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New fluorometric enzyme immunoassay for 17β-estradiol by homogeneous reaction using biotinylated estradiol

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Abstract

A new fluorometric enzyme immunoassay for 17β-estradiol (E2) using biotinylated estradiol (BE) as a probe ligand, is described. In this method, E2 is detected indirectly by a solid-phase avidin–biotin binding assay, in which the biotin is immobilized on a microtiter plate (biotin-plate). After the competitive reaction between E2 and BE for the anti-E2 antibody in solution, the free E2 and BE are separated from the bound forms by means of ultrafiltration. The concentration of BE in the solution is determined from the reaction between the biotin immobilized on the plate and the free BE for the limited biotin binding sites of avidin conjugated with horseradish peroxidase (avidin–HRP), which is added to the solution. The enzymatic reaction of HRP was measured by a fluorometric analysis with the QuantaBlu™ Fluorogenic Peroxidase Substrate (QFPS) in order to detect of the avidin–biotin binding with a high degree of sensitivity. The detection limit and linear range for the determination of E2 were 0.12 nM and from 0.12 to 25 nM, respectively. The relative standard deviations (R.S.D.) for the E2 assay were between 2.2 and 9.1% ($n = 3$). The cross-reactivity for several other estrogens was also evaluated.

Keywords: Fluorometric enzyme immunoassay; 17β-Estradiol; Biotin-immobilized microtiter plate; Solid-phase avidin–biotin binding assay

1. Introduction

A great deal of interest has developed regarding the effects of endocrine disruption caused by artificial and natural chemicals that are present at low concentrations in the environment with respect to interactions with the natural hormone system. The focus of much of this research has been on endocrine disrupting chemicals (EDCs) that are suspected of influencing the reproductive system of wild life and humans [1–5]. Among the natural estrogens and estrogen mimicking chemicals, E2 shows the strongest estrogenic activity. The affinity of E2 for the estrogen receptor is ca. 10^3–10^4 times higher than those of EDCs, such as DDT, alkylphenol and phthalate ester. Thus, it is suspected that even a low concentration of E2 might be capable of disrupting the normal hormone system of animals [6–8]. Moreover, estrogens that are excreted by human cannot be completely removed in a general sewage plant and, as a result, they are into the treated water. It has been reported that the plasma vitellogenin levels of fish living in rivers around sewage plants are relatively high due to the presence of estrogen [9,10]. Therefore, the concentration of E2 excreted from living bodies and present in the environment needs to be determined accurately.

Enzyme-linked immunosorbent assay (ELISA) is an excellent method for determining E2 rapidly and sensitively [11,12]. Unlike a radio immunoassay (RIA) in which a radioactive isotope is used [13,14], ELISA does not entail serious problems in handling and disposal, and it does not require the use of reagents that are harmful to human health. In the case of ELISA with a microtiter plate in which an immune complex is analyzed, the free antigen (or free antibody) can be easily separated from the bound form on the plate surface. Thus, it permits a great number of samples to be processed at the same time. However, in the case where a plastic microtiter plate is used as a solid phase, a significant excess of antigen (or antibody) against the

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immobilized antibody (or antigen) on the plate is frequently required in the solid-phase binding process in comparison with the homogeneous process [15]. In addition, the binding reaction between antigen and antibody on a plate surface is slower than that in solution. Homogeneous methods are simpler and faster, and separation or washing steps are not required. Therefore, a homogeneous enzyme immunoassay (HEIA), based on measuring changes in enzyme activity caused by the formation of an immune complex in a solution, is regarded as an excellent strategy for overcoming the disadvantages associated with ELISA [16–18]. Dhar et al. reported on the use of a HEIA for estradiol using estradiol-3-*o*-carboxymethyl ether as a hapten [19].

We previously reported on a spectrophotometric enzyme receptor binding assay for benzodiazepine drugs using a biotinylated ligand (biotin-1012S) and biotinylated bovine serum albumin immobilized on a microtiter plate (biotin-plate) [20]. In this method, benzodiazepine drugs were determined indirectly by means of the solid-phase avidin–biotin binding assay after homogeneous binding between a probe ligand and the receptor. The uptake of avidin conjugated with the enzyme onto the biotin-plate is inhibited by the addition of biotin-1012S as a probe ligand. Thus, the detection of benzodiazepine drugs can be achieved by the competitive reaction between biotin-1012S and benzodiazepine drugs for the limited binding sites of the receptor. Since the affinity between avidin and biotin is high (association constant: $10^{15}\,M^{-1}$), avidin–biotin binding is negligibly influenced by other sample matrix molecules. Therefore, an assay using avidin–biotin binding is generally very reproducible. In addition, this method can also be readily applied to many other assay systems, if the appropriate biotinylated ligands for a receptor and an antibody can be prepared.

In this study, we describe a new fluorometric enzyme immunoassay for E2 based on the combination of a homogeneous reaction between a probe ligand and the antibody and the avidin–biotin binding assay. By utilizing a monoclonal antibody and an avidin–biotin binding system, it is possible to reduce cross-reactivity in the assay system. In most immunoassay kits, a ligand directly conjugated with an enzyme is used. However, a direct labeling of the ligand with a macromolecule, such as an enzyme, generally affects the affinity and the selectivity of the ligand for the protein. Therefore, in this method, a biotinylated estradiol, a low-molecular weight compound, is employed as a probe ligand for the immunoassay. Mares et al. also reported on an assay in which biotinylated estradiol is used [21]. Nevertheless, they did not investigate the effect of spacer strength between the biotin and ligand and the binding behaviors of the biotinylated ligand for avidin or an antibody extensively. Hence, we investigated these issues extensively. The separation of free ligands from the bound forms with an anti-E2 antibody (B/F separation) was investigated by ultrafiltration. The enzyme activity of avidin conjugated with horseradish peroxidase (avidin–HRP) was determined using the QuantaBlu™ Fluorogenic Peroxidase Substrate (QFPS). The fluorometric enzyme immunoassay was optimized via the use of two biotinylated 17β-estradiols (BEs) with different lengths of spacers between the biotin and the E2 portion. The cross-reactivities of several other estrogens were also evaluated using this system.

2. Experimental

2.1. Apparatus

Fluorescent intensity measurements were performed using a Fluoroscan Ascent FL Thermo Labsystems (ThermoBioAnalysis Japan, Tokyo, Japan) with a 320 nm excitation filter and a 405 nm emission filter. The concentration of biotinylated estradiol was determined using a V-550 UV/Vis spectrophotometer (Jasco Co., Tokyo, Japan). pH measurements were carried out using a Horiba pH meter M-13.

2.2. Reagents

Albumin from Bovine Serum Crystallized (BSA), 17β-estradiol (E2), estrone (E1), 17α-estradiol and diethylstilbestrol (DES) were purchased from Wako Pure Chem. Co. (Osaka, Japan). (+)-Biotin *N*-hydroxysuccinimide ester (NHS-biotin), 6-ketoestradiol-6-(*o*-carboxymethyl)oxime (6-keto-E2), 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxy-2,5-pyrrolidinedion (NHS), biotinamidocaproyl-bovine serum albumin (biotin-BSA, 11 mol biotin/mol albumin), estriol (E3) was purchased from Sigma Chem. Co. (MO, USA). Biotinyl-3,6-dioxaoctannediamine (biotin-PEO-amine), avidin conjugated horseradish peroxidase (2 mol peroxidase/mol avidin) and QuantaBlu™ Fluorogenic Peroxidase Substrate Kit were obtained from Pierce Rockford (IL, USA). *p*-Nonylphenol (*p*-NP) was purchased from Kanto Chem. Co. (Tokyo, Japan). Mouse anti-estradiol (6-BSA) monoclonal antibody was provided by Lab Vision Co. (CA, USA). Phosphate buffer solution of pH 7.4 was prepared with 0.1 M KH_2PO_4 and 0.1 M NaOH and sodium chloride was then added to a concentration of 0.1 M. This buffer solution is abbreviated hereafter as PBS. All chemicals were prepared from analytical grade reagents and all solutions were prepared using deionized water purified with a Millipore Milli-Q system.

2.3. Preparation of biotinylated estradiol

3,17β-Dihydroxy-1,3,5,(10)-estratriene-6α-amine (6α-amino-E2) used in the assay was synthesized according to the procedure reported by Tiefenauer et al. [22]. The structure of 6α-amino-E2 was determined by IR, ^{1}H NMR and SI-MS.

Two BEs having spacers of different lengths were prepared as follows: the BE with a shorter spacer (BE-S) was prepared by mixing 2 mM 6α-amino-E2 and 2 mM NHS-biotin in 0.1 M phosphate buffer (pH6.5)–DMF (7:3%, v/v) followed by stirring overnight at 4 °C. The BE with a longer spacer (BE-L) was prepared by mixing 1 mM 6-keto-E2, 1 mM biotin-PEO-amine, 5 mM NHS and 5 mM EDC in CH_2Cl_2, followed by stirring overnight at 4 °C. These products were separated from unreacted reagents and by-products by thin-layer chromatography (silica gel 60 F254 alumina sheet, Merck). The irritant used for thin-layer chromatography was a mixture of chloroform + methanol (8:2%, v/v). After developing, the portion of the silica gel to which BE was adsorbed was stripped from the thin-layer sheet and BE was extracted with ethanol. The solu-

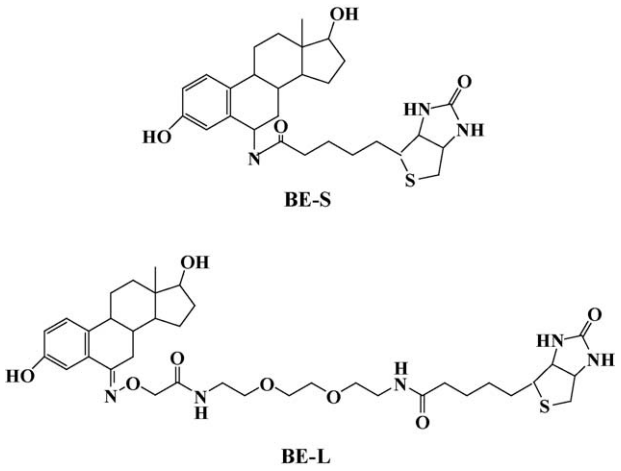

Fig. 1. The structures of biotinylated estradiols (BEs).

tion was centrifuged and filtered (pore size: 0.2 μm, Toyo Roshi, Tokyo, Japan) to remove residual silica gel. After evaporating the solvent, the residue was dissolved in water. The concentration of BE-S and BE-L was determined by comparing the absorbance of the ligands at 200 and 210 nm with that of estradiol, respectively. The structures of BE-S and BE-L are shown in Fig. 1.

2.4. Preparation of biotin-immobilized microtiter plate (biotin-plate)

Two hundred microliters of 0.002% biotin-BSA in PBS was placed into each well of a microtiter plate (Iwaki Tissue Culture Wave, Japan), followed by overnight incubation at 4 °C. The microtiter plate was washed three times with a solution of 0.1% BSA in PBS (PBS-B), then 200 μl of 1% PBS-B was then added to each well, followed by incubation for 1 h at 4 °C, to reduce non-specific binding. The plate was washed thrice with 0.1% PBS-B, prior to use.

2.5. Preparation of a sample solution for immune reaction

Two hundred and fifty microliters of a 200 mg/l E2 antibody solution was diluted to 10 mg/l with PBS. The diluted antibody solution was dialyzed for 10 h at 4 °C with dialysis tube (MWCO; 500, spectra/por CE molecularporous membrane, Spectrum Medical Industries, Inc., CA, USA) to eliminate sodium azide from the solution. This is because sodium azide, when present in the antibody solution interferes with the HRP activity [23,24]. After dialysis, the solution was stored at 4 °C.

Sample solutions were prepared as follows: a mixture of 25 μl of 10 nM BE-L, 25 μl of a solution containing various concentrations of an antigen and 200 μl of 1 mg/l E2 antibody was incubated for 30 min at room temperature. After ultrafiltration (NMWL; 10000, Ultrafree-MC Centrifugal Filter Device, Millipore, USA) to remove the E2 antibody, BE-L and E2 bound with the antibody, the filtrate was used as the sample solution in the solid-phase avidin–biotin binding assay.

2.6. Solid-phase avidin–biotin binding assay

One hundred microliters of a sample (or biotin, BE) solution, 50 μl of 0.1% PBS-B and 50 μl of 0.1 mg/l avidin–HRP were in turn placed into a sample tube, followed by incubation for 20 min at 4 °C. The solution was transferred into each well of the biotin-plate and further incubated for 20 min at 4 °C. After removing the free avidin–HRP in the well by washing five times with PBS, 100 μl of a QFPS solution and 50 μl of 0.05% H_2O_2 were added sequentially to the wells followed by incubation for 30 min at room temperature. The reaction was stopped by adding 50 μl of QuantaBlu™ stop solution and the fluorescent intensity of the solution was measured (320 nm for excitation, 405 nm for emission). A schematic illustration of the overall assay is shown in Fig. 2.

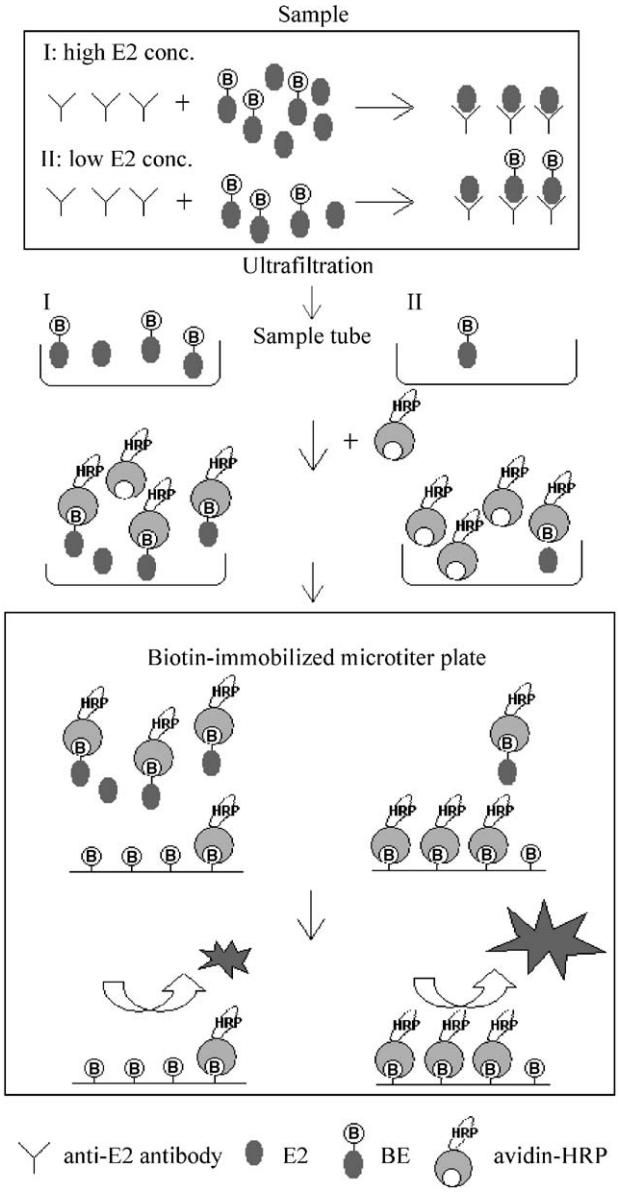

Fig. 2. Schematic diagram of the enzyme immunoassay for E2 using BE and the biotin-plate.

3. Results and discussion

3.1. Avidin–biotin binding assay

It is well known that avidin binds irreversibly to biotin. Therefore, once all the binding sites in avidin are occupied by free biotin in solution, avidin cannot bind to biotin immobilized on the plate. That is, the amount of avidin–HRP on the plate is dependent on the concentration of biotin in the solution. Biotin can be detected from the changes in the fluorometric response obtained from enzymatic reaction of avidin–HRP bound to the biotin moiety on the biotin-plate. We prepared two dose–response curves for biotin by following procedures: (1) 100 μl of biotin solution, 50 μl of 0.1% PBS-B and 50 μl of 0.1 mg/l avidin–HRP was directly added to the biotin-plate, i.e., the competitive reaction between biotin in a solution and biotin immobilized on the plate was performed for avidin–HRP. (2) The solution, which included 100 μl of biotin, 50 μl of 0.1% PBS-B and 50 μl of 0.1 mg/l avidin–HRP was incubated for 20 min in the sample tube and the solution was then transferred to the biotin-plate. The dose–response curves obtained from both of the above procedures are shown in Fig. 3. The IC_{50} values, as estimated from curves a and b were 6.4 and 0.87 nM, respectively. Hence, the sensitivity for the case (2) is about 10 times higher than case (1) of the competitive reaction. Since the reaction between biotin and avidin–HRP is not rapid, a large amount of biotin is needed in order to form a significant amount of avidin–HRP–biotin complex and to suppress the binding of avidin–HRP with biotin immobilized on the microtiter plate. By reacting avidin–HRP with biotin for 20 min prior to its introduction to the well immobilized with biotin, even less biotin would form a significant amount of avidin–HRP–biotin complex.

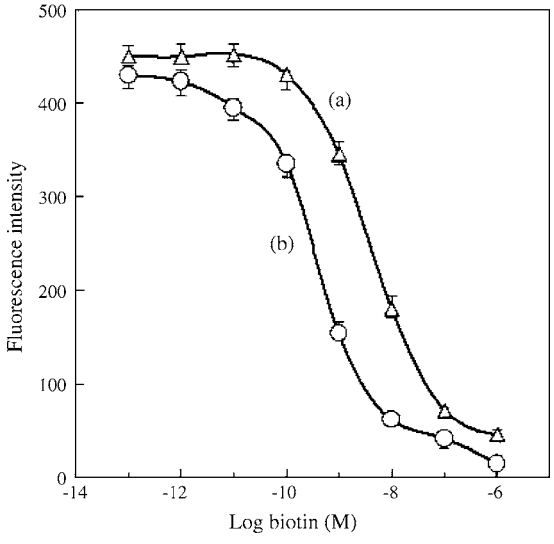

Fig. 3. Dose–response curves for biotin obtained from (a) the competitive reaction between biotin and biotin immobilized on the plate for avidin–HRP and (b) the consecutive reaction of biotin to biotin immobilized on the plate for avidin–HRP by incubating the solution that included 100 μl of biotin, 50 μl of 0.1% PBS-B and 50 μl of 0.1 mg/l avidin–HRP for 20 min in the sample tube before transferring to the biotin-plate.

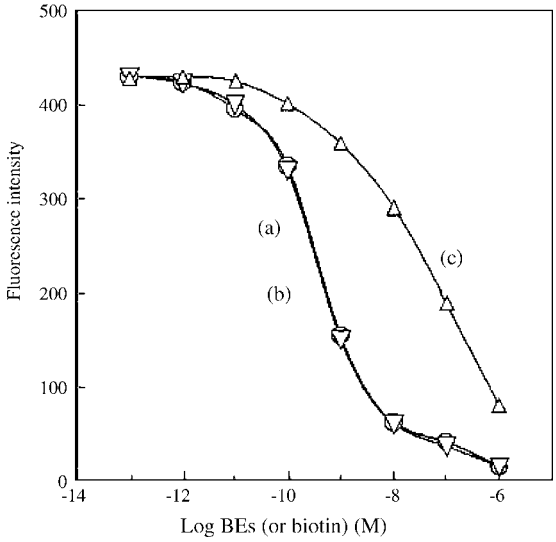

Fig. 4. Dose–response curves for BEs by the solid-phase avidin–biotin binding assay: (a) biotin, (b) BE-L and (c) BE-S.

3.2. Solid-phase avidin–biotin binding assay for BEs

Fig. 4 shows the dose–response curves for BEs obtained from the solid-phase avidin–biotin binding assay. Although the dose–response curve for BE-L (curve b) was exactly the same as that of biotin (curve a), a shift to higher concentration was found in the case of BE-S. This indicates that the affinity of BE-L for avidin is the same as that of biotin, whereas the affinity between BE-S and avidin is weaker than that of biotin. BE-S can be hydrolyzed to biotin and estradiol by heating for 3 days at 80 °C in an alkaline solution. The dose–response curve obtained from the BE-S solution after hydrolysis corresponded to that of biotin. Hence, it appears that the length of spacer in BE-S may be too short to compensate for sufficient binding with the binding site of avidin. It is known that the binding sites are located 0.8–0.9 nm below the surface of the avidin molecule [25]. Although we did not investigate the affinity of BE-S with the antibody, the affinity appears to be low. Lacorn et al. prepared some biotinylated estradiol ligands prepared from 6α-amino-E2. They reported that all of these ligands showed poor binding for an E2 antibody and that BE-S, which has the shortest spacer among the compounds examined, showed the poorest binding for the antibody [26]. Hence, it is difficult to combine BE-S with both avidin–HRP and the E2 antibody. Consequently, all of the following experiments were performed by using BE-L.

3.3. E2 assay based on the avidin–biotin binding method

The concentration of anti-E2 antibody was optimized to the solid-phase avidin–biotin binding assay. The concentration of BE-L employed was 10 nM to be finally 1 nM considering the sensitivity of the methods, that is, as shown in Fig. 4 the presence of 1 nM BE-L on the avidin–biotin binding assay indicates large decrease of fluorescence intensity, and it also exists on the position that the inclination on the dose-response curves for BE-L is largest. Hence, BE-L concentration of 1 nM is suitable for

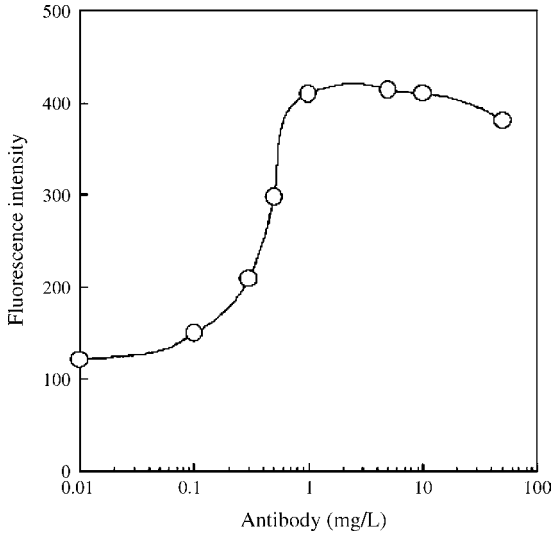

Fig. 5. Effect of dilution of the antibody on the fluorescent intensity by the solid-phase avidin–biotin binding assay at 10 nM BE-L.

competitive reaction with E2 for anti-E2 antibody. Two hundred microliters of antibody solution at various concentrations was incubated with 25 μl of 10 nM BE-L and 25 μl of PBS for 30 min at room temperature and then subjected to ultrafiltration to separate free BE-L from the bound form with the antibody. The filtrate was used in the solid-phase avidin–biotin binding assay. Fig. 5 shows the relationship between the antibody concentrations and the fluorescent intensity obtained in the solid-phase avidin–biotin binding assay. The result indicates that all of the free BE-L (10 nM) in the solution binds with the above 1 mg/l of antibody because its fluorescence intensity is in agreement with that of the solid-phase avidin–biotin binding assay performed without BE-L (or biotin). Therefore, the E2 assay was carried out using 1 mg/l of antibody.

The dose–response curve for the detection of E2 obtained from competitive reactions between various concentrations of E2 and BE-L for the antibody is shown in Fig. 6. The IC_{50} value,

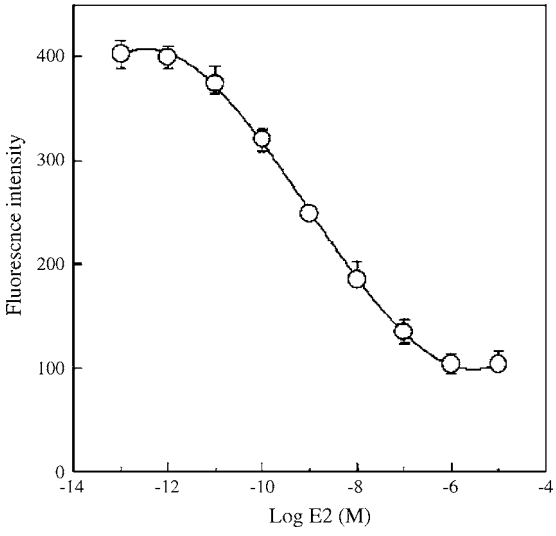

Fig. 6. Dose–response curve for the detection of E2 by the solid-phase avidin–biotin binding assay.

the concentration of antigen required to inhibit BE-L binding by 50% was 0.87 nM. The detection limit for E2 defined as the concentration corresponding to three times the deviation in the signal for the fluorescence intensity obtained without E2, that is, with only BE-L was 0.12 nM and the detection range was from 0.12 to 25 nM. The reproducibility of each measurement at the concentration within the detection range was 2.2–9.1% ($n = 3$) as the relative standard deviation.

3.4. Cross-reactivity studies in this immunoassay

The cross-reaction caused by a non-analyte, a non-true antigen, species for the antibody sometimes prevents the accurate determination of the analyte in an immunoassay. E1 and E3, as well as E2 are natural female estrogens, and it has been reported that the relative level of E1 in river water is in the range of 4–20 times that of E2 and that of E3 was almost the same as that of E2 [27,28]. DES has been used in the synthesis non-steroid estrogen for medical treatments from 1938s to 1970s, and *p*-nonylphenol that originates in plastic products, industrial detergents and insecticides, are also known to show estrogen activity [29]. Therefore, the cross-reactivity of these compounds were investigated in this immunoassay system. When several test compounds instead of E2 were incubated with 10 nM BE-L in a 1 mg/l antibody solution, some of the above compounds showed the cross-reactivities for the antibody. The cross-reactivities were calculated from (IC_{50} of E2/IC_{50} of the test compound) × 100 and are summarized in Table 1. The affinities of E2 derivatives, i.e., 6-keto-E2 and 6α-amino-E2, for the antibody were estimated to be similar with that of E2 in this assay system. Since Lacorn et al. reported poor antibody binding of biotinylated estradiol prepared from 6α-amino-E2 [26], this difference may be due to not the use of its biotinylated derivative but, rather, 6α-amino-E2 itself. The cross-reactivities for E1 and E3 were 0.1 and 0.01%, respectively, and the change in the fluorometric response caused by the other test compounds in Table 1 were within the error ranges of the E2 assay. Compared with a commercial E2 ELISA Kit, in which the ligand is directly conjugated with the enzyme, the extent of cross-reactivity of this assay is low. For example, in the case of the E2 ELISA Kit (code No. 300-0771) developed by Wako Co., the cross-reactivities

Table 1
Cross-reactivities studies with several test compounds for E2 in the enzyme immunoassay

Compound	IC_{50} (nM)	Cross-reactivity (%)
E2	0.87	–
6α-amino-E2	0.87	100
6-keto-E2	0.87	100
E1	851	0.1
E3	6607	0.01
17α-E2	$>10^4$	<0.009
DES	$>10^4$	<0.009
p-NP	$>10^4$	<0.009

E2: 17β-estradiol; E1: estrone; E3: estriol; 6α-amino-E2: 3,17β-dihydroxy-1,3,5,(10)-estratriene-6α-amine; 6-keto-E2: 6-ketoestradiol-6-(*o*-carboxymethyl)oxime; 17α-E2: 17α-estradiol; DES: diethylstilbestrol; *p*-NP: *p*-nonylphenol.

for E1 and E3 are 1.3 and 0.6%, respectively [30]. In the E2 EIA Kit (YK170) developed by the Yanaihara Co. (Shizuoka, Japan), the corresponding values for E1 and E3 are 0.94 and 0.16%, respectively [31]. These values are significantly larger than those obtained for our own method. The difference may be due to the conjugated ligand used in the assays as well as the type of antibody used. That is, it may be due to labeling with a low-molecular weight compound (biotin: M.W. 244.3) instead of labeling with macromolecule such as enzyme for a probe ligand of the immunoassay. Therefore, in this assay, E2 and its derivatives can be determined regardless the presence of these test compounds.

4. Conclusions

A new fluorometric immunoassay for E2 based on a solid-phase avidin–biotin assay was developed. In order to successfully achieve the E2 assay, two biotinylated E2 derivatives (BE-S and BE-L) with different lengths of spacer between the biotin and E2 portion were prepared as a tracer. The detection range for E2 was between 0.12 and 25 nM. The relative standard deviations for each measurements were 2.2–9.1% ($n = 3$). The cross-reactivity of E1, E3 and other test compounds for the E2 assay were 0.1, 0.01% and within the error ranges, respectively. Since this assay is based on a homogeneous reaction using a low-molecular weight ligand, a positive error caused by non-specific binding and the adsorption of analyte to the antibody and the solid phase could be reduced. Moreover, a high affinity between avidin and biotin used at the detection step in this assay is scarcely interfered by the sample matrix. This method can also be readily applied to other immunoassay systems, if the appropriate antigen can be biotinylated. The measurement of estradiol in river and ponds by this method is currently underway.

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