Development and validation of fluorescent receptor assays based on the human recombinant estrogen receptor subtypes alpha and beta

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Abstract

This article describes the development and validation of two fluorescent receptor assays for the hRec-estrogen receptor subtypes alpha and beta. As a labelled ligand an autofluorescent phyto-estrogen (coumestrol) has been used. The estrogen receptor (ER) belongs to the nuclear receptor family, a class of soluble DNA binding proteins, mainly present in the cytoplasm of the cell, that act as ligand-activated enhancer factors. It consists of two different forms, expressed as ER-α (66 kDa) and ER-β (59 kDa). The ER-α is mainly located in the uterus and the ER-β can be found in vascular tissue. Detection and identification of compounds having estrogenic effects is of importance in drug discovery programmes within the pharmaceutical industry for their search for ER-subtype selective (ant)agonists which may prove to be of therapeutic value in treating a variety of estrogen-linked pathologies (breast cancer, osteoporosis, cardiovascular disease, type II diabetes and Alzheimer disease).

Furthermore, interactions of (xeno-)estrogens with the endogenous hormonal system of the exposed organism can affect embryos, gonads, and reproductive behaviour. The latter can eventually lead to reduced reproduction and deterioration of a population. For that reason, monitoring of (xeno-)estrogens in food products and in the environment, attracts considerable attention by health councils throughout the world.

The following characteristics were obtained for the human recombinant (hRec) estrogen receptor-beta assay, which is suitable for ER subtype selective drug-discovery purposes (IC$_{50}$ values for 17-β-estradiol and genistein were 5.1 nM and 25 nM, respectively): goodness of fit (R^2) was always >0.98 ($\bar{x} = 0.9933$, $n = 10$). LLOQ of the assay is typically ≥500 picomolar, whereas the ULOQ of the assay is ≤20.0 nanomolar.

For the hRec-estrogen receptor-alpha assay, which is suitable for monitoring of (xeno-)estrogens (IC$_{50}$ values for 17-β-estradiol and genistein were 0.68 nM and 65 nM, respectively) the following characteristics were obtained: goodness of fit (R^2) was

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always >0.96 ($\bar{x} = 0.9838$, $n = 10$). LLOQ of the assay is typically ≥ 200 picomolar, whereas the ULOQ of the assay is ≤ 5.0 nanomolar.

1. Introduction

The most potent naturally occurring estrogen in humans is 17-β-estradiol, followed by its metabolites estrone and estriol. These steroidal hormones induce transcriptional activity through binding to the estrogen receptor (ER) and play a vital role in the development and functioning of the female reproductive system and the maintenance of bone mineral density and cardiovascular health. Chemical modifications of the natural estrogens have yielded highly potent compounds that are used for regulation of the menstrual cycle and for the control of fertility (e.g. ethinyl estradiol). Also non-steroidal compounds have been identified as estrogens. Some of these compounds occur naturally in plants (phyto-estrogens) whereas many others are chemically synthesized (e.g. diethyl stilbestrol, DES) [1]. Estrogen antagonists, such as tamoxifen, compete with 17-β-estradiol for binding to the estrogen receptor but fail to induce transcriptional activity. Tamoxifen has been successfully used in the treatment of ER-positive breast cancers [2].

The estrogen receptor belongs to the nuclear receptor family, a class of soluble DNA binding proteins, mainly present in the cytoplasm of the cell, that act as ligand-activated enhancer factors. It consists of two different forms, expressed as ER-α (66.4 kDa) and ER-β (53.4 kDa). The ER-α is mainly located in the uterus and the ER-β can be found in vascular tissue. Both receptor subtypes contain two functional domains, a Ligand Binding Domain (LBD) and a DNA-Binding Domain (DBD) [3,4]. Stimulation of processes in (cardio)vascular tissue and bone tissue (ER-beta) by estrogens, can have important health benefits, however stimulation of other tissues (ER-alpha), such as breast and uterus, can increase the risk of cancer at these sites [5]. The latter stimulates the search for ER subtype-selective antagonists [2,6]. Furthermore, the search for ER-subtype selective (ant)agonists may prove to be of therapeutic value in treating a variety of estrogen-linked pathologies like menopausal symptoms, osteoporosis, cardiovascular disease, type II diabetes, Alzheimer disease and urinary incontinence [6].

Interactions of (xeno-)estrogens with the endogenous hormonal system (ER-alpha) of the exposed organism can affect embryos, gonads, and reproductive behaviour. The latter can eventually lead to reduced reproduction and deterioration of a population. Reports of feminized male fish and abnormal sexual development of reptiles and birds have been published [7,8]. Due to these alarming hazardous effects, environmental (xeno-)estrogens, are attracting considerable attention by health councils throughout the world [9].

In this article, we describe the development and validation of two, non-radioactive receptor assays for the human recombinant estrogen receptor (hRec-ER) subtypes alpha and beta with fluorescence detection based on the principle used by Janssen et al. for the membrane-bound benzodiazepine receptor [10,11]. Receptor assays can be used as a highly selective analytical method, based on a certain affinity towards the receptor, to detect and quantitate both endogenous and exogenous compounds in a variety of matrices [12]. Since the development of receptor assays in the 1970s, mostly radioactive labelled compounds were used as a ligand. Due to practical limitations related to the use of radioactivity, nonradioactive ligands, such as autofluorescent ligands or fluorescent-labeled ligands, have been used for different receptor-types [13–15].

Validation of the receptor assays was conducted according to the updated Washington Conference report by Shah et al. [16], which is generally accepted as one of the most important guidelines for bioanalytical methods validation. Furthermore, development and validation of the receptor assays were carried out under controlled and monitored environmental conditions, conducted in accordance with ISO 9001:2000 guidelines.

2. Experimental

2.1. Reagents and materials

Reagent grade sodium phosphate ($Na_2HPO_4 \cdot 2H_2O$ and $NaH_2PO_4 \cdot H_2O$), potassium phosphate (K_2HPO_4 and $KH_2PO_4 \cdot 3H_2O$), sodium chloride, HPLC grade MeOH and EtOH were purchased from VWR (Roden, The Netherlands). Polyethylene glycol 6000 (PEG 6000) and dithiothreitol (DTT) were purchased from Duchefa (Haarlem, The Netherlands). Chicken egg albumin, grade III (ovalbumin) and bovine gamma globulin (Cohn fraction, 99%) were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). The used estrogen receptor ligands were a gift from the University Centre for Pharmacy (Department of Analytical Chemistry and Toxicology, Groningen, The Netherlands) and were either of reagent grade or pharmacopoeial quality.

The human recombinant receptor subtypes alpha and beta were obtained through Sopachem B.V. (Wageningen, The Netherlands) from Panvera (Madison, WI, USA). Demineralized water, was further purified by a Millipore instrument (Millipore, Amsterdam, The Netherlands).

2.2. HPLC system

The chromatographic system consisted of a Shimadzu (Shimadzu's Hertogenbosch, The Netherlands) SCL-10A system controller, a Shimadzu FCV 10 AL Low-pressure gradient flow, a Shimadzu LC 10 AD solvent delivery module, a Shimadzu CTO 10 AS column-oven, an automatic degasser (Shimadzu DGU 14 A), a 96 wells plate configured autoinjector (Shimadzu SIL 10 AD) and a Shimadzu RF-10 Axl fluorescent detector. Peak areas were integrated by the Shimadzu CLASS-VP software (version 6.10).

Separation was performed using a 125 mm × 4 mm i.d. column packed with 5-μm Lichrospher 100 RP-18 (VWR). The mobile phase consisted of a potassium phosphate (50 mM, pH 6.8)—MeOH buffer (35:65, v/v). The flow rate was 0.8 ml/min. Column temperature was at a constant 30 °C. The fluorescent detector was set at $\lambda[ex] = 379$ nm and $\lambda[em] = 436$ nm, respectively. The limit of detection (LOD) for the fluorescent ligand was 5 pM (injection volume was 100 μl).

2.3. Saturation and binding experiments

The principles used for the development of the hRec-ER assays are based on the technology described by Janssen et al. [10,11]. Due to the fact that the estrogen receptors are soluble, the procedure of Janssen et al. was adjusted in order to be able to collect the receptors on a filter after incubation. Filters of a Multiscreen MABV NOB filtration plate (Millipore) were prewetted by pipetting 250 μl assay buffer (Phosphate buffered saline, pH 7.4 (PBS) with 8 mM DTT and 0.1% ovalbumin). Vacuum (400 mbar) was applied by the Multiscreen vacuum manifold (Millipore). Coumestrol, a native fluorescent phytoestrogen was used as the fluorescent ligand. For the saturation experiments 20 μl fluorescent ligand (FL, 0.1–4 nM [hRec ER-α] or 0.1–10 nM [hRec ER-β] final concentration, respectively) in PBS was pipetted in duplicate in the wells of the filtration plate. For the determination of the total binding 20 μl assay buffer, and for the determination of non specific binding 20 μl PBS containing 10 μM 17-β-estradiol, were added, respectively. To this mixture 160 μl of the receptor suspension (480 fmol receptor protein) was added and the plate was placed on an orbital micro-plate shaker (800 rpm) for 1 h at ambient temperature. After the incubation, the receptor protein was precipitated by adding 50 μl of a PBS solution containing 1% gamma globulin and 36% PEG 6000. After 15 min of incubation on an orbital micro-plate shaker (800 rpm), the solution was aspirated. The filtration plate was washed three times with 250 μl assay buffer. After the final aspiration step the FL was dissociated from the receptor protein by adding 200 μl of a 10 μM 17-β-estradiol solution in PBS. After 1 h of incubation on an orbital micro-plate shaker (800 rpm) the solution was aspirated and collected in a standard 96-wells plate. A capmat (Micronic, Lelystad, The Netherlands) was placed onto the micro-titer plate to prevent evaporation during analyses. Hundred μl (100 μl) aliquots of the collected fractions were directly injected into the HPLC system.

2.4. Binding experiments

The filtration plate was prewetted by pipetting 250 μl assay buffer. Vacuum was applied by the Multiscreen vacuum manifold. 20 μl of a 17-β-estradiol

solution (0.01–50 nM final concentration) in PBS and 20 μl of the FL solution (1.2 nM, final concentration) was pipetted in duplicate in the wells of the filtration plate (for the blank, 20 μl PBS pH 7.4 instead of the FL solution were added). To this mixture 160 μl of the receptor suspension was added and the plate was placed on an orbital micro-plate shaker (800 rpm) for 1 h at ambient temperature, after which the procedure was conducted as described in Section 2.3.

2.5. Data analysis

Saturation and inhibition curves were fitted with the program SigmaPlot V6.00 (Chicago, IL, USA). For fitting of the specific binding of the saturation curve a hyperbola—single rectangular, two parameter equation (Eq. (1)) was used,

$$Y = \frac{aX}{b + X} \tag{1}$$

where b equals the dissociation constant, K_d of the FL, whereas the non-specific binding was fitted by linear regression. The specific binding was calculated by subtracting the non-specific binding from the total binding.

Inhibition curves were fitted by a sigmoidal logistic four-parameter equation (Eq. (2)) describing a one-site binding model:

$$Y = Y_0 + \frac{a}{1 + (x/x_0)^b} \tag{2}$$

3. Results and discussion

3.1. Saturation experiments

In order to determine the dissociation constant (K_d) of the FL for the estrogen receptor, saturation experiments were performed as shown in Fig. 1.

The calculated K_d-values for the FL were 3.4 ± 0.6 for the hRec ER-α and 4.9 ± 0.8 nM for the hRec ER-β, respectively. The latter shows that the affinity of the FL for the hRec ER-α and the hRec ER-β is almost the same. For use in the fluorescent receptor assay we have chosen a final concentration for the FL of 1.2 nM. This value corresponds with $0.35 \times K_d$ for the ER-α and $0.25 \times K_d$ for the ER-β.

From the saturation curve it can be calculated that the non-specific binding of the FL (at a concentration of 1.2 nM) was in both cases <22% (21.7 and 17.8%, respectively), relative to the total binding.

3.2. Binding experiments: selectivity and non-specificity

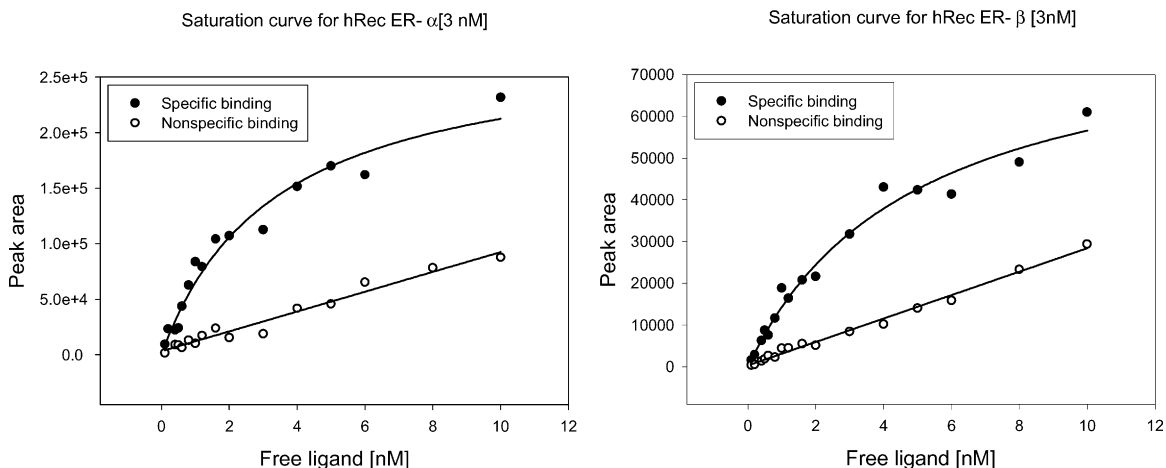The FL was tested as a label by determining calibration curves for four structurally divergent

Fig. 1. Saturation curves for the hRec ER-α and hRec ER-β, respectively. The specific binding was calculated by subtracting the non-specific binding from the total binding.
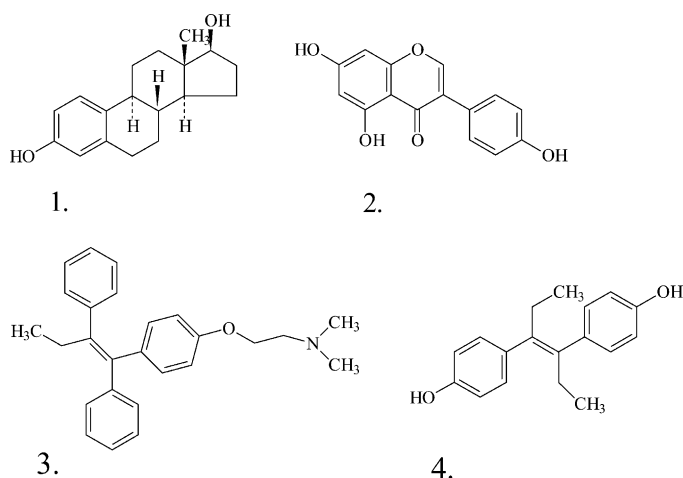
Fig. 2. Chemical structure of several ligands for the estrogen receptor: (1) 17-β-estradiol; (2) genistein; (3) tamoxifen; (4) diethylstilbestrol.

(xeno-)estrogens with different affinities for the two receptors, namely, 17-β-estradiol (1, female hormone), genistein (2, phytoestrogen), tamoxifen (3, anti-estrogen) and diethylstilbestrol (4, synthetic estrogen). Structure formulas are shown in Fig. 2.

The corresponding inhibition curves are shown in Fig. 3. The IC$_{50}$ values for the four ligands were calculated from the calibration curves and are represented in Table 1.

As can be seen from Fig. 3 the affinities of the several ligands differ (as expected) substantially from each other. Furthermore, each ligand has a different affinity towards the hRec ER subtypes. The latter is expressed by the IC$_{50}$ values given in Table 1. The table shows that diethylstilbestrol, tamoxifen and

17-β-estradiol show a higher affinity for the hRec ER-α, whereas genistein shows a higher affinity for the hRec ER-β.

The calculated relative binding affinities (RBA values) in Table 1, are comparable to those given in the literature [5,17,18].

In comparison to immunoassays, receptor assays are capable of quantifying multiple analytes in a complex matrix. Receptor assays are therefore classified as highly selective, rather than specific. However, non-specific binding (or rather non-selective binding or non-selective adsorption), due to matrix effects, should be quantified and should be as low as possible (preferable < 25% of the total binding). For the hRec ER-α and the hRec ER-β assays the non-specific
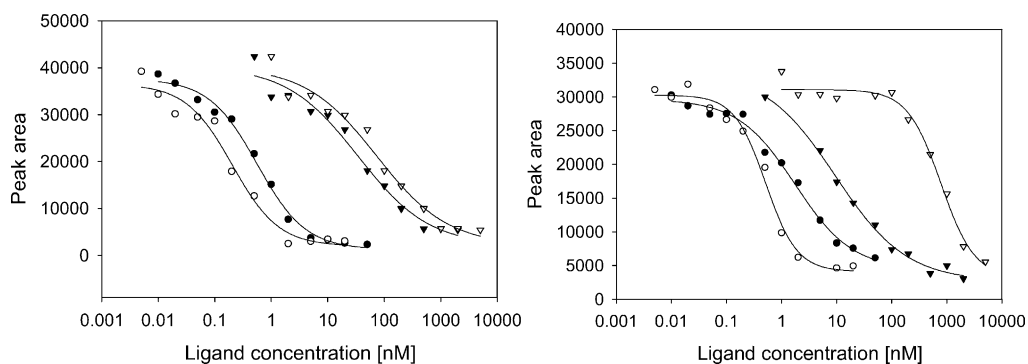

Fig. 3. Calibration curves of 17-β-estradiol (●), diethylstilbestrol (○), genistein (▼), and tamoxifen (▽) for the hRec ER-α and the hRec ER-β, respectively.

Table 1
IC$_{50}$ and RBA values of several ligands for the hRec ER-α and the hRec ER-β

Ligand	IC$_{50}$ (nM) for the hRec ER-α	RBA	IC$_{50}$ (nM) for the hRec ER-β	RBA	RBA hRec ER-β: ER-α
Diethylstilbestrol	0.3	252	0.8	641	2.5
17-β-Estradiol	0.7	100	5.1	100	(1)
Genistein	65	1.1	25	20	19
Tamoxifen	138	0.5	1204	0.4	0.9

RBA: relative binding affinity. RBA was calculated as the ratio of IC$_{50}$ values, where the RBA value of 17-β-estradiol was arbitrarily set to 100.

binding of the FL was generally between 15 and 20% (relative to the total binding) for concentrations of FL < 2 nM.

3.3. Goodness of fit and lack of fit testing of the calibration model

As explained in Section 2.5 for fitting of the inhibition (calibration) curves a sigmoidal, logistic four-parameter equation describing a one-site binding model, was used. In order to verify the choice of the calibration model, ten inhibition assays were performed for either assay. Goodness of fit (goodness of description) was expressed as the coefficient of (multiple) determination R^2 and lack of fit was expressed as the goodness of prediction, (Q^2) [19]:

$$Q^2 = 1 - \left(\frac{\text{PRESS}}{\text{SS}_{\text{tot}}} \right)$$

where PRESS is the prediction residual error sum of squares, and SS$_{\text{tot}}$ is the total sum of squares. From the equation can be seen that a low PRESS value results in a high Q^2 value. In practice, for logistic curves, Q^2 values exceeding 0.9 indicate that there is no significant lack of fit.

For the hRec ER-α assay, the goodness of fit was highly significant ($\bar{x} = 0.9838$, $n = 10$), whereas the goodness of prediction showed no significant lack of fit ($\bar{x} = 0.9610$, $n = 10$).

For the hRec ER-β assay, the goodness of fit was highly significant ($\bar{x} = 0.9933$, $n = 10$), whereas the goodness of prediction showed no significant lack of fit ($\bar{x} = 0.9870$, $n = 10$).

Furthermore, one-way analysis of variance (ANOVA) showed no significant difference between the calibration curves for both assays at a 95% confidence interval ($n = 10$, $P = 0.9997$ [hRec ER-α] and $P = 0.9997$ [hRec ER-β], respectively).

3.4. Precision, accuracy and sensitivity

A summary of the results on precision and accuracy for the hRec ER-α assay, as derived from the back calculated concentrations versus the nominal value of two concentration levels (0.5 and 2.0 nM 17-β-estradiol), is given in Table 2.

A summary of the results on precision and accuracy for the hRec ER-β assay, as derived from the back calculated concentrations versus the nominal value of two concentration levels (2.0 and 10.0 nM 17-β-estradiol), is given in Table 3.

From Tables 2 and 3 can be seen that the between-run R.S.D. values were, as can be expected, slightly higher than the within-run R.S.D. values,

Table 2
Summary of precision and accuracy of the analytical method for the hRec ER-α assay ($n = 10$)

Nominal concentration (nM)	Back calculated concentration from the curve (nM)	Bias[a] (%)	Intra-assay precision R.S.D. (%) (within-run)	Inter-assay precision R.S.D. (%) (between-run)
0.500	0.472	−6.8	7 ($n = 5$)	15 ($n = 5$)
2.00	1.91	−4.5	10 ($n = 5$)	12 ($n = 5$)

[a] Bias (%) was calculated as $(x - \mu)/\mu$, where $\bar{x}$: mean value and μ: nominal value. Data was subjected to the Grubbs test [20] at a two sided 95% confidence interval for detection of outliers.

Table 3
Summary of precision and accuracy of the analytical method for the hRec ER-β assay ($n = 10$)

Nominal concentration (nM)	Back calculated concentration from the curve (nM)	Bias[a] (%)	Intra-assay precision R.S.D. (%) (within-run)	Inter-assay precision R.S.D. (%) (between-run)
2.00	2.00	−0.1	12.4 ($n = 5$)	19.5 ($n = 5$)
10.0	10.1	0.7	3.9 ($n = 5$)	5.4 ($n = 5$)

[a] Bias (%) was calculated as $(x − \mu)/\mu$, where $\bar{x}$: mean value and μ: nominal value. Data was subjected to the Grubbs test [20] at a two sided 95% confidence interval for detection of outliers.

although all below 20% at the given concentration levels. The lower limit of quantitation (LLOQ) of the developed assays, which is defined as the lowest concentration of the standard curve which can be measured with acceptable accuracy and precision [16], was determined by defining the first mean 17-β-estradiol concentration that significantly differs from the lowest calibrator concentration calculated by a one-tailed dependent two-sample t-test at a 95% confidence level. Accuracy should be between 75 and 125%, and the R.S.D. (%) should be less than 25%. The upper limit of quantitation (ULOQ) of the developed assays, which is consequently defined as the highest concentration of the standard curve which can be measured with acceptable accuracy and precision, was determined to be the highest calibrator that could be back-calculated from the curve with an accuracy between 75 and 125% and a R.S.D. (%) less than 25%. A summary of the LLOQ and ULOQ values is given in Table 4.

The LLOQ values for the hRec ER-α and hRec ER-β were calculated by a one-tailed dependent two-sample t-test at a 95% confidence level ($\alpha = 0.10$). Calculated $p(t)$-values were 0.097 and 0.0003, respectively. Table 4 shows that precision values for the LLOQ of 17-β-estradiol are not within the criteria set by Shah et al. [16]. However, due to a greater inherent imprecision of receptor assays in comparison

to chromatographic assays (especially at the LLOQ), the calculated values may be accepted [21]. In order to monitor the imprecision of the developed receptor assays, quality control samples were implemented. The proposed acceptance criteria that 67% of the measured QC samples should have an accuracy between 75 and 125% [16], were readily met (75% were accepted, $N = 24$).

3.5. Freeze-thaw stability of the estrogen receptor

The experiments to investigate the stability of the estrogen receptor after three freeze-thaw cycles (2 h at −80 °C and 10 min at ambient temperature after thawing at 20 ± 2 °C) showed a 10% decrease in specific binding for the of hRec ER-α, and a 63% decrease in specific binding for the hRec ER-β. In both cases the nonspecific binding was higher than >25% (relative to the total binding) and therefore unacceptable for further use, from which can be concluded that, repeated freezing and thawing (especially for the hRec ER-β) should be avoided.

3.6. Long-term stability of the estrogen receptor

In order to determine the stability of the hRec ER-α and hRec ER-β the specific binding (relative to the total binding) of the FL was determined during 6 months after arrival and stored at three different temperatures: $+5 \pm 1$, $−20 \pm 2$ and $−80 \pm 3$ °C. In general, a non-specific binding of <25% (relative to the total binding) is acceptable for our assays.

Fig. 4 shows that the hRec ER-α retained its high binding capacity for at least 5 months when stored at $−80 \pm 3$ °C, approximately 10 days when stored at $−20 \pm 2$ °C, and approximately 3 days when stored at $+5 \pm 1$ °C. Long-term stability testing of the hRec ER-β gave comparable results (data not shown).
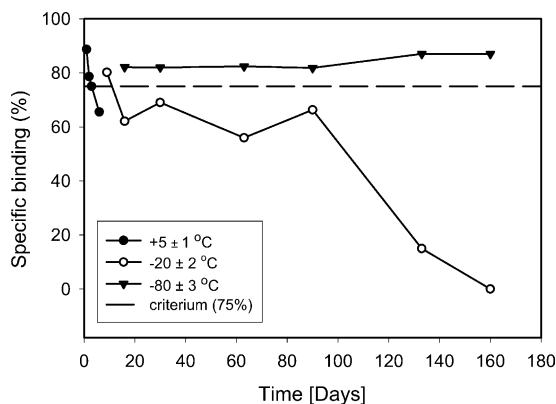
Table 4
Summary of LLOQ and ULOQ values for the hRec ER-β and hRec ER-α assay ($n = 10$)

Receptor subtype	LLOQ (nM)	Bias and R.S.D. (%)	ULOQ (nM)	Bias and R.S.D. (%)
hRec ER-α	0.2	16 and 42	5.0 ($n = 9$)[a]	14 and 14
hREc ER-β	0.5	0.8 and 39	10	15 and 19

[a] Data was subjected to the Grubbs test [20] for detection of outliers. Outliers were excluded at a two sided 95% confidence interval.

Fig. 4. Long-term stability of the hRec ER-α stored at $+5 \pm 1$, -20 ± 2 and $-80 \pm 3\,^{\circ}$C. Stability is measured as the specific binding of the fluorescent ligand to the receptor. Criterion for acceptance of the minimum specific binding in the assay is 75% (relative to the total binding) and is depicted as a dotted line in the figure.

3.7. Robustness and ruggedness testing

The robustness of an analytical method is defined as a measure of its capacity to remain unaffected by small but deliberate variations in method parameters [22]. Although robustness testing is not a part of the validation procedure mentioned in the Conference Report [16], it provides an indication of its reliability during normal usage. Furthermore, Hartmann et al. [23] indicated that considering the amount of time wasted for problem-solving during a routine application, robustness testing certainly has an impact for bioanalytical methods that are used over longer periods of time and/or in different laboratories. We have tested the robustness of the developed assays by deliberate variation of the fluorescent ligand concentration (1.0, 1.2 and 1.4 nM), the DTT concentration in the assay buffer (6, 8 and 10 mM) and the hRec ER concentration (2.5, 3.0, 3.5 nM for the ER-α and 2.0, 2.5 and 3.0 nM for the ER-β). For the hRec ER-α, ANOVA showed no significant difference between the calibration curves for the tested parameters at a 95% confidence interval ($P = 0.7927, 0.9708$, and 0.3725, respectively). For the hRec ER-β, ANOVA showed no significant difference between the calibration curves for the tested parameters at a 95% confidence interval ($P = 0.6413, 0.9732$, and 0.9032, respectively).

Ruggedness is expressed as the lack of influence on test results of operational and environmental vari-

ables of the method [22]. For instance, technician A—technician B variations or laboratory A—laboratory B variations (i.e. different HPLC systems with better or equal specifications). In order to obtain ruggedness information concerning the hRec ER-α assay an independent technician (technician A) performed the assay according to the standard operation procedure used in our laboratory. A two sided paired two sample t-test between the results of technician A in comparison to the average outcome of the validation-runs carried out by technician B, showed, at a 95% confidence level, no significant difference ($P = 0.2608$).

4. Conclusion

Two fully validated assays for the estrogen receptor subtypes alpha and beta using HPLC with fluorescence detection have been obtained. Both validated fluorescent receptor assays are highly sensitive (LLOQ of 200 and 500 pM, respectively), fast (assay time < 4 h and analyses < 4 min/run), precise (R.S.D. < 20%), accurate (bias < 15%), robust, rugged and stable for up to 6 months when stored at $-80\,^{\circ}$C. It has been shown that several ligands show different affinities for the receptor subtypes. The assays can be used to search for subtype selective (ant-)agonists for use in estrogen-linked pathologies and for monitoring of (xeno-)estrogens in food products and in the environment.

References

[1] F. Murad, J.A. Kuret, in: A. Goodman Gilman, et al. (Eds.), The Pharmacological Basis of Therapeutics, eighth ed., Permagon Press, Republic of Singapore, 1991, pp. 1384.

[2] C. Palmieri, G.J. Cheng, S. Saji, M. Zelada-Hedman, A. Warri, Z. Weihua, S. van Noorden, T. Wahlstrom, R.C. Coombes, M. Warner, J.-A. Gustafson, Endocrine Rel. Cancer. 9 (2002) 1–13.

[3] P. Walter, S. Green, G. Greene, A. Krust, J.M. Bornert, J.M. Jeltsch, A. Staub, E. Jensen, G. Scrace, M. Waterfield, P. Chambon, Proc. Natl. Acad. Sci. 88 (1985) 7889–7893.

[4] G.G.J.M. Kuiper, E. Enmark, M. Pelto-Huikko, S. Nilsson, J.A. Gustafson, Proc. Natl. Acad. Sci. 93 (1996) 5925–5930.

[5] M.J. Meyers, J. Sun, K.E. Carlson, G.A. Marriner, B.S. Katzenellenbogen, J.A. Katzenellenbogen, J. Med. Chem. 44 (2001) 4230–4251.

[6] S. Nilsson, G. Kuiper, J.-A. Gustafson, Trends Endocrinol. Metab. 9 (1998) 387–395.

[7] J.P. Sumpter, S. Jobling, Environ. Health Perspect. 103 (1995) 173–178.

[8] B. Hock, M. Seifert, Nat. Biotechnol. 17 (1999) 1162–1163.

[9] Dutch National Institute of Inland Water Management and Waste Water Treatment (RIZA) and the Dutch National Institute for Coastal and Marine Management (RIKZ), Estrogens and Xeno-estrogens in the Aquatic Environment of The Netherlands, RIZA/RIKZ-Report No. 2002.001, February 2002.

[10] M.J. Janssen, K. Ensing, R.A. de Zeeuw, PCT/NL96/00418; Eur. Patent 96 935 577.5 2120; US Patent 09/297.027 (1996).

[11] M.J. Janssen, K. Ensing, R.A. de Zeeuw, Anal. Chem. 73 (2001) 3168–3173.

[12] J. Smisterova, K. Ensing, R.A. de Zeeuw, J. Pharm. Biomed. Anal. 12 (1994) 723–745.

[13] N. Baindur, D.J. Triggle, Med. Res. Rev. 14 (1994) 591–664.

[14] N. Baindur, D.J. Triggle, Drug Develop. Res. 33 (1994) 373–398.

[15] J.C. McGrath, S. Arribas, C.J. Daly, Trends Pharmacol. Sci. 17 (1996) 393–399.

[16] V.P. Shah, K.K. Midha, J.W.A. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551–1557.

[17] G.G.J.M. Kuiper, B. carlsson, K. grandien, E. Enmark, J. Haggblad, S. Nilsson, J.-A. Gustafsson, Endocrinology 138 (1997) 863–870.

[18] U. Schopfer, Ph. Schoeffter, S.F. Bischoff, J. Nozulak, D. Feuerbach, Ph. Floerscheim, J. Med. Chem. 45 (2002) 1399–1401.

[19] A.B. Umetri, User's Guide to MODDE, Version 4.0, Umeå, Sweden, 1997.

[20] F.E. Grubbs, G. Beck, Technometrics 14 (1972) 847–854.

[21] J.W.A. Findlay, W.C. Smith, J.W. Lee, G.D. Nordblom, I. Das, B.S. DeSilva, M.N. Khan, R.R. Bowsher, J. Pharm. Biomed. Anal. 21 (2000) 1249–1273.

[22] United States Pharmacopeia, Section ⟨1225⟩, Validation of Compendial Methods, United States Pharmacopeial Convention, Rockville, 1995.

[23] C. Hartmann, J. Smeyers-Verbeke, D.L. Massart, R.D. McDowell, J. Pharm. Biomed. Anal. 17 (1998) 193–218.