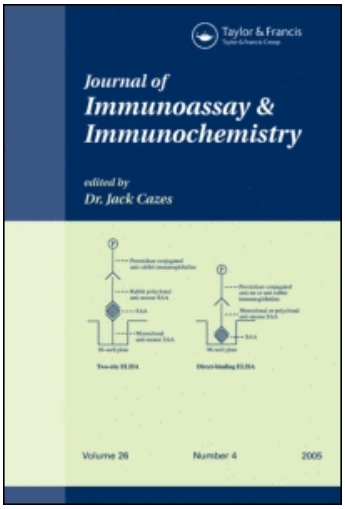

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Sensitive ELISA for Determination of Serum E_2 Using a New Tracer E_2-Biotin

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ABSTRACT

A new tracer conjugate of E_2-Biotin, with different spacers, was synthesized at position 3 in the estradiol molecule for first time. Immunoreactivity of the tracer was determined by reacting with the anti-E_2 monoclonal antibody. The monoclonal antibodies raised against E_2 were characterized for its use in ELISA detection systems of serum E_2. The purified antibody has a high affinity and specificity for E_2. The antibody and tracer were used for establishing a competitive ELISA for estradiol (E_2). The experimental results showed that the dose-response curve of the assay covered a range

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of 33–20,000 pg/mL ($n = 8$). The detection limit is 28.3 pg/mL ($S/N = 3$). The intra- and inter-assay coefficients of variation for the assay of serum samples ranged from 5.7 to 13.2% and from 5.3 to 10.6%, respectively. Precoated microtiter plates were dried at 4°C and they were stable for up to 3 months.

Key Words: Estradiol; EIA; HRP; Serum; E_2-Biotin; Avidin; Serum E_2.

INTRODUCTION

The assay of E_2 in serum or plasma has great value for clinical endocrinological investigation in women. For research and treatments for diseases such as hormone-dependent cancer,[1] osteoporosis,[2] and cardiovascular disease,[3] the long-term accurate clinical monitoring of E_2 in the circulation is required. For determining functional infertility[4] and predicting ovulation and hormone-dependent disease,[5] it is also necessary to apply short-term intensive monitoring of E_2. The serum E_2 levels fluctuate over a range of approximately 40–270 pg/mL during the menstrual cycle in premenopausal women, and decreases to approximately 4–14 pg/mL in postmenopausal women. Therefore, developing an accurate assay for E_2 is very essential for health care in women.

In clinical studies, the most common methods for determining E_2 in serum are radioimmunoassays (RIA), with extraction of E_2 from complex sample matrices by an organic solvent, but this procedure will produce organic and radioactive waste, which will have a negative impact on the environment. For the replacement of RIA, direct methods, i.e., without extraction of E_2, have been described in several papers. Direct enzyme immunoassays (EIA) for E_2 in plasma or serum have been developed for decades.[6–13] Different methods have been tested as using conjugates of E_2 with penicilinase[7] or with horseradish peroxidase (HRP),[8,12,13] or with alkaline phosphatase.[9] Other researches focused on using conjugates of E_2 with Biotin[6,10] at position 6, and Biotin-Avidin/Streptavidin amplification system. In the case of assay with enzyme-labeled E_2, the small efficient working ranges always could not cover the relatively broad range of E_2 concentrations in serum. In the case of assay with Biotin-labeled E_2 at position 6, measuring the broad range of E_2 concentrations had been achieved and characterized. However, the synthesis of the conjugate E_2-Biotin at position 6 is very hard to carry out. Now, we designed and synthesized a new tracer E_2-Biotin at position 3 using a simpler and shorter procedure. Although E_2-protein conjugates

at position 6 or 7 can keep more immunogenic information of E_2, recent research works on the interaction between Biotinylated E_2 and the paratope of anti-E_2 antibody have demonstrated that the highest immunoreactivity of anti-E_2 is not necessary for the specific modified E_2 that was used as a protein-coupled hapten for immunization. Therefore, the new method using E_2-Biotin at position 3 and 3-specific anti-E_2 antibody would be easier to achieve and have a broader application.

It is our aim to develop a simpler EIA for determining serum E_2 accurately. For this purpose, a highly specific monoclonal antibody against E_2 ($McAbE_2$) was raised by 3-specific complete antigen, and a new tracer conjugate of E_2-Biotin at position 3 with different spacer was prepared. The simpler assay was established with a Biotin-Avidin amplification system for enhancing the sensitivity.

EXPERIMENTAL

Apparatus

The experiments were performed using an automatic enzyme-kinetic analytical system from Tecan Instruments (Austria). Absorbance and reference wavelengths were set at 450 nm and 492 nm, respectively.

Materials and Reagents

96-Well microtiter plates were purchased from Tianjin plastic Instrumental Company (Tianjin, China). Bovine serum albumin (BSA, Mr 68000), estradiol, estrone, estriol, di-*tert*-butyldicarbonate, 1,2-diaminoethane, 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, *iso*-butylchloroformate, biotin, trifluoroacetic acid, and 3,3′,5,5′-tretramethylbenzidine (TMB) were obtained from Sigma (St. Louis, MO, USA). Avidin-horseradish peroxidase (Avidin-HRP) and the second antibody (sheep anti-mouse immunoglobins), were purchased from Huamei Chemicals (Beijing, China). Estradiol-3-carboxymethyl ether (E_2-3-CME) was prepared by using E_2 and bromoacetic acid according to Dhar's method.[17] Biotinylation reagents were synthesized by acylation of diamines according to a previously described procedure.[15] Other reagents were of analytical grade from China; deionized water was used throughout.

Buffers

The coating buffer consisted of sodium carbonate (60 mmol/L, pH 9.6). The phosphorate buffer saline (PBS) consisted of 8.0 g NaCl, 2.9 g Na_2HPO_4, $12H_2O$, 0.2 g KH_2PO_4, 0.2 g KCl solved in 1 L deionzed water (pH 7.4). The blocking buffer was made of 0.8 g glutin in 100 mL PBS. The washing solution consisted of 500 μL Tween 20 in 1 L PBS. The Enzyme buffer was PBS containing 4% calf serum. The substrate buffer was 0.1 mol/L (pH 6.0) phosphorate buffer (PB).

Preparation and Characterization of Antibody

With our previous works on preparation of Anti-E_2 monoclonal antibody ($McAbE_2$), ascites fluid was produced by injecting the positive hybridoma cells into the BALB/c mice for 2 weeks or more, and purified by a modified *n*-caprylic acid-saturated ammonium sulfate method. The purified $McAbE_2$ with internally labeled proteins run on the SDS-PAGE CE gave an indication that the molecular weight of $McAbE_2$ was about 1.64×10^5. The purity of $McAbE_2$ shown by the two bands on the SDS-PAGE gel was essentially pure ($> 95\%$) and good enough for the immunoassay. The affinity constant K_{aff}, determined by Beatty's method,[18] was 1.5×10^9 L/mol.

The second antibody was also purified by a modified *n*-caprylic acid-saturated ammonium sulfate method.

Preparation of Conjugates of E_2-Biotin

The conjugates of E_2-Biotin, with various spacers, were synthesized for analyzing the relationship of the affinity and the structural demand on the spacer group. A typical procedure was briefly introduced as follows. The conjugation of E_2-3-CME to 3-biotinylaminopropylammonium trifluoroacetate was carried out by a mixed anhydride method of Boudi and Fiet,[16] with modification as follows: 0.36 g of E_2-3-CME (1.1 mmol) and 0.2 mL of triethylamine was dissolved in 3.0 mL dioxane, and 0.14 mL of *iso*-butylchloroformate was added slowly with stirring. Stirring was continued at 5°C for 30 min and, into this solution, a solution consisting of 0.37 g of 3-biotinylaminopropylammonium trifluoroacetate (1.0 mmol) and 0.3 mL triethylamine in 1.0 mL DMSO was added. After this, the mixture was stirred slowly at room temperature for 4 h and then was dropped in 15 mL water (4°C). The precipitated solid

was washed with cold water and dried in vacuo, and the conjugate of E_2-Biotin was purified by column chromatography using CH_2Cl_2-MeOH (9:1, v/v) as eluent. The characteristic ^{1}H-NMR (DMSO-d_6) data are as follows: δ 0.662 (s, 3H, $\underline{CH_3}$ in E_2), 4.122 and 4.133 (2 m, 2H, CO-$\underline{NH}$ in Biotin), 4.29 (s, 2H, CO-$C\underline{H}_2$-O in E_2-Biotin).

Characterization: Dilution Test for Affinity Constant

This test was used to determine the affinity of the $McAbE_2$ against the biotinylated E_2 derivatives with different spacers. After binding of the respective E_2-Biotins to the Avidin-coated microtiter plates, the $McAbE_2$ was incubated first with the antigens and then with a goat anti-mouse IgG-HRP conjugate. Subsequently, the signal generation was carried out by addition of TMB. The measured OD_{450} values were corrected for the blanks (same components, but without E_2-Biotin) and related as percentage to the value of the position equivalent antibody. Dilution test of $McAbE_2$ was performed in concentrations of 20, 40, 80, 160, and 320 ng/mL of E_2-Biotins. The typical procedure was described as follows: First, 150 µL of respective E_2-Biotins, in a final concentration of 320 ng/mL in assay buffer, was added to the wells. After 30 min of incubation at 37°C, the wells were washed three times with washing buffer. Then, 150 µL of 2.5–50 µg/mL $McAbE_2$ solution was pipetted into the coated wells. After one hour of incubation at 37°C, the wells were washed three times. After that, 150 µL of an anti-mouse IgG-HRP solution (1:200 diluted with assay buffer) was added and incubated again for one hour at 37°C and the wells were washed three times with washing buffer and two times with deionized water. Finally, 150 µL of substrate solution (1.5 µL 30% H_2O_2 and 10 µL 6 mg/mL TMB in 1 mL PB) was added into the wells and the enzyme reaction was allowed to develop in the darkness for 15 min at room temperature. The reaction was stopped by adding 50 µL of 2 mol/L H_2SO_4.

Samples and Standards

In this assay, the different concentrations of estrogen-free human serum, obtained from healthy volunteers, were used as a matrix for standards and samples to eliminate the possible interferences from the serum. The standards (20–20,000 pg/mL) and samples were prepared in this matrix and stored at 4°C before use.

Immunoassay for E_2

First, 150 μL of McAbE_2 solutions (1500-fold diluted with PBS) were added to each well, which were coated by goat anti-mouse IgG. After incubation for 1 h at 37°C, the wells were washed three times with washing buffer. The second step, the standards or unknown samples (100 μL) diluted with PBS (1:1) were added to the wells and the plates were incubated for 30 min at 37°C. The third step, 50 μL of E_2-Biotin conjugate solution (2 ng/mL in PBS) was added and the plates were further incubated for 1 h at 37°C. The competitive immunoreactions were stopped by washing the plates three times. The fourth step, 150 μL of Avidin-HRP (200-fold diluted with enzyme buffer) was added and incubated for 30 min. After this, the plates were washed three times by washing buffer and two times by deionized water. The final step, 150 μL of substrate solution (1.5 μL 30% H_2O_2 and 10 μL 6 mg/mL TMB in 1 mL PB) was added to the wells and the enzyme reaction was allowed to develop in the darkness for 15 min at room temperature. The reaction was stopped by adding 50 μL of 2 mol/L H_2SO_4.

Spectrometric Detection of Enzyme Activity

The plates with enzyme reaction solution were put into the detection chamber of the automatic microplate reader and shaken for 15 s before measurement. The absorbance and reference were measured at 450 nm and 492 nm, respectively.

Data Analysis

The affinity constant (K_{aff}) was calculated with data from the dilution test by Betty's method. The standard curves were constructed by plotting B/B_0 vs. log concentration of the standards with a four-parameter model.

RESULTS AND DISCUSSION

Design and Synthesis of New Tracer and Complete Antigens

Regarding the molecular interaction between the epitope represented by the steroidal E_2 molecule and the paratope of a specific mono- or

poly-clonal anti-E_2 antibody, a main aspect is the *specificity-to-structure* relationship. For different goals, the relative complete antigens were synthesized for producing different kinds of specific antibodies. For increasing the specificity of complete antigens in the chemical structure, the better way for the synthesis of complete antigen was derivatization and binding protein of E_2 at position 3 used in this article. In synthesizing the tracers used in immunoassay for E_2, one critical point is to keep the antigenicity of E_2 (the relativity between complete antigen and its tracers was restrictively required), so the tracers would be derivatized and synthesized at position 3; another important thing is the chemical structure of the spacer group linking the Biotin residue to the E_2 ring system. Therefore, in this work, tracers with different chain-lengths were synthesized for minimizing steric hindrances between the Biotin moiety and the antibody. The structures of the tracers are shown in Fig. 1.

ELISA Dilution Test

The results of the ELISA dilution test for K_{aff} are listed in Table 1. The smallest K_{aff} was found in tracer 1, due to its smallest spacer group. K_{aff} for tracer 2–4 was almost identical, being nearly 8.5×10^8, but smaller than E_2-OVA and E_2-BSA's. This phenomenon was due to the different binding format between tracers and complete antigens with McAbE_2. According to the previous work by G. Giraudi, large molecules such as complete antigens or conjugates of haptens with carrier proteins or enzymes can bind to different sites at same time, so that it is more strongly bound than the small molecules, such as haptens or hapten

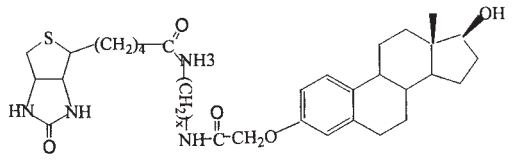

Figure 1. The structure of tracer 1, 2, 3, 4 ($x = 2, 3, 4, 5$).

Table 1.

No.	Tracer 1	Tracer 2	Tracer 3	Tracer 4	E_2-BSA	E_2-OVA
K_{aff} (mol/L)	7.9×10^7	8.5×10^8	8.6×10^8	8.4×10^8	1.5×10^9	1.5×10^9

derivatives, to the antibodies. Therefore, the affinity of conjugates of E_2 with carrier proteins becomes stronger than biotinylation E_2 derivatives. But the affinity constant of tracer 2–4 was about 8.5×10^8, also good enough for immunoassay application. The tracer 2 was used for establishing an assay for measurement of serum E_2.

Optimization of Concentrations of McAbE_2 and E_2-Biotin Conjugate

Figure 2 shows that the optical concentrations of McAbE_2 and E_2-Biotin for this assay were 2.5 μg/mL and 1.0 ng/mL, respectively. The concentration of the second antibody for coating was also investigated; it showed that the most optimal concentration was 10 μg/mL (500-fold diluted in coating buffer).

Optimization of Spectrometric Measurement

Figure 3 shows the signal-to-noise radio (SNR), calculated by measuring absorbance of the standards and references with 0–10% calf

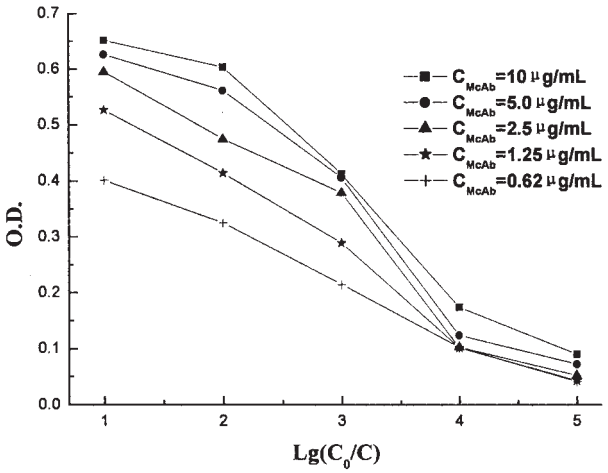

Figure 2. E_2-Biotin conjugate dilution curves at different McAbE_2 concentrations from 10 to 0.62 μg/mL. The primary concentration of the conjugate was 100 ng/mL. The concentration of the second antibody was 1000-fold diluted purified sheep anti-mouse IgG.

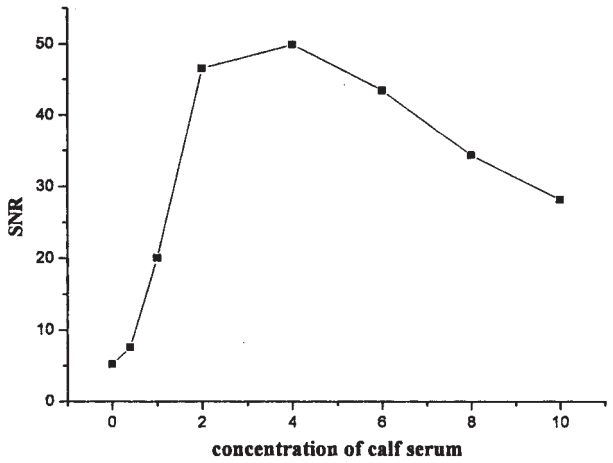

Figure 3. The curve of SNR with different concentration of calf serum in enzyme buffer. The results shown are the mean value of four measurements.

serum in enzyme buffer. There is a peak for SNR at 4% calf serum in enzyme buffer. This indicates that the appropriate amount of calf serum in enzyme buffer can prevent plate wells from nonspecific absorption of Avidin-HRP, but much calf serum would increase the backgrounds of the references. In this assay, we chose PBS containing 4% calf serum as enzyme buffer.

In order to further optimize the spectrometric measurement, the absorbance was measured at different enzyme reaction times, i.e., 3, 6, 10, 15, 20, and 30 min. Figure 4 shows that the absorbance signal could reach the maximum when the reaction time was above 20 min. To save time, while maintaining relatively high sensitivity, a reaction time of 15 min was used.

Immunoassay for Serum E_2

Calibration Curve

The dose-response curve was measured under optimized conditions discussed above, and the results are shown in Fig. 5. The RSD ($n = 8$) for each point was calculated. The detection limit of the assay was 28.3 pg/mL calculated by $3\sigma/N$, and the effective working range was 33.3–10,000 pg/mL.

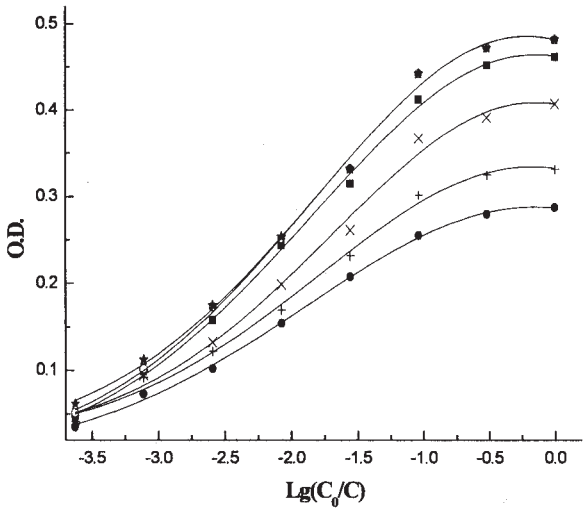

Figure 4. The standards curve with the different enzyme reaction times. The data were measured at 3 min (●), 6 min (+), 10 min (×), 15 min (■), 20 min (○), and 30 min (*), respectively. The results shown are the mean values of triplicate measurements.

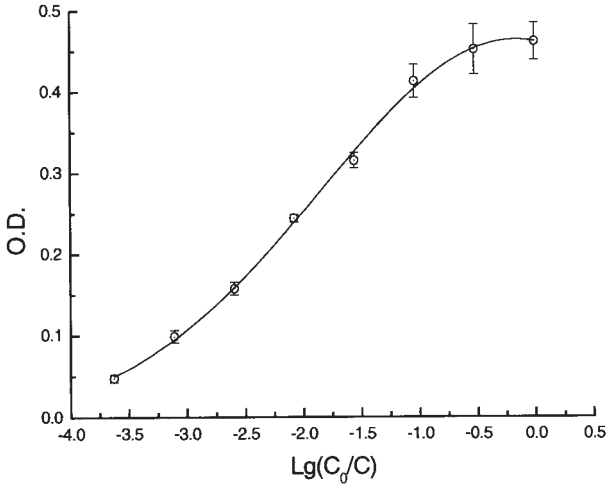

Figure 5. Calibration curve of the assay for E_2. The primary concentration of E_2 was 10 pg/mL, and the results shown were the mean value of eight measurements.

Specificity

The cross reactivities of McAbE_2 in this assay were E_2 100%, E_1 0.2%, E_3 0.5%, and not observed for progesterone. The results were measured and calculated by the 50%-D Method.[19] These results showed that, in the effective working range for measuring E_2, this assay was free of interferences from E_1, E_3, and other steroids existing in the serum samples and satisfied the needs for determination of serum E_2.

Precision

Intra-assay precision was calculated from 10 replicate measurements and inter-assay precision was calculated from 8 consecutive assays of a single sample. The results are shown in Table 2.

Analytical Application

Three different mimetic serum samples were measured and the results are listed in Table 3. The results showed that this is a reliable method for the determination of serum E_2. For measuring a great deal of samples in clinical studies, this method is better than RIA from the point of view of short assay time and freedom from pollution from radioisotopes.

Table 2. Precision of E_2 determination by the assay.

Serum sample	No. of determination	E_2 concentration Mean ± SD (pg/mL)	CV (%)
Intra-assay			
1	10	96.2 ± 12.7	13.2
2	10	489 ± 28	5.7
3	10	1067 ± 114	10.7
4	10	12454 ± 1250	10.0
Inter-assay			
1	8	136 ± 15	11.0
2	8	573 ± 30	5.2
3	8	1528 ± 115	7.5
4	8	9453 ± 1005	10.6

Table 3. Recoveries of samples in this assay.

Sample content (pg/mL)	Added (pg/mL)	Results (pg/mL)	R.S.D. ($n=8$)	Recovery (%, $n=8$)
0	50	45.7	11.5	91.4
0	250	238	5.6	95.3
0	1000	975	7.8	97.5

According to the results shown above, the use of a new tracer in a direct EIA for E_2 has several advantages over the use of an E_2-horseadish peroxide conjugate in that it allows a lower limit of detection and obviates matrix effects observed with the enzyme label. The results also showed improvement compared with the assay with E_2-Biotin tracer at position 6. Since the synthesis of the new tracers was simpler to perform than the other methods; this assay will have a broader application in several areas, such as clinical investigation and environmental analysis. So, this assay is an analytically more reliable, technically simpler, and less time-consuming method for directly determining E_2 in serum.

CONCLUSION

The biotinylation E_2 tracers were synthesized with different spacers by a simple and rapid procedure; these tracers were characterized by a dilution test for their K_{aff} with McAbE_2. Results showed that the tracer 2–4 was suitable for immunoassay for E_2. Furthermore, the preparation procedures of E_2 tracers could also be used for preparations of other steroids tracers.

A simpler and more direct EIA using E_2-Biotin tracer for serum E_2 was established and evaluated. The results have demonstrated that this assay is good both for their specificity and precision. The sensitivity of this method (about 30 pg/mL) is lower than that achieved by RIA (0.3 pg/mL) and it is not possible to use this assay for measuring E_2 concentration in postmenopausal women. However, it is possible to apply this method for monitoring of E_2 concentration in premenopausal women during the menstrual cycle, where E_2 levels is about 40–270 pg/mL. As a fast and direct method, it also can be used in other areas such as environmental analysis, food analysis, and tissue analysis.

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