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MICROWELL BASED SOLID PHASE COMPETITIVE ENZYME IMMUNOASSAY FOR ESTRADIOL AND ESTRIOL

BY

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B.S. June 1985, Fu-Jen University

A Thesis Submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE

CHEMISTRY

OLD DOMINION UNIVERSITY
August 1990

Approved by:

Dr. James H. Yuan (Director)

Mark S. Elliott

ABSTRACT

A sensitive and simple microwell based competition enzyme immunoassay for the quantitative determination of estradiol (E_2) and estriol (E_3) was developed. Microwells coated with antibody were incubated with antigen followed by adding horseradish peroxidase (HRPO) conjugate. The assay, which can be performed within two hours at room temperature, involved simultaneous incubation of E_2- or E_3-HRPO conjugate and serum sample in polystyrene microwells coated with anti-E_2 or anti-E_3 gamma globulin fraction.

Gamma globulin was isolated from whole anti-serum by DEAE-cellulose chromatography. A carbodiimide coupling method was utilized to prepare the E_2- and E_3-HRPO conjugates.

The detection limit of the E_2 assay is 2.0 pg/mL and 6.0 ng/mL for the E_3 assay. Precision studies involving pooled serum samples with three different levels of E_2 and E_3 were performed. Intra-assay coefficients of variation of 15.0%, 8.83%, and 8.13% were obtained for E_2 at 20.48 pg/mL, 79.14 pg/mL, and 424.6 pg/mL, and of 12.4%, 9.63%, and 9.08% for E_3 at 49.19 ng/mL, 153.4 ng/mL, and 505.7 ng/mL. Inter-assay coefficients of variation of 18.04%, 10.40%, and 8.70% were obtained for E_2 at 20.57 pg/mL, 78.67 pg/mL, and 422.0 pg/mL, and of 15.06%, 12.33%, and 9.84% for E_3 at 47.76 ng/mL, 153.4 ng/mL, and 504.7 ng/mL. Values for total E_2 and total E_3 so determined correlated well with those

determined by radioimmunoassay with correlation coefficient of 0.955 and 0.959 respectively.

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Chapter I

INTRODUCTION

A. Background

Estrogens are responsible for the development and maintenance of the female sex organs and secondary sex characteristics. They also participate in the regulation of the menstrual cycle and in the maintenance of pregnancy. In women, estrogen is secreted mainly by the ovarian follicles and during pregnancy by the placenta.

Research in recent years has shown that biosynthesis of estrogens differs qualitatively and quantitatively during pregnancy from the nonpregnant state. In the nonpregnant state, the ovaries are the main site of estrogen synthesis; estradiol is secreted in microgram quantities, and estriol is only a byproduct of estradiol metabolism. During pregnancy the placenta is the major source of estrogens; estriol is produced in milligram quantities, and estradiol is produced in microgram amounts.

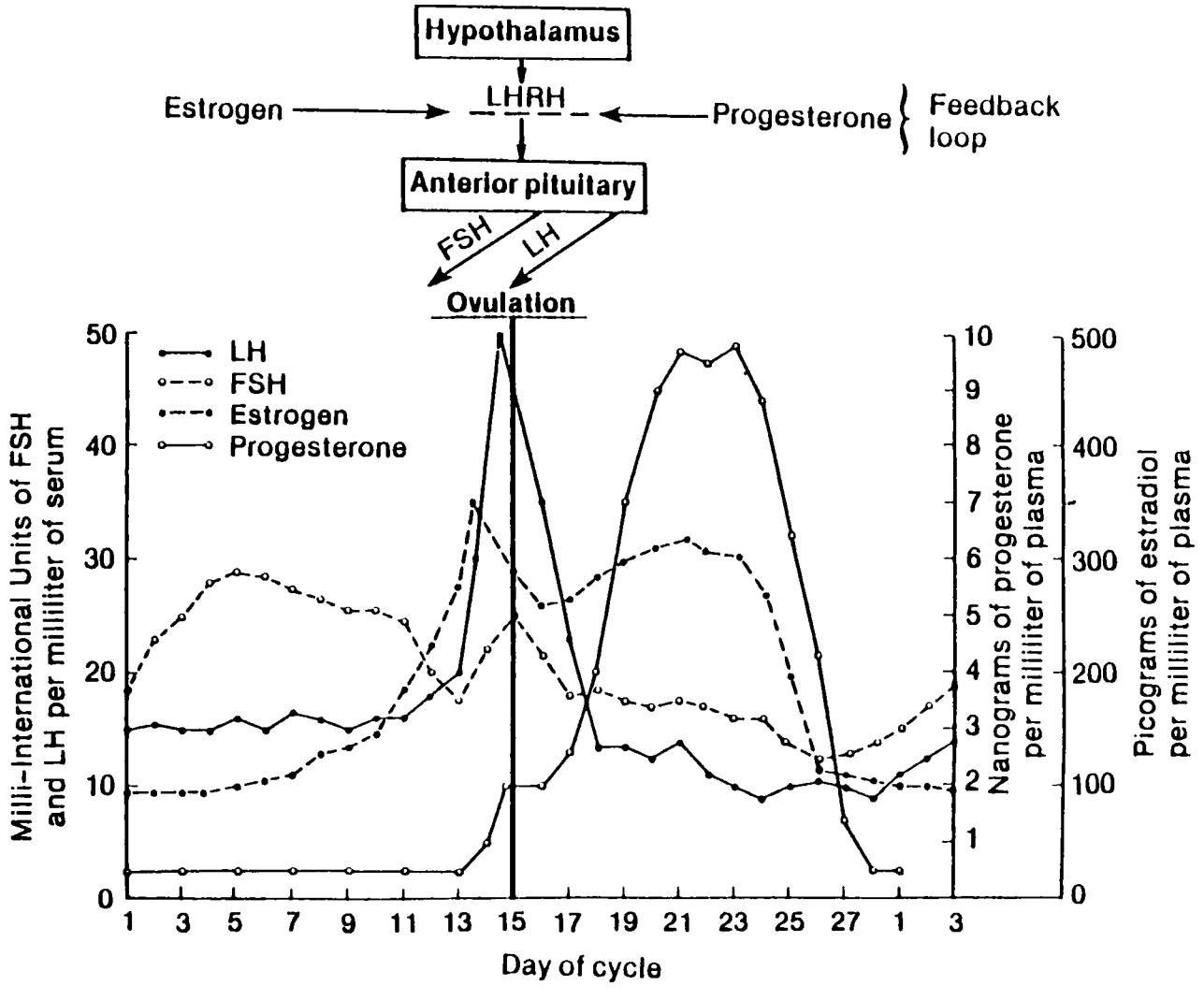
Estradiol (E_2) is the most potent ovarian estrogen, and it participates in the regulation of the menstrual cycle. Early in the cycle when the levels of estrogen and progesterone are relatively constant and low, the follicle-

stimulating hormone (FSH) levels are rising and high and the luteinizing hormone (LH) levels are low. These high levels of FSH stimulate the follicular growth and its output of estrogens, particularly estradiol. By days 7 and 8, the rise of estradiol is at a rapid rate, and it reaches its first peak before ovulation. The rising levels of estradiol result in a negative feedback to the hypothalamus and pituitary gland and cause a fall in FSH levels because of the inhibitory action of estradiol on FSH release. Concurrently, the rise in estradiol triggers a rapid rise in LH (positive-feedback effect). Estradiol reaches a maximum on the day before LH peak (1). During mid-cycle there is a peak of LH, which leads to maturation of the graafian follicle and its rupture, releasing the ovum (ovulation) 16 to 24 hours after LH peak. Before the LH surge and before ovulation, the estradiol drops considerably and then rises again after ovulation. The ruptured follicle becomes the corpus luteum. The progesterone released by the corpus luteum begin to rise, causing an inhibition of the secretion of LH. A sharp increase in progesterone follows, reaching a maximum in 8 or 9 days after the LH peak (days 23 to 25 of the cycle). As estradiol and progesterone increase, FSH and LH decline through out the luteal phase. As the corpus luteum regresses, the levels of both estradiol and progesterone begin to diminish. The removal of the inhibitory effect of these two compounds results in the

increase of FSH, which stimulates the growth of a new crop of follicles in the ovary (2). During the menstruation phase, estradiol, progesterone, and LH are at relatively constant but low levels; whereas, FSH is the only hormone present in elevated and rising levels (Figure 1).

Circulating estradiol is largely unconjugated; it is strongly bound to a specific globulin, sex hormone binding globulin (SHBG), and loosely bound to albumin. Only 2-3% of total estradiol circulates in the biologically active unbound form.

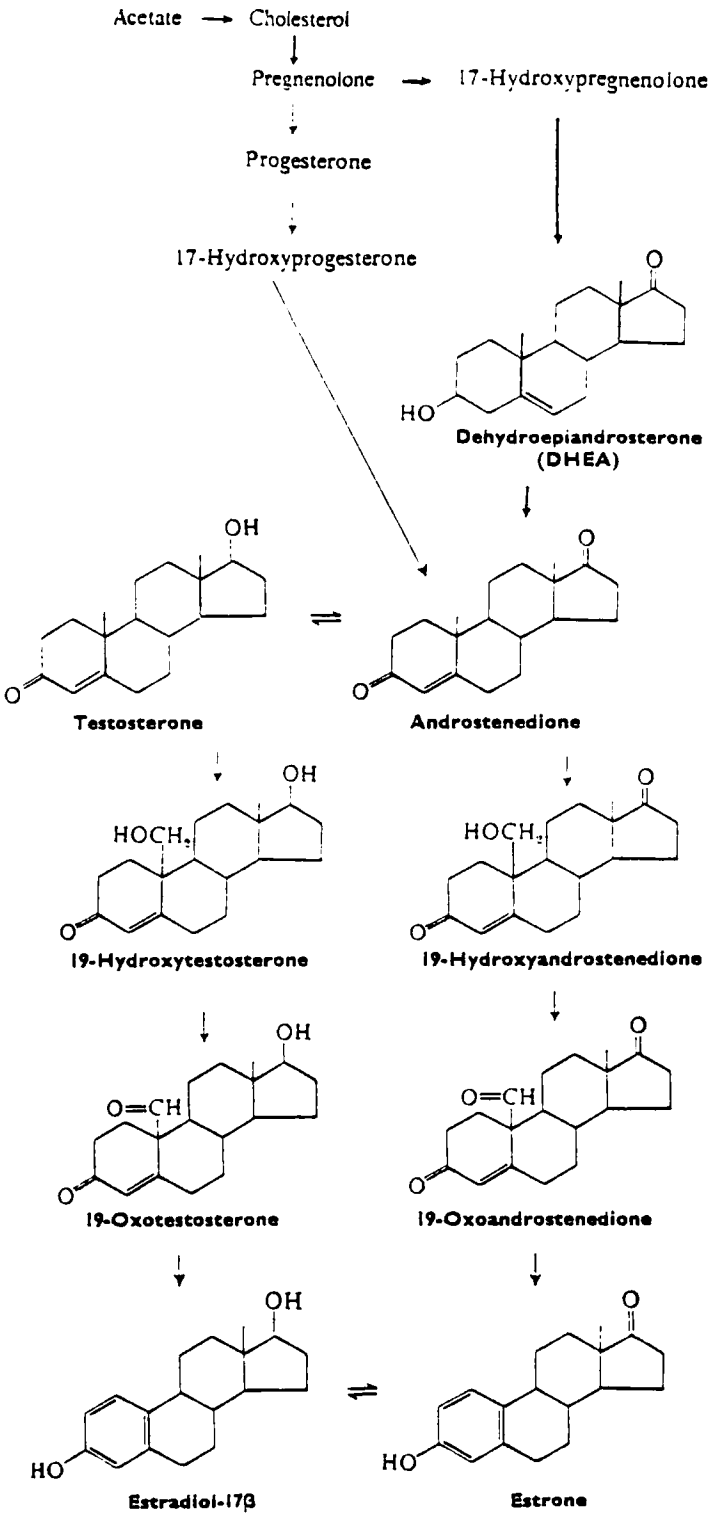
The concentration of estradiol increases in primary ovarian hyper-function (feminizing tumors) and secondary ovarian hyper-function. The main cause of primary ovarian hyper-function is estrogen-secreting tumors. Granulosa and thecal cell tumors are the most common of the estrogen-producing tumors (3). It decreases in primary ovarian hypofunction (including menopause) and secondary ovarian hypofunction (4). In primary ovarian hypofunction, because of the lack of estrogenic feedback on the hypothalamic-pituitary axis, primary ovarian hypofunction is characterized by increased levels of gonadotropins in association with decreased estrogen levels. Secondary ovarian hypofunction may be attributed to hypothalamic, pituitary, or constitutional disturbance. Resulting in a decreased estrogen level. This includes tumors of the pituitary and necrosis resulting from postpartum

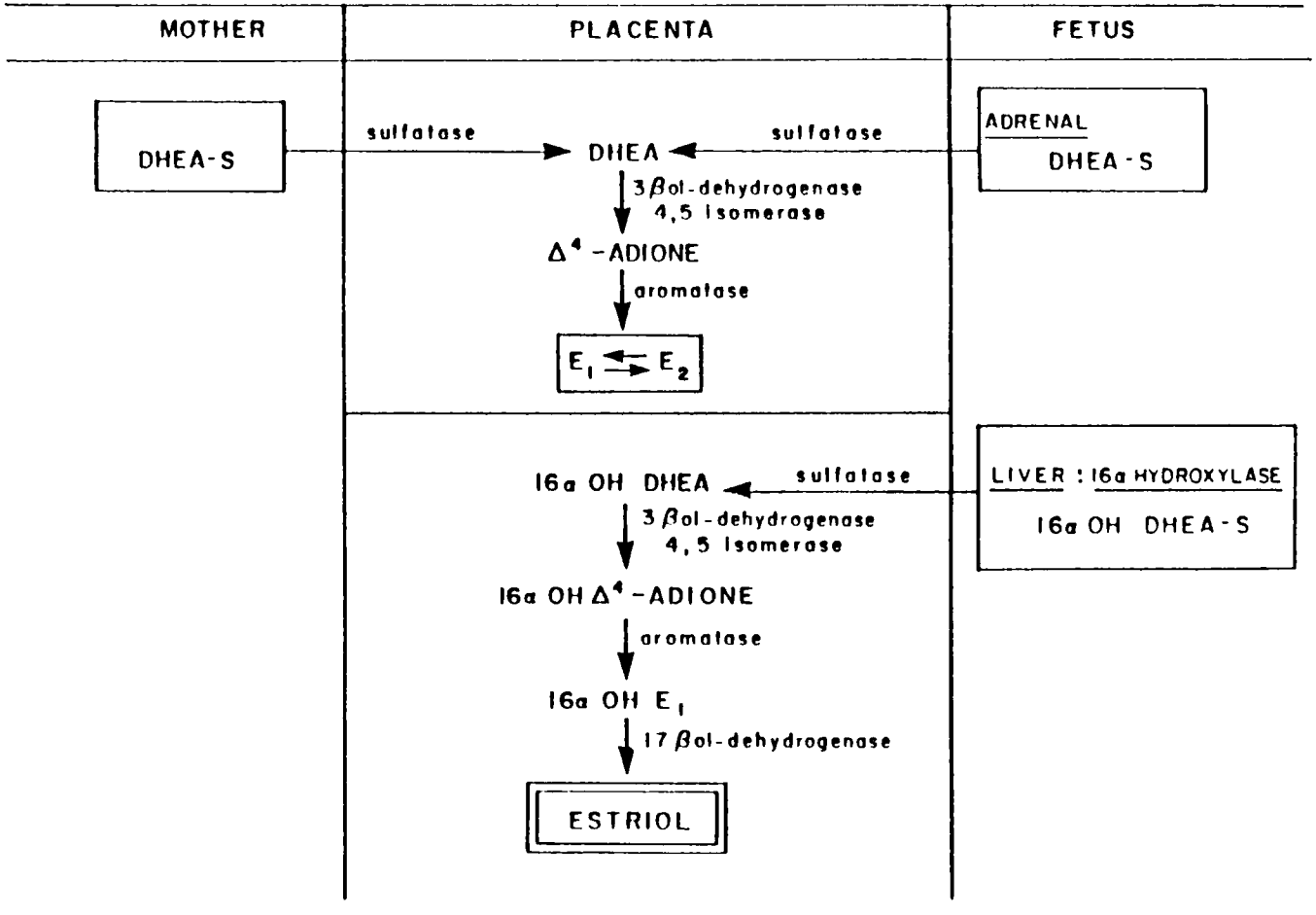

Hypothalamus
LHRH
Estrogen
Progesterone
Feedback loop
Anterior pituitary
FSH
LH
Ovulation
LH
FSH
Estrogen
Progesterone
Milli-International Units of FSH and LH per milliliter of serum
Nanograms of progesterone per milliliter of plasma
Picograms of estradiol per milliliter of plasma
Day of cycle

hemorrhages.

Acetate, cholesterol, progesterone, and testosterone can all serve as precursors of estrogens. The ovary possesses a highly active aromatase system that rapidly converts testosterone to estrogens. The first biochemical event in the aromatization of testosterone is hydroxylation of the C-19 methyl group to produce 19-hydroxytestosterone. This hydroxylated compound is further oxidized to 19-oxotestosterone. The C-19 carbon atom and the C-1 hydrogen atom of this intermediate are eliminated as formaldehyde. The resulting 3-oxo-androst-1(10),4-diene aromatizes spontaneously to estradiol (Figure 2).

Both the fetus and the placenta cooperate in estriol biosynthesis in pregnancy. The placenta, as opposed to the ovary, cannot accomplish de novo synthesis of estrogens from precursors such as acetate, cholesterol, or progesterone and has to be provided with adrenal C-19 steroid precursors of either maternal or fetal origin (Figure 3). The fetal adrenals provide dihyroepienadrostenedione sulfate (DHEA-S), which is 16-alpha-hydroxylated in the fetal liver. The fetal 16-alpha-OH DHEA-S is then hydrolyzed by a placental sulfatase, and the 16-alpha-DHEA formed is further metabolized by the placenta via the hydroxysteroid dehydrogenase/isomerase enzyme system to 16-alpha-OH androstenedione (16-alpha-OHA). The C-19 product is then aromatized by the aromatizing enzyme system (19-hydroxylase,


Acetate
Cholesterol
Pregnenolone
17-Hydroxypregnenolone
Progesterone
17-Hydroxyprogesterone
Dehydroepiandrosterone (DHEA)
Testosterone
Androstenedione
19-Hydroxytestosterone
19-Hydroxyandrostenedione
19-Oxotestosterone
19-Oxoandrostenedione
Estradiol-17β
Estrone


MOTHER
PLACENTA
FETUS
DHEA-S
sulfatase
sulfatase
DHEA
ADRENAL
DHEA-S
3βol-dehydrogenase
4,5 isomerase
Δ4-ADIONE
aromatase
E1 ⇄ E2
16α OH DHEA
sulfatase
LIVER : 16α HYDROXYLASE
16α OH DHEA-S
3βol-dehydrogenase
4,5 isomerase
16α OH Δ4-ADIONE
aromatase
16α OH E1
17βol-dehydrogenase
ESTRIOL

19-oxidase, and 10,19-desmolase) to estrogen. The 16 OH-estrone thus formed is then converted to estriol by the placenta 17-beta-dehydrogenase which reduces the keto group at C-17. Estriol is then secreted into maternal and fetal circulation, where its measurement can be used as an indicator of fetoplacental status (5).

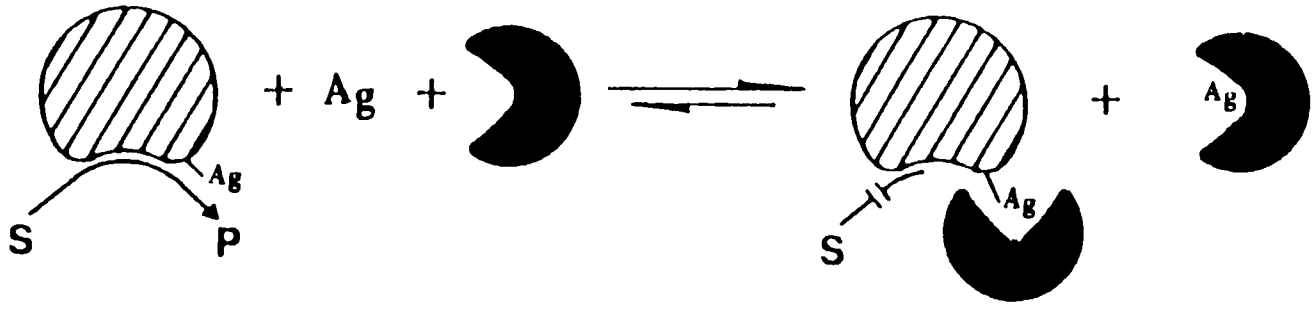
Because the pathways for estriol formation during pregnancy involve both fetus and placenta, estriol measurements can be a sensitive clinical indicator of fetoplacenta status. Estriol levels during pregnancy, however, are influenced by many factors other than fetal well-being. These include fetal weight;placental enzyme deficiencies (e.g. sulfatase); primary or secondary fetal adrenal hypoplasia (e.g. anencephaly, congenital adrenal hypoplasia, exogenous adrenal hormone therapy) (6); and changes in maternal intestinal flora (antibiotic use), maternal renal excretion (affecting both blood and urine levels), and maternal liver function. All these potential confounding factors must be considered before an abnormal estriol level can be attributed to deterioration of fetoplacental function. Even in the absence of all these factors, a low estriol value should always be evaluated with caution and in conjugation with other independent indices of fetal well-being, such as ultrasound assessment, amniotic fluid evaluation of fetal maturity, oxytocin challenge test, or nonstress tests.

The range of normal serum and urinary estriol levels during pregnancy is very wide (7). An isolated single estimation of estriol in a high risk pregnancy has therefore only limited clinical application unless it is definitely subnormal. Serial measurements (i.e.,tests performed daily or weekly) to evaluate the trend of estriol production are more meaningful (8).

A large number of radioimmunoassays (RIA) have been developed for the determination of the most widely differing antigens and haptens (9-11). In RIA, the antigen and a constant amount of radioactively labeled antigen compete for a limited number of antibody-binding sites. Addition of unlabeled antigen to the assay results in a net increase in the total antigen (labeled plus unlabeled) but, because of competition for antibody-binding sites, a decrease in the proportion of labeled antigen that will be bound by the antibody. Since this method involves measurement of the radioactivity of the labeled antigen-antibody complexes, the excess free antigen marked with a radioactive label has to be removed from the test mixture (bound-free separation). Radioimmunoassays do however have several disadvantages due to the use of radioactive isotopes as markers. Laboratories that handle radioactive substances must comply with special regulations governing the use, storage and disposal of these materials. This results in higher costs for a laboratory using radioactive isotopes. Other disadvantages, mainly

concerning the principle employed, are the relatively short half-life (60 days) of the most commonly used radioisotope (iodine-125) and the decomposition of sensitive substances as a result of radiolytic processes. This situation promoted the development of new immunoassays using nonradioactive markers. The use of enzymes as markers yielded highly promising results in terms of performance in the clinical chemistry laboratory and eliminating the problem of storage and disposal.

Enzyme immunoassays (EIA) are the developmental successor to radioimmunoassays. In enzyme immunoassays a distinction is made between a homogeneous test principle and a heterogeneous test principle. The homogeneous enzyme immunoassay is referred to as the enzyme-multiplied immunoassay technique (EMIT) (Figure 4). Binding of the antibody to the enzyme-labeled antigen changes the enzyme activity of the label enabling the antibody-bound label to be distinguished from the unbound labeled antigen. This dispenses with the need for phase separation. As there is no need for a bound/free separation step, EMIT is quickly and easily done with automated equipment. There are, nevertheless, other problems which may occur in the homogeneous test. Because it does not need the phase separation, the enzyme activity is liable to interference from serum constituents. Inhibition of the enzyme activity result in only small absorbance differences (0.1-0.2 A)



Reaction

No Reaction

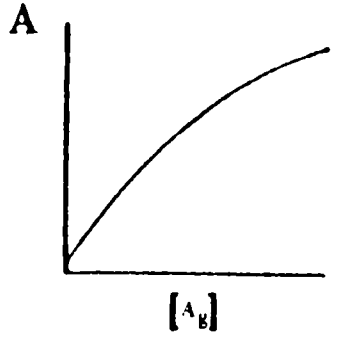
= Enzyme-Labeled

= Antibody

Ag = Antigen

S = Substrate

P = Product

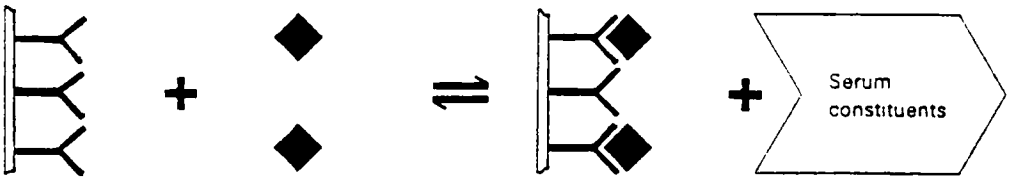


between an inhibited reaction and noninhibited reaction; therefore, measurements are difficult.

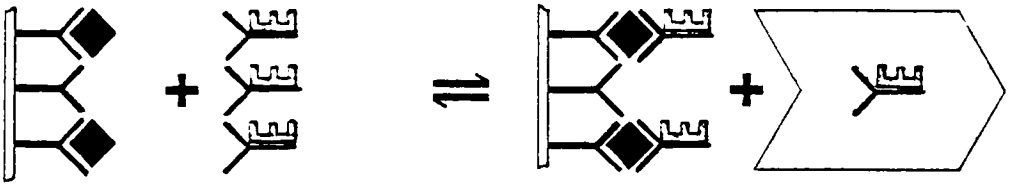
The heterogeneous enzyme immunoassays (or ELISA-enzyme linked immunosorbent assays) are those procedures that require the physical separation of the antibody-antigen complex from the unbound constituents in order to determine the enzyme activity associated with free reactant separated from the bound labelled reactant after the immunological incubation. ELISA usually have an antibody immobilized on to a solid support, and the ligand is labeled with the enzyme. There are two kinds of ELISA that have been already developed : sandwich and competitive.

In the sandwich technique (Figure 5), the antibody of the desired specificity is immobilized to a solid surface in the first step. The solid phase may be the wells in a microwell or a plastic test tube. The microwell or test tube is washed to remove all unreacted materials. In the second step, the fluid containing the antigen is reacted with the immobilized antibody. All nonreacting material is washed away. The third step is to react the enzyme-labeled antibody with the antigen that has now been immobilized by the antibody on the solid phase. All unreacted enzyme-labeled antibody is then washed away and substrate with appropriate cofactors is added so that the enzyme on the antibody can then convert the substrate to the product. The amount of product is then measured by a color change or

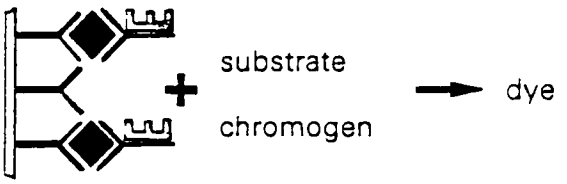
1st immunological reaction

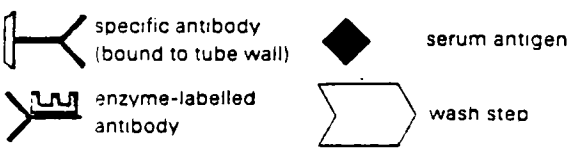


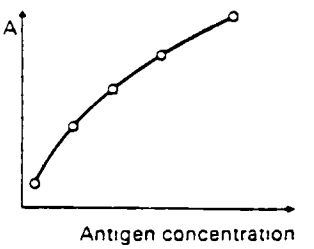
2nd immunological reaction



Indicator reaction



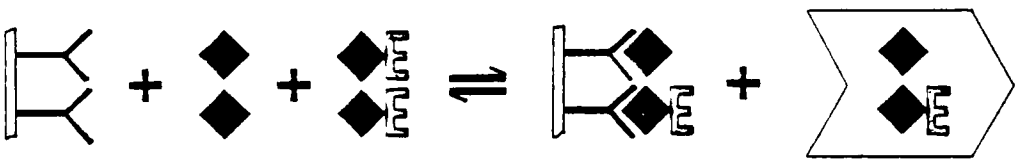




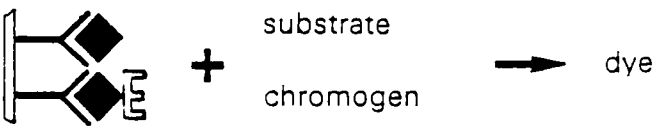
color reaction. The intensity of the color is directly proportional to the amount of antigen that has been immobilized on the solid surface by reaction with the antibody. Because there are two different antibodies that must bind to the antigen, only large antigens, such as proteins, can be measured by this system.

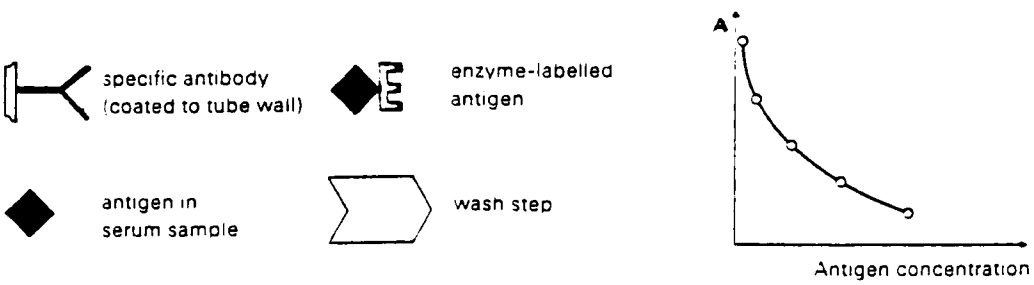
In the competitive technique (Figure 6), the enzyme-labeled antigen is mixed with the test solution containing an unknown amount of the antigen. The solution containing the labeled and unlabeled antigen is allowed to react with a limited amount of antibody bound to a solid matrix. One removes unbound antigen (both labeled and unlabeled) by washing and measures the amount of labeled antigen by determining the amount of enzyme bound to the solid surface. The concentration of the antigen present in the test sample is inversely proportional to the intensity of the color measured. The possible mathematical expression for the relationship between the concentration of antigen and the absorbance measured was suggested by Gupta (12) and Maggio (13). Since this method has to be compared to those of other methods or reference method and requires at least the same sensitivity, specificity and practicability. The ability to obtain these features depends on the choice of the enzyme used for coupling. It should have a high specific activity and a good stability after chemical modification. Escherichia coli beta-D-galactoside-

Immunological reaction



Indicator reaction





galactohydrolyase and horseradish peroxidase were reported to have high specific activities and stabilities suitable for enzyme immunoassay (14, 15). The molecular weight of horseradish peroxidase is 40,000. Seven isozymes have been described by Kay, et al. (16); and Strickland, et al. (17). All contain protohemin IX as prosthetic group. Neutral and amino sugars account for approximately 18% of the enzyme. Weinryb (18) indicates that the "active site" involves apoprotein as well as the heme group. The enzyme exhibits a high specificity and its activity is observed with H_2O_2 (19). It is quite stable. As a lyophilized, dry powder, it may be stored several years refrigerated (20). Peroxidase acts upon hydrogen peroxide as follows (21):

$$H_2O_2 \xrightarrow{\text{Peroxidase}} H_2O + O^{\cdot}$$

$$O^{\cdot} + TMB_{red} \longrightarrow TMB_{ox} + H_2O$$

The chromogen used is tetramethylbenzidine (TMB). Since TMB is noncarcinogen, it becomes more popular than benzidine (22). The pKa of TMB (tetramethylbenzidine) is 4.5. It is colorless in the reduced form and becomes blue color upon oxidation. To stop the enzyme reaction, 2.0 N sulfuric acid is added. As the pH is changed, the TMB_{ox} turns to yellow color.

B. Statement of problem

The goal of the research project is to develop a competitive enzyme immunoassay system for serum estradiol and estriol determination. The goals of this study are (a) to develop a simple and quick method to isolate gamma globulin from whole anti-serum, (b) to determine the optimal concentration of gamma globulin for coating the well, (c) to produce the enzyme (HRPO) conjugate, (d) to determine the best conditions for estradiol and estriol assays (e) to test the cross reaction, and (f) to investigate the performance characteristic of the developed method and compare it with other methods which have already been developed.

Chapter II

EXPERIMENTAL

A. Materials

The following materials were purchased from Sigma: Diethylaminoethyl (DEAE) cellulose (medium mesh); Sephadex LH-20; horseradish peroxidase (HRPO), type VI-A; sodium phosphate (monobasic, anhydrous); succinic anhydride; Trizma base (Tris [hydroxymethyl] aminomethane); polyoxyethylene-sorbitan monolaurate (Tween 20); 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide HCl (EDC); activated charcoal (untreated powder, 100-400 mesh); sodium barbital buffer; testosterone; 3,3',5,5'-tetramethylbenzidine (TMB); 17-alpha-estradiol; 17-beta-estradiol. 1,6-Hexanediamine was obtained from Eastman Kodak Co. Sodium hydroxide pellets and sodium chloride were products of Baker Chemical Co. Silica gel thin-layer chromatographic plates (Gel GF 20x20) and glycerin were supplied by Fisher Scientific Company. Absolute methanol was obtained from EM Science. Dimethyl sulfoxide (DMSO) was product of Mallinckrodt, Inc. Econo-Columns were purchased from Bio-Rad. Spectra/por dialysis tubing (molecular weight cutoff 8,000), Sepraphore III cellulose acetate membranes, and Supor-200 (modified

polysulfone) membrane filters (0.20 um) were purchased from American Scientific Products. Immulon I, Immulon II, and Immulon IV removable strips were products of Dynatech Laboratories. Rabbit antisera against estradiol and estriol were gifts of Neo-Bio Meditech Company. The pooled serum was obtained from Norfolk Community Hospital, Norfolk, Virginia. All other chemical were all reagent grade. The commercially available radioimmunoassay kits, Coat-A-Count estradiol and Coat-A-Count estriol, were purchased from Diagnostic Products Corporation. The serum samples for comparison studies were obtained from Maryview Medical Center, Portsmouth, Virginia.

B. Equipment

A Beckman Microzone Cell model R-101 coupled with an EC-400 power supply was used for protein electrophoresis. A Varian model 216 scanning spectrophotometer coupled with an Apple computer was used for densitometric scan of electrophoretic pattern and calculation of peak areas. High speed centrifugation (15,000 rpm) was performed using a Beckman model J2-21 refrigerated centrifuge, while, low speed centrifugation (3,000 rpm) was performed using a Beckman model TJ-6 centrifuge equipped with a Beckman TJ-R refrigeration unit. A Milton Roy model 1201 spectrophotometer was used for all absorbance readings. All pH measurements were performed with a Corning Digital 110 pH

meter. A Precision Scientific water bath was used for all temperature controlled incubations. An ISCO model UA-5 Absorbance/Fluorescence monitor coupled with a 10-mm light path flow cell was used for monitoring column effluent, and a Pharmacia Frac-100 fraction collector was used for effluent collection. A Travemol-Genen Tech Diagnostic (multi-crystal, 12-well) Gamma Counter was used for radioimmunoassay.

C. Methods

1. Preparation of DEAE-cellulose resins

For DEAE-cellulose column chromatography, the resin was prepared according to Stanworth (23) with minor modifications. Fifty grams of DEAE-cellulose were suspended in one liter of 0.10 N HCl and stirred for ten minutes at room temperature. The resin was then filtered on a 300-mL glass fritted funnel (coarse) and again washed with one liter deionized water. The process was repeated with one liter of 0.10 N NaOH and one liter deionized water. Finally, the resin was equilibrated in 5.0 mM sodium phosphate buffer, pH 6.5 and stored at 4°C.

2. Isolation of gamma globulin fraction from whole antiserum

Lyophilized rabbit anti-estradiol or anti-estriol powder was reconstituted with deionized water. One milliliter of the antiserum was diluted further to a final

volume of 10 mL with deionized water and dialyzed against four liters deionized water for four hours at 4°C, followed by four liters of 5.0 mM sodium phosphate buffer, pH 6.5, overnight with three changes of the same buffer. The anti-serum was then applied to a DEAE-cellulose column (1 x 24 cm) which was pre-equilibrated with the same buffer. The gamma globulin fraction was eluted from the column with the same buffer and concentrated to approximately 1.0 mL with an Amicon Diaflo system (model 52) equipped with a YM-05 ultrafiltration membrane.

3. Preparation of assay solutions

Solution A: 57 mg urea-hydrogen peroxide (Sigma U-1753 with hydrogen peroxide content 35%) dissolved in 100 mL of buffer containing 0.10 M citric acid and 0.10 M Na_2HPO_4, pH 5.0.

Solution B: 20 mg TMB completely dissolved in 1.0 mL DMSO, 10 mL glycerin, and 40 mL methanol. The solution is then diluted with deionized water to a final volume of 100 mL.

4. Preparation of E_2-3-hemisuccinate and E_3-3-hemisuccinate

The procedure described by Exley and Woodhams (24) was used for the preparation of E_2 and E_3 hemisuccinate derivatives. A round bottom flask containing a mixture of 0.50 g E_2, 1.5 g succinic anhydride, and 7.5 mL pyridine was incubated in a 50°C water bath for 80 minutes. The mixture

was then cooled to room temperature and evaporated to dryness under reduced pressure. The dried compound was dissolved in 50 mL chloroform and washed with 150 mL deionized water three times. The organic layer was dried over anhydrous sodium sulfate and re-evaporated to dryness. The crude material was partially purified using a sephadex LH-20 column. The column was first equilibrated and then eluted with toluene, methanol 85:15 (v/v). Fractions (3.0 mL) were collected with a fraction collector. These fractions were analyzed by thin layer chromatography with a solvent system of benzene, ethylacetate, acetic acid [60:40:0.5 (v/v/v)].

5. Preparation of Estradiol-3-hemisucimyl-peroxidase (E_2-HRPO) and Estriol-3-hemisucimyl-peroxidase (E_3-HRPO)

One part (0.5 mmole, 0.18 mg) of E_2-3-HS, one part (0.5 mmole, 0.06 mg) of 1,6-hexanediamine, 3.0 mL of water, and 1.2 parts (0.6 mmole, 0.12 mg) of EDC were mixed and stored overnight (20 hours) at 4°C with the pH maintained between 5.0 - 5.5. Horseradish peroxidase, 0.5 part (0.25 mmole, 10 mg) and one part (0.5 mmole, 0.10 mg) EDC were then added to the mixture and incubated at 4°C for an additional five hours. The sample was dialyzed against running water for eight hours at room temperature. To stabilize the HRPO activity, horse serum albumin was added to a final concentration of 10 mg/mL. The sample was

further dialyzed at 4°C against phosphate buffered saline (PBS) for 16 hours with one change of the buffer. E_3-3-HS was prepared in the same manner.

6. Preparation of E_2 and E_3 standard sera

Pooled serum was treated with activated charcoal at 0.2 g per mL for 24 hours at 4°C with gentle mixing (25). The charcoal was removed by centrifugation at 20,000 x g for 30 minutes at 4°C; the serum obtained was further filtered through two layers of glass filter followed by twice with supor-200 polysulfone membrane (0.2 um) filter. The steroid-free serum thus obtained was stored at -20°C until further use. To prepare the E_2 standard sera, an appropriate amount of E_2 solution (3 ug/mL in Ethanol) was pipetted into the steroid-free serum to give E_2 standard sera with final concentrations of 0, 10, 100, and 1000 pg/mL. Similarly, E_3 solution (100 ug/mL in Ethanol) was added to the prepared steroid free serum to give E_3 standard sera with final concentrations of 0, 10, 100 and 1000 ug/mL, respectively.

7. Preparation of gamma globulin coated microwells

The concentration of isolated gamma globulin was determined using its absorption at 280 nm with an extinction coefficient, $E_{1\%}$, of 13.5 (17). The gamma globulin was diluted with coating buffer to 500 ng/mL for E_2 assay (18 ug/mL for E_3 assay). The microwells were then incubated at room temperature for 16 hours, and these were washed with

PBS containing 0.3 % Tween-20 three times and followed with deionized water three times.

8. The E_2 and E_3 assay procedures

The serum samples (standard, control or unknown) were added (25 uL for E_2 and 10 uL for E_3) to gamma globulin coated microwells and followed with 50 uL of the appropriate enzyme-conjugate, 50 uL testosterone (10 ug/mL), 75 uL 0.1 M sodium phosphate buffer, pH 7.4, and deionized water to a total volume of 200 uL. The mixture was incubated at room temperature for 90 minutes, and the microwells were washed with 0.1 mM sodium phosphate buffer, pH 7.4, containing 0.1 % Tween-20 three times and deionized water three times. Solution A (substrate), 100 uL, and Solution B (chromogen), 100 uL, were added to these microwells and let stand at room temperature for color development for 30 minutes. After the color developed, 50 uL of 2.0 N sulfuric acid was added to stop the reaction, and the absorbance at 450 nm was measured.

9. Study of the optimal testosterone concentration in the standard assay

For the determination of total serum E_2 or E_3 concentration, it is necessary to prevent binding of these steroid hormones to the serum steroid binding proteins, sex hormone binding globulins (SHBG). This was accomplished by the addition of testosterone to the reaction mixture to block their binding to SHBG. For determining the optimal

concentration of testosterone to be included in the standard assay procedure, a series of testosterone concentrations was included in E_2 and E_3 assay procedures. In E_2 assay, 25 uL standard serum was added into each microwell (in E_3 assay, the serum volume was 10 uL) followed by 50 uL of the appropriate enzyme-conjugate, 50 uL of different concentrations of testosterone (0, 0.5, 1.0, 2.0, 4.0, 10.0, 20.0, 40.0 ug/mL), and 75 uL (or 100 uL for E_3 assay) of 0.1 mM sodium phosphate buffer, pH 7.4. The microwells were incubated at room temperature for 60 minutes and washed with 0.1 mM sodium phosphate buffer, pH 7.4, three times and deionized water three times. For color development, 100 uL of Solution A and 100 uL Solution B were added to each well. The wells were incubated at room temperature for 30 minutes. Finally, 50 uL of 2.0 N sulfuric acid was added to each microwell to stop the color development, and the absorbance at 450 nm was determined.

10. The study of cross reactivity

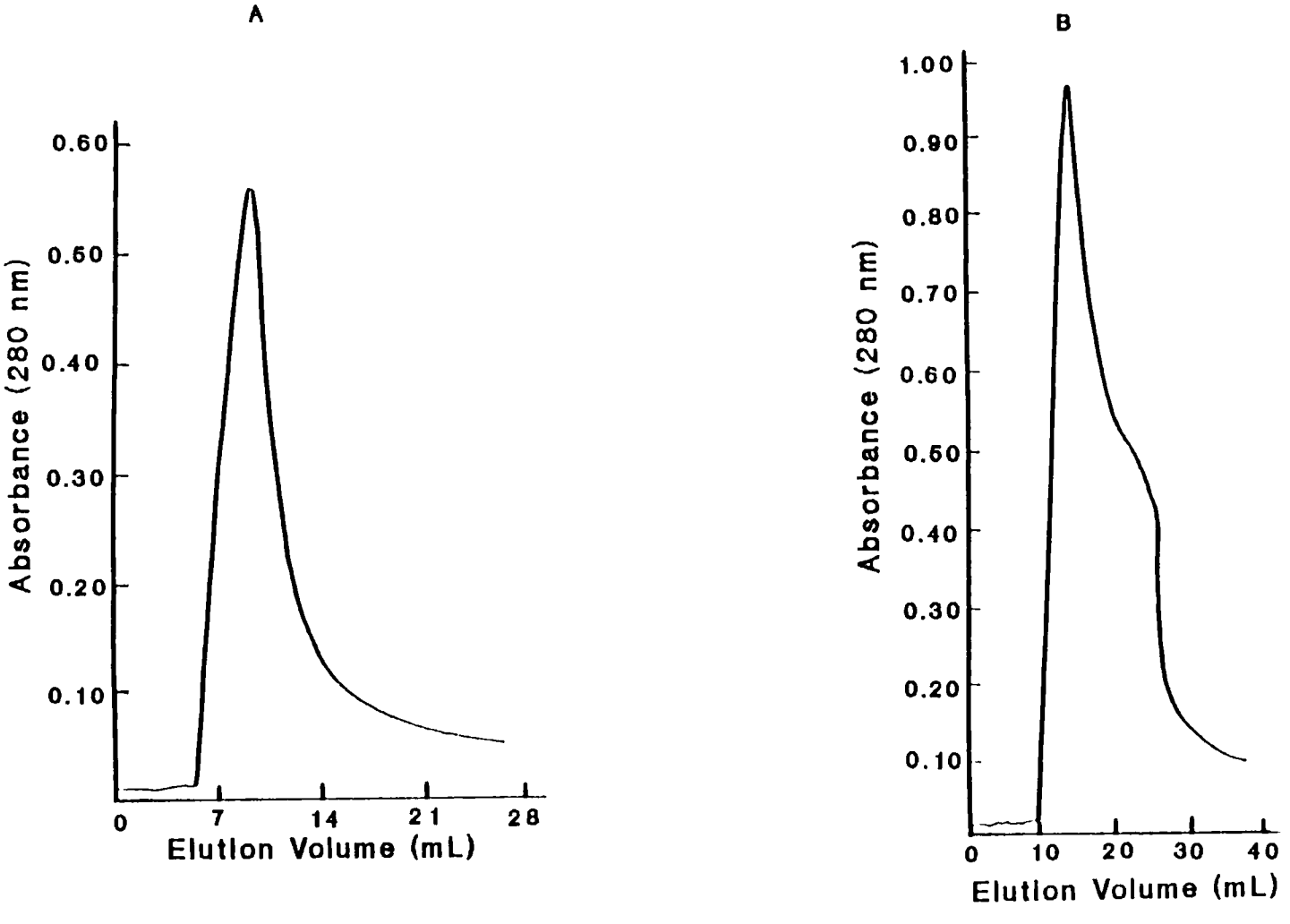
Solutions of potential cross reacting compounds such as 17-beta-estradiol, estriol, 17-alpha-estradiol were prepared in 100 mM sodium phosphate buffer, pH 7.4. In the anti-E_2 gamma globulin coated wells, the assay was performed by the addition of 25 uL of E_2 standard (or solutions of cross reacting compound) in 100 mM sodium phosphate buffer and 50 uL E_2-HRPO conjugate. The assay was also carried out for anti-E_3 gamma globulin coated wells in a similar way.

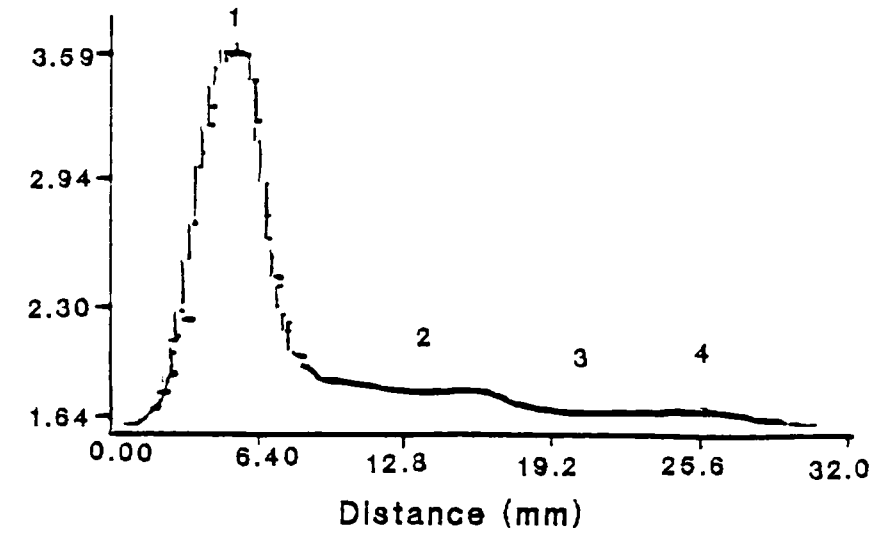
Chapter III

RESULTS

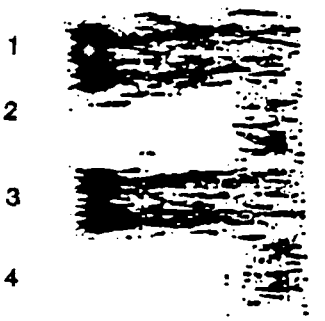
A. Isolation of the gamma globulin from whole anti-serum

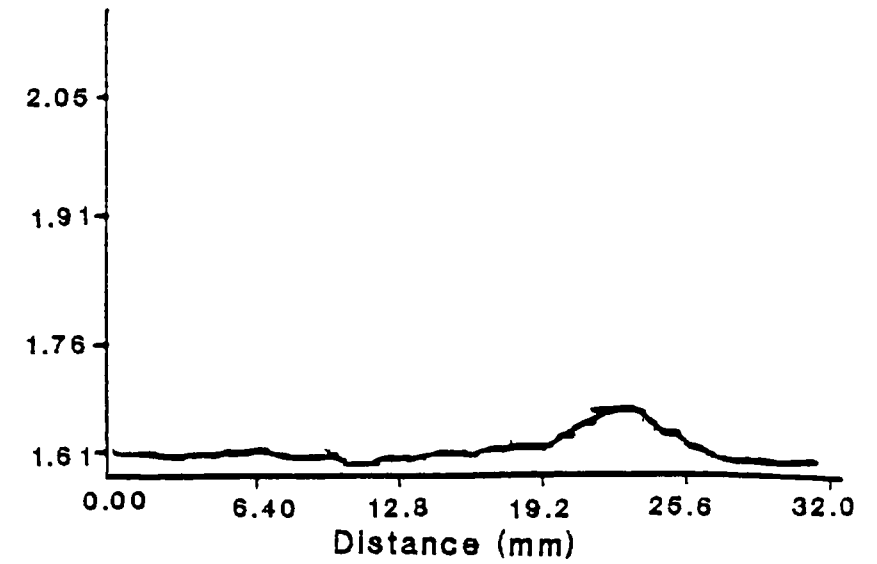
The rabbit whole anti-serum, 0.5 mL, was diluted to 4.0 mL with deionized water and dialyzed against deionized water at 4°C for eight hours and followed with 5.0 mM sodium phosphate buffer, pH 6.5, for 16 hours. It was then loaded onto a DEAE-cellulose column (1 x 24 cm) which was pre-equilibrated with 5.0 mM sodium phosphate buffer, pH 6.5. The gamma globulin was eluted from the column with the same buffer and monitored at 280 nm. Figure 7 represents the elution profile anti-estradiol gamma globulin fraction and anti-estriol gamma globulin fraction from DEAE-cellulose columns. Each of these gamma globulin fractions was then concentrated to about 1.0 mL using an Amicon ultrafiltration system equipped with a YM-05 membrane. The rabbit whole anti-serum and the purified gamma globulin fractions were then analyzed by Beckman microzone electrophoresis system using a cellulose acetate membrane. The results are presented in Figures 8 and 9. The peak 1, 2, 3, and 4 represent albumin, alpha globulin, beta globulin and gamma globulin. For anti-estradiol, there was only 5.0% gamma

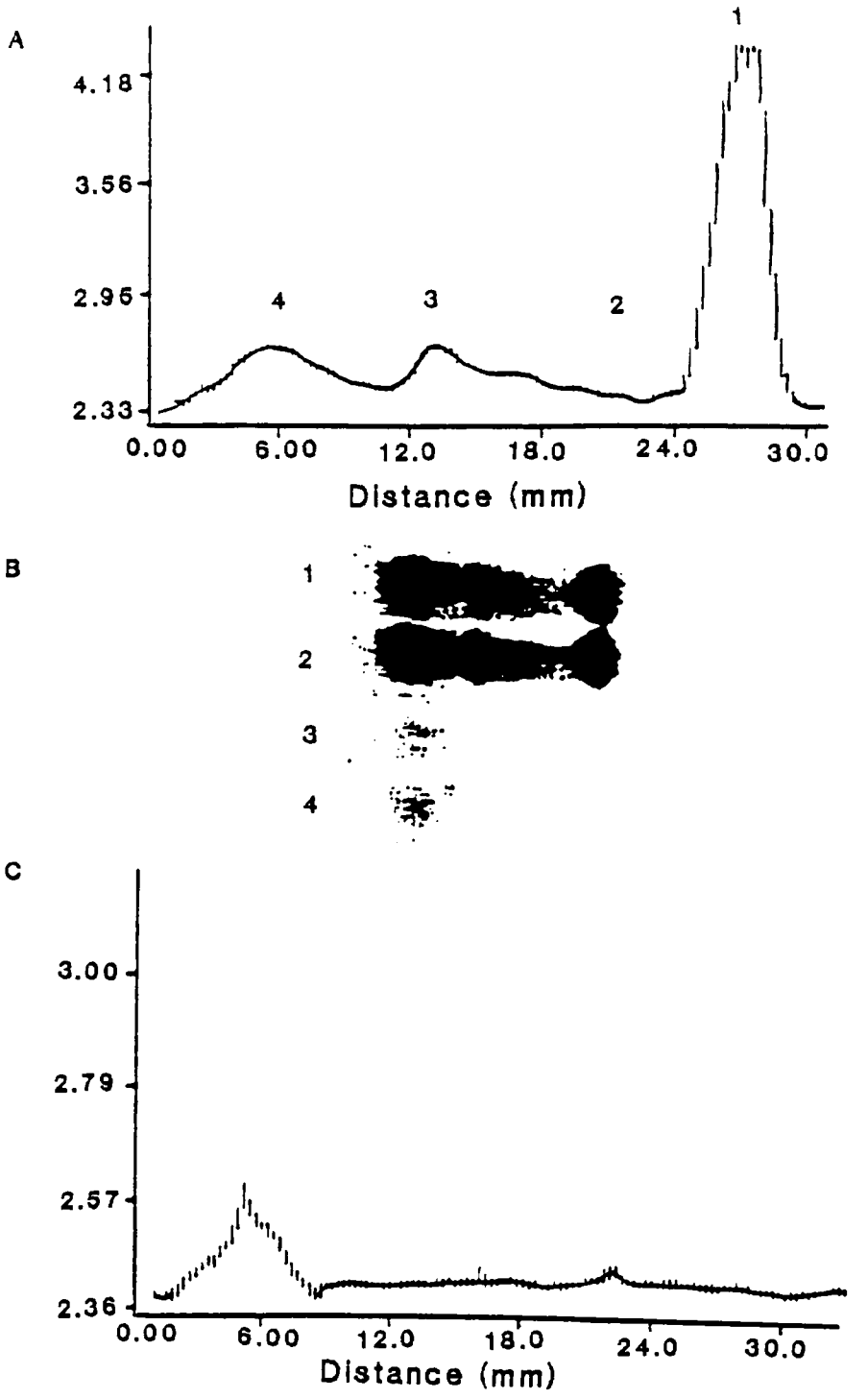

A
0.60
0.50
0.40
0.30
0.20
0.10
Absorbance (280 nm)
0
7
14
21
28
Elution Volume (mL)
B
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
Absorbance (280 nm)
0
10
20
30
40
Elution Volume (mL)


1
3.59
2.94
2.30
1.64
2
3
4
0.00
6.40
12.8
19.2
25.6
32.0
Distance (mm)

B


1
2
3
4


2.05
1.91
1.76
1.61
0.00
6.40
12.8
19.2
25.6
32.0
Distance (mm)


A
4.18
3.56
2.95
2.33
1
4
3
2
0.00
6.00
12.0
18.0
24.0
30.0
Distance (mm)
B
1
2
3
4
C
3.00
2.79
2.57
2.36
0.00
6.00
12.0
18.0
24.0
30.0
Distance (mm)

globulin fraction in the whole anti-serum, but more than 77.5% purity was achieved in the purified anti-estradiol gamma globulin fraction. For anti-estriol, there was only 20.4% in the whole anti-serum, and more than 87.4% purity was obtained in the purified anti-estriol gamma globulin fraction.

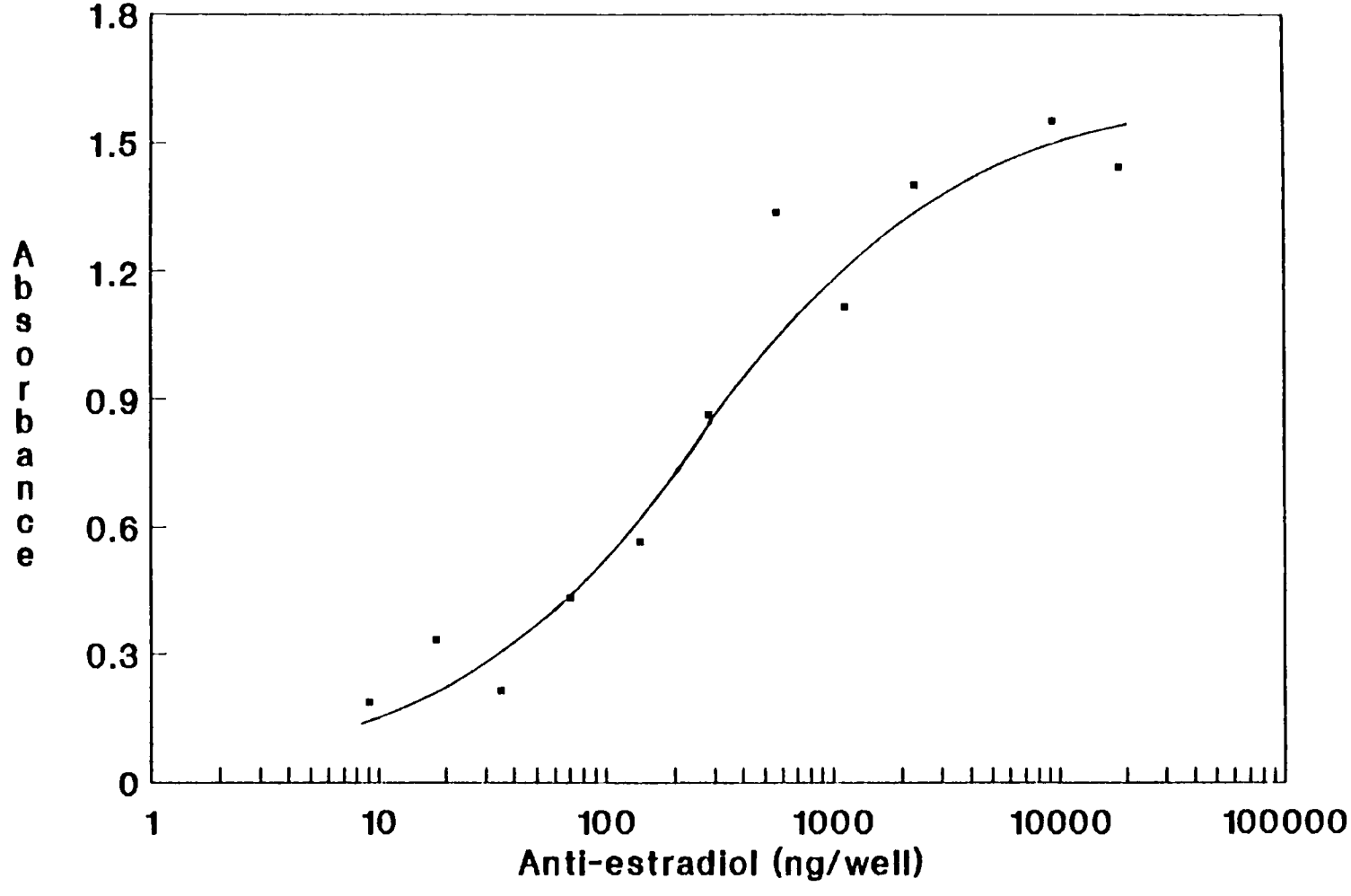
B. Study of the optimal concentration of gamma globulin for microwell coating

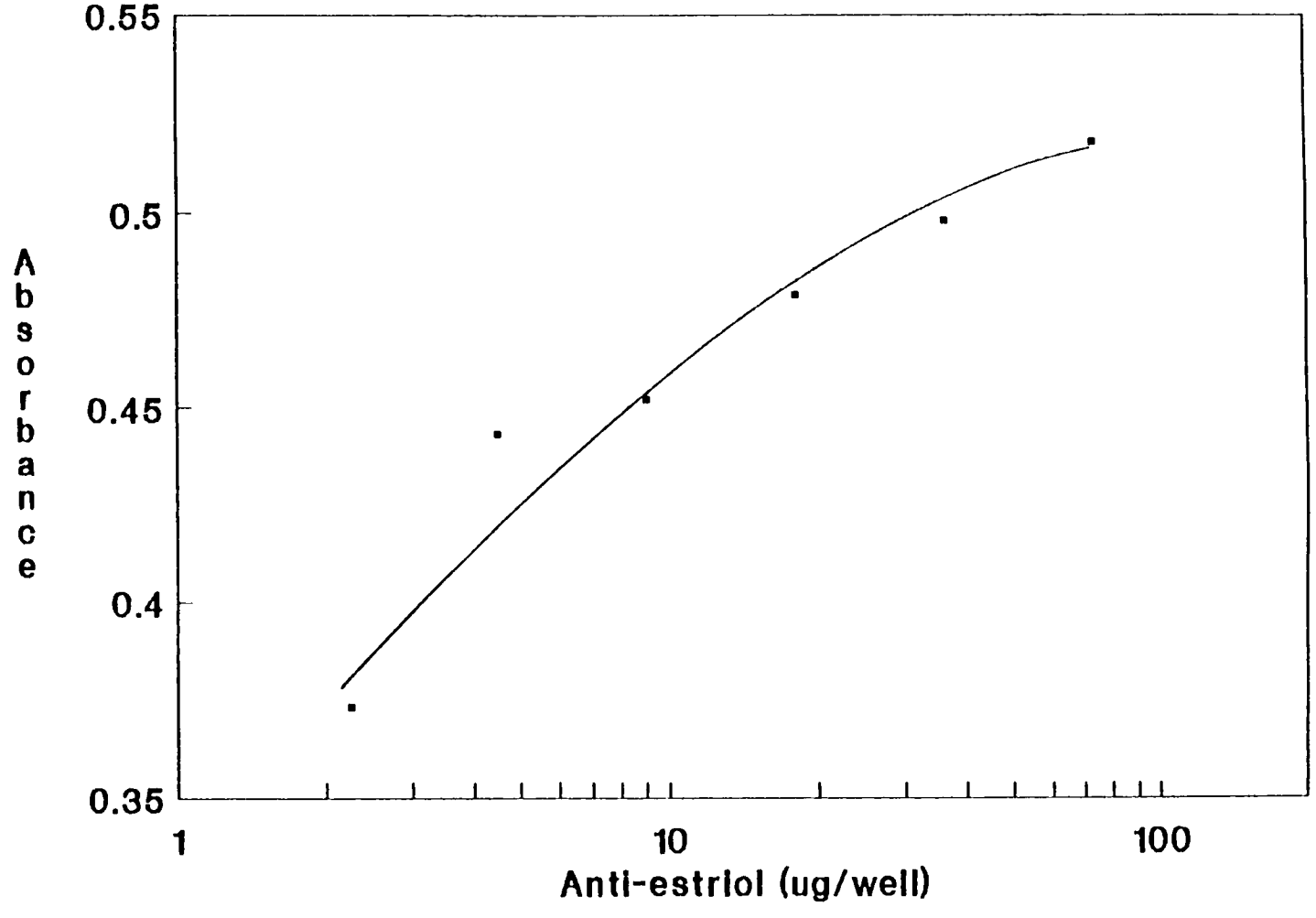
The concentrations of purified anti-estradiol and anti-estriol gamma globulins were determined by measuring the absorbance at 280 nm and calculated by using an extinction coefficient, $E_{1\%}$, of 13.5 (26). The concentration was then diluted to the desired concentration. In studying the optimal anti-estradiol gamma globulin concentration for microwell coating, diluted purified anti-estradiol gamma globulin was pipetted into the microwells to give 9.0, 18, 35, 70, 141, 281, 563, 1125, 2250, 4500, 9000, 18000 ng/well. These microwells were incubated at room temperature for 16 hours and then washed with PBS containing 0.3% Tween 20 three times and dried. To each of these microwells, 200 uL of E_2-HRPO were added and incubated at room temperature for 60 minutes. After the incubation the microwells were emptied and washed with 100 mM sodium phosphate containing 0.1% Tween-20. For color development, solutions A and B, 100 uL of each , were added to each microwell and let stood at room temperature for 30 minutes.

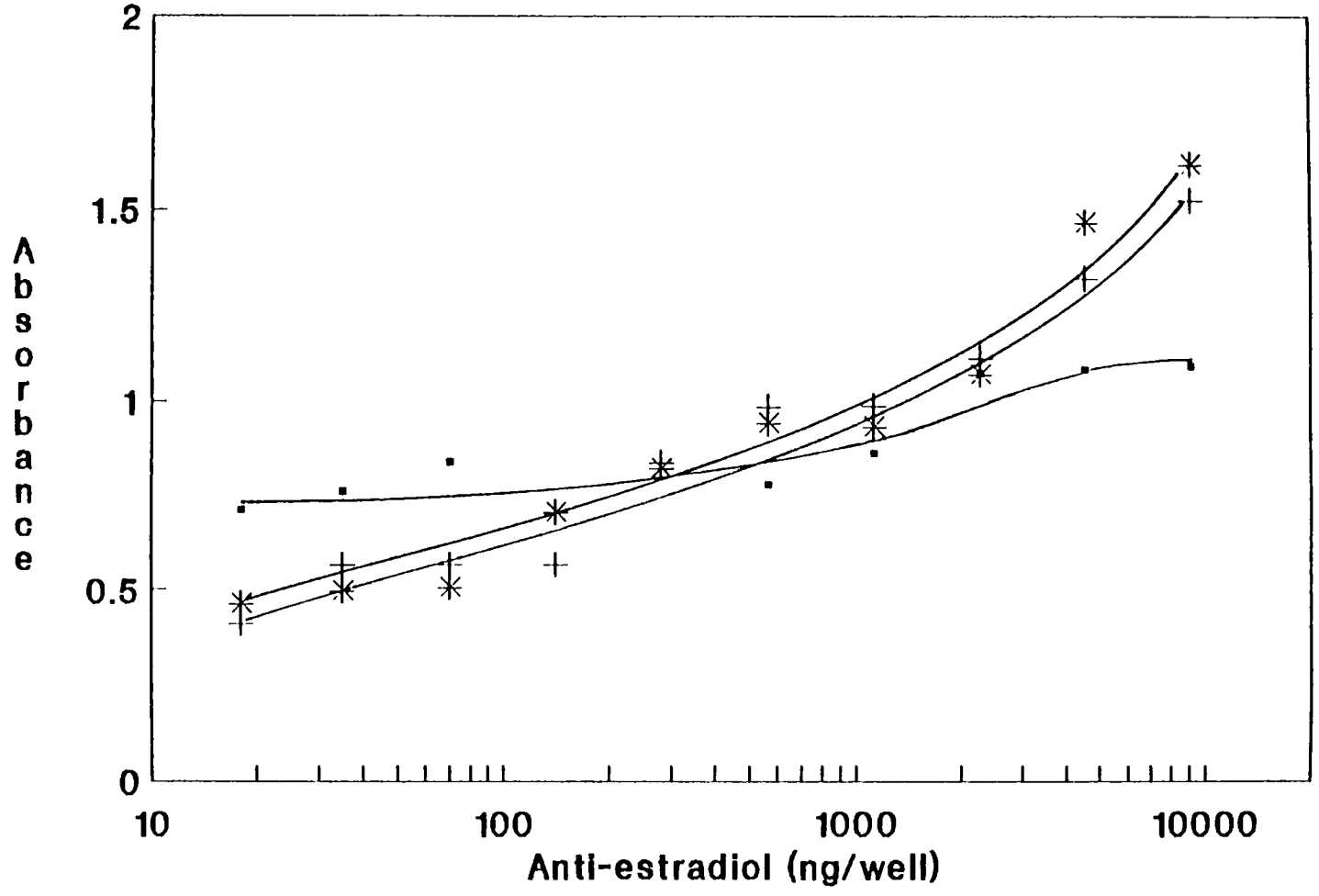
The reaction was stopped with the addition 50 uL of 2.0 N sulfuric acid, and the absorbance at 450 nm was measured. The data obtained were plotted as absorbance at 450 nm versus the amount of gamma globulin coated in the microwell in semi-log scale as illustrated in Figure 10. The amount of anti-estradiol gamma globulin for microwell coating was decided to be 500 ng/well. Similarly, anti-estriol gamma globulin fraction was coated in the following concentrations : 1.13, 2.25, 4.50, 9.00, 18.0, 36.0, 72.0, 144.0, and 288.0 ug/well. The results obtained were treated in the same manner and are shown in Figure 11. The amount of anti-estriol gamma globulin for microwell coating was decided to be 18 ug/well.

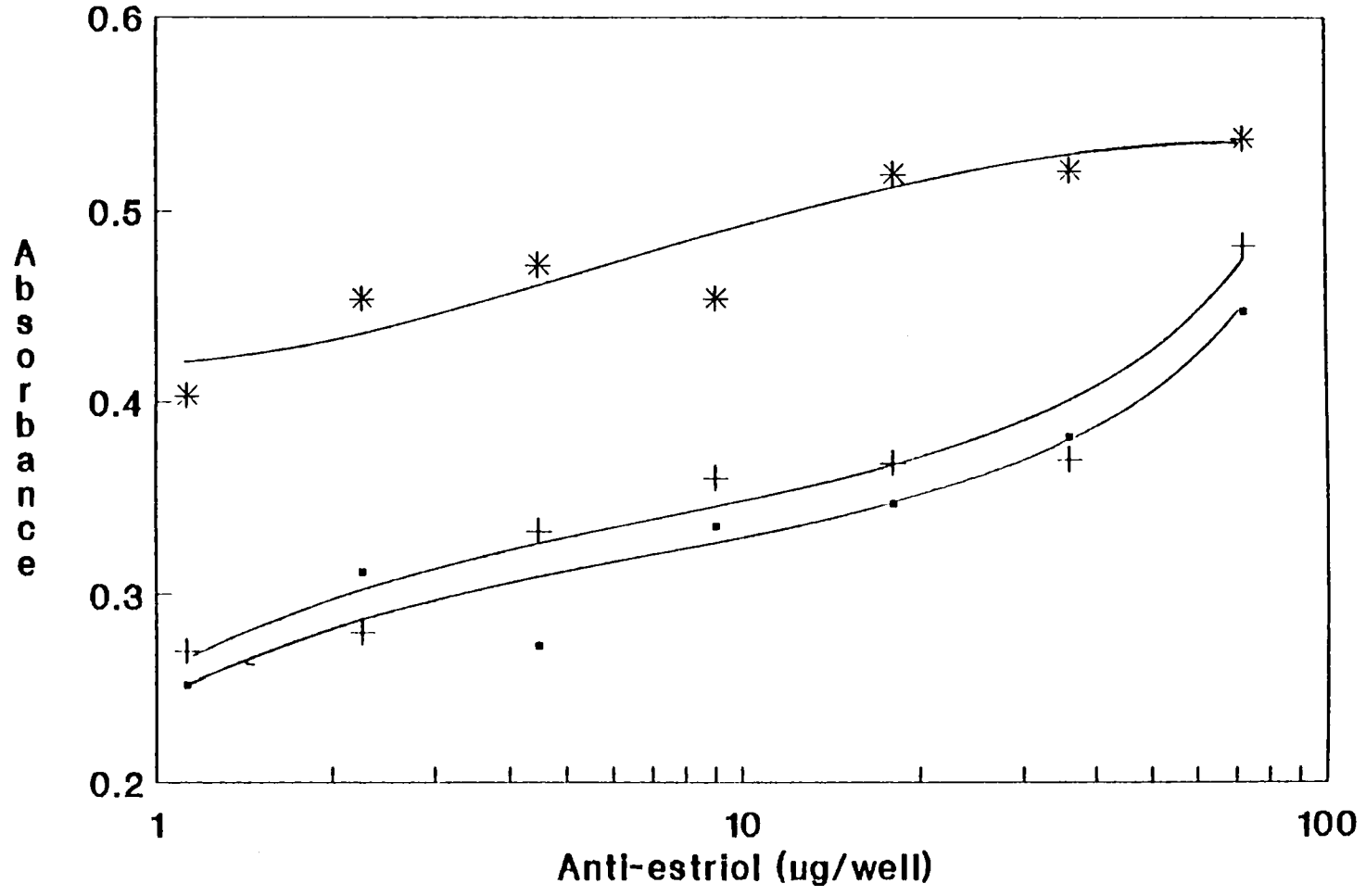
C. The comparison of Immulon I, II, and IV microwells

Three different types of Immulon microwells are currently available from Dynatech Laboratories. To decide the best microwell for anti-estradiol and anti-estriol gamma globulin coating, a series of these microwells were coated with appropriate purified gamma globulin to give various concentrations, and the titration studies on these coated microwells were made. Figure 12 is the comparison of the three types of microwells, Immulon I, II, and IV, coated with anti-estradiol gamma globulin. Figure 13 is the comparison of these microwells coated with anti-estriol gamma globulin. Both of these comparisons displayed that the immulon II microwell is the best microwell for the


1.8
1.5
1.2
0.9
0.6
0.3
0
Absorbance
1
10
100
1000
10000
100000
Anti-estradiol (ng/well)


0.55
0.5
0.45
0.4
0.35
Absorbance
1
10
100
Anti-estriol (ug/well)


2
1.5
1
0.5
0
Absorbance
10
100
1000
10000
Anti-estradiol (ng/well)


0.6
0.5
0.4
0.3
0.2
Absorbance
1
10
100
Anti-estriol (ug/well)

assay.

D. Study of estradiol and estriol assays

1. Optimal concentration of testosterone

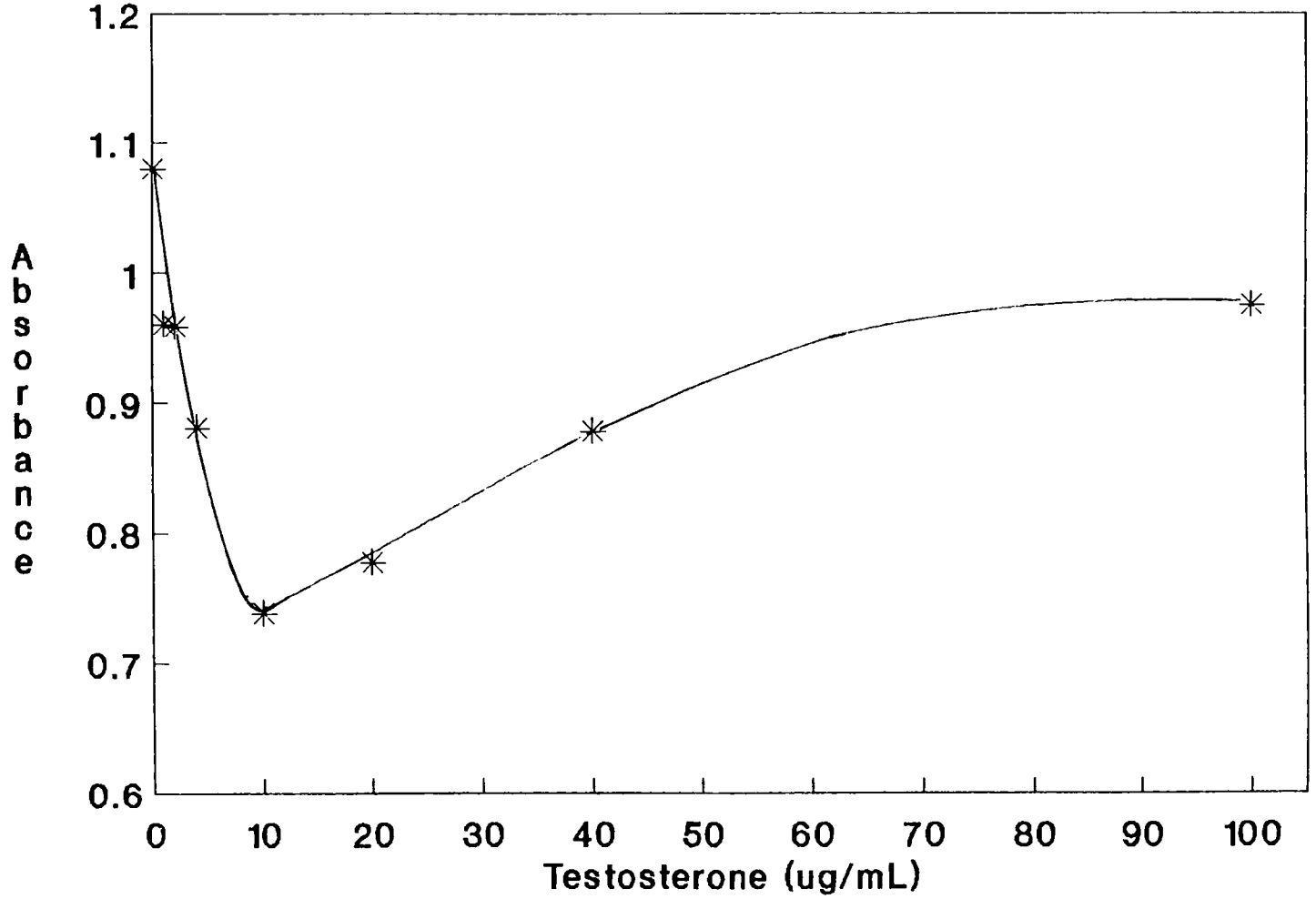
Figures 14 and 15 show the effect of the concentration of testosterone on estradiol and estriol assays respectively. In estradiol assay, the maximum absorbance change was observed at 10 ug/mL of testosterone in the assay mixture. Whereas, the maximum absorbance change was observed at 2.5 ug/mL of testosterone in estriol assay.

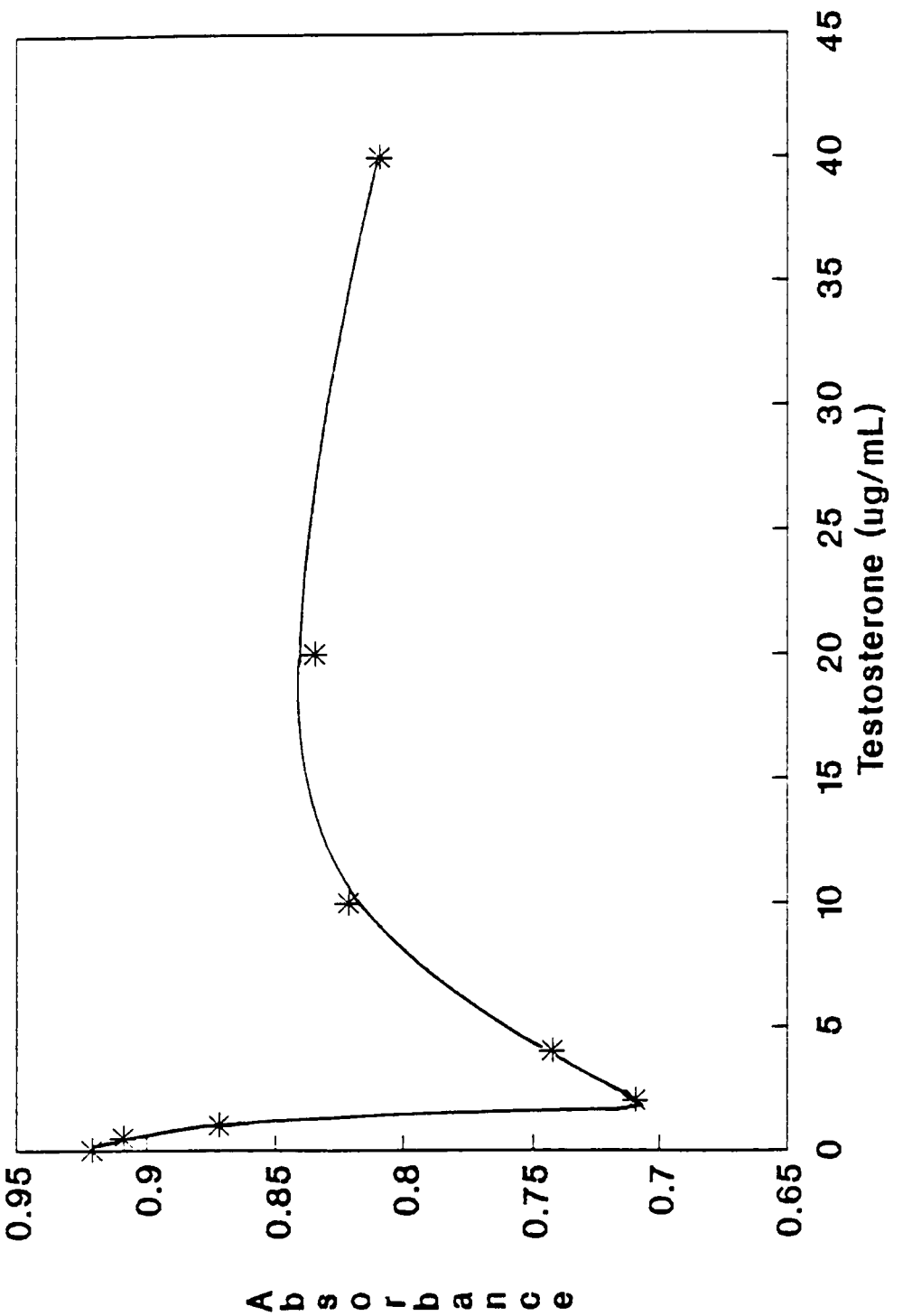
2. Optimal incubation time and temperature

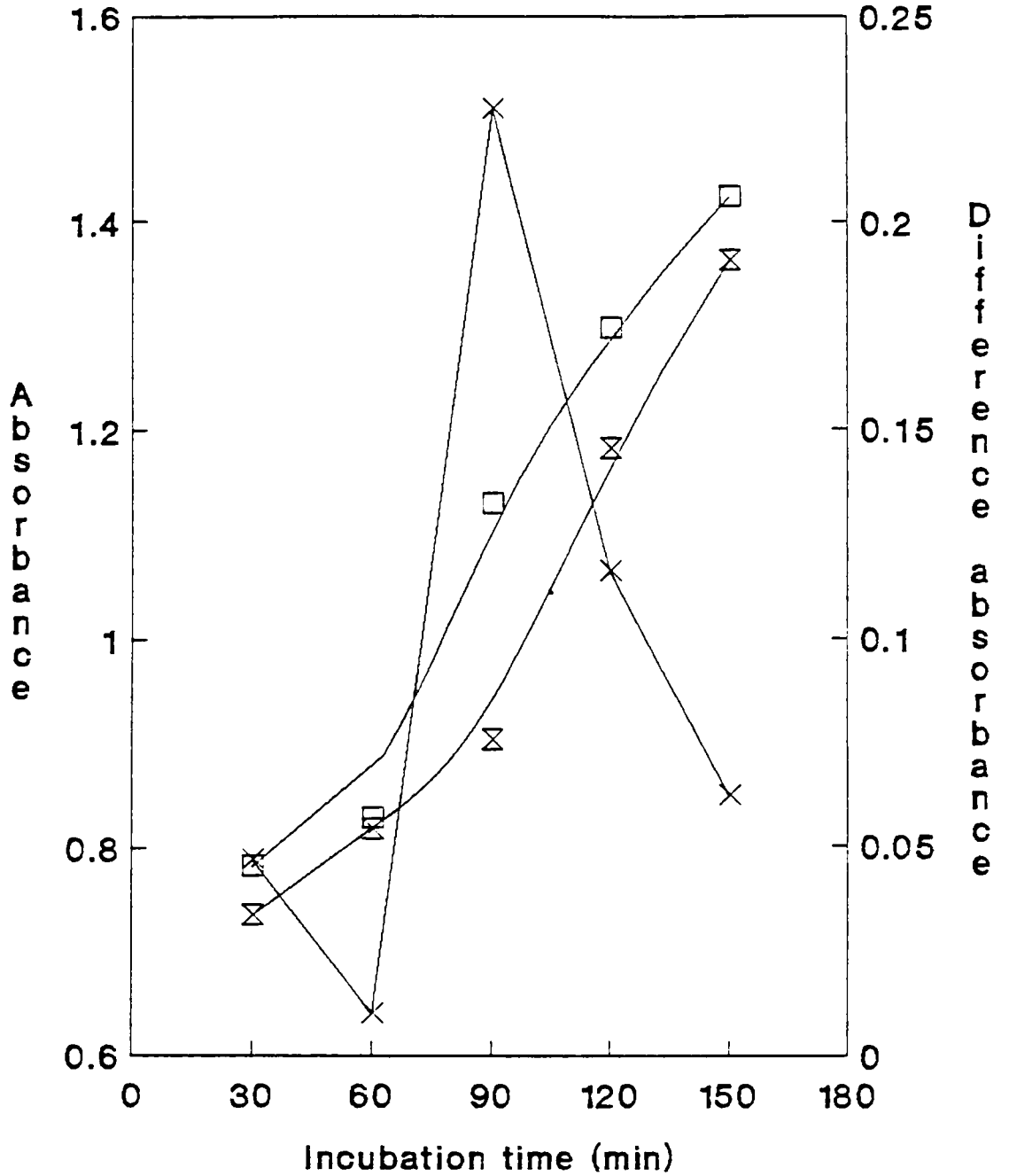
Studies on the optimal incubation time and incubation temperature were carried out. These studies were performed at 25°C and 37°C for 30, 60, 90, 120, and 150 minutes. Figures 16 and 17 illustrate the results obtained for estradiol assay at different incubation time at 25°C and 37°C respectively. These results indicate that the maximum change in absorbance was achieved after 90 minutes incubation at 25°C (Figure 16). For estriol assay, the results obtained are summarized in Figures 18 and 19, and the best result for this assay was obtained after 60 minutes incubation at 25°C.

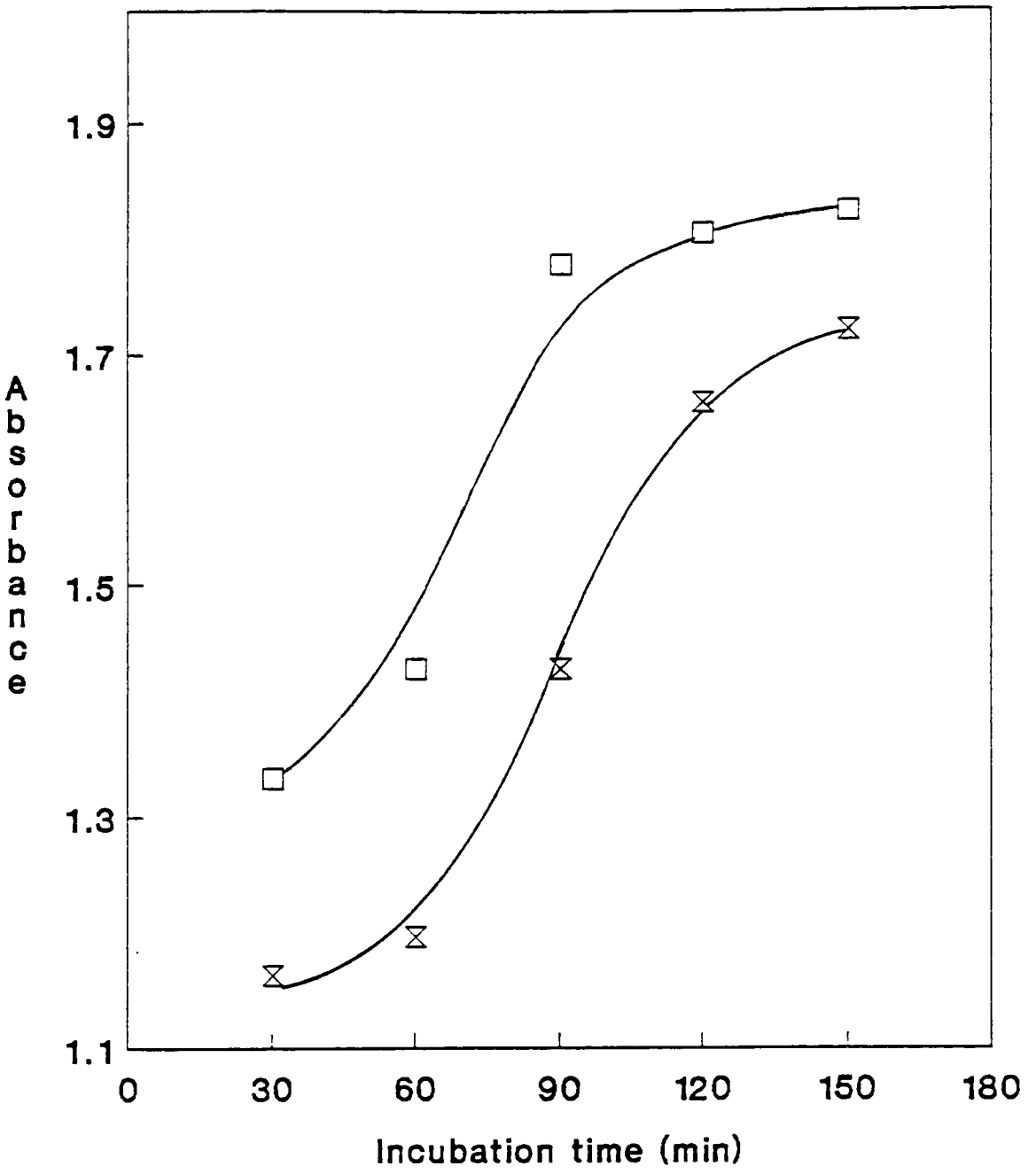
3. Selection of incubation buffer

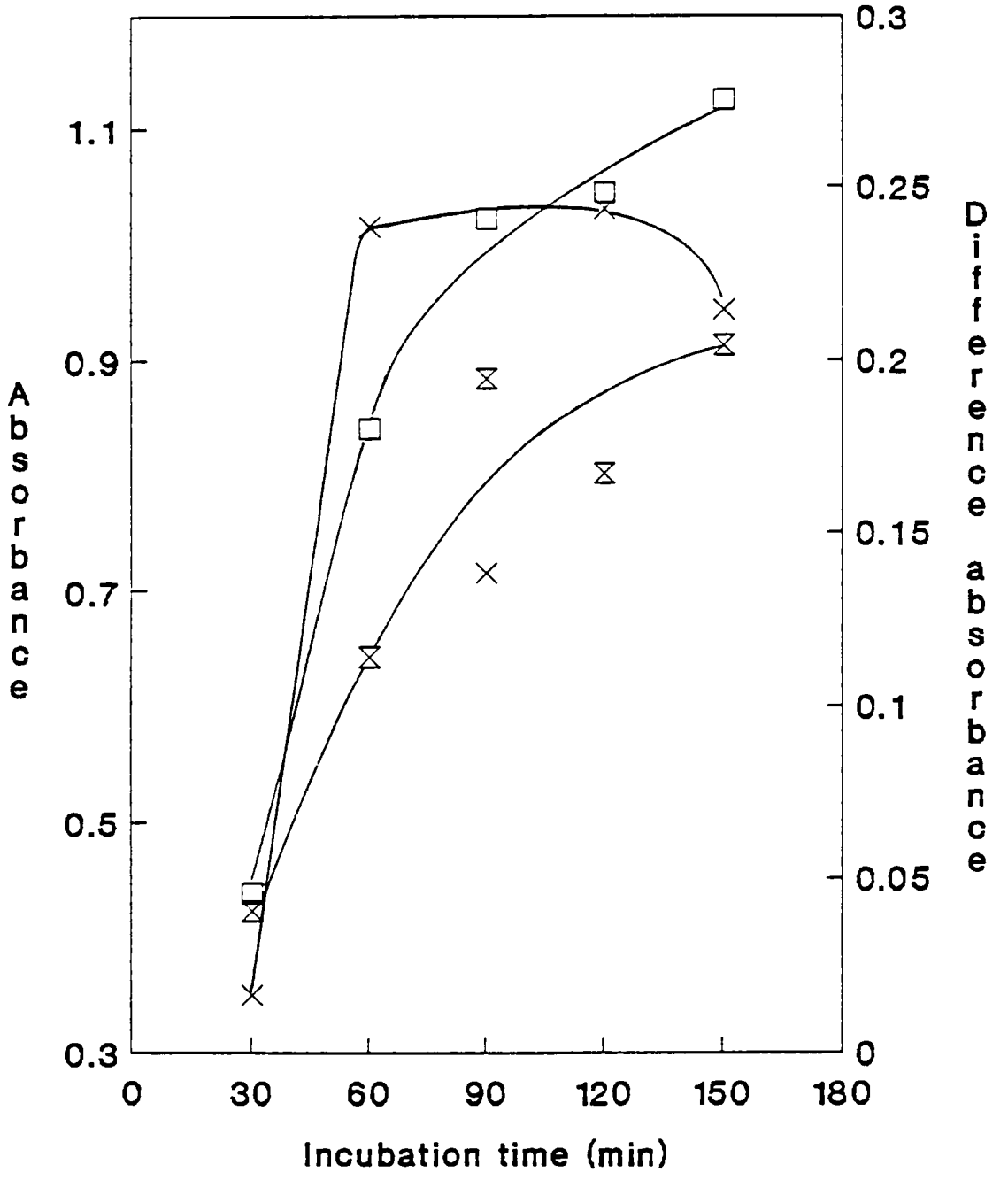
Three different buffers [100 mM sodium phosphate buffer, 5.0 mM sodium phosphate buffer, and phosphate buffered saline (PBS)] were studied as buffer system for the

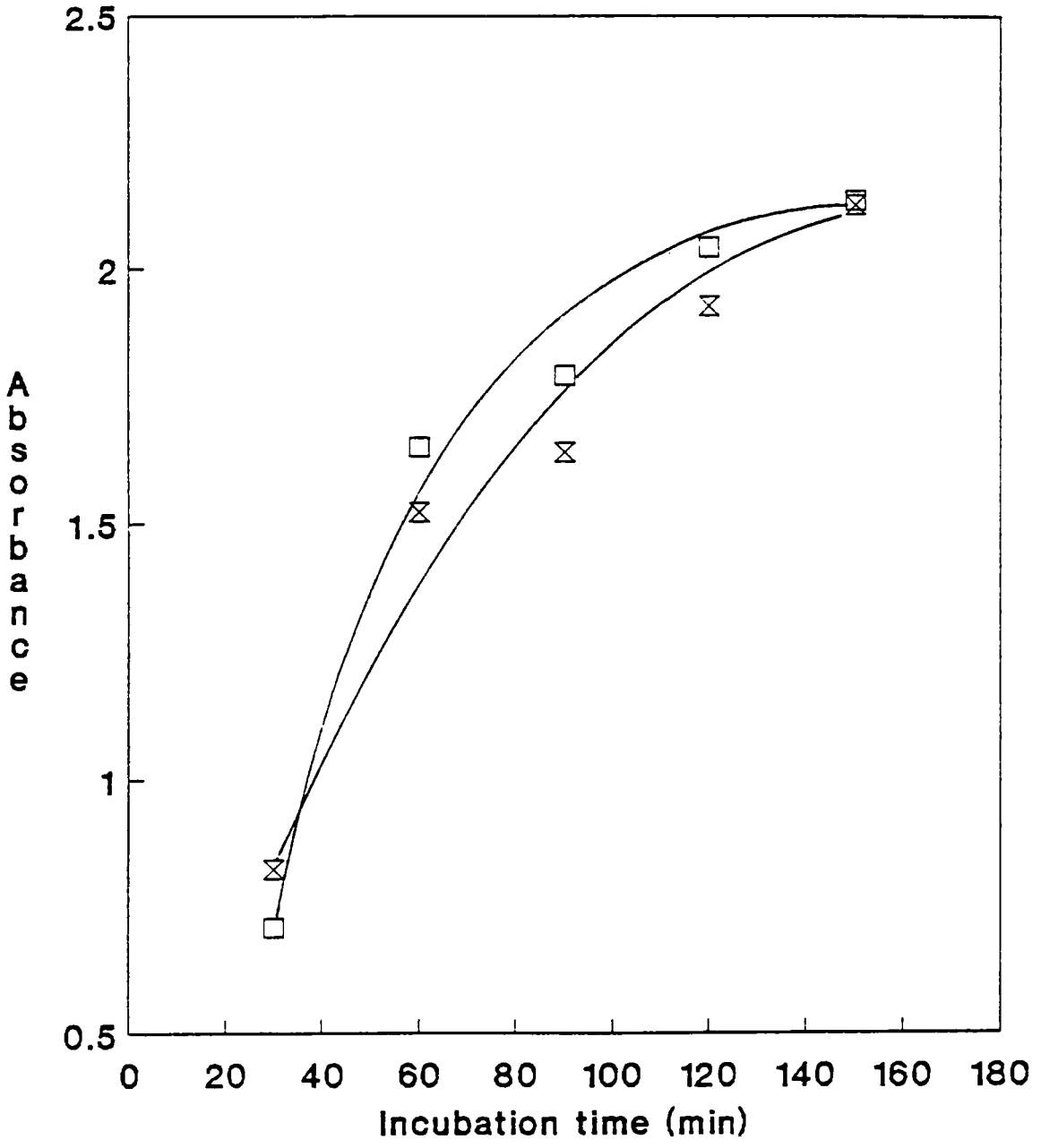

1.2
1.1
1
0.9
0.8
0.7
0.6
Absorbance
0
10
20
30
40
50
60
70
80
90
100
Testosterone (ug/mL)


0.95
0.9
0.85
0.8
0.75
0.7
0.65
Absorbance
0
5
10
15
20
25
30
35
40
45
Testosterone (ug/mL)


1.6
1.4
1.2
1
0.8
0.6
Absorbance
0.25
0.2
0.15
0.1
0.05
0
Difference absorbance
0
30
60
90
120
150
180
Incubation time (min)


1.9
1.7
1.5
1.3
1.1
Absorbance
0
30
60
90
120
150
180
Incubation time (min)


Absorbance
1.1
0.9
0.7
0.5
0.3
0.3
0.25
0.2
0.15
0.1
0.05
0
Difference absorbance
0
30
60
90
120
150
180
Incubation time (min)


2.5
2
1.5
1
0.5
Absorbance
0
20
40
60
80
100
120
140
160
180
Incubation time (min)

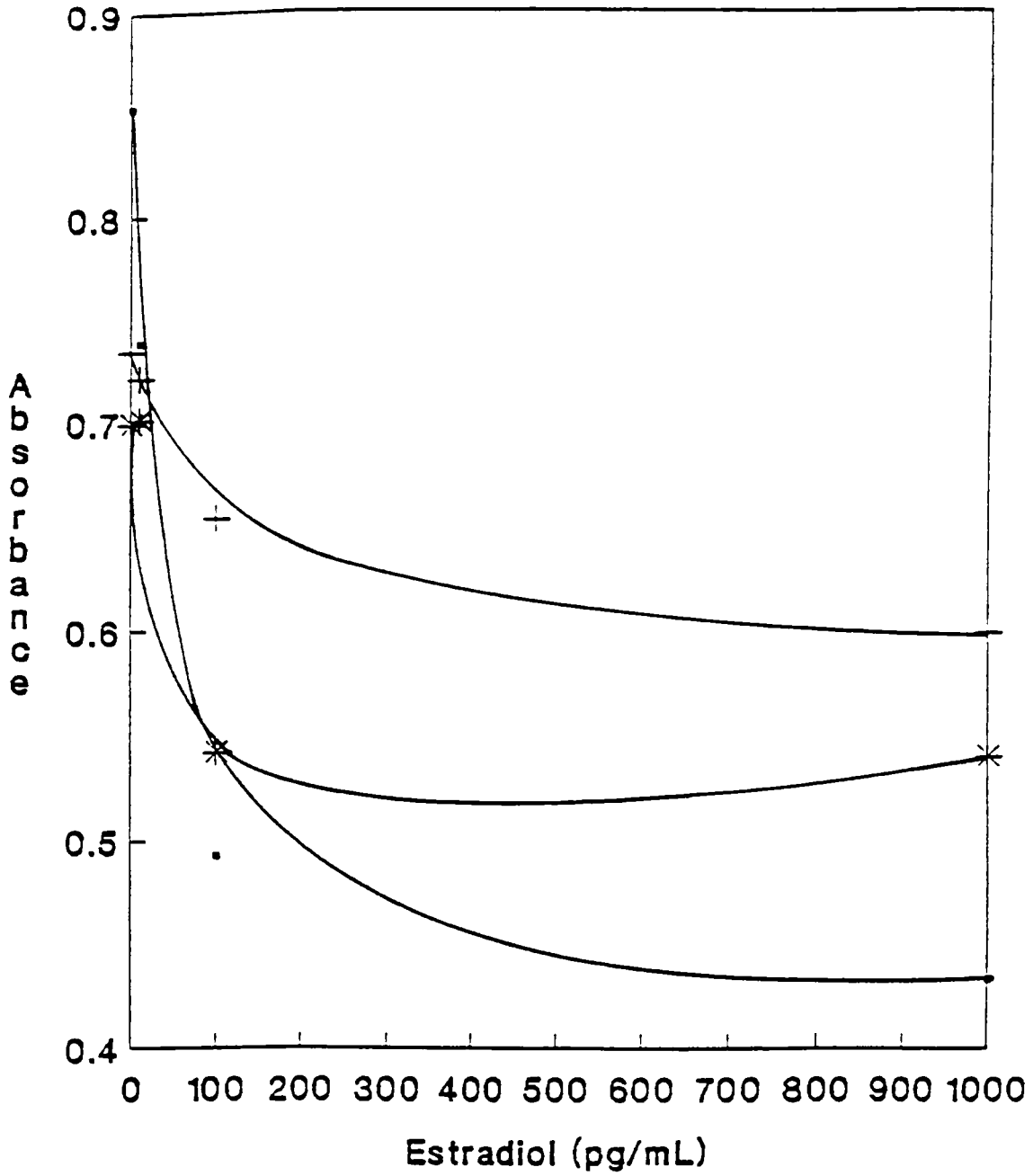
estradiol and estriol assays. The study involved the assay, estradiol or estriol, in an appropriate buffer system, and the results obtained were plotted as absorbance versus concentration of either estradiol or estriol. Under the assay conditions, it was found that the greatest absorbance change was observed with 100 mM sodium phosphate buffer in both estradiol and estriol assays as shown in Figures 20 and 21.

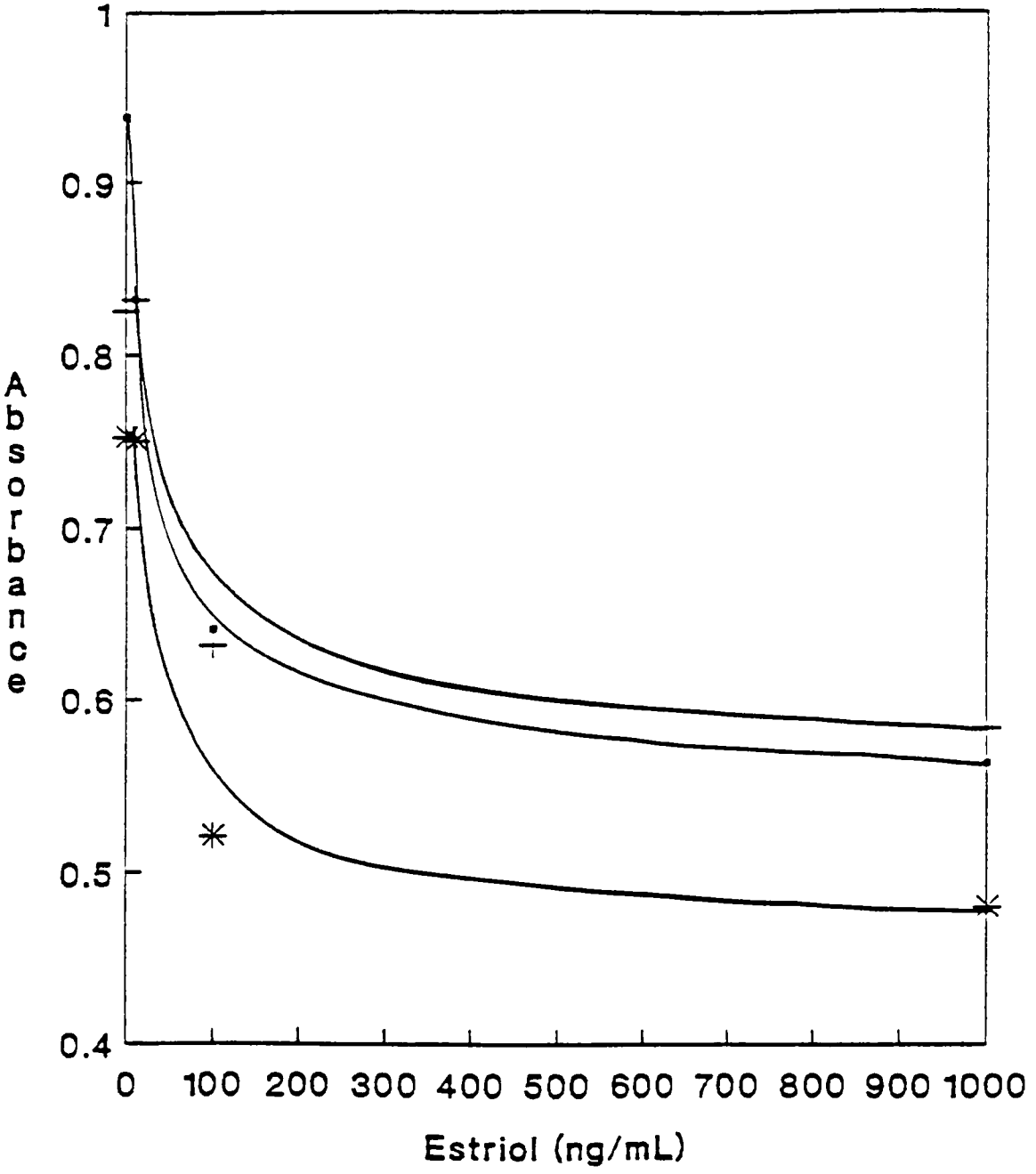
4. Selection of washing solution

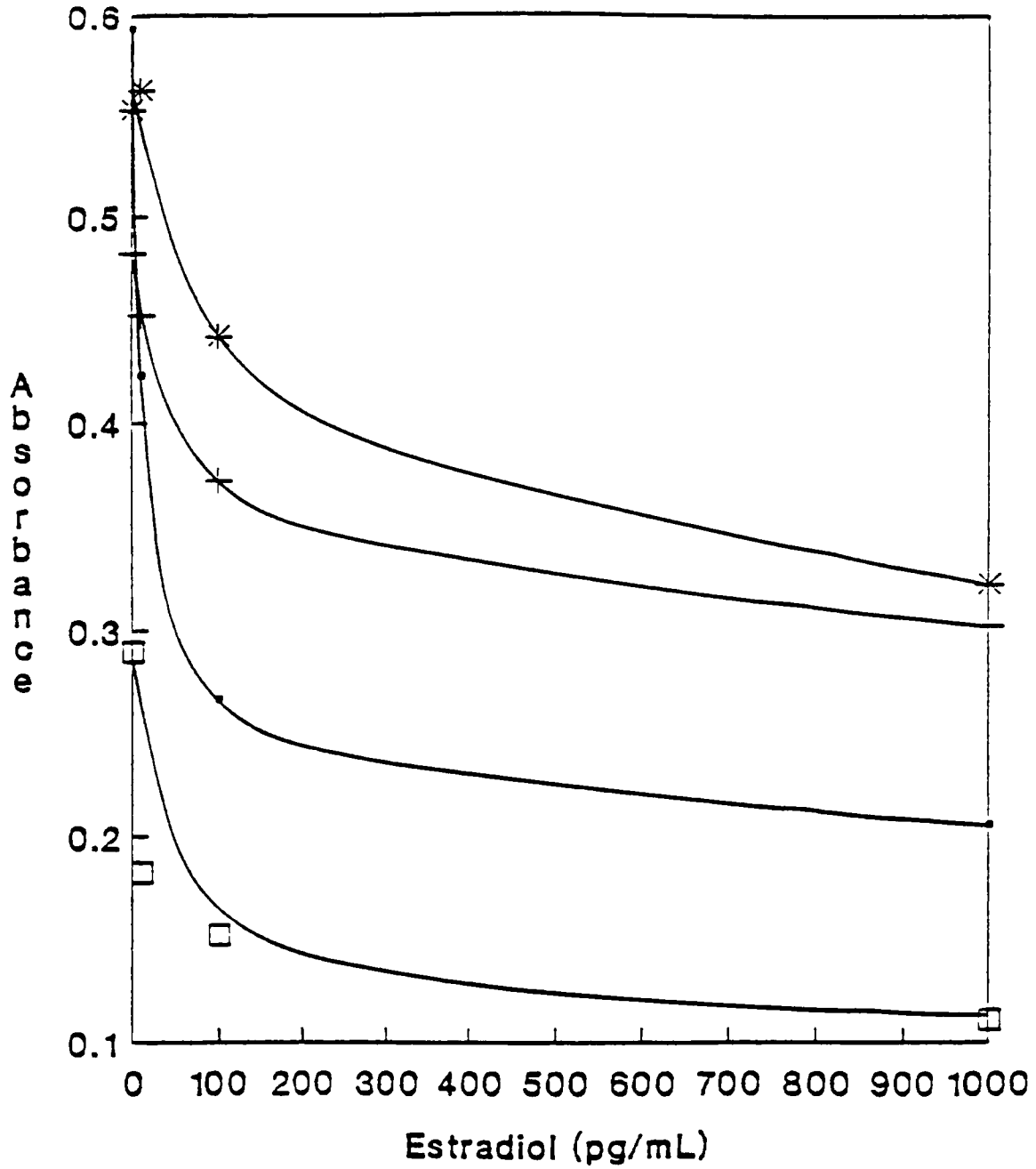
Four different buffer systems [0.1% Tween-20 in 100 mM sodium phosphate buffer, 0.1% Tween-20 in 5.0 mM sodium phosphate buffer, 0.05% Tween-20 in 5.0 mM sodium phosphate buffer, and 0.05 % Tween-20 in phosphate buffered saline (PBS)] were studied as possible washing solution for either estradiol or estriol assays. The study indicated that 0.1% Tween-20 in 100 mM sodium phosphate buffer, pH 7.4, gave the best results for both estradiol and estriol assays as illustrated in Figures 22 and 23.

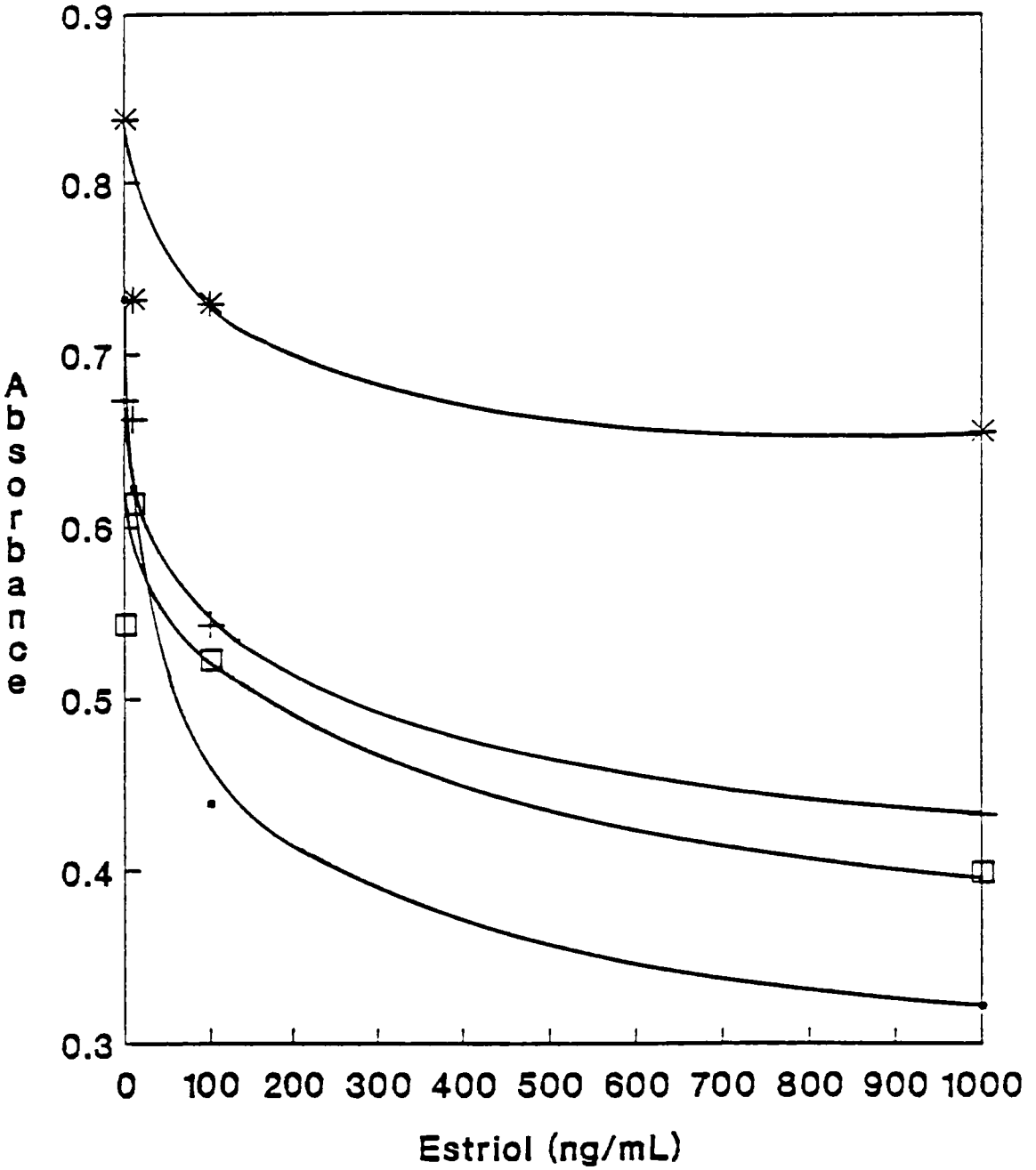
5. Standard curves for competitive enzyme immunoassays of estradiol and estriol

For estradiol assay, six concentrations of serum standards were prepared by adding stock estradiol solution to steroid-free serum to give final concentrations of 0, 10, 25, 100, 500, 1000, and 3000 pg/mL; these were used for established the standard curve. The results obtained from estradiol assay by the procedure described in the method


0.9
0.8
0.7
0.6
0.5
0.4
Absorbance
0
100
200
300
400
500
600
700
800
900
1000
Estradiol (pg/mL)


1
0.9
0.8
0.7
0.6
0.5
0.4
Absorbance
0
100
200
300
400
500
600
700
800
900
1000
Estriol (ng/mL)

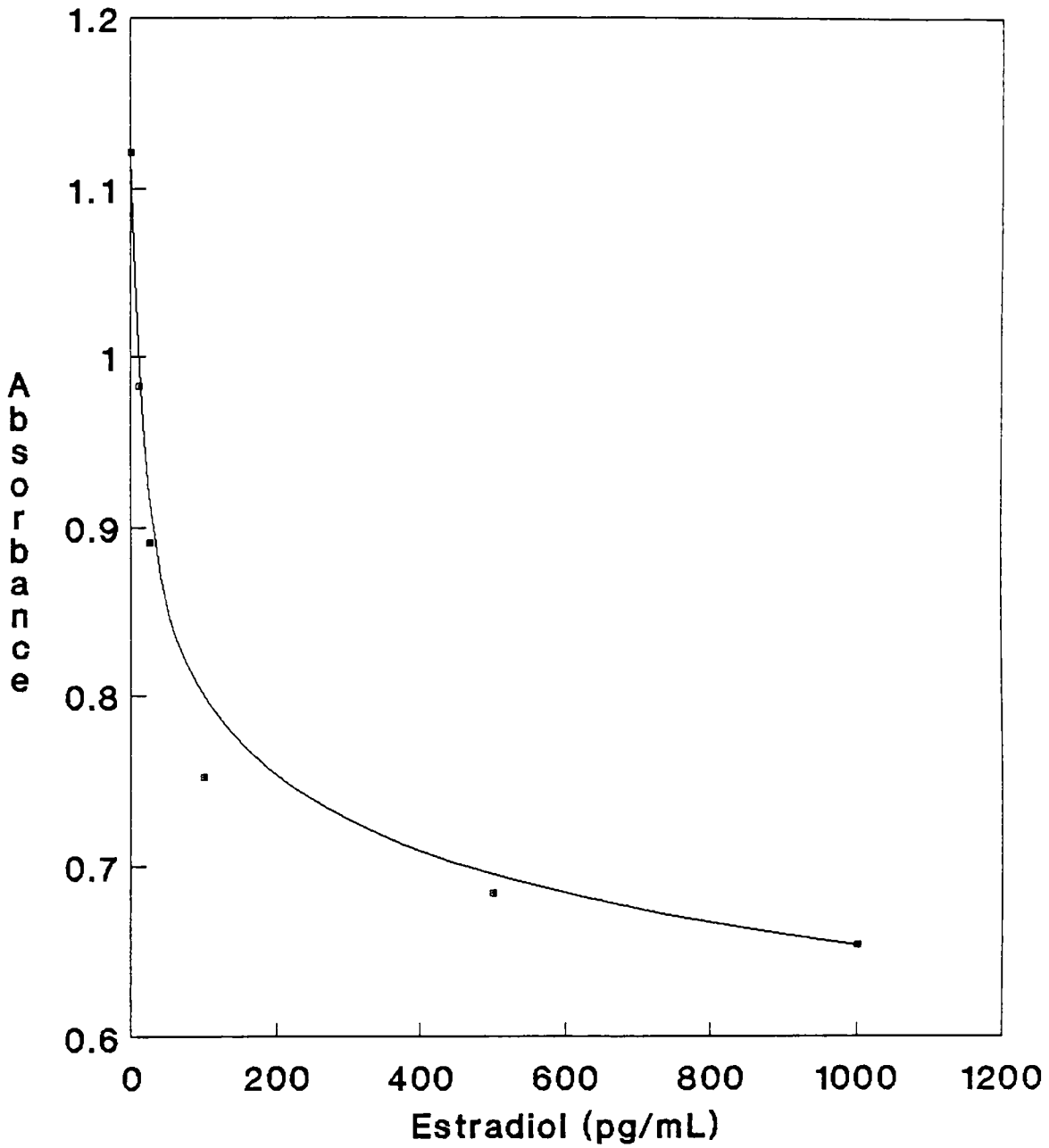

0.6
0.5
0.4
0.3
0.2
0.1
Absorbance
0
100
200
300
400
500
600
700
800
900
1000
Estradiol (pg/mL)

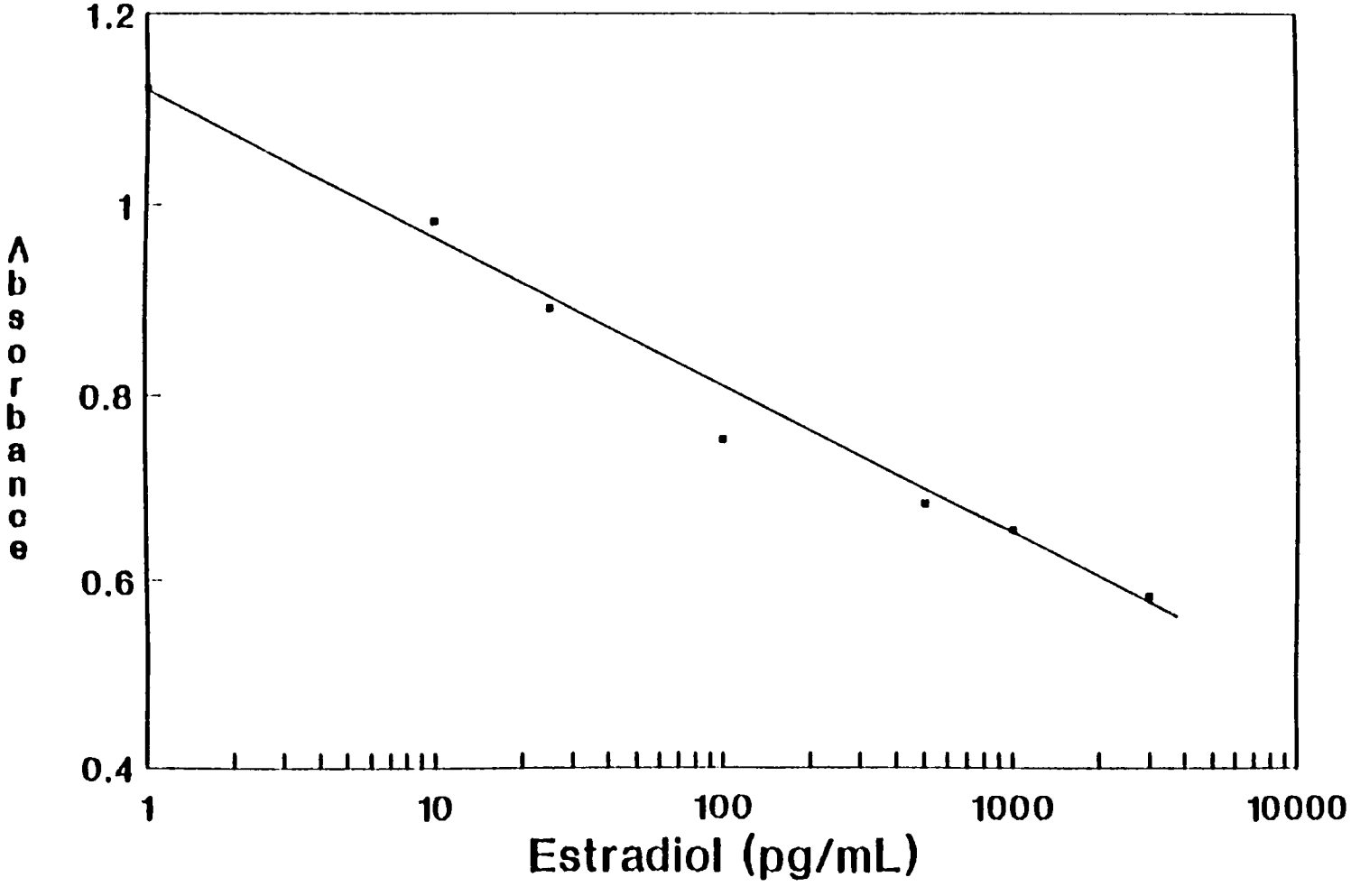

0.9
0.8
0.7
0.6
0.5
0.4
0.3
Absorbance
0
100
200
300
400
500
600
700
800
900
1000
Estriol (ng/mL)

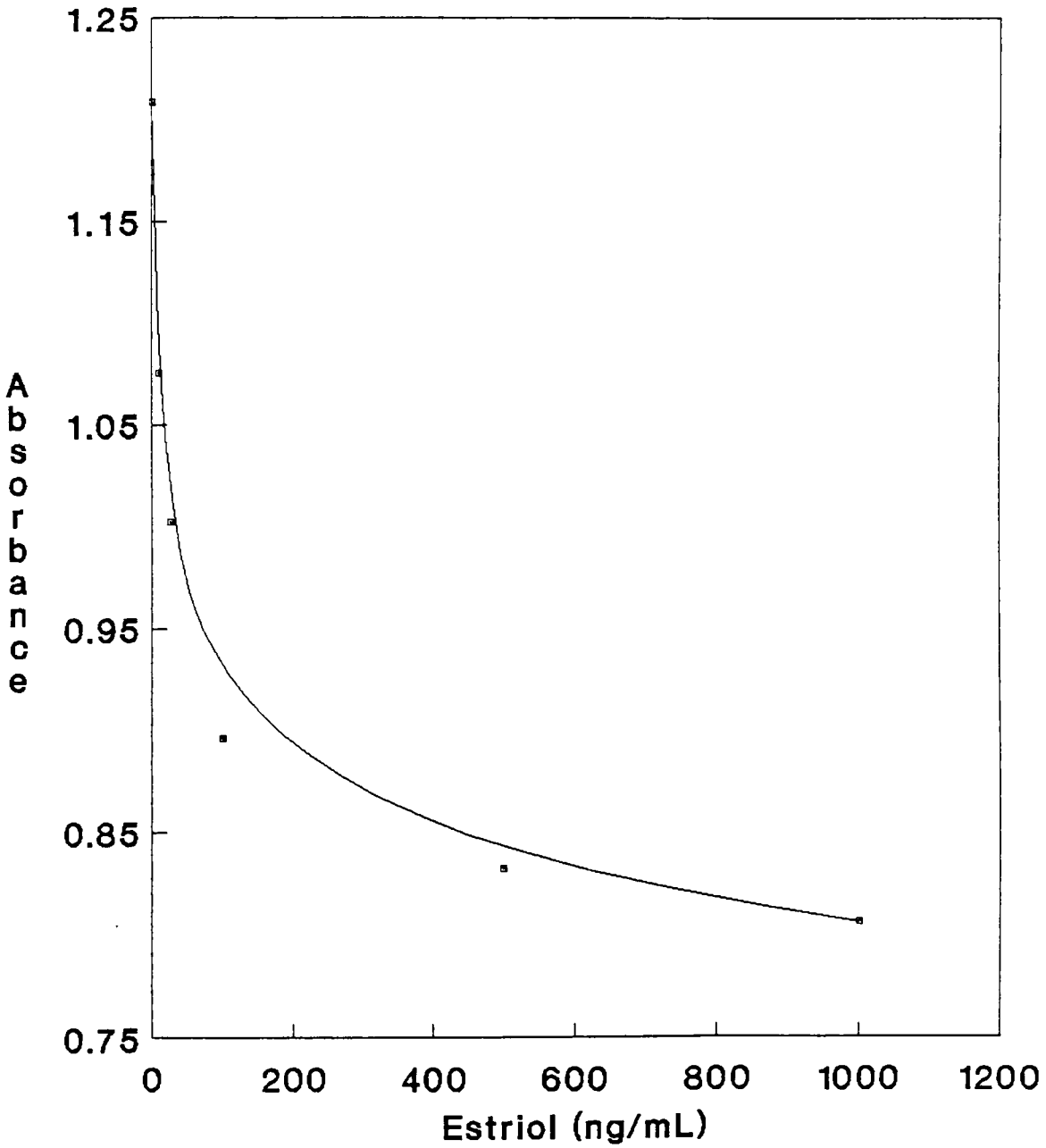
section were plotted as absorbance at 450 nm versus estradiol concentration (Figure 24). When the results were plotted in a semi-logarithmic scale of logarithm of estradiol concentration versus absorbance at 450 nm, a linear relationship was observed in the concentration range studied and is shown in Figure 25. For estriol assay, serum standards containing 0, 10, 25, 100, 500, and 1000 ng/mL of estriol were used for establishing the standard curve. The results obtained were treated in a similar fashion and are shown in Figure 26. When the results were plotted in a semi-logarithmic scale, again a linear relationship was observed in the concentration range studied as shown in Figure 27.

E. Study of cross reactivities

The following compounds were tested for possible cross-reaction with the anti-estradiol antibody used in the total estradiol assay: testosterone, 17-alpha-estradiol, and estriol. Less than 0.01% of cross reactivity was observed with testosterone at 3 ng/mL. The cross reactivities of 17-alpha-estradiol and estriol were 0.42% and 0.15% at 10 ng/mL (Table 1). Testosterone, 17-alpha-estradiol, and 17-beta-estradiol were tested for possible cross reaction with anti-estriol antibody used in the estriol assay. Less than 0.01% of cross reactivity was obtained with testosterone at 3 ug/mL. The cross reactivities of 17-alpha-estradiol and 17-beta-estradiol were 0.10% and 0.23% at 10 ug/mL (Table 2).


1.2
1.1
1
0.9
0.8
0.7
0.6
Absorbance
0
200
400
600
800
1000
1200
Estradiol (pg/mL)


1.2
1
0.8
0.6
0.4
Absorbance
1
10
100
1000
10000
Estradiol (pg/mL)


1.25
1.15
1.05
0.95
0.85
0.75
Absorbance
0
200
400
600
800
1000
1200
Estriol (ng/mL)

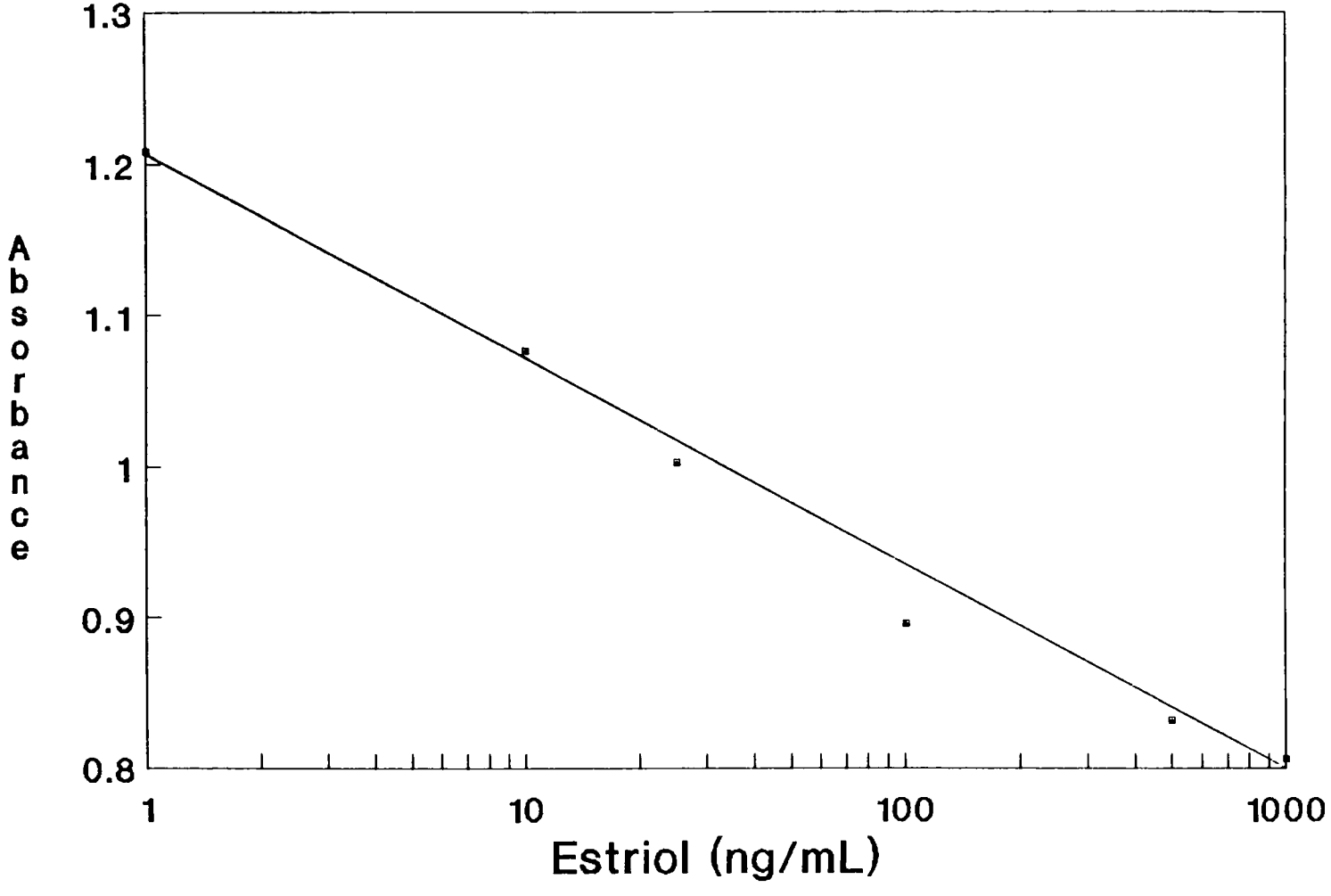

1.3
1.2
1.1
1
0.9
0.8
Absorbance
1
10
100
1000
Estriol (ng/mL)

Table 1. Specificity of anti-E_2 gamma globulin fraction

Compound	Concentration (pg/mL)	% Cross Reactivity Equivalent to E_2
17-alpha-Estradiol	10000	0.42%
Estriol	10000	0.15%
Testosterone	3000	<0.01%

Table 2. Specificity of anti-E_3 gamma globulin fraction

Compound	Concentration (ng/mL)	% Cross Reactivity Equivalent to E_3
17-alpha-Estradiol	10000	0.10%
17-beta-Estradiol	10000	0.23%
Testosterone	3000	<0.01%

F. Study of performance characteristics

1. Study of recovery

Two different levels of E_2 and E_3 sera were used for the analytical recovery studies. Experiments were performed by mixing E_2 solution with known concentration of E_2 standard serum. The total E_2 concentrations were measured and percentage of recovery determined. Recoveries of 103% and 94% for 20.0 pg/mL and 410 pg/mL were obtained (Table 3). The same experiment was performed for E_3 assay. Recoveries of 104% and 93% for 20.0 ng/mL and 410 ng/mL were obtained (Table 4).

2. Study of precision

Precision was performed by using three different concentrations of pooled sera. Intra-assay coefficients of variation were assessed by determining 21 replicates of the same sample in a single experiment. The data obtained were then subjected to statistical analysis for the calculation of the coefficients of variation. For intra-assay precision study of E_2, coefficients of variation of 15.0%, 8.83%, and 8.13% were obtained for 20.48 pg/mL, 79.14 pg/mL, and 424.6 pg/mL respectively. For E_3, coefficients of variation of 12.4%, 9.63%, and 9.08% were obtained at 49.19 ng/mL, 153.4 ng/mL, and 505.7 ng/mL respectively. Inter-assay coefficients of variation were performed by determining the sample on 15 consecutive days, and the data obtained were then subjected to statistical analysis for

Table 3. Recovery of total E_2 assay

Expected Value (pg/mL)	Observed Value (pg/mL)	Recovery %
20.00	20.60	103
410.0	385.4	94

Table 4. Recovery of total E_3 assay

Expected Value	Observed Value ng/mL	Recovery %
20.00	20.8	104
410.0	381.3	93

coefficient of variation. The results for E_2 assay were found to be 18.0%, 10.4%, and 8.73% at 20.57 pg/mL, 78.67 pg/mL, and 422.0 pg/mL. For E_3 assay, the results showed 15.1%, 9.08%, and 12.3%, and 9.84% at 47.76 ng/mL, 153.4 ng/mL, and 504.7 ng/mL. The results of the intra- and inter-assay precision studies are summarized in Tables 5 and 6.

3. Study of detection limit

The detection limit was determined by measuring 24 replicates of blank serum sample against a standard curve. The standard deviation of blank samples was calculated. Detection limit equals to the concentration determined by mean absorbance minus 2.6 times the standard deviation as described by international federation of clinical chemistry (27). The detection limits thus determined were 2.0 pg/mL for E_2 assay and 6.0 ng/mL for E_3 assay.

G. Comparison study

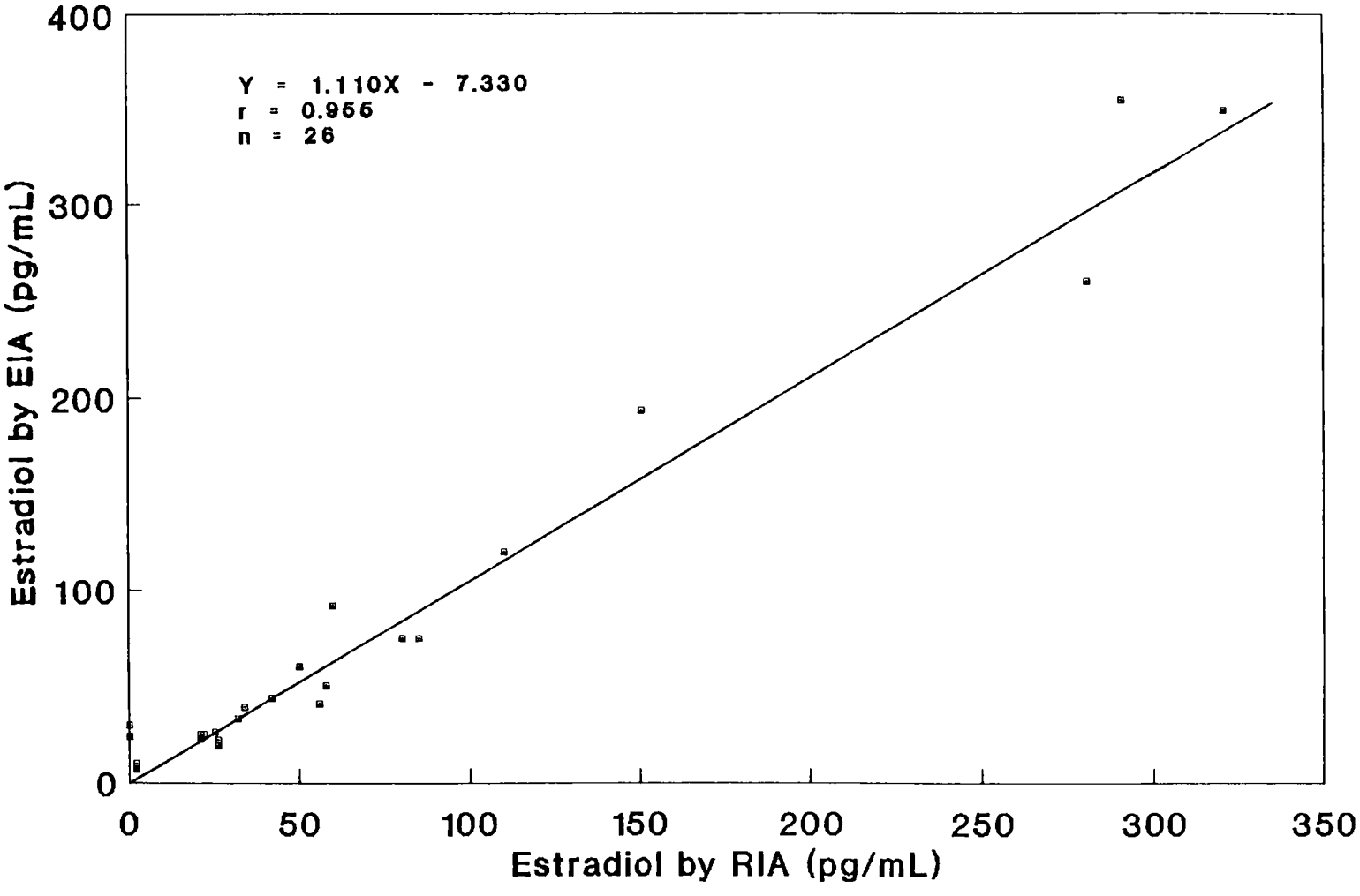
To establish the validity of the developed enzyme immunoassays, comparison study between the developed enzyme immunoassay and a commercially available radioimmunoassay was carried out. Serum samples obtained from hospital laboratories were subjected to EIA and RIA for the determinations of both total E_2 and E_3 concentrations. The serum total E_2 concentrations obtained by EIA method were plotted against those obtained by RIA method and are shown in Figure 28. These data were also subjected to statistical

Table 5. Precision of total E_2 assay

	Mean pg/mL	S.D. pg/mL	CV %
Intra-assay (n=21)			
Sample A	20.48	3.07	15.0
Sample B	79.14	6.99	8.83
Sample C	423.6	34.4	8.13
Inter-assay (n=15)			
Sample D (triplicate)	20.57	3.71	18.0
Sample E (triplicate)	78.67	8.18	10.4
Sample F (triplicate)	422.0	36.8	8.73

Table 6. Precision of total E_3 assay

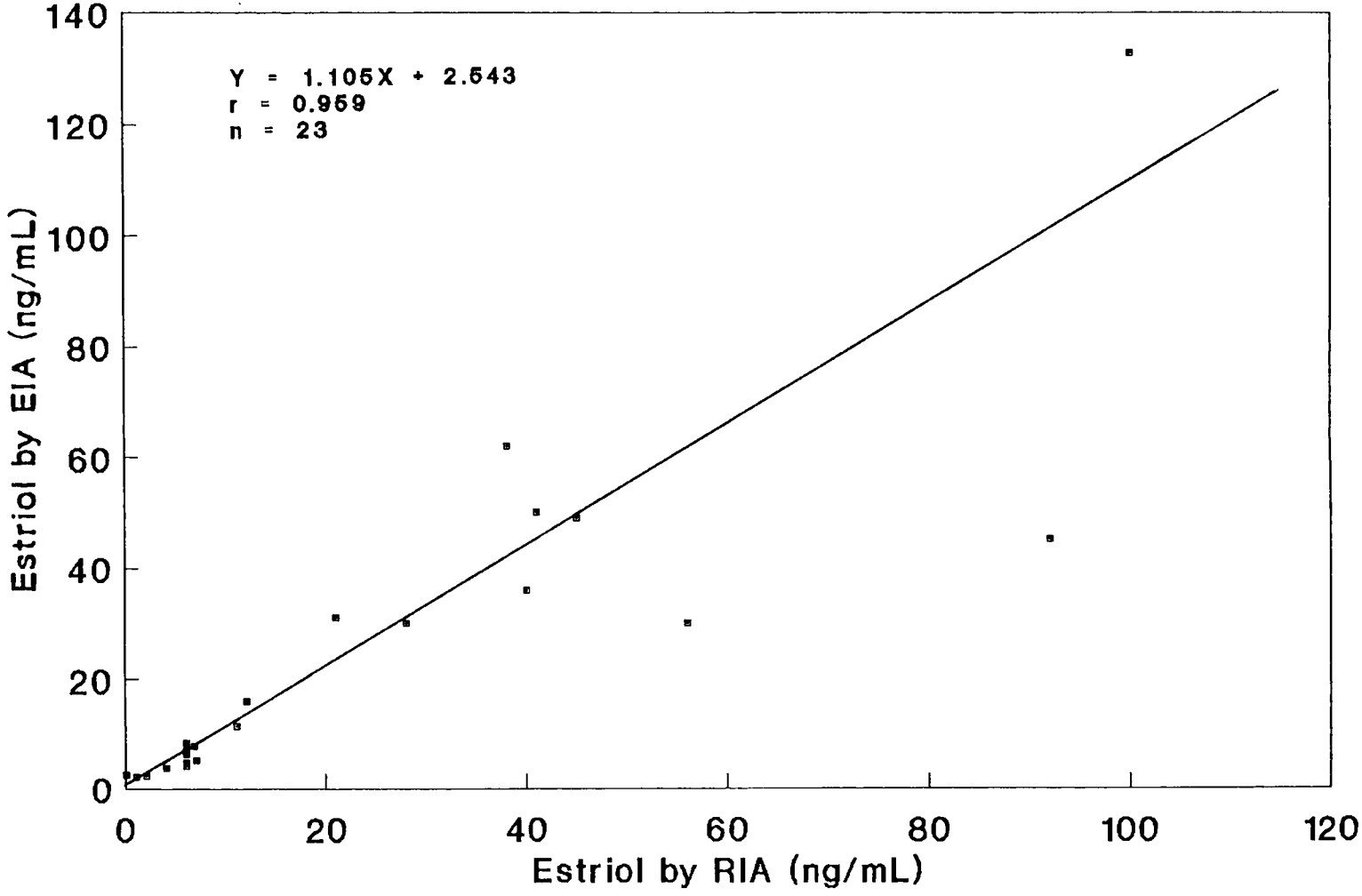
	Mean ng/mL	S.D. ng/mL	CV %
Intra-assay (n=21)			
Sample A	49.19	6.084	12.4
Sample B	153.4	14.78	9.63
Sample C	505.6	45.91	9.08
Inter-assay (n=15)			
Sample C (triplicate)	47.76	7.191	15.1
Sample D (triplicate)	153.4	18.91	12.3
Sample E (triplicate)	504.7	49.66	9.84


Y = 1.110X - 7.330
r = 0.956
n = 26
Estradiol by EIA (pg/mL)
400
300
200
100
0
0
50
100
150
200
250
300
350
Estradiol by RIA (pg/mL)

analysis by linear regression, and the results obtained were listed in Table 7. Similarly, the data obtained from the determination of total serum E_3 were plotted in Figure 29 and the statistical analysis were listed in Table 7.

Table 7. Statistical data obtained for comparison of E_2 and E_3

E_2:	
Intercept:	7.330
Slope:	1.110
Correlation Coefficient:	0.959
E_3:	
Intercept:	-2.544
Slope:	1.105
Correlation Coefficient:	0.959


Y = 1.105X + 2.543
r = 0.959
n = 23
Estriol by EIA (ng/mL)
Estriol by RIA (ng/mL)
0
20
40
60
80
100
120
140

Chapter IV

DISCUSSIONS

The adsorption of bio-molecules to a polystyrene surface is due to intermolecular attraction forces (Van der waals forces), to be distinguished from "true" chemical bonds, i.e. covalent bonds (through electron sharing) and ionic bonds (through stoichiometric charges of opposite signs). Intermolecular attraction forces are based on intramolecular electric polarities of which two types can be distinguished: alternating polarities (AP) and stationary polarities (SP), i.e. dipoles. AP arises when molecules approach each other, thereby creating disturbances in each other's electron clouds. This causes synchronously alternating polarities in the molecules, which may establish a bond between them. AP mediated binding is a common substance property, which is obviously stronger the larger the molecules. In addition to the AP attraction forces, molecules may possess SP through which they can bind to each other simply by bedding dipole against dipole. Compared with SP, AP attraction is inversely proportional to the seventh power of the distance, whereas SP attraction is inversely proportional to only the second power of the

distance (28, 29). Hence, the former has a much shorter range than the latter. In general, van der Waals mediated bonds are about 100 times weaker than ionic and covalent bonds. However, among SP mediated bonds, the hydrogen bond takes up an exceptional position because it is up to 10 times stronger than the others and because of its crucial importance for the properties of water and for the specific behaviors of bio-molecules. Hydrogen bonds may be called hydrophilic bonds, as opposed to AP mediated bonds which are called hydrophobic bonds. The AP mediated attraction is also called hydrophobic interaction.

Polystyrene is a common solid-phase support in ELISA as well as in many radioimmunoassays. In 1967, Catt and Tregear reported the adsorption of antibody to polymeric surfaces and developed a new method of solid-phase radioimmunoassay (30). Cantarero reported that the amount of protein binding varied for different proteins at a given input concentration and under constant conditions of time and temperature (31). The maximum proportion of proteins adsorbed did not correlate with the net charge of the protein at the adsorption pH. It was suggested that the adsorption of proteins to a surface such as polystyrene occurred through hydrophobic bonds and that charge played a minor role. A low concentration of antigen may not be detected if the concentration of antibody used for coating the microwells is either too high or too low. It is

important, therefore, to find the optimal antibody concentration for microwell coating (32). The optimal concentrations of purified gamma globulins from anti-estradiol and anti-estriol sera for the microwell coating (Immulon II) were found to be 500 ng/mL and 18 ug/mL, respectively (Figures 11 and 12).

The lability of the protein structure makes it imperative that the pH be kept within certain limits and that the denaturing effects be avoided. Any analytical method capable of detecting one-hundredth to one-thousandth of the concentration initially applied to the column may be used for the monitoring of the proteins in the eluate, thus the monitoring of the absorbance at 280 nm was routinely used (33).

The basis of ion exchange chromatography is the electrostatic attraction between oppositely charged ions, one of which is an electrolyte and the other a synthetic resin polymer. DEAE-cellulose, a weakly basic anion exchange polysaccharide backbone, contains diethylaminoethyl positively charged functional groups associated with a small mobile anion counter-ions. The counter-ion can be exchanged reversibly with other ions of the same charge, such as negatively charged protein molecules, without physically changing the matrix. The electrostatic interactions taking place between the DEAE group of the resin and the protein are equilibrium processes involving diffusion of the charged

protein to the resin surface and then to the charged exchange site. Finally, diffusion away from the exchange resin takes place upon elution with an appropriate buffer system. The rate of movement of a given ion down the column is a function of its ionizibility, the ionic strength and the concentration of counter ions in the elution buffer, and their relative affinity for charged site on the resins. By adjusting the pH and the ionic strength of the elution buffer, the protein ions held by electrostatic attraction on the resin are eluted differentially to yield the desired separation.

Serum proteins are separated electrophoretically into five different fractions. Albumin having the greatest negatively charged surface migrates most rapidly toward anode; whereas, gamma globulin fraction migrates the least. It was reported that the gamma globulin fraction could be obtained by using DEAE-cellulose with 0.01 M sodium phosphate buffer (pH 7.5) containing 0.015 M sodium chloride as the elution buffer system (23). However, a procedure of simpler handling and better yield was obtained with the use of 5.0 mM sodium phosphate buffer, pH 6.5 as the buffer for the elution of gamma globulin fraction from DEAE-cellulose column. By selecting a column buffer with proper pH and ionic strength, the majority of the serum proteins from the whole anti-serum are bound to the DEAE-cellulose exchange column with the desired gamma-globulin fraction eluted

straight from the column. For the chromatographic separation of serum proteins on DEAE-cellulose it has been found best to elute with 5.0 mM sodium phosphate buffer, pH 6.5 (34).

After concentration, the eluent fraction was easily detected by electrophoresis, and the yield was calculated as percent of gamma globulin fraction present in the sample. Only 5% of the whole anti-estradiol was found to be gamma globulin, and 78% of gamma globulin fraction was found in the DEAE-cellulose purified fraction. Similarly, for estriol, 20% of the whole anti-estriol was found to be gamma globulin, and 87% of gamma globulin fraction was in the DEAE-cellulose purified fraction.

It was in 1972 that Van Weemen and Schuurs first introduced the immunoenzymatic method for the measurement of estrogens (35, 36, 37). Since then, many researchers have perfected the enzyme immunoassays of steroids using various enzymes as labeling agents. All assays are based on the competition between free steroid and enzyme labeled steroid for a limited number of antibody binding sites. The performance of a new method, such as enzyme immunoassay for estrogen, has to be compared to those of other methods or reference method and requires at least the same sensitivity, specificity and practicability. To obtain these features depends first on the choice of the enzyme used for coupling. Secondly, it depends on the choice of the coupling reaction,

i.e. what steroid derivative should be used for the enzyme coupling and what are the best reaction conditions for preparing a labeled antigen that is still immunoreactive (35, 37). In the present study, the estrogen derivatives, E_2-3-HS and E_3-3-HS were prepared via succinic anhydride reaction by which the 3-hydroxyl group forms an ester linkage with the succinate derivative and subsequently forms an amide bond with 1,6-hexanediamine to form E_2-3-succinyl-6-aminohexamine (E_2-3S-6AHA) and E_3-3-succinyl-6-aminohexamine (E_3-3S-6AHA) respectively. The products, E_2-3S-6AHA and E_3-3S-6AHA, were then used for coupling to horseradish peroxidase via carbodiimide reaction for the formation of amide linkage between the amino group of the steroid derivative and a carboxyl group of the enzyme (38).

The results of comparison of adsorption capacity among Immulon I, Immulon II, and Immulon IV show that immulon II has the best adsorption capacity for gamma globulin. These observations are consistent with the manufacturer's report in which Immulon I was suggested for adsorption of antibodies and larger molecular weight compounds. Immulon I was said to allow for less non-specific binding and therefore provide a relatively low background. Thus, Immulon I is utilized widely for sandwich ELISA assay for which low background is critical in measuring minute amounts of analyte. Immulon II was designed, as the manufacturer reported, to enhance protein uptake and is generally

suggested for antigens or proteins in the range of 10,000 to 180,000 molecular weight. Immulon IV was reported to be the improved version of Immulon I.

In the selection of incubation buffer, all buffers contain sodium phosphate. The reason is to mimic physiological condition. The molarity may be a factor to effect the incubation and washing.

In men, the reference range for total serum estradiol is reported to be 8-36 pg/mL. However, in women, three reference ranges for total serum estradiol through the menstrual cycle were established; they are: follicular phase, 10-90 pg/mL; midcycle, 100-500 pg/mL; luteal phase, 50-240 pg/mL. The concentration range of estradiol chosen for establishing a standard curve is 0, 20, 50, 100, 200, 500, 1000, and 3000 pg/mL.

In men and nonpregnant women, the reference for total serum estriol is less than 2 ng/mL. The serum concentrations of total serum estriol through pregnancy are: 24-28 weeks, 30-170 ng/mL; 28-32 weeks, 40-220 ng/mL; 32-36 weeks, 60-280 ng/mL; 36-40 weeks, 80-350 ng/mL. The concentrations of estriol chosen for making a standard curve are 0, 20, 100, 200, 500, and 1000 ng/mL. The definition of sensitivity in international federation of clinical chemistry is "The ability of an analytical method to detect small quantities of the component. It has no numerical value. The corresponding measured quantity is the detection

limit. Detection limit is the smallest single result which, with a stated probability, can be distinguished from a suitable blank. The limit may be a concentration or an amount and defines the point at which the analysis becomes just feasible" (39). It is necessary to determine the detection limit which will serve three purposes: (a) To characterize the detectability of the method as compared with other methods for the same analyte; (b) to avoid frequent misuse of the method in attempts to measure values near or lower than the detection limit; and (c) to recognize those results which should be reported as "lower than detection limit" rather than as numerical values. Here the detection limit is an insurmountable barrier; whereas, values above the working range can usually be determined by using diluted specimens or smaller sample volumes. The determination of detection limits is to select suitable blank samples and determine the mean and S.D. of apparent blank-sample results. The detection limit would be approximately equal to mean blank result + 2.6 S.D. More precise or rigorous calculations are rarely necessary if it is remembered that the detection limit should be used as a warning sign (KEEP OFF) rather than a justification for continually using a method at the lower levels than it may be able to measure.

Most serum estradiol methods measure both free and bound fractions of the unconjugated steroid. The RIA method

of Tulchinsky and Abraham for the measurement of unconjugated estriol requires the extraction of the serum with diethyl ether (40). Although double isotope-derivative methods (41) or gas chromatography with electron capture detection (42) are sufficiently specific and sensitive, these methods find limited utility in the clinical laboratory. Radioimmunoassay is widely used but still has the problems of isotope hazard, waste disposal and expensive instrument requirement and is limited to large hospital laboratories or central reference laboratories.

Both serum total E_2 and E_3 values, obtained from the current competitive EIA methods, were compared with determinations on identical specimens by a commercially available RIA method (Diagnostic Products Coat-A-Count estradiol and estriol). For total E_2 assay, on 26 specimens, the result is about 10% higher than RIA methods. The reasons are: (a) Most E_2 concentrations of being chosen samples are at lower range; and (b) the sample number is not enough. However, the agreement was excellent with a correlation coefficient of 0.955. Likewise, for total E_3 assay, on 23 specimens, excellent agreement was also obtained with a correlation coefficient of 0.959. In EIA method, intra-assay coefficients of variation of 15.0%, 8.83%, and 8.13% were obtained for E_2 at 20.48 pg/mL, 79.14 pg/mL, and 424.6 pg/mL, and of 12.4%, 9.63%, and 9.08% for E_3 at 49.19 ng/mL, 153.4 ng/mL, and 505.7 ng/mL. Inter

assay coefficients of variation of 18.04%, 10.40%, and 8.70% were obtained for E_2 at 20.57 pg/mL, 78.67 pg/mL, and 422.0 pg/mL, and of 15.06%, 12.33%, and 9.84% for E_3 at 47.76 ng/mL, 153.4 ng/mL, and 504.7 ng/mL. In RIA method, intra-assay coefficients of variation is from 18.3 to 5.6 for E_2 assay and from 12.2 to 8.2 for E_3 assay. Inter-assay coefficients of variation is from 20.0 to 7.3 for E_2 assay and from 16.3 to 8.4 for E_3 assay (11, 43, 44, 45, 46).

The EIA method has the following advantages: longer shelf life; no isotopic hazard; no requirement of a major instrument. The competition enzyme immunoassay procedure for E_2 and E_3 proposed here offers several advantages over the existing RIA. It is simple and quick, and it can be performed at room temperature in just 2 hours. A low-cost microplate reader can be used instead of a more expensive gamma counter which is costlier to the laboratory. The EIA method involves no radioactive hazardous material, requires no elaborate training and is easier for technologists to handle.

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