CONSIDERATIONS ON THE WORKING RANGE IN BIOASSAY DOSE-RESPONSE CURVES: CURVE FIT AND ASSAY BACKGROUND RESPONSE

Haedrich J[1]*, Stumpf C[1], Podestat U[1], Kotz A[1], Wahl K[1] and Malisch R[1]

[1]European Union Reference Laboratory for Dioxins and PCBs in Feed and Food, Freiburg, Germany

Introduction

Bioanalytical response data follow the shape of a hyperbolic receptor-binding curve, although the intracellular mechanisms involved from formation of the aromatic hydrocarbon receptor (AhR)-ligand complex to luciferase gene expression are manifold and complex. When plotted on a semi-log scale to accommodate a wide range of concentrations until saturation, data show a sigmoidal relationship to concentration. Typical calibration curves are therefore sigmoidal in shape, for which the 4-parameter logistic function providing an accurate depiction of the relationship between response (expressed in relative light units, RLUs) and analyte concentration is generally considered the best suitable model. Within the scope of this work, Hill's equation[1,2] being mathematically analogous to the logistic equation is fitted to the response data. It defines a response as a function of the minimum response (d), the maximum response (a), the concentration required to evoke a response half-way between the minimum and maximum (c, being EC_{50}), and a parameter (b) that describes the steepness of the curve. If the unspecific assay background response is subtracted from the response data obtained from a calibration standard dilution series, d becomes zero, resulting in a simplified 3-parameter Hill equation.

$$y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^b} \qquad y = \frac{a}{1 + \left(\frac{x}{c}\right)^b}$$

4-parameter Hill equation 3-parameter Hill equation

This study suggests criteria for assessing the quality of calibration curves and requirements for assay working ranges in bioanalytical screening of feed and food samples, especially at low levels of contamination.

Materials and methods

Fitting dose-response curves by minimizing the sum of squared residuals

The calibration curve is usually fit to concentration-response data pairs by minimizing the sum of squared residuals (SSR). Model curve parameters are optimized in an iterative process to achieve minimal bias of the calibrator concentrations over the maximum usable calibration range. SSR regression, however, assumes that the variance is the same for all values of x (homoscedastic data), which is in reality rarely the case for bioanalytical response data. As a result, low-quality data points exhibiting a higher variation, generally found in the upper part of dose-response curves, influence the fit to the same extent as high-quality data points typically located in the lower part of the curves. Although after performing SSR regression the coefficient of determination (R^2) frequently suggests a perfect fit, this may not be the case in the low concentration range where SSR regression is often far from ideal. R^2 has thus recently been discarded as a quality-of-fit criterion from EU legislation[3,4].

Weighted sum of squared residuals regression

The noise (standard deviation or variance) of bioanalytical response data generally increases with the response (heteroscedastic data). The quality of the curve fit may therefore be improved if heteroscedasticity is taken into account. This can be done by placing less "weight" on responses exhibiting higher variation. Weighted sum of squared residuals (WSSR) regression reflects that the variance of the response data is a function of the magnitude of the response:

$$WSSR = \sum_{i=1}^{N} w_i \left[y_i - \hat{y}_i \right]^2$$

As weighing factor w_i, the inverse variance $w_i = 1/\text{variance}\ (y_i)$ of replicate response data at each concentration i may be used, y_i being the observed standard response, while $\hat{y}_i$ = response predicted by the curve model.
Calculations on response data sets generated from series of 2,3,7,8-TCDD standard dilutions were performed in custom-tailored Microsoft Excel® data sheets using the "Solver" add-on provided by Microsoft Excel®.

Chemically activated luciferase gene expression (CALUX) assay

Five recombinant rat and mouse hepatoma cell lines stably transfected with dioxin (AhR)-responsive firefly luciferase reporter genes were included in this study. Three cell lines contained four, and two cell lines contained 20 dioxin responsive elements (DREs): H4IIe rat hepatoma cells (4 DREs) were obtained from Biodetection Systems (BDS), Amsterdam (NL). H4L1.1c4 rat and H1L6.1c2 mouse hepatoma cells (both 4 DREs), and new "3rd generation" H1L7.5c3 mouse and H4L7.5c2 rat hepatoma cells (both 20 DREs)[5] were obtained from MS Denison, Department of Environmental Toxicology, University of California Davis, Davis (USA).
Exposure of these cells to dioxins and other AhR-active dioxin-like compounds results in induction of luciferase expression in a dose-, AhR-, and chemical-specific manner. The level of reporter gene expression correlates with the overall concentration of the Ah inducers present[6]. Individual concentrations of 2,3,7,8-TCDD standard dilution series were added to incubation medium and the mixtures subsequently added in triplicate to 96-well plates, each containing the CALUX mouse or rat hepatoma cells. Cells were exposed for 24 or 48 h, followed by lysis of the cells and measurement of luciferase activity in a Berthold Centro LB 960 microplate luminometer.

Results and discussion

Assessing the quality of the fit: bias and imprecision of back-fitted calibrator concentrations

A sound calibration curve is a prerequisite to sound assay performance. Special attention should therefore be paid to the quality of the fit, especially in the designated working range, to keep lack-of fit errors as small as possible. Key metric for evaluating suitability of the curve is the extent of agreement of nominal calibrator concentrations with back-calculated concentrations read from the fitted curve[7]. These predicted concentrations can be expressed as recovery (or as bias) at each concentration level. Although bias and imprecision associated with the calibrators will underestimate the true bias and imprecision when analyzing samples, they are a good front-line check of whether given requirements are met[7].
Both SSR and WSSR regression were applied to the concentration-dependent response data, to fit the simplified 3 parameter Hill equation. Results from SSR and from WSSR regression are displayed in figure 1, showing relative bias plots of back-fitted calibrator concentrations read off exemplary dose-response curves obtained after exposure of various CALUX cell lines to TCDD dilution series. Dotted lines indicate a maximum acceptable bias of 15% as suggested by the authors, from which bias-based assay working ranges were derived (table 1).
WSSR regression aligns the calibration curve more closely to data of low variation in all cell lines evaluated. The resulting improved fit exhibiting smaller calibrator bias values especially in the lower part of the curves considerably reduces the risk for over- and especially for underestimation of BEQ results in unknown feed and food samples for which low maximum levels have been set by EU law[8,9]. In each cell line, response-based triplicate coefficients of variation (RLU-CVs) were below 10% for each calibrator thus meeting current legal EU requirements[3,4] (CV<15%), spanning a purely RLU-CV based assay working range all along each of the curves (table 1). Although triplicate response data from the same TCDD standard are statistically dependent, calibrator imprecision (or precision) expressed as concentration-based CVs may be assessed, as optionally suggested by the new EU criteria[3,4]: instead of averaging triplicate RLUs before conversion, each individual RLU value is converted to a concentration. In the cell lines investigated, these concentration-based CVs often tend to explode at the lower and upper ends of dose-response curves yielding smaller assay working ranges if the CV<15% criterion is applied (table 1).
Results in table 1 and bias plots in figure 1 show that WSSR regression provides a better curve fit than SSR regression, leading to broader assay working ranges if a maximum bias of 15% is to be observed, extending towards lower concentrations. Lower endpoints of working ranges established under observance of the suggested bias-restriction were between 1 pmol/L for H4IIe cells and as low as 0.1 pmol/L for H4L7.5c2 cells. Under the conditions applied at EU-RL, the legal requirement for tolerable imprecision (RLU-CVs<15%) was easily met in each cell line from the lowest calibrator (0.1 or 0.3 pmol/L) all the way up to the highest. The more restrictive (but only optional) EU criterion for concentration-CVs not to exceed 15%, however, shifts lower ends of the working ranges somewhat up to the next higher calibrator, in most of the cell lines.

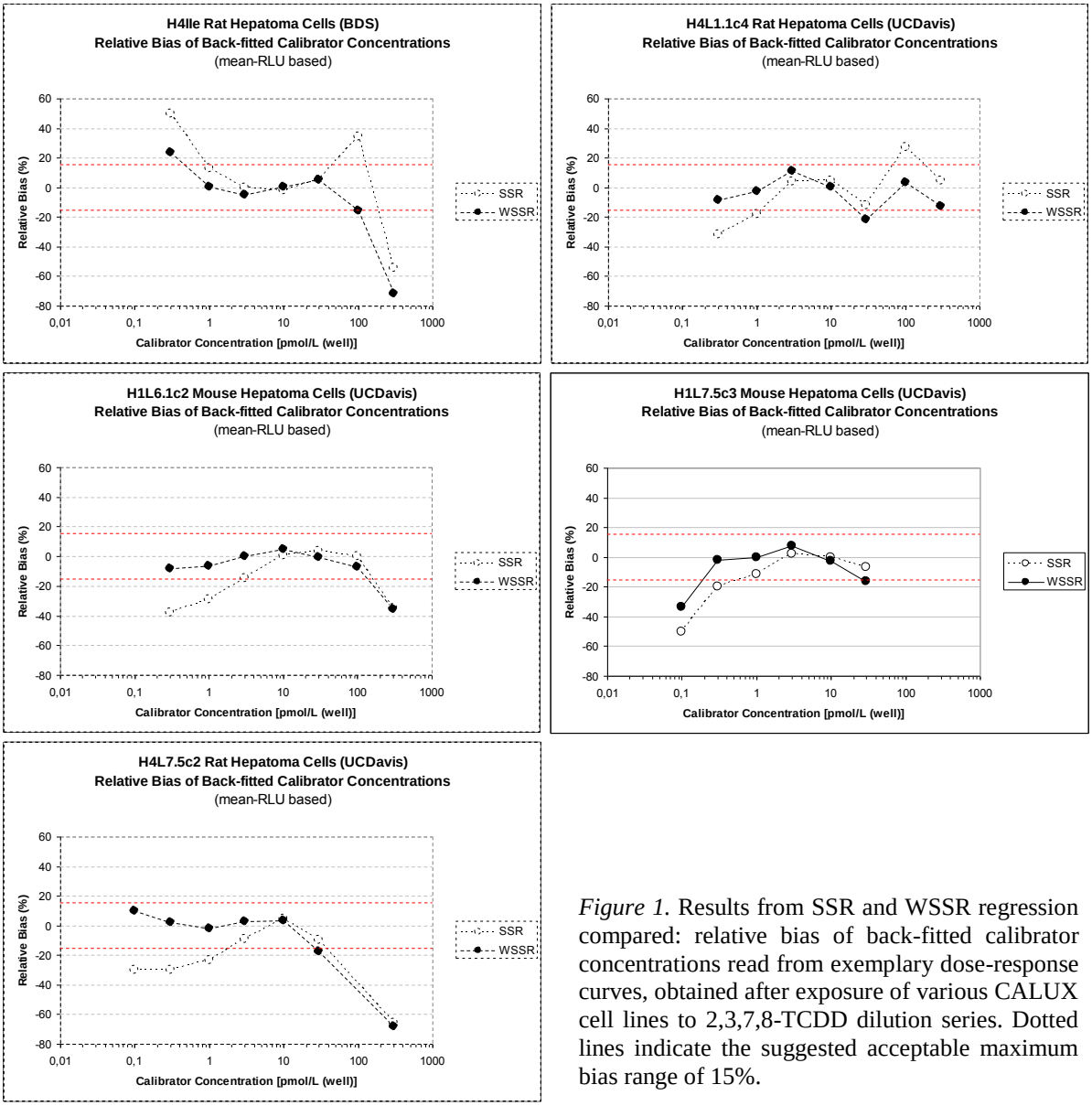

Figure 1. Results from SSR and WSSR regression compared: relative bias of back-fitted calibrator concentrations read from exemplary dose-response curves, obtained after exposure of various CALUX cell lines to 2,3,7,8-TCDD dilution series. Dotted lines indicate the suggested acceptable maximum bias range of 15%.

Table 1. Suitability of dose-response curves: Assay working ranges for various cell lines after SSR and WSSR regression, based on the following criteria: RLU-CVs <15% (required by EU law), concentration-based CVs <15% (optional); requirements suggested by the authors: bias of back-fitted concentrations <15%, unspecific assay background contribution to results < 50%. Assay concentrations are in pmol/L.

Criterion	Regression	H4IIe Rat	H4L1.1c4 Rat	H1L6.1c2 Mouse	H1L7.5c3 Mouse	H4L7.5c2 Rat
RLU-based CVs <15%	SSR/WSSR	0.3 - 300	0.3 - 300	0.3 - 300	0.1 - 300	0.1 - 300
Conc.-based CVs <15%	SSR/WSSR	0.3 - 30	1.0 - 30	1.0 - 100	0.3 - 10	0.3 - 30
Calibrator bias <15%	SSR	1.0 - 30	1.0 - 30	3.0 - 100	0.3 - 30	3.0 - 30
	WSSR	1.0 - 100	0.3 - 30	0.3 - 100	0.3 - 30	0.1 - 10
Background contribution <50%	WSSR	1.8 - 300	0.8 - 300	1.5 - 300	1.4 - 300	0.5 - 300

In the dose-response curve obtained from H4L7.5c2 rat hepatoma cells, the EU legal requirement of RLU-CV<15% is met within a range of 0.1-300 pmol/L. All requirements for imprecision and bias are met in a range of 0.3-10 pmol/L, suggesting H4L7.5c2 cells containing 20 DREs in principle most suitable for screening samples with low levels of contamination. This is remarkable because for CALUX cell lines with 4DREs, the lower end of working ranges (previously: "LOQ") is considered to be close to 1 pmol/L.

Unspecific assay background contribution to results

While absolute induction is driven by the efficiency of gene expression, fold induction is the ratio between the response induced by a calibration standard, or by AhR-active dioxin-like compounds present in a sample extract, and the unspecific assay background response. The latter's contribution to results should be reproducible and well controlled. In some CALUX cell lines, however, it may be as high as 70% (fold induction as low as 1,4) at 1 pmol/L. In figure 2a, fold induction is plotted vs calibrator concentrations in the lower range of dose-response curves of various CALUX cell lines. The authors suggest that besides restrictions to imprecision and bias in assay working ranges, relative background contribution to results should be limited to 50%. Based on this requirement, lower ends of assay working ranges of the cells investigated in this study are e.g. 1.8 pmol/L for H4IIe rat cells and 0.5 pmol/L for H4L7.5c2 rat cells (figure 2b and table 1).

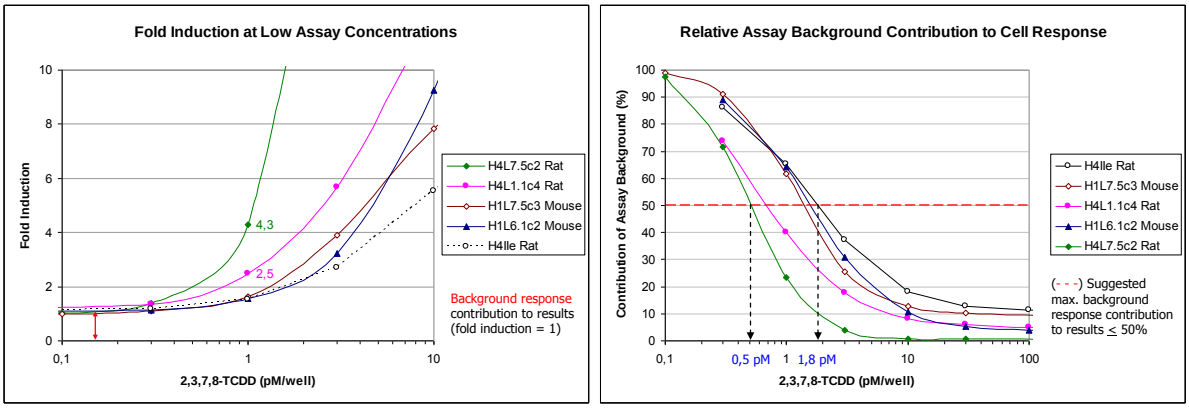

Figure 2a. Fold induction at low assay concentrations; various CALUX cell lines. *2b.* Relative assay background contribution to cell response at low assay concentrations: a maximum relative background contribution of 50% is proposed as a further requirement for setting lower ends of assay working ranges.

Acknowledgements

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