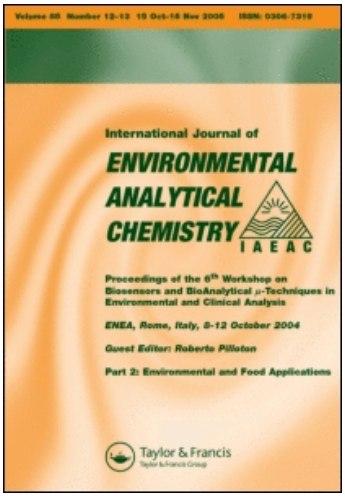

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A novel microplate reader-based high-throughput assay for estrogen receptor binding

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Coumestrol is a well-known ligand for the estrogen receptor (ER). The compound itself is fluorescent, and its fluorescence intensity at 408 nm increases upon binding to the ER. Here we describe a novel binding assay in 96-well plate format for estrogenic compounds, based on the competition between fluorescent coumestrol and estrogenic compounds for binding to the ligand binding domain (LBD) of the ER-alpha. Displacement of coumestrol was measured as a decrease in fluorescence intensity using a Victor^2 1420 multilabel reader. Competitive binding curves for the well-known estrogenic compounds, 17β-estradiol (E_2), ethinylestradiol, 4-nonylphenol, 4-octylphenol, genistein, bisphenol A, tamoxifen and diethylstilbestrol were constructed by using 7–10 different concentrations of the compounds and a fixed concentration of ER-α-LBD (14 nmol) and coumestrol (100 nmol). IC_{50} values and relative potencies (compared to E_2) of the estrogenic compounds were determined. The assay was validated by comparing the relative potencies to those from standard radioligand binding assays in the literature. Within day and between day variations were determined and the performance of the assay was assessed by determining the coefficients of variation and Z' values. The present fluorescent binding assay has proven to be fast and easy, and allows accurately quantifying the binding affinity of estrogenic ligands. The method is also suitable as a high-throughput screening assay for ER ligands.

Keywords: Estrogen receptor; Binding assay; Microplate reader; Ligand-binding domain; High-throughput screening

1. Introduction

Over the last decade, there has been growing concern about environmental chemicals that have potential estrogenic activity and cause reproductive dysfunction [1]. High risk to wildlife and humans has increased the need for practical screening methods to identify estrogenicity of industrial and environmental chemicals. Furthermore, there is a requirement for the generation of high quality (and quantity) data in

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a short time with low cost in drug discovery (hit identification) and development, which makes high throughput screening an essential part of this process. Various *in vitro* and *in vivo* assays have been used to evaluate the estrogenic potential of chemicals and to investigate their mechanism of action. *In vitro* binding affinity of chemicals to the estrogen receptor (ER) is conventionally determined by a competitive binding assay in which a radioactive ligand and the test compound compete for binding [2]. Although it is a sensitive technique, it has limitations such as the need to use radiochemicals, facilities to handle such compounds, and personnel training to prevent exposure of researchers to radiation. Recently, several high throughput screening methods for ER-binding have been reported. Bolger *et al.* [3] and Parker *et al.* [4] described a fluorescence polarization-based ER binding assay based on competition of fluorescein-labeled estradiol (E_2) and estrogen-like chemicals for binding to the ER. Several advantages of this assay over conventional binding assays are reported such as their being fast and less labour intensive. Furthermore separation of free and bound tracer ligand is not required. Another ER binding assay was reported by Usami *et al.* [2] in which a biosensor measures the binding between a ligand (E_2) immobilized on the sensor chip and a high molecular weight interactant (human recombinant ER) as a rise in the sensogram due to the surface plasmon resonance. Finally, Schobel *et al.* [5] reported a high resolution screening assay for ER binding using an online HPLC-MS-biochemical detection system, based on the methodology reported by Oosterkamp *et al.* [6] using coumestrol, a well known ligand for the ER [7, 8]. Coumestrol itself is fluorescent, and its fluorescence at 408 nm increases upon binding to the ER. Coumestrol was used as the tracer ligand in this assay for postcolumn-receptor affinity detection. ER binding was detected by a decrease in coumestrol fluorescence following competition for binding to the ER between coumestrol and analytes eluting from the LC column. Both the coumestrol and the ER solutions were continuously pumped into two reaction coils where the test compound and the ER interact in the first coil for 30 s and the ER reacts with coumestrol for 30 s in the second. The structure of biologically active compounds in crude plant extracts was elucidated by connecting a MS to the HPLC and biochemical detection system.

Here we describe a microplate reader assay for determining estrogen receptor binding based on the competition of fluorescent coumestrol with estrogenic compounds for binding to the ligand binding domain (LBD) of the ER-alpha. The LBD was obtained from recombinant E.coli BL21 (DE3) cells-expressing His_6-ERα LBD. The binding of test chemicals is determined as a reduction in the coumestrol fluorescence at 405 nm. Competitive binding curves for the well-known estrogenic compounds, 17β-estradiol (E_2; natural estrogen), ethinylestradiol (synthetic estrogen), 4-nonylphenol (NP; xenoestrogen), 4-octylphenol (OP; xenoestrogen), bisphenol A (BPA; xenoestrogen), genistein (phytoestrogen), tamoxifen (antagonist) and diethylstilbestrol (DES; synthetic estrogen) were plotted. IC_{50} values were calculated and the relative potencies (relative to E_2) of the estrogenic compounds were determined. The relative potencies were compared to those found in a conventional radioligand binding assay with [^{3}H]-E_2 reported in the literature to validate the method. Within day and between days variations were determined by using controls and the performance of the microplate reader-based assay was evaluated by determining the coefficients of variation and Z' values.

2. Experimental

2.1. *Chemicals*

Coumestrol was obtained from Fluka AG (Buchs, Switzerland). Blocking reagent for ELISA was from Boehringer Mannheim (Almere, The Netherlands). NP and OP were purchased from Acros Organics (Geel, Belgium) and BPA was from Aldrich. (^{3}H)-labelled E_2 was obtained from Amersham (Buckinghamshire, UK). All other chemicals were obtained from Sigma (St. Louis, MO, USA). Recombinant E.coli BL21(DE3)-expressing His6-ERα LBD were used for preparation of ERα LBD. The transformed cells were kindly supplied by Dr. Marc Ruff from Laboratoire de Biologie et Genomique Structurales, Illkirch, France.

2.2. *Preparation of ERα LBD*

Bacteria were grown according to Eiler *et al.* [9], but without estradiol in the medium. The cells were pelleted at 4000 *g* for 60 min at 4°C and the pellet was subsequently resuspended in a buffer containing 150 mM NaCl, 10 mM NaH_2PO_4 and 400 mg L^{-1} blocking agent for ELISA (pH 7.4; adjusted with KOH). The pellet was washed three times with 40 mL of the same buffer by centrifugation at 4000 *g* for 15 min at 4°C. After the last washing step, the pelleted cells were dissolved in 40 mL of the same buffer. Partial purification of the ER-α was performed by use of three French Press cycles (1000 PSI) on ice and sonification was carried out (Branson sonifier 250; output 7, 30% duty cycle, 2×10 cycles) to break up the cells. The soluble ERα LBD was obtained in the supernatant by ultracentrifugation (100.000 *g*) at 4°C for one hour and was stored at −80°C.

2.3. *Functional characterization of ERα LBD*

Functional characterization of the partially purified LBD was done by measuring its E_2 binding ability in a saturation radioligand binding assay as described by Eiler *et al.* [9] with minor modifications. The saturation binding analysis was done by incubating the protein with increasing concentrations of radiolabeled E_2 for 210 min at 4°C, which gave the total binding. To obtain the non-specific binding, similar incubations were done in the presence of 10^{-5} M non-labeled E_2. Bound and free ligand were separated by dextran-coated charcoal (4% Norit A charcoal, 0.4% dextran T-70 in the binding buffer (10 mM NaCl, 10 mM Tris-HCl, 1.5 mM EDTA, 1 mM DTT, 10% Glycerol, 0.4 g L^{-1} blocking reagent, pH 7.4)). The LBD concentration, B_{max}, was determined by using GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

2.4. *Fluorescence emission spectra of coumestrol and coumestrol-LBD complex*

Emission spectra of coumestrol and coumestrol-LBD complex were recorded after excitation at 355 nm. Emission spectra were obtained in a LS50-B luminoscence spectrometer (Perkin-Elmer, Boston, MA). Coumestrol was used at 100 nM and LBD was used at 1.5 and 15 nM final concentrations in the assay.

2.5. *Estimating tracer ligand equilibrium time*

The dissociation of coumestrol from the LBD-coumestrol complex was first studied by incubating the LBD (14 nM) with coumestrol (100 nM) for 30 min, until equilibrium was established. After adding E_2 (10^{-5} M) to the incubation mixture the fluorescence was recorded over time and the coumestrol dissociation time-course was plotted. From this, the equilibration time was calculated according to the rule mentioned by Hulme and Birdsall [10] by multiplying the half-time of the coumestrol dissociation process by five.

Furthermore, competitive binding curves for several competitors with different affinities for the ER-LBD were constructed at several time points to determine the optimum time for the competitive binding reactions.

2.6. *Effect of unbound coumestrol on the measurement of coumestrol-LBD complex*

Since coumestrol itself is fluorescent, and the free coumestrol is not separated from the coumestrol-LBD complex in the present assay, we investigated whether fluorescence of free coumestrol effects the measurements of the fluorescence of the complex. To achieve this goal, increasing concentrations of E_2 (in dimethyl sulfoxide; DMSO) were incubated with coumestrol (100 nM), and LBD (14 nM) in binding buffer in a total volume of 410 μL for 60 min at room temperature in eppendorf tubes. Control tubes, containing the same amount of DMSO as vehicle for E_2, were included in the experiment. At the end of the incubation period, 205 μL of the mixture from every incubation, containing both the bound and unbound coumestrol, were transferred to a black, flat-bottom polypropylene 96-well plate (Greiner, Cat #655209). 200 μL dextran-coated charcoal was added to the remaining 205 μL incubation mixture, mixed and left on ice for 5 min. The mixture was centrifuged at 14000 *g* for 5 min and the supernatants, containing only the bound coumestrol-LBD complex, were transferred to a 96-well plate. The fluorescence in both plates was determined in Victor^2 1420 multilabel reader at 355 nm excitation and 405 nm emission.

2.7. *Competitive binding curves*

Competitive binding curves for 8 known estrogenic compounds against coumestrol were recorded to evaluate the performance of the binding assay in the microplate reader. The curves were obtained by preparing serial dilutions of test compounds ranging from 10^{-11} to 10^{-5} M in DMSO. The LBD was introduced into the assay in binding buffer at a final concentration of 14 nM. Coumestrol stock solution was prepared in DMSO and dilutions were made in binding buffer. The final concentration of coumestrol in the assay was 100 nM. A negative control (binding buffer, equal to 0% inhibition) and a positive control (10 μM E_2, equal to 100% inhibition) were included in the assay on every day. Aliquots of 5 μL of the test compounds at various concentrations were pipetted into a 96-well plate that already contained 180 μL binding buffer in every well. The 96-well plate was gently but thoroughly mixed after pipetting 10 μL coumestrol into each well. Finally 10 μL of the LBD was pipetted into the wells. After an incubation time of 60 min at room temperature, the fluorescence was recorded with a Victor^2 1420 multilabel reader at 355/405 nm.

IC_{50} values were calculated by using GraphPad Prism software. To compare binding affinities of the tested estrogenic compounds to those reported in the literature, relative binding affinities (RBA) were calculated by using 17β-E_2 as standard ($RBA_{E_2} = 100$). The calculation was done according to the equation given below:

$$\mathrm{RBA} = \left(\frac{\mathrm{IC_{50}(E_2)}}{\mathrm{IC_{50}(competitor)}}\right) \times 100$$

2.8. *Assessing the performance of the assay*

To assess the assay performance, coefficients of the variation (CV) were determined for positive and negative controls both for within day and between day experiments. The Z' factor, introduced into the literature by Zhang JH *et al.* [11] as a simple and dimensionless parameter to evaluate the overall quality of high throughput assays, was also determined for the present method. The Z' factor is defined as the ratio of separation band to the dynamic range of the assay based on the positive and negative control data of the assay. It is formulated as:

$$Z' = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{|\mu_{c+} - \mu_{c-}|}$$

in which μ_{c+} and μ_{c-} represent the means of the positive and negative control signal, respectively. The standard deviations of the signals are denoted as σ_{c+} and σ_{c-} respectively.

"Within day" CV and Z' factors were determined using 20 positive and negative control samples. Data for the calculation of "between day" CV and Z' factors were obtained from 27–30 well incubations performed on 7 different experiment days.

2.9. *Statistical analysis*

Kruskal-Wallis test was used to statistically analyze the differences between the fluorescence of the coumestrol-LBD complexes in binding buffer with different concentrations of DMSO compared to binding buffer alone and the effect of free coumestrol on the measurement of fluorescence of coumestrol-LBD complex. IC_{50} values were calculated after plotting the competitive binding curves of individual competitiors by using GraphPad Prism (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. *Spectral characteristics of coumestrol and coumestrol-LBD complex*

Binding buffer alone and LBD in the binding buffer did not show a significant emission after excitation at 355 nm. Coumestrol, in binding buffer, emitted light at 437 nm when excited at 355 nm. The emission spectrum of the coumestrol-LBD complex in binding buffer showed two peaks, at 408 and 441 nm (figure 1).

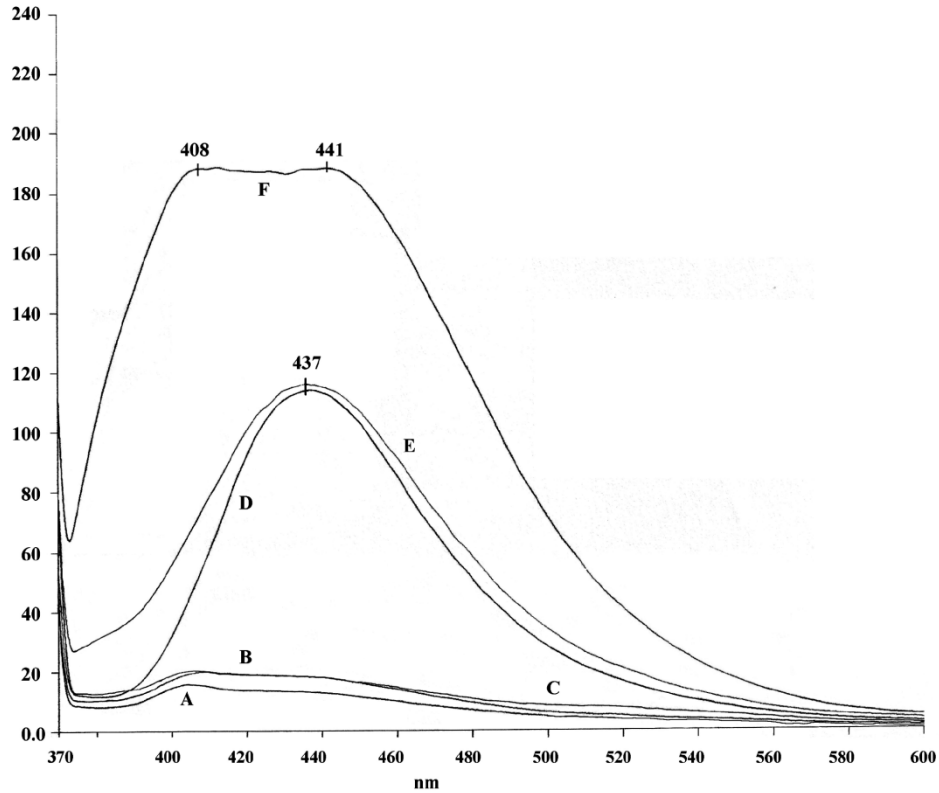

Figure 1. Emission spectra of binding buffer, coumestrol, coumestrol-LBD complex and coumerol-LBD complex at an excitation wavelength of 355 nm at RT and pH 7.4. (A) Binding buffer; (B) LBD at a concentration of 1.5 nM in binding buffer; (C) LBD at a concentration of 15 nM in binding buffer; (D) Coumestrol at a concentration of 100 nM in binding buffer; (E) Coumestrol (100 nM) and LBD (1.5 nM); (F) Coumestrol (100 nM) and LBD (15 nM) in binding buffer.

3.2. *Optimization of incubation time and temperature*

We have investigated the time- and temperature-dependent formation of coumestrol-LBD complex. As shown in figure 2, the difference between the fluorescence of coumestrol and LBD + coumestrol is bigger at room temperature (RT; 20°C) than at 4°C. This means that the dynamic range of the assay will be wider at RT than at 4°C. Since incubation at RT does not interfere with binding of competitive ligands and some of the competitors are not very soluble at 4°C, we decided to perform all incubations at RT.

Coumestrol binding to the LBD reaches equilibrium at around 15–30 min (both in the presence and absence of competitor, E_2 and remains stable for up to 180 min (figure 2). Similar experiments were also performed with low affinity ligands, such as NP and OP, and the equilibrium was reached around 30 min as well (data not shown). The half-time for dissociation of coumestrol from the LBD was 1.4 min as determined by plotting the displacement of tracer ligand vs time in the presence of excess concentration of high affinity competitor ligand, E_2 (figure 3). From this, the overall tracer ligand equilibration time can be estimated as $5 \times 1.4\,\text{min} = 7\,\text{min}$.

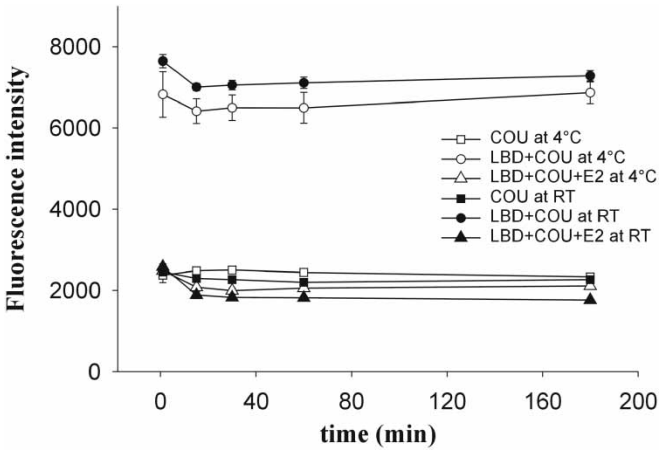

Figure 2. Effect of time and temperature on fluorescence intensity of LBD + coumestrol. The fluorescence of coumestrol, and LBD + coumestrol (with and without E_2) were recorded over time at RT and 4°C.

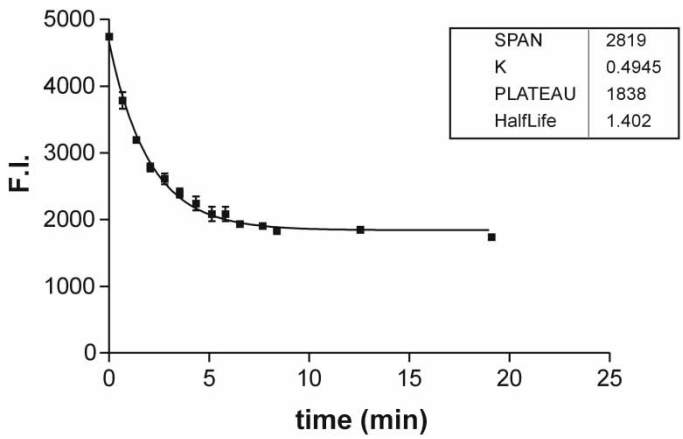

Figure 3. Coumestrol dissociation time-course initiated by addition of a non-fluorescent competitor, showing reversal of binding. The half time $t_{1/2} = 1.4$ min. From this, the overall tracer ligand equilibration time can be estimated and equals $5 \times 1.4 = 7.0$ min.

Taking all of the above data into account, we decided to perform the incubation for 60 min to accommodate even ligands with the lowest affinity.

3.3. *Effect of DMSO on binding*

The presence of DMSO in the incubation mixture was necessary for the solubility of coumestrol and the competitor ligands. Therefore the effect of DMSO on coumestrol binding to LBD and on the stability of LBD up to 3 h of incubation at RT was also investigated. No significant effect of DMSO was found when present up to 3.7% in the assay system by the Kruskal-Wallis analysis ($p = 0.13$) (figure 4). We decided to add 2.4% DMSO into the assay medium, which was sufficient for the solubility of ligands and had no unwanted effects on the LBD and the ligand binding.

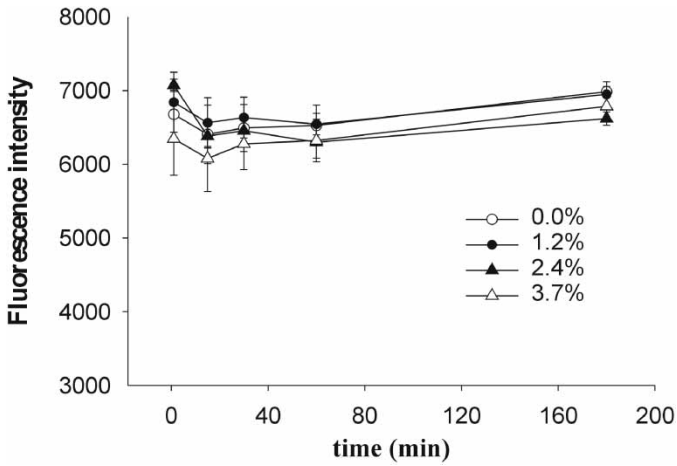

Figure 4. Stability of the coumestrol-LBD complex with various concentrations of dimethylsulfoxide (DMSO) over time. No statistically significant differences were found between any of the groups at any time point (Kruskal-Wallis test, $p = 0.129$).

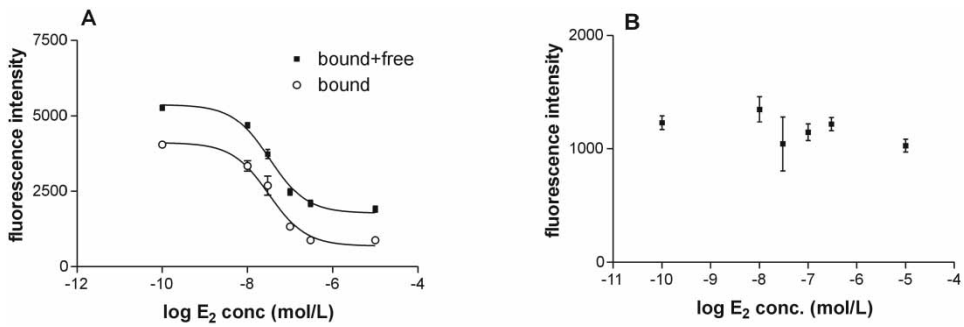

Figure 5. Effect of unbound coumestrol on the fluorescence of coumestrol-LBD complex. (A) Competitive binding curves were obtained from two different experimental procedures. Filled squares represent fluorescence intensity from the experiment where free and bound coumestrol are not separated. Open circles represent the fluorescence intensity from the experiment where free and bound coumestrol are separated and only bound coumestrol was measured. (B) By subtracting the fluorescence intensity of "bound + free" from "bound" coumestrol, the fluorescence intensity belong to the free coumestrol was obtained at various concentrations of the E_2. No statistically significant difference was found between the means of the data (Kruskal-Wallis test, $p = 0.22$).

3.4. *Effect of unbound coumestrol on the fluorescence of coumestrol-LBD complex*

To examine the effect of (natively fluorescent) unbound coumestrol on the fluorescence of coumestrol-LBD complex, an optimized 96-well plate-format binding assay was also performed with and without separating free and LBD-bound coumestrol. As can be seen in figure 5A, two displacement curves were obtained representing "free + bound" and "bound" coumestrol. IC_{50} values were found to be very comparable; 33.3 nM and 32.1 nM respectively. Figure 5B shows the differences between "free + bound" and "bound" at each data point, which represents the fluorescence of unbound coumestrol. No statistically significant difference was found between the mean values of free coumestrol ($p = 0.22$).

3.5. *Establishing the assay performance*

To establish the assay performance, "within day" and "between day" variability and Z' factors were determined by using replicated positive and negative controls. Negative controls represent the fluorescence intensity of coumestrol-LBD complex in binding buffer (equal to 0% inhibition) and positive controls represent the fluorescence of coumestrol-LBD complex in the presence of 10 μM E_2 (equal to 100% inhibition). Low CV values were obtained from both negative and positive controls analyzed both in an experiment day and in different experiment days (table 1). Z' values of 0.73 and 0.66 obtained from within day and between day experiments, respectively, indicate the high quality of the present assay.

3.6. *Competitive binding curves of estrogenic compounds*

Competitive binding curves for eight known estrogenic compounds were constructed. Table 2 displays the IC_{50} values ± S.D. determined from those curves which are quite reproducible. The representative displacement curves (figure 6) from 3 different experimental days show the high reproducibility of the assay for 17β estradiol. To compare the binding affinities of the tested compounds to the values reported in the literature, RBAs were calculated and listed in table 2. RBAs from various radioligand-binding assays in the literature (where mostly ERα was used as the

Table 1. Within day and between day performances of the fluorescent ligand-binding assay by using negative (binding buffer, equal to 0% inhibition) and positive control (10 μM E_2, equal to 100% inhibition) samples.

	Mean ± SD (CV%)	*n*
Within day		
(−) control	6326 ± 190 (3%)	20
(+) control	1726 ± 222 (12%)	20
Z' factor	0.73	
Between day		
(−) control	5947 ± 296 (5%)	27
(+) control	2043 ± 150 (7%)	30
Z' factor	0.66	

Table 2. Comparison of IC_{50} values and relative binding affinities (RBA) of eight estrogenic compounds in the present fluorescence microplate reader assay and radioligand binding assays from literature.

	IC_{50} (nM) ± SD	RBA_f[a]	$RBA_{radioligand}$	Reference
E_2	26.4 ± 2.2	100	100	
EE_2	20.9 ± 1.5	126	117[b], 85[b]	[13, 17]
BPA	20476 ± 7110	0.13	0.023[b], 0.1[b]	[12, 13]
NP	4235 ± 1382	0.62	0.018[b], 0.05[b]	[12, 13]
OP	6849 ± 654	0.39	0.07[b], 0.005[b]	[12, 13]
Genistein	3545 ± 992	0.74	0.01[b], 0.59[b]	[13, 17]
Tamoxifen	113	23.4	2.33[b], 16[c]	[5, 13]
DES	32	82.5	175[b], 141[b]	[13, 17]

RBA_f: Relative binding affinity obtained from the present, fluorescence-based binding assay. $RBA_{radioligand}$: Relative binding affinity obtained from the conventional radioligand binding assay. [a]Recombinant E. coli BL21(DE3)-expressing His6-ERα LBD was used in the present study. [b]ERα was used in the mentioned assays. [c]ERβ was used as the receptor source.

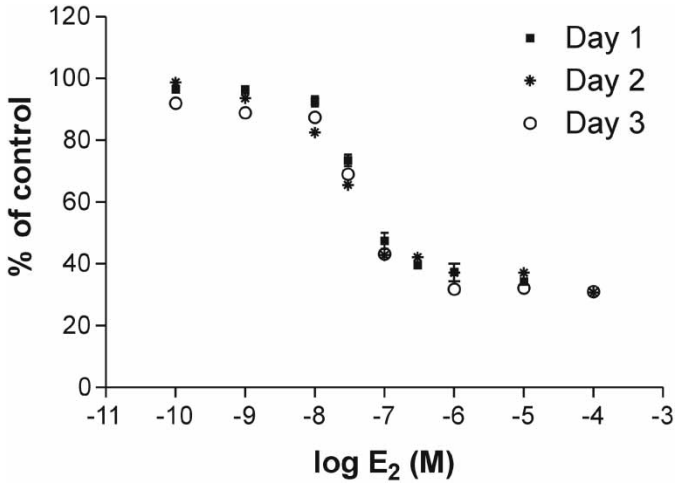

Figure 6. Representative competitive binding curves for estradiol from three different days. The values are given as the percentage of control (without E_2).

receptor source) are also listed in table 2. The obtained RBAs from the present assay are in good agreement with the data from the literature except for NP and OP were higher RBAs were obtained and DES where a slightly lower RBA was obtained.

4. Discussion

The aim of the present study was to develop and evaluate a simple, economic and accurate method for assessing estrogenic activity based on a competitive ligand-binding assay. Coumestrol was used as the tracer ligand because it has different fluorescent characteristics in its unbound and bound forms [5, 6].

After determining the spectral characteristic of coumestrol alone and the "coumestrol + LBD" complex, our preliminary studies showed that LBD alone had very low fluorescence, whereas coumestrol gives a significant fluorescence response. However, incubation of coumestrol with LBD gave 3–4 times higher fluorescence response compared to the fluorescence of coumestrol alone. This enhancement most likely is due to specific binding of coumestrol to the LBD because adding excess amount of E_2 to the incubation decreased the fluorescence back to the coumestrol alone values.

The present incubations were done in polypropylene 96-well plates in order to reduce the nonspecific binding of LBD (or ER) to the surface. Nonspecific binding was further reduced by adding casein as blocking reagent ($0.4\,g\,L^{-1}$) to the binding buffer. The binding buffer was also supplemented with 10% glycerol and DTT (1 mM) in order to keep LBD stable and functional over the incubation period.

Since many competitor estrogenic ligands are not very soluble in aqueous buffer, similar to coumestrol, introduction of an organic solvent into the assay appeared to be necessary. The competitor ligands were dissolved and introduced into the incubation in DMSO so that the final DMSO concentration was 2.4% in the assay. The effect of this amount of DMSO on the binding and the stability of coumestrol-LBD complex

was evaluated and as shown in figure 4, no inhibitory effect of DMSO on coumestrol binding was found at the indicated concentration up to 180 min incubation.

Optimal incubation conditions for the microplate assay, such as the reaction time and incubation temperature, were also determined. To evaluate the optimal reaction temperature, the binding assay was performed both at RT and 4°C with various competitors. The advantage of the former condition is that the background (coumestrol alone) fluorescence is lower and the fluorescence intensity of coumestrol-LBD complex is higher which gives a wider dynamic range. Experiments performed at 20–25°C showed that incubation at RT does not seem to interfere with the binding and the stability of the LBD up to 180 min incubation (figure 2). Since some of the competitors, such as NP, have solubility problems at 4°C we decided to perform the incubations at RT. Performing the incubations at RT is an advantage of the present assay both for solubility and unnecessity of a temperature controller compared to conventional ER binding assays that usually measure displacement of a radioactive ligand from ER at low temperatures [12].

In competitive receptor binding studies, large changes in the concentration of radioligand or other 'indicator' ligand should be avoided in order to allow the reaction to proceed according the law of mass action. As a rule of thumb, no more than 10% of ligand should be receptor-bound in any situation. Therefore, the fractional occupancy of the LBD was calculated. Approximately, 74% of the LBD (14 nM) will be occupied when 100 nM coumestrol is used as the ligand, which means that 10 nM of coumestrol will be bound and the criterion is therefore met.

To screen large numbers of samples in a short time is essential for high throughput screening (HTS). In the present study, coumestrol equilibrium time was found to be theoretically 7 min and a 30 min incubation time proved to be sufficient for several low-affinity ligands. One hour incubation time was chosen to include a two-fold 'safety margin' to accommodate even ligands with the least affinity. With an analysis time for one sample of less than 1 min, this means a throughput of 480 samples per 8 h working day. The present assay can easily be automated making it a real high throughput assay.

The performance of the assay, determined as within day and between-day variability, was examined by using control samples. Negative controls (equal to 0% inhibition) and positive controls (10 μM E_2, equal to 100% inhibition) were used for this aim. CV and Z' values were calculated to assess the quality of the assay. The Z' factor has been widely used as a measure of assay performance and relevancy for screening compounds [11–15]. A Z' factor between 0.5 and 1.0 indicates that the assay has large separation between the bound and unbound coumestrol (basal values), with small variations in these values. As can be seen in table 1, low CVs (3–12%) and high Z' values (0.73 and 0.66 respectively) were obtained from both within day and between day experiments indicating the high quality of the assay. These results are comparable to what Schobel *et al.* [5] obtained in a high resolution screening assay by using an online HPLC-MS biochemical detection system. Although the present assay has several advantages over this online ER binding assay, such as being simple, relatively inexpensive and requiring smaller amounts of ER, the online binding assay undoubtedly has the advantage of identification of biologically active compounds by coupling to a MS.

IC_{50} values of eight pure compounds from various chemical classes were determined in the present study by using the reported 96-well format binding assay. IC_{50} values could be determined with good reproducibility. The IC_{50} values of our

present assay for the tested compounds are 4–9 times higher than in a conventional radioligand binding assay using the same source for LBD as in this study (results not shown). This probably is largely due to the relatively high concentration of coumestrol (compared to the tracer amounts of radioligand normally applied) in our present assay against which the test compounds have to compete for binding to the estrogen receptor. Our assay seems comparable sensitive as another homogeneous assay that uses coumestrol as a fluorescent probe (IC_{50} of E_2 and tamoxifen for ER-beta: 15 and 95 nM respectively; [5]).The RBAs of the tested compounds were calculated relative to E_2, which gave us the possibility to compare our data to those reported in the literature [5, 12, 13–17]. Among many *in vitro* and *in vivo* assays reported in the literature, we used the results from cell free binding assays because these are most similar to our assay. However, important differences still remain such as the use of commercially available human ERα and β or rat uterus cytosol containing the ER, in the referred papers whereas ERα-LBD was used in the present study. Bearing in mind the different techniques and receptor sources, a reasonably well agreement of the RBAs from the present fluorescence-based binding assay with values reported in literature from conventional radioligand binding assay was obtained for ligands with a high affinity (E_2, EE_2, DES). For some ligands with a low affinity (e.g. NP, OP), the differences seem to be larger. This possibly may be due to a relatively larger influence of non-specific binding with low-affinity ligands. The non-specific binding may greatly differ in the various assays which may result in different receptor binding.

The data for the present study clearly show that coumestrol is an excellent tracer ligand for determining ER binding activity. Advantages of the present assay over conventional radioligand binding assay, where [^{3}H]-E_2 is used as the reporter molecule, are its short assay time and high capacity for samples due to the use of 96-well plates. This makes the assay suitable for high-throughput screening of compounds with potential estrogenic activity. The assay is also less laborious compared to a radioligand binding assay because no separation step is needed between unbound and bound tracer compound. Since the incubations are carried out at room temperature, and radioligand is not used as the reporter molecule, no specific facility such as cold room and/or a radioisotope laboratory is required. Furthermore the present assay has the advantage of requiring small amounts of ER, which is a drawback in several reported HTS assays for ER binding [4, 5].

In conclusion, a novel high-throughput microplate reader assay is introduced for determining the ER binding activity, which is simple, rapid, and sensitive. The assay represents a significant alternative for previously reported HTS assays for ER binding by being robust, non-laborious, and inexpensive. These features make the assay valuable both for screening environmental estrogenic compounds and for drug discovery and developmental aspects.

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References

[1] T. Colburn, D. Dumanoski, J.P. Myers, *Our Stolen Future*, Droemer Knaur, Munich, Germany (1996).
[2] M. Usami, K. Mitsunaga, Y. Ohno, *J. Steroid Biochem. Mol. Biol.*, **81**, 47 (2002).
[3] R. Bolger, T.E. Wiese, K. Ervin, S. Nestich, W. Checovich, *Environ. Health Perspect.*, **106**, 551 (1998).
[4] G.J. Parker, T.L. Law, F.J. Lenoch, R.E. Bolger, *J. Biomol. Screening*, **5**, 77 (2000).
[5] U. Schobel, M. Frenay, D.A. van Elswijk, J.M. McAndrews, K.R. Long, L.M. Olson, S.C. Bobzin, H. Irth, *J. Biomol. Screening*, **6**, 291 (2001).
[6] A.J. Oosterkamp, M.T. Villaverde Herraiz, H. Irth, U.R. Tjaden, J. van der Greef, *Anal. Chem.*, **68**, 1201 (1996).
[7] T.E. Kute, C. Linville, G. Barrows, *Cytometry*, **4**, 132 (1983).
[8] K. Nelson, E.J. Pavlik, J.R. van Nagell, M.B. Hanson, E.S. Donaldson, R.C. Flanigan, *Biochemistry*, **23**, 2565 (1984).
[9] S. Eiler, M. Gangloff, S. Duclaud, D. Moras, M. Ruff, *Protein Expression and Purification*, **22**, 165 (2001).
[10] E.C. Hulme, N.J.M. Birdsall, In *Receptor-Ligand Interactions: A Practical Approach*, E.C. Hulme (Ed.), p. 80, Oxford University Press, Oxford (1992).
[11] J.H. Zhang, T.D.Y. Chung, K.R. Oldenburg, *J. Comb. Chem.*, **2**, 258 (2000).
[12] A.J. Murk, J. Legler, M.M.H. van Lipzig, J.H.N. Meerman, A.C. Belfroid, A. Spenkelink, Burg B. van der, G.B.J. Rijs, D. Vethaak, *Environ. Toxicol. Chem.*, **21**, 16 (2002).
[13] B. Gutendorf, J. Westendorf, *Toxicology*, **166**, 79 (2001).
[14] J.A. Schwartz, D.F. Skafar, *Biochemistry*, **32**, 10109 (1993).
[15] C.W. Scott, D.E. Wilkins, S. Trivedi, D.J. Crankshaw, *Anal. Biochem.*, **319**, 251 (2003).
[16] E. Silva, N. Rajapakse, A. Kortenkamp, *Environ. Sci. Technol.*, **36**, 1751 (2002).
[17] H.A. Harris, A.R. Bapat, D.S. Gonder, D.E. Frail, *Steroids*, **67**, 379 (2002).