HAPTEN-RADIOIMMUNOASSAY: A GENERAL PROCEDURE FOR THE ESTIMATION OF STEROIDAL AND OTHER HAPTENIC SUBSTANCES

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ABSTRACT

Hapten-radioimmunoassay may be used for sensitive and specific quantitation of low molecular weight substances which can be made immunogenic by conjugation to a protein. This method, which involves radioiodination of the protein-portion of the conjugate, has been used for quantitation of non-conjugated estrogenic steroids.

Radioimmunoassay procedures have been applied to the estimation of proteins and large polypeptides (1, 2) since these substances elicit specific antisera and contain tyrosine residues which may be radioiodinated to give preparations with high specific activity. This approach has been extended to similar labeled peptides which, although non-immunogenic by themselves become immunogenic when conjugated to a protein (3, 4). Non-immunological radioassays have been developed for estimation of certain steroids and vitamins for which specific naturally occurring binding agents were available (5-13). In this approach, termed competitive protein binding (5, 6), saturation analysis (7, 8) or radio-ligand binding (9), the labeled steroid or vitamin (ligand) has been allowed to compete with varying amounts of unlabeled ligand

for available reactive sites on the binding agent. The obvious limitation of this approach as a general method is the need for specific, stable and avid binding agents and radioisotopically labeled, high specific activity ligands. This communication describes a radioimmunoassay procedure applicable for any small molecular weight substance which can act as a hapten (immunogenic following conjugation to a protein). In this approach the protein portion of the conjugate is radioiodinated and used in combination with antibodies specific for the hapten for radioimmunoassay of free hapten. The feasibility of the method was established in the following preliminary studies with estradiol-17β (E2) which was conjugated to bovine serum albumin (BSA) through its chloro-derivative. That antibodies against steroid hormones can be obtained by immunization with steroid-protein conjugates has been well documented (14-18). The double antibody radioimmunoassay and radioiodination procedures used in this study were similar to those reported from this laboratory for several protein hormones (19-21).

METHODS

One gram of E2 was dissolved in 200 ml dry ether and phosgene gas bubbled through the solution for one hour at room temperature. Twenty-four hours later, nitrogen gas was bubbled into the solution for two hours to remove excess phosgene. Ether was then evaporated in vacuum and the resulting chloro-derivative was crystallized from acetone. It was not determined whether the resulting chlorocarbonate was at the 3, the 17 or both of these carbons. One hundred five mg of BSA (Pentex, crystalline) was dissolved in 2.5 ml of water and the pH was adjusted to 9.0 by the addition of 9.48 ml of 1N NaOH. One hundred mg of the chloro-derivative dissolved in 2 ml of dioxane was added gradually to the protein solution, and stirring was continual for 4 hours at 0°C. The product (E2-BSA) was dialyzed first against 60% aqueous dioxane and then exhaustively against water, and lyophilized.

For the preparation of antiserum each rabbit was injected in the foot pads and multiple subcutaneous sites with 1 ml of complete Freund's adjuvant containing 1 mg of E2-BSA. This procedure was repeated twice at three week intervals and after the third injection the animals were bled weekly.

E2-BSA was radioiodinated with ^{131}I by a modification of the method of Greenwood et al (22) as described previously (19-21). A double antibody radioimmunoassay procedure was employed in the presence of excess BSA in order to suppress antibodies directed against this portion of the conjugate. Each assay tube contained 0.5 ml of 5% BSA in 0.01 M phosphate-buffered 0.14 M NaCL, pH 7.0 (PBS) with variable amounts of the test steroid and 0.2 ml of antibody diluted in 1:400 normal rabbit serum. The tubes were left at 4°C for 24 hours and then 0.1 ml of E2-BSA-^{131}I (0.2 mμg) was added. After an additional 24 hours at 4°C, 0.2 ml of sheep anti-rabbit gamma globulin serum was added at a dilution capable of maximally precipitating the rabbit gamma globulin in each tube. After three days the tubes were centrifuged and the radio-activity of the precipitates was counted. For standards, various steroids in an amount not to exceed 10 mg were dissolved in 1 ml of ether to which was added 2 ml of ethanol followed by 5% BSA-PBS to give a final volume of 100 ml. All subsequent dilutions were also made in 5% BSA-PBS.

RESULTS

As shown in Table 1, with anti-E2-BSA added at a dilution of 1:4000, 31% of E2-BSA-^{131}I with a specific activity of 330 mC/mg was bound by antibody. More extensive iodination resulted in a loss in the immunoreactivity of the conjugate. This suggests that as the specific activity of the conjugate increased, iodination of the phenolic ring A of the estradiol occurred which decreased its reactivity with the antiserum.

TABLE 1

IMMUNOREACTIVITY OF E2-BSA-^{131}I CONJUGATES PREPARED WITH DIFFERENT SPECIFIC ACTIVITIES

Iodination Conditions				
Amount of Conjugate μg	Amount of Chloromine-T μg	Amount of I^{131} mc	Resulting Specific Activity mc/mg	% of Conjugate-^{131}I bound To antibody
2.5	60	1.0	330	31.1
2.5	60	6.25	1845	9.9
1.0	60	6.25	4304	4.8

As illustrated in Figure 1, increasing amounts of free estradiol in PBS-5% BSA resulted in a progressive decrease in the amount of antibody-bound E2-BSA-^{131} I. (The amount of radioactivity bound to antibody in buffer-control tubes was considered as 100%). A significant inhibition was obtained by 0.1 mμg of estradiol-17β (the amount which inhibited to the minimum value of the 95% confidence limits of the PBS-BSA control tubes).

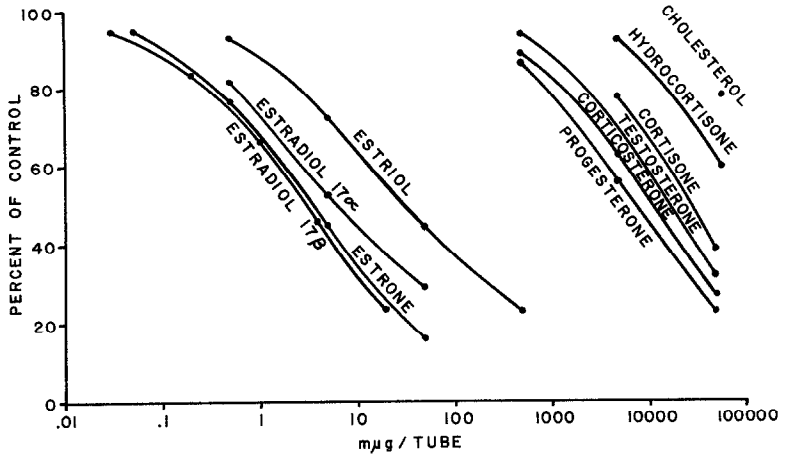

Figure 1. Inhibition by various steroids of the reaction between E2-BSA-^{131} I and anti-E2-BSA in the presence of excess BSA. See text for details.

The relative potencies of other steroids tested, read at 78% inhibition (Figure 1) and expressed in percent equivalents of estradiol-17β, are as follows: estrone, 81; estradiol 17α, 52; estriol, 12; progesterone, 0.03; corticosterone, 0.02; testosterone, 0.01; cortisone, 0.008; hydrocortisone, 0.002; and cholesterol, 0.0006. With the exception of the estrogenic compounds, other steroids contained one 3,300th to one 167,000th the activity.

DISCUSSION

The assay just described was not presented as a definitive procedure for estrogenic steroids but rather an example of the feasibility of hapten-radioimmunoassay. Many improvements could be made to the assay. Thus, conjugates should be prepared at a single position on the steroid molecule, preferably at a site other than the 3 or 17 carbon to increase chances for obtaining a more specific antiserum (23). The ideal labeled preparation should have one steroid conjugated to a polypeptide with many tyrosine residues, such as the copolymer polytyrosine. Since, as indicated by the results in Table 1, iodination of the phenolic ring A of estradiol likely occurred, attempts could be made to protect the steroid during iodination by reacting it with insolubilized antibody, and then recovering the fully immunoreactive labeled conjugate by elution. Attempts could also be made to obtain better antisera by immunization of more rabbits and by absorption of the antisera with insoluble conjugates of other steroids. Studies in progress (G.D. Niswender, T. Higgins, A.R. Midgley, Jr.) have indicated that it will be possible to use these procedures to develop a highly sensitive and specific assay for testosterone (24).

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24. Antisera from rabbits immunized with testosterone-17-BSA have been found to react strongly with testosterone-17-rabbit serum albumin (RSA)-^{131}I and very poorly with testosterone-3RSA-^{131}I. The converse is also true, i.e., antibodies to testosterone-3BSA react almost exclusively with testosterone-3-RSA-^{131}I and not with testosterone-17-RSA-^{131}I.