# Direct Radioimmunoassay of 17β-Estradiol in Ether Extracts of Bovine Sera

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Anabolic estrogens such as 17β-estradiol or 17β-estradiol benzoate are used to promote growth and increase feed efficiency in food-producing cattle. This paper describes a technique to produce a more specific antibody to 17β-estradiol by intradermal immunization using microquantities of 6-(carboxymethyl)-17β-estradiol oxime bovine serum albumin and the development of a radioimmunoassay (RIA) procedure to measure directly the amounts of 17β-estradiol in ether extracts of bovine serum without using cleanup procedures. Results demonstrated that a specific and sensitive antibody was produced, and a titer of 1:10 000 was used in the RIA procedure. Antibody cross-reactivity with β-estradiol metabolites and other anabolic estrogens was negligible. The untreated bovine sera showed 0–24 pg of apparent 17β-estradiol/mL, while 0–31 pg/mL "total estrogens" had been reported in the literature. This assay can measure 5–100 pg in 20–250 μL/sample. This method can be used before or immediately after slaughter to monitor the residual amounts of estradiol used in the treatment of cattle.

## INTRODUCTION

17β-Estradiol or its benzoate salt in combination with other hormones are used as anabolic drugs in cattle to increase feed efficiency and promote rapid growth. Estradiol is also physiologically present in humans and animals. Therefore, administration of estrogens and other anabolic drugs may lead to increased levels of 17β-estradiol in blood and tissues.

Long-term ingestion of edible tissues containing amounts of estradiol in excess of physiological level may lead to a possible health hazard. The concensus of the members of the International Agency for Research in Cancer (IARC, 1979) reported that laboratory animal studies demonstrated tumor effects of administered 17β-estradiol. The members of IARC further reported that no human epidemiological studies have shown direct cancer effect from steroid hormones, but there is evidence that suggests that steroid hormones may stimulate carcinogenesis. Furthermore, the exogenous estrogen may seriously influence the human hormonal environment if intake is equal to or greater than the amounts produced endogeneously (IARC, 1979). Such hormonal imbalance may lead to sexual abnormalities as in premature sexual development in prepubertal children. These health concerns led us to study the development of a screening method for estradiol.

The endogenous levels of "total estrogens" at various physiological states of the cow have been measured by RIA and reported by various investigators. Henricks and coworkers (1971) reported 0.5–25 pg/mL of plasma estrogens, wherein the maximum value occurred just before estrus. Henricks (1976) again reported levels of 4–25 pg/mL up to 39 days after mating. From 14 days to the day of parturition, estrogen levels increased from 500 to 2700 pg/mL and then decreased to a range of 15–88 pg/mL, 3–40 days postpartum. The antibody used in this study had 100% binding with 17β-estradiol or estrone and approximately 10% binding with estriol or 17α-estradiol. Monk et al. (1979) also observed plasma estrogen levels of 3–31 pg/mL in an estrus cycle using an antibody that exhibited binding with 17β-estradiol, 17α-estradiol, or estrone at 100%, 50%, or 50%, respectively. Using the same procedure and antibody, Mollett et al. (1976) observed that when 17β-estradiol and progesterone were injected into nonlactating cows, there was a 14-fold estrogen increase (from 38 to 534 pg/mL) from 0 to 8 days after treatment, which dropped to 76 pg/mL at the 35th day. Similarly, Erb and co-workers (1976) administered the above hormones to lactating cows and observed a 17-fold increase (87–1447 pg/mL) of estrogen at the 14th day and gradually decreased to 100 pg/mL at the 43rd day. These studies suggest that the blood estrogen level can be an indicator of 17β-estradiol use in animals. Studies by Henricks (1976) did not show increased plasma estrogen levels after treatment with nonestrogenic compounds such as melengesterol acetate, pregnant mare serum gonadotropin, or prostaglandin $F_2\alpha$. In these investigations, antibodies used cross-reacted with estradiol metabolites (estrone, estriol, 17α-estradiol) and therefore measured total estrogens.

The objectives of this present study were (1) to produce an antibody with high specificity to 17β-estradiol and to use an intradermal immunization technique utilizing small doses of immunogen for antibody production, (2) to design a relatively rapid radioimmunoassay (RIA) procedure that can directly measure the amount of 17β-estradiol in ether extracts of bovine serum without chromatographic or solvent partition cleanup procedures prior to RIA, and (3) to provide a simple approach to manipulation of RIA data.

## MATERIALS AND METHODS

**Materials:** 17β-Estradiol-6-CMO-BSA (carboxymethyl oxime bovine serum albumin), from Steraloids Inc. (Wilton, NH); Freund's complete adjuvant, from Difco (Detroit, MI); 17β-estradiol, estrone, estriol, equilin, equilinin, gelatin, Triton-X, activated charcoal, from Sigma (St. Louis, MO); New Zealand white rabbits; diethylstilbesterol (DES), from Aldrich Chemicals (Milwaukee, WI); zearalenone, zearalanol (zeranol), from International Minerals and Chemical Corp. (Terre Haute, IN); ether (anesthesia grade, 100-mL volume), from Fisher Scientific (King of Prussia, PA); Dextran T-70, from Pharmacia (Piscataway, NJ); glass-distilled methanol, toluene, from Burdick and Jackson (Muskegon, MI); Liquifluor, from New England Nuclear (Boston, MA); [2,4,6,7-$^3$H]estradiol from Amersham (Arlington Heights, IL); IEC Centra-7R centrifuge, from International Equipment Co. (Needham Hts., MA); IKA-VIBRAX shaker, from Tekmar Co. (Cincinnati, OH); LS8100 scintillation counter, from Beckman (Fullerton, CA).

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Table I. Percent Cross-Reactivity of Antiestradiol[a]

| compd | concn, pg | | |
|---|---|---|---|
| | 100 | 500 | 1000 |
| 17β-estradiol | 100 | | |
| 17α-estradiol | 0 | 0 | 0 |
| estrone | 7.5 | 5 | 5 |
| estriol | 5.0 | 6.2 | 5 |
| 17β-estradiol benzoate | 0 | 21 | 25 |
| zearalanol (zeranol) | 2.0 | 0 | 0 |
| zearalenone | 2.0 | 0 | 0 |
| zearalanone | 0.05 | 0 | 0 |
| zearalenol | 0 | 0 | 0 |
| DES | 0 | 0 | 0 |
| equilin | 0 | 0 | 0 |
| equilenin | 0 | 0 | 0 |

[a] Batch No. 62.

**Antisera Production.** 17β-Antiestradiol was produced in six male New Zealand white rabbits according to the procedure reported by Vaitukaitis (1981). Two doses (150 or 300 μg) of 17β-estradiol-6-CMO-BSA were suspended in 5 mL of physiological phosphate buffer solution, pH 7.0 (5.38 g of $NaH_2PO_4 \cdot H_2O$, 8.66 g of $Na_2HPO_4$, and 9.0 g of NaCl/L) and then emulsified with 5 mL of Freund's complete adjuvant by sonicating for 30 s. Two milliliters of emulsion was injected intradermally at 50 sites on the clipped back of each of the six rabbits, three rabbits receiving 30 μg of the immunogen and the other three receiving 60 μg each. From the 6th week, serum samples were collected from the central ear artery weekly and the antibody titers were measured. At the 11th week each rabbit received subcutaneous booster injections 1.1 mg of estradiol conjugate in 2 mL of emulsion at 10 sites. Rabbit sera were collected at the 10th day after the booster injections, and the antibody titers were measured again. Blood (50–60 mL) was collected from each rabbit on the 12th day by cardiac puncture. Following this procedure, the antibody production was terminated and the rabbits were sacrificed. The antisera were separated from the red cells and fibrin by allowing the blood to clot for at least $^1/_2$ h and centrifuged at 1500 rpm in a table top clinical centrifuge. The antisera were immediately frozen or freeze-dried and stored in 100-μL aliquots in a −80 °C freezer.

**Antiserum Titer and Specificity.** Aliquots of 100 μL were diluted with phosphate-buffered saline (PBS) to 1:100, 1:1000, 1:5000, and 1:10 000 dilutions. Binding with [$^3$H]estradiol (sp act. 12 500 cpm/30 pg) was measured weekly to determine the titer. The antiserum with the highest titer was further characterized for cross-reactivity and used in subsequent immunoassays. The antibody dilution that yielded a 50% binding with [$^3$H]estradiol was used for subsequent immunoassays and to determine cross-reactivity of the 17β-antiestradiol with some estradiol metabolites and other anabolic estrogens (Table I). Each compound was assayed at 100-, 500-, and 1000-pg amounts, and the percent cross-reactivity was determined from the calibration standard of 17β-estradiol.

**Sample Preparation.** Bovine serum samples (0.25 mL) were extracted in conical glass tubes with 2 mL of ether by mixing in the IKA shaker at 1600–1800 rpm for 15 min. After mixing, the tubes were allowed to stand for 10 min to allow separation of the aqueous and ether layers. The aqueous layer was quickly frozen in dry–ice acetone and the ether layer decanted into silanized 12 × 76 mm glass tubes. The ether was blown off under a stream of nitrogen at 40 °C. The extraction procedure was repeated once. The dried extracts were then assayed for estradiol content. The ether blanks were treated similarly and determined for nonspecific binding. The calibration standards and samples were analyzed in duplicate.

**Radioimmunoassay.** *Reagents*: Phosphate-buffered saline (PBS) contained 0.041 M $NaH_2PO_4 \cdot H_2O$, 0.061 M $Na_2HPO_4$, 0.9% NaCl, 0.1% $NaN_3$, and 0.1% gelatin adjusted to pH 7.0 with 1 N NaOH. [$^3$H]Estradiol was diluted such that 50 μL contained 12 000 cpm (420 cpm/1 pg). Dextran-coated charcoal was prepared by suspending 12.5 mg of Dextran T-70 in 25 mL of PBS. After stirring briefly, 125 mg charcoal was added; the resultant mixture was stirred for another 10 min and chilled at 4 °C prior to use. Scintillation cocktail contained 160 mL of Liquifluor and 25 g of Triton-X in 3.78 L (1 gal) of toluene. The scintillation fluid was always mixed thoroughly prior to use.

*Procedure*: Estradiol standard (1 pg/μL) in PBS was pipetted into 12 × 76 mm tubes in 0-, 10-, 25-, 50-, 75-, 100- and 150-pg amounts. PBS was added to make up a total volume of 400 μL. Total counts and blank tubes for nonspecific adsorption of charcoal were set aside containing 600 and 400 μL of PBS, respectively. PBS (400 μL) was also added to assay tubes containing dried extracts of bovine serum samples. Antiestradiol (50 μL of 1:10 000 dilution, batch #62) were added to all tubes (except total counts and blank tubes), mixed gently, and preincubated at room temperature for 30 min. The tracer, [$^3$H]estradiol (50 μL), was added to all tubes, mixed gently, and incubated at 37 °C for 2 h. Incubation may also be carried out overnight for 16 h at 4 °C. The assay tubes were further incubated at 4 °C for another 30 min. Dextran-coated charcoal (200 μL) was added rapidly to all tubes except the total counts to which 200 μL of PBS was previously added. The charcoal solution was stirred continuously and kept at 4 °C while pipetting. The assay tubes were mixed gently, equilibrated at 4 °C in an ice bath for 10 min, and centrifuged at 3200 rpm (1750*g*) for 12 min at 4 °C. Aliquots of 400 μL were immediately pipetted out from each tube and transferred into 7-mL scintillation vials. Scintillation fluid (5 mL) was added, and the vials were tightly capped, inserted into a test tube rack, and then mixed vigorously in the IKA shaker for 5–10 min. Each vial was counted for 2 min in the scintillation counter.

The calibration standard was plotted manually as estradiol concentration vs. bound/total cpm × 100 (% B/T) or concentration vs. total/bound cpm (T/B) or by use of simple linear regression equation $y = a + bX$ where $y$ = total cpm/bound cpm and $X$ = estradiol concentration. The cpm values of the blank tubes were subtracted from total cpm and bound cpm prior to calculating B/T or T/B ratios. The estradiol content of the unknowns was determined from the manual plots and the equation of the line to compare results.

## RESULTS AND DISCUSSION

The antibody production procedure described by Vaitukaitis (1981) utilizing small doses of immunogen yielded a relatively high titered antibody from responding rabbits. Three rabbits individually received 30 μg while the other three received 60 μg each. five out of six animals generated antibodies against 17β-estradiol-6-CMO-BSA, while one animal never responded throughout the study. One rabbit had to be terminated prior to the completion of the study due to an infection problem. Booster injections were given to induce secondary antibody production at the 11th week after the drop in the primary antibody production leveled off. Ten days after the booster injections, the serum of one rabbit yielded 63% binding, while two animals had 40% binding and one had <20% binding at 1:10 000 dilution. These results indicate that antibody production

Table II. 17β-Estradiol Levels in 0.25 mL of Serum of Six Heifers Calculated from Regression Equations

| sample no. | endogenous level[a] | | | calcd[b] 50-pg equiv | | | mean[c] | |
|---|---|---|---|---|---|---|---|---|
| | n | mean | SD | n | mean | SD | % rec | SD |
| 1 | 6 | 4.4 | 1.92 | 4 | 50.35 | 6.29 | 101 | 12.7 |
| 2 | 6 | 2.5 | 1.95 | 4 | 52.65 | 9.26 | 105 | 18.4 |
| 3 | 6 | 3.2 | 1.72 | 4 | 51.3 | 9.90 | 103 | 19.2 |
| 4 | 6 | 3.1 | 1.10 | 4 | 46.7 | 4.95 | 93 | 9.9 |
| 5 | 6 | 3.9 | 1.51 | 4 | 51.1 | 8.63 | 102 | 17.7 |
| 6 | 6 | 1.3 | 1.97 | 4 | 52.2 | 4.67 | 104 | 8.5 |

[a] Range 0–6.1 pg/0.25 mL. [b] Calculated 50-pg values obtained by substracting measured total estrogens with measured endogenous estrogens.[a] [c] % recovery = calcd 50-pg amt[b]/50 × 100.

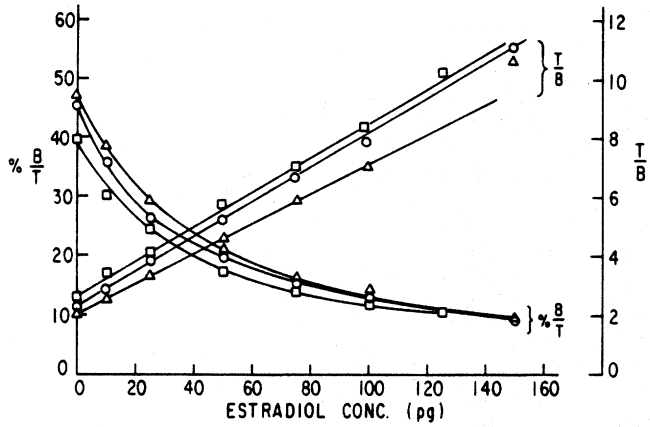


Figure 1. Manually plotted calibration curves of three sets of RIA data showing linear plots of T/B vs. estradiol concentration and the hyperbolic curves of percent B/T vs. estradiol concentration.

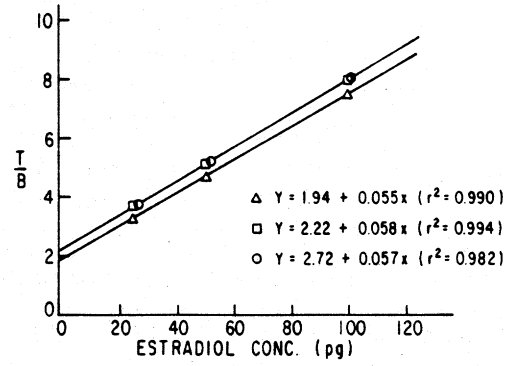


Figure 2. Regression lines from three sets of RIA data shown in Figure 1.

was stimulated by 30- or 60-μg doses. The higher antibody titer displayed by one rabbit can be attributed to the individual response of the animal. Antiserum from this animal was selected for use in subsequent immunoassays and therefore characterized for cross-reactivity. This antiserum had high specificity for 17β-estradiol and low cross-reactivity with other estrogenic compounds (Table I). Therefore, there was no need to further purify the antiserum because such manipulation usually results in an increase in antibody specificity but a decrease in titer. On the basis of chemical structure of these compounds, the antibody appears to have specificity for the steroidal A, B, and D rings, requiring a phenolic moiety of the A ring, cyclic aliphatic at the B ring, and 17β-hydroxyl at the D ring.

The radioimmunoassay data were screened by plotting bound/total cpm × 100 (% B/T) vs. concentration and total cpm/bound cpm (T/B) vs. concentration. The former yields a hyperbolic curve and the latter a straight line as shown in Figure 1. The hyperbolic curve can also provide information as to the amount of antigens required to saturate the antibody binding sites. Concentration of antigen used in the calibration standard must be adjusted below this saturation point (i.e., concentration where percent B/T plateaus). The equation of the line $y = A + bX$ was determined by using a simple regression equation where $y$ = T/B and $X$ = concentration. The lines in Figure 2 derived from three sets of analyses show parallelism and statistical analysis proved that the slopes of these lines are not significantly different at $P < 0.01$. There are many approaches to data reduction or processing of radioimmunoassay data as reviewed by Rodbard and Hutt (1974), Rodbard (1979), Cernosek (1979a, 1979b, 1979c), and Chase (1979a, 1979b, 1979c, 1980). The simple approach of data reduction by using a linear regression is satisfactory when handling a small number of samples per assay. Computer programs are also available and are interfaced with more recent models of scintillation counters.

Bovine serum samples analyzed in these sets of assays yielded a range of 0–6.1 pg/0.25 mL of serum when derived from the regression equations (Figure 2). Mean values are shown in Table II. These values had been corrected for nonspecific binding in reagent and serum blanks. The amount of endogenous estradiol in the serum samples showed high variability shown by high SD values in Table II. This is apparently due to the influence of nonspecific binding of reagents and other sample components at assay concentration below 25 pg. Estradiol levels (0–24 pg/mL) in these samples are within the range (0.5–33 pg of total estrogens/mL) of those reported by Henricks and coworkers (1971) and Monk et al. (1975). The sample set was also read from manually plotted standard curves and yielded similar results of 0–24 pg/mL of serum. These manual plots allow a rapid screen of the RIA standard curves, and preliminary estimation of estradiol in the samples can be quickly obtained.

Fortification of serum samples with [$^3$H]estradiol showed an extraction efficiency of ≥95% after double extraction with ether. Sera from the same six heifers fortified with 50 pg of estradiol showed 50.8–60.3 pg of total 17β-estradiol measured by RIA procedure and calculated from linear regression equations. Estimated equivalent of 50 pg of estradiol fortified in samples ranged from 43.2 to 57.2 pg with corresponding recoveries of 86–118%. Table II shows mean estimates of estradiol added at 50-pg amounts ranged from 46.7 to 52.6 pg with mean recoveries of 93–105%. Similar data read from manual plots shows 85–105% recoveries. Variabilities within and between assays were less than 10% and 20%, respectively. One-way analysis of variance showed that results from linear regression lines vs. manually calculated data were not significantly different at $P < 0.01$. The bovine sera were also fortified with 100 pg of estradiol and recoveries were 75–90% (manual estimation showed 78–89% recoveries). This analysis suggests that the assay was losing sensitivity at 100-pg concentration, which can be attributed to decrease in the binding capacity of the antibody as a result of saturation of its binding sites at higher dose and is well illustrated

by the hyperbolic calibration curve. Sample size then should be adjusted to contain to <100 pg of estradiol to obtain the most reliable data at conditions defined by this assay protocol. Should a sample contain >100 pg, the sample must be assayed again in smaller volume or an appropriate dilution must be made prior to analysis. Bovine serum should contain <100 pg/mL of normal physiological amounts of extradiol. However, one may elevate the assay range by increasing the concentration of the tracer ([$^3$H]estradiol) and antibody, but higher sensitivity can be achieved by reducing the amounts of tracer and antibody. The optimum conditions described in this assay were selected from systematically tested assay conditions including preincubation (binding of cold antigen with antibody) time and temperature; incubation (competition of tracer and cold antigen with antibody binding sites) time and temperature; and charcoal concentration vs. adsorption time and temperature. The sensitivity of the assay at the conditions described in this paper were equal or better than the assay incubated at 4 °C for 16 h. Results were also better than those obtained by assays without preincubation or incubation periods shorter or longer than 2 h at 37 °C. It is important to carry out the charcoal absorption step at 4 °C, and the precise time should be followed. Higher temperature or longer exposure to charcoal even after centrifugation resulted in stronger absorption of the free ligand, significantly decreasing bound counts and consequently losing assay sensitivity. This is presumably attributed to the stripping of weakly bound ligand from the antibody.

In conclusion, this study shows that ether extracts of bovine serum can be measured for 17β-estradiol by RIA without using chromatographic or solvent partition cleanup procedures. Use of such steps would increase time and cost of analysis and may lead to loss of compound analyzed. The ether extraction procedure is rapid and simple. Twenty samples, in duplicate, can be extracted twice with ether in 1 h, and their subsequent analysis with RIA can be accomplished in $4^1/_2$ h by one person. However, sample throughput can be increased by use of automated systems. This RIA analysis of ether extracts of bovine serum has the potential for use as a screening technique to detect the presence of higher than normal levels of estradiol in treated animals prior to slaughter. Presence of high level of estrogens can be confirmed by use of other chromatographic techniques. This procedure, used postslaughter, may be capable of predicting incurred residue levels of estradiol in tissue if the correlations with sera content were determined.

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**Registry No.** 17β-Estradiol-6-CMO-BSA, 35048-47-6; 17β-estradiol, 50-28-2; 17β-estradiol benzoate, 50-50-0.

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