**125I-Labeled Gonadoliberin of High Specific Activity and Immunoreactivity: Method of Iodination and Rapid Separation**

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We describe optimum conditions for iodinating gonadoliberin with use of relatively large proportions of Na125I. Products of the iodination are separated on an anion-exchange resin (Amberlite IRA-400). The 125I-labeled gonadoliberin thus obtained has a high specific activity (1400 to 1590 Ci/g); because of the conditions of iodination, we believe that the predominant species of the labeled decapeptide is the mono-iodinated one. Our separation and purification of the labeled substance on anion-exchange resin is rapid, economical, and less cumbersome than the use of a Biogel P-2 column. There is no adsorption of the labeled hormone onto the resin, as evidenced by analytical recovery studies with tritium-labeled gonadoliberin. Paper-strip chromatoelectrophoresis showed no free Na125I or radiolabeled damaged peptide fragments after purification on the resin. When antisem was used at a concentration 32-fold that used in the regular assay procedure, only 4% of the radioactivity remained in the free form, indicating the high immunoreactivity of the labeled hormone.

**Additional Keyphrases:** ion-exchange chromatography · peptide hormone · radioassay

Gonadoliberin (gonadotropin-releasing factor) is a decapeptide, pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2 (I, 2), produced in the hypothalamus, which regulates the anterior pituitary hormones lutropin (luteinizing hormone) and follitropin (folllicle-stimulating hormone). Because it is present in peripheral circulation in very minute quantities, its measurement by radioimmunoassay (RIA)3 requires 125I-labeled gonadoliberin of high specific activity. The higher the specific activity, the less labeled hormone is needed, and consequently even the very minute amount of the hormone in a biological sample can be measured (3).

Because only one tyrosine is present in each molecule of gonadoliberin, iodination will result in either mono-iodinated or di-iodinated hormone. The formation of a tri-iodophenolic compound is improbable, owing to steric hindrance in the molecule. Substitution of an iodine atom in the histidine ring of gonadoliberin requires vigorous reaction conditions, which might damage the immunoreactivity. This limited capacity of the hormone to accept the iodine substituent imposes certain delicate but precise conditions for iodination, which need to be optimized. For separation of 125I-labeled gonadoliberin from other unwanted products of iodination, among the different methods that have been used are those involving chromatography on Sephadex G-25 (4), Biogel P-2 (5), carboxymethyl-cellulose (6), or anion-exchange resin (7). In this paper, we describe optimized and rapid microscale methods for the efficient labeling of gonadoliberin with Na125I and the purification of the radiolabeled hormone to obtain a product of high specific activity, immunoreactivity, and femtomole sensitivity in radioimmunoassay systems.

**Materials and Methods**

**Iodination of Gonadoliberin**

Gonadoliberin was iodinated essentially by the Chloramine-T oxidation procedure of Greenwood et al. (8), with necessary modifications. Two different quantities of gonadoliberin were used for labeling under similar experimental conditions. In the first labeling procedure we used 5 μg of gonadoliberin in 5 μL of Tris acetate buffer (0.2 mol/L, pH 7.3); in the second, we used 330 ng of gonadoliberin in 5 μL of the same Tris acetate buffer (these are hereafter referred to as Product 1 and Product 2, respectively). In each case we used 2 μCi of Na125I (cat. no. IMS-300; Amersham/Searle Corp., Arlington Heights, IL 60005) in 10 μL of phosphate buffer (0.4 mol/L, pH 7.5) and 50 μL of Tris acetate buffer (0.5 mol/L, pH 7.3). The reaction was started with 20 μL of freshly prepared Chloramine T (2.5 g/L in the 0.2 mol/L Tris acetate buffer), continued for 60 s with gentle agitation, and terminated by adding 50 μL of freshly prepared aqueous sodium metabisulfite (2.5 g/L). The reaction vial was gently agitated, by tapping, for another 15 s and then 100 μL of transfer solution (KI, 10 g/L in sucrose solution, 180 g/L) was added.

**Separation of Iodination Products on Biogel P-2 Column**

A disposable Biogel P-2 column (0.9 x 15 cm), equilibrated with 0.2 mol/L Tris acetate buffer and coated with 2 mL of a 2 g/L solution of bovine serum albumin in 100 mL of 0.2 mol/L Tris acetate buffer, was used. About half of the reaction mixture from the Product 1 iodination procedure was transferred to the Biogel P-2 column and eluted with 0.2 mol/L Tris acetate buffer containing 2.5 g of bovine serum albumin per liter. Thirty 0.5-mL fractions were collected in disposable plastic tubes precoated with a drop of albumin solution (20 g/L of 0.2 mol/L Tris acetate buffer). We measured the radioactivity of 10-μL aliquots with a gamma counter and determined the immunoreactivity of the various fractions in the radioactive peaks by short incubation experiments. The labeled hormone thus purified is designated Product 1A.

**Immunoreactivity of Product 1A Fractions by Short Incubation**

Fractions from the Biogel P-2 column were checked for immunoreactivity in terms of percent B/T binding by short incubation. We incubated 1 mL of a mixture of 2.5 mL of normal rabbit serum in 100 mL of PBS, excess anti-gonadoliberin (1:20 000 final dilution), and ~20 000 cpm of Product 1A from different fractions for 2 h at 37 °C and for 16 h at 4 °C. Bound 125I-labeled gonadoliberin was separated by adding excess (100 μL) goat antirabbit γ-globulin (Antibodies Incorporated, Davis, CA 95616) and incubating for another 6 h at 4 °C. The tubes were centrifuged at 4 °C and 2000 x g for...
30 min and the supernatant fluid was poured off. The precipitate was washed with 800 µL of a mixture of 1 mL of normal rabbit serum in 100 mL of PBS at 4 °C, and the tubes were centrifuged again under the same conditions. The second supernate was pooled with the first. Radioactivity in both the precipitate and supernate tubes was counted with the gamma counter.

Only fractions with high radioactivity were checked for immunoreactivity in this way. After the degree of binding to antiseraum was determined by short incubation, the fractions with high immunoreactivity were pooled. The specific activity, least detectable dose, mid-point of the displacement curve, and immunoreactivity in terms of percent B/T were determined with the use of the pooled fractions of Product 1A by setting up the RIA under the same conditions as described below for Product 1B and Product 2.

Separation of 125I-Labeled Gonadoliberin from Unwanted Products of Iodination by Use of Anion-Exchange Resin

Three grams of anion-exchange resin (Amberlite IRA-400; Mallinckrodt, Inc., St. Louis, MO 63147) was placed in each of two disposable plastic tubes and washed with 5 mL of the albumin/PBS mixture; 2 mL of this same mixture was added to one of the tubes, followed by the other half of the Product 1 iodination mixture. The tube was gently vortex-mixed and allowed to stand at 4 °C, with occasional mixing, for about 1 h. The supernate was then transferred to the second tube containing washed Amberlite IRA-400 and subjected to the same treatment it received in the first tube. This “Product 1B” was subsequently evaluated by RIA.

Product 2 was purified under the same conditions as Product 1B.

Adsorption of Gonadoliberin onto Anion-Exchange Resin

We weighed 3 g of Amberlite IRA-400 into a disposable plastic tube and washed it with 5 mL of albumin/PBS mixture. Excess buffer was poured off and the resin weighed again, to determine the amount of buffer still present. In another disposable plastic tube, 26.8 pg (516.4 pCi) of 3H-labeled gonadoliberin (spec. act. 19.2 Ci/g; New England Nuclear, Boston, MA 02118) in 50 µL of PBS was diluted to 2 mL with a 1 g/L solution of albumin/PBS. Duplicate 200-µL (51.6 pCi; 2.7 pg) portions of this diluted material were placed in 5 mL of Aquasol (Beckman Instruments, Inc., Fullerton, CA 10591) and their radioactivities counted with a liquid scintillation counter equipped with automatic quench correction (Model LS-350; Beckman). The remaining 1.6 mL (412.8 pCi; 21.6 pg) was transferred to the disposable plastic tube containing Amberlite IRA-400 and mixed, and the tube and contents were weighed again. The tube was kept overnight at 4 °C with occasional mixing, and, as before, the radioactivity in 200 µL was then counted in duplicate to evaluate any adsorption of gonadoliberin to the resin.

Paper-Strip Chromatoelectrophoresis of Product 2 Purified on the Resin

Five microliters of Product 2 that had been purified on the anion-exchange resin and mixed with plasma was electrophoresed for 16 h on Whatman No. 3 MM paper (2.5-cm width), with use of 0.1 mol/L barbital buffer, pH 8.6, at 100 V and 6 °C (9). The strips were dried at room temperature under a hood for 30 min and then cut into pieces 0.5-cm wide. The radioactivity in each piece was counted for 1 min with a Biogamma (Beckman) counter.

RIA of Gonadoliberin

We set up in duplicate an 11-point displacement curve, using the albumin/PBS system as diluent. The total volume in each tube was 800 µL, containing 3 mL of normal rabbit serum per liter as carrier, 25 mmol of ethylenediaminetetraacetate, anti-gonadoliberin (Miles Laboratories, Inc., Elkhart, IN 46515) at a final dilution of 80,000-fold, 7.5 µL of goat anti-rabbit gamma-globulin (Antibodies Incorporated), and about 5000 cpm of 125I-labeled gonadoliberin. Unlabeled gonadoliberin was used in the range of 2.5 pg to 2.5 ng. The assay mixture was incubated at 4 °C for 24 h, then the tubes were centrifuged (4 °C, 2000 × g, 40 min) and the supernatant fluid was poured off. The precipitate was washed with 600 µL of albumin/PBS solution at 4 °C and tubes were centrifuged again (same conditions). The second supernate was pooled with the first. Radioactivity in both the precipitate and supernate tubes was counted with the gamma counter. The RIA was also set up and processed with use of anti-gonadoliberin at final dilutions ranging from 1250- to 160,000-fold, each dilution being twice the previous one.

Measurement of Specific Activity of 125I-Labeled Gonadoliberin

The specific activities of Product 1A, 1B, and 2 were measured by comparing the binding (percent B/T) of increasing quantities (10, 20, 40, 80, 160, and 320 × 102 cpm) of labeled gonadoliberin with a constant but small quantity (5000 cpm) of labeled gonadoliberin added to increasing quantities (2.5 pg to 2.5 ng) of unlabeled gonadoliberin. We drew displacement curves for labeled (percent B/T vs cpm of 125I-labeled gonadoliberin) and unlabeled (percent B/T vs pg of hormone) gonadoliberin. However, in plotting the displacement curve for 125I-labeled gonadoliberin, we corrected by subtracting 5000 cpm (the constant small quantity of 125I-labeled gonadoliberin added to increasing quantities of unlabeled gonadoliberin) from the actual respective number of counts per minute used. In the region where the curves paralleled each other, the mass (picograms) of unlabeled gonadoliberin and the counts per minute of labeled gonadoliberin were read at the same percent B/T value. This value for counts per minute was taken as equivalent to the mass value of gonadoliberin. Three sets of values were used. The mean value for cpm/pg of gonadoliberin was calculated, and the specific activity of 125I-labeled gonadoliberin was expressed as Ci/g.

Results

Table 1 shows the salient features of the method of iodination and separation of Products 1A (n = 4), 1B (n = 4), and 2 (n = 12), where n is the number of iodinations from which the data are derived.

On the Biogel P-2 column, the iodination mixture (Product 1) gave two peaks. By short incubation, the immunoreactivity (B/T) of the fractions in the second radioactive peak (69-75%) was threefold that of those in the first peak (22%) (Figure 1). For four such similar but separate iodinations, we observed identical patterns in the distribution of radioactivity and immunoreactivity of different fractions. When only gonadoliberin or [3H]gonadoliberin or Na125I was eluted on a Biogel P-2 column, the Na125I eluted earlier than either the unlabeled or the tritium-labeled gonadoliberin. Product 2, purified

<table>
<thead>
<tr>
<th>Product</th>
<th>Amt. of hormone used</th>
<th>Amt. of Na125I used</th>
<th>Molar ratio</th>
<th>Method of sepn.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 µg</td>
<td>2 mCi</td>
<td>4.2:1</td>
<td>Bio-Gel P-2 column</td>
</tr>
<tr>
<td>1A</td>
<td></td>
<td></td>
<td></td>
<td>Anion-exchange resin</td>
</tr>
<tr>
<td>2</td>
<td>330 ng</td>
<td>2 mCi</td>
<td>1/3.6</td>
<td>Anion-exchange resin</td>
</tr>
<tr>
<td>1B</td>
<td></td>
<td></td>
<td></td>
<td>Anion-exchange resin</td>
</tr>
</tbody>
</table>
on Amberlite IRA-400, yielded only one radioactive peak when eluted from a Biogel P-2 column, and this peak corresponded with that of gonadoliberin and \textsuperscript{\textit{\textit{[3]}}}Hgonadoliberin.

There was an apparent 48% adsorption of \textsuperscript{\textit{\textit{[3]}}}Hgonadoliberin on Amberlite IRA-400. When corrections were made for the amount of buffer retained by the resin and consequent dilution of \textsuperscript{\textit{\textit{[3]}}}Hgonadoliberin, there was no loss of \textsuperscript{\textit{\textit{[3]}}}Hgonadoliberin by adsorption (Table 2).

The binding (B/T) of pooled fractions of Product 1A (those with maximum immunoreactivity on short incubation) under the conditions described was 33% (SEM 3) (Table 3). Under the same assay conditions, a similar binding of 34% (SEM 3) could also be obtained for Product 1B (Table 3).

From four separate iodinations, the specific activity of Product 1A (purified on Biogel P-2 column) varied from 120 to 170 Ci/g. This specific activity was determined only for those pooled fractions with maximum immunoreactivity as determined by short incubation. From a similar number of iodinations, the specific activity of Product 1B varied from 200 to 280 Ci/g (Table 3).

Under our assay conditions, Product 2 twice purified on Amberlite IRA-400 showed binding (B/T) of 50% in all our experiments (Table 3). From 12 iodinations the specific activity of Product 2 purified on Amberlite IRA-400 increased to 1400–1590 Ci/g (Table 3). When stored at 4 °C on Amberlite IRA-400, there was no significant loss in the immunoreactivity of \textsuperscript{125}I-labeled gonadoliberin for about three weeks. The labeled gonadoliberin gave a displacement curve that was superimposable on the displacement curve obtained with unlabeled gonadoliberin (Figure 4).

On paper-strip chromatoelectrophoresis, Product 2 gave only one peak, at the origin, and showed no peaks corresponding to damaged \textsuperscript{125}I-labeled gonadoliberin or free radiiodine (Figure 2). \textsuperscript{125}I-labeled gonadoliberin purified on Amberlite IRA-400 was found to be 96% bindable at a final anti-gonadoliberin dilution of 2500-fold (Figure 3).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Product 1A,} & \textbf{Product 1B,} & \textbf{Product 2,} \\
$\textbf{n} = 4$ & $\textbf{n} = 4$ & $\textbf{n} = 12$ \\
\hline
Specific act, Ci/g & 140 (10.8) & 235 (17.0) & 1475 (18.8) \\
Least detectable dose, pg & 10.4 (0.6) & 7.1 (0.4) & 3.3 (0.1) \\
Immunoreactivity, B/T & 33 (3) & 34 (3) & 50 (3) \\
Midpoint of displacement curve, pg & 60 (8) & 75 (10) & 95 (10) \\
\hline
\end{tabular}
\caption{Comparison of \textsuperscript{125}I-Labeled Gonadoliberin Characteristics$^a$}
\end{table}

\textit{$^a$ Nos. in parentheses are SEMs.}

\begin{table}[h]
\centering
\begin{tabular}{|l|c|}
\hline
Vol. of albumin/PBS diluent retained by resin & 1.45 mL \\
Vol. of tritiated hormone transferred & 1.6 mL \\
Dilution factor, (1.45 + 1.6)/1.6 & 1.91 \\
\hline
cpm from 200 \textmu L of \textsuperscript{3}H-labeled hormone & \\
solution: & \\
1) Pre-resin treatment & 2810 \\
2) Post-resin treatment (apparent & 1441 (radio-hormone adsorption = 48\%) \\
3) Post-resin treatment, corrected & 2752 \\
dilution & \\
Percent of radio-hormone adsorbed & 2 \\
\hline
\end{tabular}
\caption{Adsorption of Tritiated Gonadoliberin by Anion-Exchange Resin (Amberlite IRA-400)}
\end{table}

Fig. 2. Chromatoelectrophoresis of Product 2 purified by anion-exchange resin (Amberlite IRA-400)

Fig. 3. Maximum bindable radioactivity of Product 1B

A similar pattern was seen for Product 2

CLINICAL CHEMISTRY, Vol. 26, No. 5, 1980 575
Discussion

Gonadoliberin possesses only one tyrosyl phenolic group on the two adjacent carbons on which one or two 125I atoms can be substituted. The theoretical specific activity of mono-iodinated gonadoliberin, calculated by using Roth's (10) equation,

\[ 130 \times 10^6/M_r \text{ of hormone monomer} \times t_{1/2} \text{ of isotope (days)} \]

will be about 1616 Ci/g (\(M_r\) = relative molecular mass). If 5 \(\mu\)g of gonadoliberin is used for iodination, the amount of Na\(^{125}\) required will exceed 8 mCi. Most workers have used up to 2 mCi of Na\(^{125}\)I to iodinate 5 \(\mu\)g of gonadoliberin. From stoichiometric considerations, if these proportions of Na\(^{125}\)I and gonadoliberin are used for iodination, the maximum specific activity of 125I-labeled gonadoliberin would be about 400 Ci/g—if all the iodine were incorporated in the gonadoliberin molecule. Moreover, both iodinated and unlabeled gonadoliberin elute in the same fractions on the Biogel P-2 column. These two factors may be responsible for the low specific activity (50 Ci/g) reported by others (5) after iodinating gonadoliberin and then separating the iodination mixture on a Biogel P-2 column. Under these conditions we obtained Product 1A with specific activities that varied from 120 to 170 Ci/g. Other workers (5) have assumed that the first radioactive peak from the Biogel P-2 column corresponded to the labeled hormone. However, we have observed that free Na\(^{125}\)I elutes first from the Biogel P-2 column and 125I-labeled gonadoliberin elutes only in the later fractions. As determined by short incubation, the later fractions, corresponding to the second radioactive peak, have a higher immunoreactivity (\(B/T\) = 69 to 75%) than those of the first radioactive peak (\(B/T\) = 22%). This gives further indirect support to our observation that 125I-labeled gonadoliberin elutes from the Biogel P-2 column after free radioactive iodine.

To obtain Product 2, the reaction mixture for iodination contains 0.25 nmol of gonadoliberin (\(M_r\) 1345.5) and 0.9 nmol of radioiodine. Na\(^{125}\)I was intentionally added in this relatively large proportion so that the concentration of iodine during iodination reaction does not decline substantially (10). Thus, excess Na\(^{125}\)I per mole of the peptide hormone and a several-hundred-fold excess of Chloramine T are present in the reaction mixture. In aqueous medium, Chloramine T, the sodium salt of N-chloro-p-toluenesulfonylamine, gives rise to sodium hypochlorite, which oxidizes Na\(^{125}\)I to an active iodinating species, iodine monochloride, as given below:

\[
\text{(H}_2\text{C} = \text{O})_2\text{SO}_2\text{Cl}^- + \text{Na}^+ + \text{H}_2\text{O} \rightarrow \text{NaClO} + \text{H}_2\text{C} = \text{O} \rightarrow \text{SO}_2\text{Cl}^- + \text{Na}^+
\]

The higher electronegativity of the chlorine atom in ClI renders the iodine positively polarized.

\[
\text{ICI} = \text{I}^- - \text{Cl}^-
\]

At the slightly alkaline pH (7.3) of the reaction mixture, the tyrosyl phenolic group is stripped of its electrons and the electron-rich oxygen contributes towards the formation of a carbocation (C\(^+\)), which facilitates the addition of the I\(^-\)--Cl\(^-\) species followed by a substitution reaction in the nucleus with elimination of HCl. Thus, a conversion of the tyrosyl residue to the 3-mono-iodotyrosine occurs. The ICI and Chloramine T are also potentially capable of reacting with other amino acids such as tryptophan and histidine. Such oxidation reactions are prevented by the slightly alkaline pH maintained in the reaction medium and also by immediate addition of excess amounts of sodium metabisulfite. It has also been observed that the oxidation of the tryptophan moiety in a protein molecule will result in the destruction of its immunoreactivity and biological activity (11).

When we used anion-exchange resin to separate the contents of the iodination mixture containing 5 \(\mu\)g of gonadoliberin (Product 1B), the specific activity (200–280 Ci/g) was higher than that obtained by Hichens et al. (5) (50 Ci/g), but not much different from that of Product 1A (120 to 170 Ci/g). This may explain why the immunoreactivity (percent \(B/T\)) of Product 1A and Product 1B is nearly the same. This specific activity (200 to 280 Ci/g) of Product 1B is still far below the theoretical specific activity. However, when we reduced the amount of iodoglucose to 330 ng and used 2 mCi of Na\(^{125}\)I, the observed specific activity (1400 to 1580 Ci/g) came very close to the theoretical specific activity (1616 Ci/g) of moniodinated 125I-labeled gonadoliberin. Also, in the absence of unlabeled gonadoliberin in the binding (\(B/T\)) of Product 2 (50%) increased nearly 50% over that of Product 1B (34%). This may be because of the high specific activity of Product 2, compared with that of Product 1B (Table 2). 125I-labeled gonadoliberin is stable at 4 °C for about three weeks after iodination.

There is essentially no adsorption of gonadoliberin by the anion-exchange resin, as shown by the experiment with [3H]gonadoliberin (Table 2). Ideally, we should have checked the adsorption of 125I-labeled gonadoliberin by anion-exchange resin. However, constant breakdown of 125I-labeled gonadoliberin and the resulting formation of free radiiodine prevent one's making any reliable conclusion. Any loss of counts will be taken as adsorption of 125I-labeled gonadoliberin on resin, whereas actually the loss of counts will be due
to liberation and subsequent adsorption of radioactive iodine.

All the free Na\(^{125}\)I and other ruptured label fragments of gonadoliberin were adsorbed on the anion-exchange resin, as shown by the elution pattern on the Biogel P-2 column of the repurified Product 2. The paper-strip chromatofocusing also failed to show any free iodine or fragments of radio-peptide. A binding capacity of 96% for the label also supports our contention that there are no damaged radioactive gonadoliberin or free radioiodine molecules left in solution after purification on anion-exchange resin.

Nett and Adams (7) have shown that the diido-gonadoliberin binds more readily than monoido-gonadoliberin to high \(M_\text{r}\) components of serum and seems to bind less readily to antisera; therefore, it is advantageous to obtain only monoido-gonadoliberin. We do not have any direct evidence to show that our method of separation of iodination products gives only monoido-gonadoliberin, but we have strong reasons to believe that the predominant iodinated species of gonadoliberin has only one atom of iodine per molecule of the hormone. Tyrosine is the only amino acid of gonadoliberin that can be iodinated under our experimental conditions. The phenolic pK (12, 13) of diiodotyrosine (6.4) is considerably less than that of monoidotyrosine (8.2) and tyrosine (10.1). Therefore, the substitution of iodine in phenolic positions 3 and 5 of tyrosine results in the formation of phenolate anion at the pH (7.3) of our iodination reaction. During separation and storage of \(^{125}\)I-labeled gonadoliberin the pH of the medium is raised to 7.8, which will further enhance the adsorption of diido-gonadoliberin anion by the anion-exchange resin.

The apparent average affinity constant (\(K_{\text{av}}\)) of this anti-gonadoliberin was calculated to be \(2.4 \times 10^{10} \text{ mol}^{-1}\), and is about the same as the \(K_{\text{av}}\) determined for most antisera used in RIA (14). To find the approximate optimum concentrations of \(^{125}\)I-labeled gonadoliberin and anti-gonadoliberin, we used Ekin's rule (15), according to which the optimum quantity of labeled antigen and antibody under the present conditions will be 179.5 pg (4/\(K\)) and 1.2 \(\times 10^{-10} \text{ mol/L} (3/\(K\)), respectively. However, we have used a much lower concentration of anti-gonadoliberin (5.1 \(\times 10^{-11} \text{ mol/L}\) and labeled gonadoliberin (1.2–2.6 pg) to obtain a sharper displacement curve in the lower ranges.

In conclusion, because one of the requirements for the detection of very minute amounts of gonadoliberin in biological fluids by RIA is to obtain moniodo\(^{125}\)I-labeled gonadoliberin of high specific activity, we have developed relatively simple, economical, and rapid methods for obtaining such labeled gonadoliberin and describe here the optimum conditions for setting up the RIA for gonadoliberin.

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Fig. 5. Apparent average affinity constant (\(K_{\text{av}}\)) of unlabeled gonadoliberin (X) and product 2 (O)


