progress in washing step, there was a decrease in binding reaching its constant binding after washing with 1 ml washing buffer twice. This finding is in good agreement with the published data\(^{(13,14)}\).

f- Stability of the coated tubes:

The results obtained demonstrated that these tubes could be stored for at least 14 months at 4°C without any appreciable reduction in binding.

Assay has been designed to give optimum sensitivity, maximum ruggedness and minimum error in the range of clinical interest for PRL. RIA was set up in the locally prepared coated polystyrene tubes. 100µl of standards or unknown samples were pipetted into the corresponding coated tubes. Then 200µl \(^{125}\text{I}-\text{PRL}\) were dispensed into all tubes (=15,000 cpm). The tubes were incubated for 3 hrs at 37°C. At the end of the incubation time the assay tubes were decanted and washed using 1 ml washing buffer twice. Then the tubes were counted in a gamma counter.

4- Validation tests for PRL coated tube RIA system:

For a valid and reliable prolactin assay, certain criteria should be achieved which might be considered also as advantages of RIA technique. These criteria included sensitivity, specificity, precision, accuracy and method comparison.

a- Sensitivity (Minimal detectable dose):

Detection limit of 0.5 ng/ml has been obtained by assaying 20 duplicates of the zero standard. The sensitivity has been determined as the concentration corresponding to mean count per minute minus its double standard deviation\(^{(14,15)}\).

Table (4): The sensitivity of PRL solid phase RIA system.

<table>
<thead>
<tr>
<th>Mean(X)</th>
<th>Mean(X) - 2SD</th>
<th>B/B₀ %</th>
<th>Apparent concentration (ng/ml)</th>
<th>Approximate sensitivity (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15200</td>
<td>14470</td>
<td>95.2</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>
b- Specificity (Cross-reactivity):

The PRL- RIA method of the present study is highly specific for PRL with an extremely low cross-reactivity to other glycoprotein hormones such as FSH, LH and β-hCG.

c- Precision:

The reliability of the present procedure was assessed by examining its reproducibility on pooled human serum samples selected to represent a wide range of PRL. The data of the intra-assay (Within Run) and inter-assay (Run to Run) precisions as illustrated in Table 5 show the consistency of the results obtained by the assays of the present work. In addition, these results are in agreement with numerous reports\(^{(15,16)}\). In conclusion, the reproducibility of the present assay is satisfactory.

Table (5): Precision profile for PRL assay.

<table>
<thead>
<tr>
<th>Serum PRL</th>
<th>Intra-assay</th>
<th>Inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ng/ml</td>
<td>SD</td>
</tr>
<tr>
<td>Pool (1)</td>
<td>5.8</td>
<td>0.50</td>
</tr>
<tr>
<td>Pool (2)</td>
<td>54.6</td>
<td>4.45</td>
</tr>
<tr>
<td>Pool (3)</td>
<td>100.7</td>
<td>9.5</td>
</tr>
</tbody>
</table>

d- Accuracy:

The assay accuracy was tested in this study by recovery and dilution tests\(^{(17)}\). Recovery test measures the concentrations in three human serum samples of different diagnostic states before and after adding known amounts of prolactin. The measurements are expressed as a percentage of the added mass(Table 6). The recovery data are in good agreement with Pillai and Bhandarkar\(^{(10)}\).

The results in Table (7) reveal the concentration of three human serum samples, and at various dilutions in the matrix of the assay (zero standards) to assess the linearity of the assays. Edwards\(^{(17)}\) stated that non-linearity indicates inaccurate calibration or inappropriate matrix or both. The results obtained show that the procedure of the present work for PRL maintains good linearity under dilution.

Table (6): Recovery test for PRL assay:

<table>
<thead>
<tr>
<th>Pooled Samples</th>
<th>PRL (ng/ml) endogenous</th>
<th>PRL added (ng/ml)</th>
<th>Observed (O)</th>
<th>Expected (E)</th>
<th>O/E %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.5</td>
<td>10 60 100</td>
<td>7.75 31.11 53.21</td>
<td>7.75 32.75 52.75</td>
<td>100 96.99 100.8</td>
</tr>
<tr>
<td>2</td>
<td>54.6</td>
<td>10 60 100</td>
<td>31.3 58.2 77.3</td>
<td>32.3 57.3 77.3</td>
<td>96.9 101.6 100</td>
</tr>
<tr>
<td>3</td>
<td>100.7</td>
<td>10 60 100</td>
<td>54.32 81.21 99.2</td>
<td>55.35 80.35 100.35</td>
<td>98.13 101.1 98.85</td>
</tr>
</tbody>
</table>
Table (7): Dilution test for PRL assay.

<table>
<thead>
<tr>
<th>Pool Samples</th>
<th>PRL (ng/ml) endogenous</th>
<th>Dilution factor</th>
<th>Observed (O)</th>
<th>Expected (E)</th>
<th>O/E%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.4</td>
<td>1:2</td>
<td>1.31</td>
<td>1.20</td>
<td>109.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:4</td>
<td>0.57</td>
<td>0.60</td>
<td>95.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:8</td>
<td>0.32</td>
<td>0.30</td>
<td>106.7</td>
</tr>
<tr>
<td>2</td>
<td>10.8</td>
<td>1:2</td>
<td>5.100</td>
<td>5.40</td>
<td>94.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:4</td>
<td>2.500</td>
<td>2.70</td>
<td>92.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:8</td>
<td>1.400</td>
<td>1.35</td>
<td>103.7</td>
</tr>
<tr>
<td>3</td>
<td>40.7</td>
<td>1:2</td>
<td>19.100</td>
<td>20.35</td>
<td>93.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:4</td>
<td>10.500</td>
<td>10.17</td>
<td>103.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:8</td>
<td>5.200</td>
<td>5.08</td>
<td>102.4</td>
</tr>
</tbody>
</table>

e- Method comparison:

The validity of this assay was tested by comparing the results of prolactin levels of 30 serum samples obtained by Siemens, IRMA technique. Results are comparable with correlation coefficients of 0.9977 as illustrated in Fig(4).

Fig(4): Regression line equation and correlation coefficient “r” between prolactin values obtained by Siemens method and the local solid phase coated tube.

CONCLUCION:

In conclusion, this coated tube solid phase RIA technique provides materials for the reliable quantitative measurement for prolactin in human serum. This assay is more than sufficient to fulfill the clinical requirement of prolactin assay.
REFERENCES