A Fast and Convenient Method for Determining Estrogen Receptor Using Rabbit Uterus as a Standard

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ABSTRACT

A fast and convenient method is described for the determination of estrogen receptors (ERs). This method involves the use of rabbit uterus as a standard. ER content of the rabbit uterus powder was determined using the conventional methods, i.e., Scatchard plot and sucrose density gradient methods. The rabbit uterus cytosol was serially diluted to give a range of protein concentrations from 1 to 0.062 mg/ml. A standard curve was thus generated with the corresponding ER concentrations, and this curve was used for the determination of ERs in breast tumors. The method involved incubating the standards with 125I-estradiol and subsequent removal of the free radiolabeled estradiol using dextran-coated charcoal. A parallel sample was also incubated with diethylstilbestrol. A standard curve was obtained between the 125I-estradiol percentage of binding and the corresponding ER concentration. Tumor cytosols were also treated in a similar manner, and the receptor content was determined from the standard curve. Excellent correlation was obtained between this method and other conventional methods. This method is simpler and less time consuming, and up to ten tumors can be analyzed at one time. It is especially useful when limited amounts of tumor tissue are available, as a concentration of only 1 mg of protein per ml is required.

INTRODUCTION

Numerous reports have shown that measurement of steroid receptors in breast cancer is useful in predicting response to endocrine therapy (1, 15, 16). During the past decade, the content of ERs and progesterone receptors in breast cancer has been shown to correlate to a high degree with response to endocrine therapy (3, 19, 24). The importance of ER and progesterone receptor determination in the management of breast cancer has increased to such an extent that these assays are done routinely on all surgically removed breast cancers.

Several assay systems have been used since the evolution of the concept of ER (15). Some of the methods used most frequently are SDG (15, 18), SSD (4), and Scatchard plot analysis (11, 21). The SDG method is time consuming, and only a limited number of samples can be studied at a given time; Scatchard plot analysis involves a multiple point assay, which is also time consuming. In addition, these methods require a large amount of tissue to maintain an adequate protein concentration. A simplified Scatchard plot assay has been introduced by Nakamura and coworkers. This method is useful when a limited amount of tissue is available (9, 10).

In 1979, Hochberg (7) first reported the synthesis of the iodinated estradiol derivative (8). Since then, its usefulness in the measurement of ER in breast tumors has been demonstrated (22). The superiority and sensitivity of γ isotopes over β isotopes are well known, thus resulting in assays which are very sensitive. In addition, γ isotopes have the advantage of higher efficiency and counting simplicity. A study done by Tercero et al. (22) comparing [3H]estradiol and 125I-estradiol warrants the use of the latter because of its cost reduction and diminished radioactive disposal problems. These investigators have also noted that, because of its higher specific activity, a smaller quantity of labeled 125I-estradiol could be used.

Our assay utilized rabbit uterus with a relatively high ER content (110 fmol/mg) as a standard. The receptor content was determined using the conventional methods, i.e., Scatchard plot and SDG methods. The rabbit uterus cytosol was serially diluted to give a range of protein concentrations from 1 to 0.062 mg/ml. The method involved incubating each cytosol dilution with 125I-estradiol with subsequent removal of the free radiolabeled estradiol using dextran-coated charcoal. A parallel sample was also incubated with diethylstilbestrol for the nonspecific binding. A standard curve was generated between the 125I-estradiol percentage of binding and the corresponding ER concentration. Tumor cytosols were also treated the same way, and the receptor content was determined from the standard curve.

MATERIALS AND METHODS

Preparation of Standard Cytosol. Rabbit uterus was used as a source of standards for generating the standard curve. Female New Zealand rabbits weighing 7 to 8 lb were anesthetized with Nembutal Sodium (Pentobarbital Sodium dose, 7.2 ml of 1:2 dilution). The abdomen was shaved, and the abdominal cavity was opened with a vertical incision. The uterus, tubes, and ovaries were identified and released from the neighboring tissues. The uterus was separated from the surrounding fat in an ice bath, rapidly frozen in liquid nitrogen (Linde liquid N2 tank), transported on dry ice to the laboratory, and stored at −70°C. The details of the procedure for preparing cytosol were as described elsewhere (14, 15, 25). The tissue was pulverized by Thermovac pulverizer (Thermovac frozen tissue pulverizer, Thermovac Industries Corp., Copiaque, N. Y.). Then, the tissue powder was homogenized (Polytron PT-10 ST homogenizer; Brinkmann Instruments, Inc., Westbury, N. Y.) with about 10 to 12 ml of 0.1 M phosphate buffer (containing 0.345 mg of sodium monophosphate and 450 ml of deionized water, pH adjusted to 7.4 by NaOH and the volume made up to 500 ml). The homogenate was centrifuged in a Beckman Model L5-75 ultracentrifuge (Palo Alto, Calif.) at 25,000 × g, 5°C, for 1 hr in a Model 60TI, 1898 rotor head. The cytosol was decanted, the residue was homogenized with 6 to 8 ml of buffer and centrifuged at the same speed for 1 hr, and the supernatant was added to the cytosol.

The protein content of the cytosol was determined by the spectrophotometric method (12). The protein concentration was then adjusted to 2 mg/ml with the phosphate buffer. Protein determination was also done...
by the method of Lowry et al. (13). (All the receptor concentrations in the text are expressed after determination of protein by the method of Lowry et al.) Aliquots of 2.5 ml were transferred to 5-ml glass vials, stoppered, and stored at -70°. The frozen cytosols were then lyophilized overnight (Unilab II; Virtis, Gardiner, N. Y.) and stored at -70°.

Preparation of Tumor Cytosols. Surgically resected breast tumors were rapidly frozen in liquid nitrogen, and cytosols were prepared in phosphate buffer (15). A protein concentration of approximately 1 mg/ml was used.

Preparation of Positive and Negative Controls. Human uterus was used as a positive control, male rat thigh muscle was used as a negative control, and cytosols were prepared in phosphate buffer (15). After determining the protein concentration, 1.5 ml were aliquoted in vials (1 mg/ml) and stored at -70° (26).

Scatchard Analysis of ER in Rabbit Uterus Cytosol. The RIANEN [³H]ER assay kit (New England Nuclear, Boston, Mass.) was used to determine the receptor content in the rabbit uterus (20). This kit contained various levels of concentration of [³H]estradiol. Scatchard plot analysis on the rabbit uterus ER was determined as described in their protocol. Subsequent protein determination was also done according to the method of Lowry et al., and the receptor content after correction with protein was estimated to be 112.4 fmol/mg of protein.

SDG Analysis of Rabbit Uterus Cytosol. Rabbit uterus cytosol (2 mg/ml) was also analyzed by the SDG method (15, 18). After incubation with [³H]estradiol, the samples were layered on a linear gradient (30 to 10% sucrose) and centrifuged for 16 hr at 425,000 x g at 4°. Fractions of 0.2 ml were collected, and the radioactivity was counted in a liquid scintillation spectrometer (Mark III; Tracer Analytic, Inc.).

SSD Analysis of Rabbit Uterus Cytosol. Rabbit uterus cytosol (2 mg/ml) was also analyzed by the SSD method (4). The cytosol was incubated with [³H]estradiol overnight at 4°. The following day, excess [³H]estradiol was removed by dextran-coated charcoal, and 0.2 ml of the supernatant was counted in a liquid scintillation spectrometer for [³H]estradiol activity. The receptor content was estimated to be 107.6 fmol/mg of protein.

Generation of the Standard Curve. The lyophilized rabbit uterus cytosol was reconstituted with 2.5 ml of deionized water. This was further diluted with 2.5 ml of phosphate buffer to give a protein concentration of 1 mg/ml. The standard curve consisted of protein concentrations of 1, 0.75, 0.50, 0.25, 0.125, and 0.062 mg/ml. These dilutions were obtained by serially diluting the cytosol solution with phosphate buffer.

Methodology. The methodology principally was based on binding estradiol with 125I (16β-iodo-3,17β-estradiol; New England Nuclear Catalogue No. NEX 144; specific activity, 2050 Ci/mmol) to the receptor. Vials, each containing fifty µl of each level of protein concentration ranging from 1 to 0.062 mg/ml were incubated with 10 µl of DES (80 pmol). After 10 min of incubation at ice-cold temperatures, 100 µl of 125I-estradiol (1 pmol) were added to all the tubes. After overnight incubation at 4°, the mixture was transferred onto the charcoal pellets and incubated for 10 min at 4°. Charcoal pellets were made fresh from charcoal slurry, which contained 0.25% Norit A.0.0025% dextran in 0.01 M Tris, pH 8.0 [Norit A: activated charcoal (untreated powder), Lot 18B-260, Sigma Chemical Co.; dextran: Grade C (clinical grade), Mann Research]. The mixture was then centrifuged at 1500 x g for 15 min, and 200 µl of the supernatant were counted in a gamma counter for 125I activity (Searle Analytic, Inc., Model 1285 automatic γ system). The nonspecific binding is represented by the tubes containing DES, and the specific binding is represented by the tubes containing only 125I-estradiol. After subtracting nonspecific binding from specific binding, the percentage of binding at each level of the concentration was calculated. A standard curve was generated using the percentage of binding on the Y-axis and the corresponding receptor concentrations on the X-axis (Chart 1).

Similarly, 250 µl of the tumor cytosols and controls were incubated in duplicate with 10 µl of DES. After 10 min of incubation, 100 µl of 125I-estradiol were added to all tubes. The rest of the procedure was as described above. The percentage of binding for ER in each specimen was calculated, and the corresponding receptor content was obtained from the standard curve.

Stability of Rabbit Uterus Cytosol. Receptor content determined from time to time by Scatchard plot analysis and SSD analysis revealed a nearly consistent receptor content of 110 fmol/mg. We have been using this cytosol for the past 4 months and have found no deterioration of the receptor concentration.

RESULTS

Lyophilized rabbit uterus powder contained 110 fmol/mg of ER, and this value of 110 fmol/mg was a mean of assays as determined by Scatchard plot analysis (112.4 fmol/mg) and the SSD method (107.6 fmol/mg) (Chart 2). The binding characteristics of the ER protein in rabbit uterus as determined by Scatchard plot analysis confirm the presence of ER, i.e., Kd, 1.3 x 10¹⁰ liters/mol; KD, 0.729 x 10⁻¹⁰ mol/liter (Chart 2). Also, SDG analysis confirmed the presence of 4S and 8S fragments in Fractions 12 and 20 as shown in Chart 3.

The standard curve used the rabbit uterus cytosol diluted to contain 0.75, 0.50, 0.25, 0.125, and 0.062 mg of protein per ml. The corresponding receptor contents were 110, 82.5, 55, 27.5, 13.75, and 6.8 fmol/mg (Chart 1). Tumors analyzed by this method varied from zero fmol/mg to high values of 97 fmol/mg. Comparison between Scatchard plot analysis and this method indicates an excellent correlation (r = 0.96) as shown in Chart 4. Similar correlation was obtained with the SSD method (r = 0.97) as shown in Chart 5.

It is generally considered that more than 10 fmol/mg of receptors is positive, less than 3 fmol/mg is negative, and between 3 and 9 fmol/mg is borderline. Eighteen breast tumors proved to be negative by the standard curve and SSD methods. There were 2 tumors that were positive by the standard curve method (11.0 and 12.2 fmol/mg) and borderline by the SSD method (8.6 and 8.1 fmol/mg), respectively. Five tumors were borderline by the standard curve method and negative by the SSD method. Of the 15 tumors analyzed by Scatchard plot analysis and the standard curve method, only one tumor was borderline by Scatchard plot analysis (8.9 fmol/mg) and positive by the standard curve method (12.2 fmol/mg), and one tumor was negative by

![Chart 1: Standard curve for ERs using rabbit uterus. Percentage of binding on Y-axis and ER concentration in fmol/ml of protein on X-axis. ERs: 110 fmol/mg, 82.5 fmol/mg, 55 fmol/mg, 27.5 fmol/mg, 13.75 fmol/mg, 6.8 fmol/mg.](chart1.png)

![Chart 2: Scatchard plot analysis for rabbit uterus ER. X-axis represents receptor content in fmol/mg, and Y-axis represents percentage of binding.](chart2.png)

![Chart 3: SSD analysis of rabbit uterus cytosol. X-axis represents receptor content in fmol/mg, and Y-axis represents percentage of binding.](chart3.png)

![Chart 4: Comparison between Scatchard plot analysis and SSD analysis. X-axis represents receptor content in fmol/mg, and Y-axis represents percentage of binding.](chart4.png)

![Chart 5: Correlation between Scatchard plot analysis and SSD analysis. X-axis represents receptor content in fmol/mg, and Y-axis represents percentage of binding.](chart5.png)
ER Determination Using Rabbit Uterus

Chart 2. Scatchard plot analysis of rabbit uterus. Receptor concentration of rabbit uterus was calculated according to New England Nuclear protocol for determination of ER. Binding capacity equals 112.4 fmol/mg of protein. $K_1 = 0.0137 \times 10^{-9}$ or $1.37 \times 10^{-9}$ liters/mol; $K_2 = 0.729 \times 10^{-10}$ or $7.29 \times 10^{-11}$ mol/liter.

Chart 3. Analysis of rabbit uterus by SDG. The lower curve represents sample treated with DES and $^{125}$I-estradiol; the upper curve represents sample treated with $^{125}$I-estradiol only.

Scatchard plot analysis (2.4 fmol/mg) and borderline by the standard curve method (4.6 fmol/mg). The stability of the lyophilized rabbit uterus over the past 4 months is shown in Chart 6. These data are based on assays set up by the SSD method and Scatchard plot analysis. The points plotted are a mean of 2 or more assays set up on consecutive days. The range was between 107 and 112 fmol/mg.

DISCUSSION

Numerous assay systems have been developed since the evolution of the concept of ER (15). Most of these assay systems require the use of $[^3]$Hestradiol which is a $\beta$ emitter (4, 11, 18, 21) and has a half-life of 12.2 years. Various assays that have been developed include the following systems.

Dextran-coated Charcoal Method (11). This is the most
widely used and requires large amounts of tissue to maintain the protein concentration of the cytosol ranging from 2 to 4 mg/ml. Technically, this is a demanding assay requiring careful attention to both temperature and time of dextran-coated charcoal exposure (17).

**SDG Method** (15, 18). The basic principle is sedimentation of ER in sucrose gradient. This assay too is technically demanding and relatively expensive because of the large amounts of ultra-centrifugation required for the separation, and therefore, only a few tumors can be analyzed at a given time.

**Electrophoresis.** Even though this is a more economical method than dextran-coated charcoal and SDG, it is cumbersome and time consuming (23).

**Gel Exclusion Column Chromatography** (6). It is not a widely applied method and is more a research tool. This approach requires extended separatory periods (17).

**Protamine Sulfate** (2). Even though this method is effective and efficient for ER analysis, it is not widely used. The ERs in this method are precipitated, and it is not certain whether they are completely protected from endogenous proteases (5).

The standard curve method developed by us utilizes iodinated estradiol which is a γ emitter with a half-life of 60 days. The apparent advantages of using 125I-labeled estradiol have already been alluded to.

The advantage of this method is that it is less time consuming, simple to perform, and as sensitive as the already existing methods. Excellent correlation was obtained when compared to existing methods as shown in Charts 4 and 5. Certain tumors did show minimal differences in the receptor content when analyzed by SSD (8.6 and 8.1 fmol/mg) and standard curve methods (11.0 and 12.2 fmol/mg), but these differences are insignificant. Also, the differences by Scatchard plot analysis (8.9 and 2.4 fmol/mg) and the standard curve method (12.2 and 4.6 fmol/mg) are likewise insignificant. This method for determination of ER has been extremely useful in our laboratory. It is faster, economical, and as sensitive as other conventional methods. Our experience has been that a large number of tumors (up to 10 tumors) can be analyzed in one assay. It completely eliminates the use of liquid scintillation counting, preparation of scintillation fluid, and the problems associated with disposal of tritiated radioactive isotopes.

**ACKNOWLEDGMENTS**

The authors wish to acknowledge the technical assistance by Carol Lee and constant encouragement by Dr. Harold Cheek. Our special thanks to Karen Marks and Catherine Higgins for their excellent assistance given during the preparation of this manuscript.

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