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Logit-log evaluation of planar yeast estrogen screens

Dinah Schick, Wolfgang Schwack*

Institute of Food Chemistry, University of Hohenheim, Garbenstraße 28, D-70599 Stuttgart, Germany

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ABSTRACT

Receptor assays like the yeast estrogen screen (YES) performed in microtiter plates normally provide dose-response curves with a sigmoidal shape in semi-log plots. Such sigmoidal plots can be linearized by the logit function resulting in logit-log plots, as mainly known for the evaluation of enzyme-linked immunosorbent assays and radioimmunoassays. Since the planar yeast estrogen screen (pYES) represents the transfer of the receptor assay YES to high-performance thin-layer chromatography (HPTLC), it was assumed to obtain sigmoidal shaped dose-response curves from the measured signals, which subsequently could be used to generate logit-log plots. However, it was observed that typical sigmoidal curves were not obtained, when peak areas were plotted against the applied amount on a logarithmic scale (log amount). Therefore, peak heights were examined in the present study, which revealed proper dose-response curves when plotted against the log amount. The presence of sigmoidal dose-response curves from HPTLC-pYES made it possible to transform the signals into logits and, therefore, to create logit-log plots with linear correlations. The logit-log plots for the estrogen active compounds (EAC) 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) provided a working range up to 500 pg/zone. Applying logit-log plots, mean recovery rates for E2 and EE2 from spiked water samples (2–20 ng/L) were determined to 90% and 108%, respectively, with ≤24% RSD. Moreover, the linear graphs allowed an easy determination of the half maximal effect dose (ED_{50}) of EAC, since the intersection of the graph with the abscissa represents the ED_{50}. Additionally, with the knowledge of the ED_{50} values, the estrogenic potential of EAC in terms of estradiol equivalent factors (EEF) could be determined, resulting in 0.64 for EE2.

1. Introduction

The yeast estrogen screen (YES), introduced by Routledge and Sumpter, is a receptor assay performed in microtiter plates to determine estrogen active compounds (EAC) in terms of effect-directed analysis. For the assay, genetically modified yeast cells containing the human estrogen receptor (hER) and the *lacZ* reporter gene encoding for β-galactosidase were employed [1]. The hER is activated by binding EAC present in a sample, and subsequently the activated hER binds to an estrogen-responsive element causing transcriptions leading to the expression of the reporter gene that results in the production of the enzyme [1]. By enzymatic cleavage, the substrate chlorophenol red β-D-galactopyranoside releases chlorophenol red to be determined by absorbance as indirect signal of the estrogenic activity of EAC [1]. Such ligand-bindings are equilibrium processes that usually show saturation, observable as dose-response curves with a typical sigmoidal shape in semi-log plots. By plotting the YES absorbance signals against the respective concentrations on a logarithmic scale, sigmoidal dose-response curves were obtained, as exemplarily shown by Routledge and Sumpter or van den Belt et al. [1,2].

The transfer of the YES assay to high-performance thin-layer chromatography (HPTLC-planar yeast estrogen screen, HPTLC-pYES) was already shown and provides the great chance to chromatographically separate individual EAC before pYES is performed directly on the HPTLC plate [3–6]. However, when HPTLC-pYES signals expressed as peak areas were plotted against the respectively applied amounts of EAC on a logarithmic scale, typical sigmoidal curves generally were not obtained. Schönborn and Grimmer reported increasing zones and signal intensities, when increasing amounts of EAC were applied, but showed the dose-response relationship for 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) only in a range of 0.5–25 pg/zone, resulting in linear graphs [3]. The dose-response curve for EE2 in a range of 0.3–100 pg/zone was shown by Spira et al., when peak areas were plotted against the applied amount on a log scale, but no real sigmoidal curve was observable [4]. However, by applying a four parametric logistic function curve-fitting model to the data, values for the half maximal effect dose (ED_{50}) were obtained [4]. Results were given in half maximal response instead of half maximal effect

* Corresponding author.
E-mail address: wolfgang.schwack@uni-hohenheim.de (W. Schwack).

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concentrations (EC_{50}), thus as the ratio of the amounts applied [4]. First, Klingelhöfer and Morlock showed a reliable and profound investigation of E2 equivalency results based on dose-response curves (showing 4 repetitions each) for six different EAC in different ranges using 4-methylumbelliferyl-β-D-galactopyranoside as substrate [5], well-suited also for quantitation of EAC in complex samples [7,8].

The presence of sigmoidal curves with upper and lower asymptotes provides the opportunity to generate logit-log plots as linear calibration curves. The transformation of sigmoidal curves to linear graphs by applying the logit function is mainly used for radioimmunoassays [9–11], and is also known from enzyme-linked immunosorbent assays [12], both performed in cuvettes or microtiter plates. Alternatively, log-log plots or four parameter logistic-log functions were also used [13]. The aim of the present study was to obtain proper dose-response curves from the signals of a recently developed HPTLC-pYES with the substrate resorufin-β-D-galactopyranoside (RGP) [6] and subsequently to apply the logit-log method. The applied HPTLC-pYES utilized the recombinant yeast strain *Saccharomyces cerevisiae* generated by McDonnell et al. [14,15], which is modified with a DNA sequence of the hER and the *lacZ* reporter gene that encodes for β-galactosidase. Enzymatic cleavage of the suitable substrate RGP releasing resorufin indicates estrogenic activity as orange fluorescent HPTLC zones [6]. Since plotting peak areas of the signals of a fluorescence scan against the amount per zone on a log scale did not result in curves with a sigmoidal shape, the peak height as signal of intensity was examined, which indeed showed saturation curves for both E2 and EE2. Hence, it was possible to demonstrate the transfer of the logit-log procedure to HPTLC-pYES, resulting in linear calibration graphs. The applicability of logit-log plots as evaluation tool in HPTLC-pYES was tested with water samples spiked with E2 and EE2, and the obtained results were compared to the results from the former publication [6]. Moreover, the presence of sigmoidal dose-response curves enabled the determination of values for the half maximal effect dose (ED_{50}), on the one hand as the point of inflection of the curve from the sigmoidal curve itself, on the other hand simply from the intersection of the linear graph of a logit-log plot with the abscissa. Thus, it was possible to determine ED_{50} values for E2 and EE2, and, therefore, the estrogenic potential of EE2 in terms of the estradiol equivalent factor (EEF).

2. Materials and methods

2.1. Chemicals and materials

HPTLC glass plates silica gel 60 RP–18 W (200 mm × 100 mm, layer thickness 200 μm), copper(II) sulfate pentahydrate (p.a.), disodium hydrogen phosphate (p.a.), L-histidine (≥99%), hydrochloric acid (37%), potassium chloride (>99%), potassium dihydrogen phosphate (p.a.) and sodium hydroxide solution (20%) were from Merck (Darmstadt, Germany). The following substances for agar plates were from Becton Dickinson (Heidelberg, Germany): yeast nitrogen base without amino acids (YNB), casamino acids [16], adenine and agar. YNB for culture media, ethanol (≥99.8%), methanol (≥99.9%), *t*-butyl methyl ether (TBME, ≥99.8%), 17β-estradiol (E2, ≥98%) and 17α-ethinylestradiol (EE2, ≥98%) were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium hydrogen carbonate and D-Glucose (anhydrous) were from BDH Prolabo Chemicals (VWR, Bruchsal, Germany). Ethyl acetate (for pesticide residue analysis), *n*-hexane (for pesticide residue analysis), L-lysine (≥98%) and magnesium sulfate heptahydrate (puriss. p.a.) were obtained from Fluka-Sigma-Aldrich (Steinheim, Germany). The substrate resorufin-β-D-galactopyranoside (RGP) was from Santa Cruz biotechnology (Dallas, USA) and dimethyl sulfoxide (DMSO, 99.98%) from Fisher Scientific (Schwerte, Germany). Acetone (≥99.8%) and sodium chloride (≥99%) were from Carl Roth (Karlsruhe, Germany). Ultrapure water was prepared using a Synergy System (Millipore, Schwalbach, Germany). For pYES, yeasts of the strain *Saccharomyces cerevisiae* BJ3505 (protease deficient, MATα, PEP4:HIS3, prb1-Δ 1.6R, HIS3-Δ200, lys2-801, trp1-Δ 101, ura3-52gal2can1) were used. The yeast strain was generated by McDonnell et al. [14,15] and the yeasts were stored in a cryo-vial at −70 °C. As optical filter for the TLC Scanner 4 (CAMAG, Muttenz, Switzerland), orange filter glass O 580 from HEBO (Aalen, Germany) was cut to shape.

2.2. Solutions, media and agars

Standard solutions of E2 and EE2 in concentrations of 1 μg/L, 10 μg/L and 100 μg/L were prepared by respectively diluting the stock solutions (1 mg/L). The solvent for standard solutions as well as stock solutions was ethanol. Growth medium was composed as described in [6] and contained 6.8 g/L YNB, 1 g/L glucose, 170 mg/L L-lysine and 100 mg/L L-histidine. Test medium was growth medium plus 112 μM $CuSO_4$. Plate agar was composed of YNB (6.7 g/L), casamino acids (10 g/L), adenine (50 mg/L) and agar (20 g/L) [6]. A stock solution of RGP (20 mg/mL in DMSO) was diluted in phosphate buffer (40.8 g/L KH_2PO_4, 42.6 g/L Na_2HPO_4, 1.2 g/L $MgSO_4{\cdot}7H_2O$, 3.7 g/L KCl, adjusted to pH 7 with 20% sodium hydroxide solution) to a concentration of 0.1 mg/mL to obtain the substrate dipping solution for pYES [6]. Stock solutions were stored at −20 °C.

2.3. Cultivation of yeast cells for pYES

The cultivation of the yeasts was performed as described in [6]. Briefly, overnight cultures were prepared by inoculation of growth medium and incubation for ≥18 h. Test cultures for pYES with a cell number of 6–8 × 10^7 cells/mL were obtained by centrifugation of the required volume of overnight cultures and re-suspending the cell pellets in test medium.

2.4. Planar yeast estrogen screen (pYES)

The pYES was executed on HPTLC glass plates silica gel 60 RP–18 W prewashed with acetone/water (9/1, v/v). The pH value of the plates was adjusted to about 6.5 with a solution of sodium hydrogen carbonate (25 g/L, pH 6.4) as described in [6]. Sample applications were performed with an Automatic TLC Sampler 4 (ATS4, CAMAG, Muttenz, Switzerland) on 10 cm × 10 cm HPTLC plates (10 mm from the lower edge, 10 mm from the left side, track distance set to automatic). To examine the relationship between dose and response, different volumes of the standard solutions were applied onto 10 tracks as 5-mm bands resulting in amounts of 3, 4, 5, 6, 10, 50, 100, 200, 500 and 1000 pg per zone for both E2 and EE2. Extracts of water samples (50 μL) were applied as 5 mm × 10 mm areas and standard solutions of E2 and EE2 for calibration as 5-mm bands with amounts of 3, 5, 10, 50, 100, 200 and 500 pg per zone [6]. After application, pYES was performed as described in [6]. Briefly, the separation of the analytes was achieved by chromatography, followed by yeast incubation (6–8 × 10^7 cells/mL, 30 °C, 4 h), substrate incubation (0.1 mg RGP/mL 0.6 M phosphate buffer, pH 7, 37 °C, 3 × 30 min), and documentation of the HPTLC plate. The plates were documented with a TLC Visualizer (CAMAG) under UV 254 nm, UV 366 nm and under white light illumination, and scanned with a TLC Scanner 4 (CAMAG) in fluorescence mode at 550/ > 580 nm (tungsten lamp). TLC instruments were controlled by the software winCATS, version 1.4.6 (CAMAG).