MODEL FOR INTERPRETATION OF COUPLED DATA FROM BIOTESTS BASED ON THE DIOXIN-LIKE RESPONSE OF AH-RECEPTORS

Thiem I, Böhmler G

Lower Saxony State Office for Consumer Protection and Food Safety, Food Institute Braunschweig (LAVES LI-BS); Dresdenstraße 2+6, 38124 Braunschweig, Germany

Introduction

Considering official food control one of the most intriguing functions of the cellular Ah-receptor is its response to dioxins and dioxin-like (dl) compounds. Several test systems described by numerous authors[1,2,3] use the biological concept. The European Comission[4] defined minimum criteria for dioxin analysis including screening with biological systems. Using the EROD-Micro-Bioassay we established and optimized a mathematical protocol to interpret multiple data points of a sample with no complete sample curve and thus no EC50 to compare with the standard curve. The results represent a larger pool of data compared to a previous publication.[5]

Materials and methods

Material

Following samples with GC/MS results for PCDD/Fs and partially for dl PCBs were tested: Beef (78), bovine liver (40), pork (1), meat (16) and liver (22) of sheep, fish (1), fish oil (3), mixtures of fish oil (7), and mixtures of bovine fat (4). One beef and the fish were obtained from NIH (Norway), one fish oil was courtesy of EURL Dioxins and PCBs (Freiburg) all other samples were measured in routine analysis of LAVES (Food Institute Oldenburg).

2,3,7,8-TCDD for standard curves and spiking was from LGC-Standards. Pesti grade solvents, Na_2SO_4 and H_2SO_4 (95%) were purchased from VWR International. Silica 60 from Applichem was used. Substrates for bioanalysis (Resorufin ethyl ether and 3,3′-Methylenebis(4-hydroxycoumarin)), BCA test kit and DMSO (cell culture tested) were from Sigma-Aldrich. All glass material and some chemicals were heated for eight hours (glass and silica 300°C; Na_2SO_4 600°C).

Reuber rat hepatoma cells (H4IIE) were courtesy of Prof. Schramm (Helmholtz Institute Neuherberg). They were cultured in DMEM with Phenolred (Biochrom), 10% FCS (Invitrogen) at 37°C, 95% humidity, 7% CO_2.

Chemical Methods

Each sample was split into two sub samples with 1g or 4g fat each. One sub sample was spiked with 20pg 2,3,7,8-TCDD, apart from 6 sub samples which were spiked with smaller TCDD concentrations. Tissue fat was extracted 2 hours after mixture with Na_2SO_4 using 300ml dichloromethane/cyclohexane 1/1 (v/v). 1g fat was cleaned up following Gizzi[6] with minor alterations: Silica column (top to bottom: 3g Na_2SO_4; 5g 22w% sulphuric acid on silica; 5g 33w% sulphuric acid on silica) eluted with 3x 2ml + 34ml n-hexane. The clean up of 4g fat was performed on a larger scale[7]. Dichloromethan was used for the transfer of the reduced extracts into glass vials with 10µl DMSO as keeper. The sample stem was prepared from the reduced extract by adding 10µl DMSO/isopropanol (3+2) and 1980µl DMEM aseptically. Then it was shaken (600rpm; 20°C, >10min).

Bioanalytical Methods

The EROD-Bioassay[8,9] was performed with H4IIE cells in 96-well plates (10000 cells in 50µl DMEM/well). 50µl sample solution and controls were added two or more hours after seeding. The final solvent concentration in DMEM was 0.5% DMSO/isopropanol 4/1 v/v. Each plate contained a full 2,3,7,8-TCDD standard curve (0.5; 0.4; 0.2; 0.12; 0.06; 0.03; 0.015; 0pg/well). All concentrations were measured fourfold. To cover a broad range of possible results the stem, ½ and ¼ of the stem concentration were tested. Further 59 sample extracts were tested at 6 concentrations as described later. After 72h incubation at culture conditions the medium was removed. Substrate incubation in DMEM with Ethoxyresorufin (30min) and transfer of 80µl to 178µl ethanol is described elsewhere[7,8]. Fluorescence was measured at 530nm for excitation and 590nm for emission. A protein test (BCA) ensured no cytotoxicity was left undetected.

Evaluation

A sample result is calculated by comparing the data to the standard curve. Is a full sample curve available the ratio of EC50s is determined[10]. Other authors[11] use only one point of several that fits best into the so called "linear" part of the TCDD curve. The European Comission[5] demands testing of at least 3 different dilutions but it does not state how a "good" fit to the standard curve is determined. There methods to calculate the limit of detection are based on the solvent blank or background response and added by 3x or 5x the standard deviation.

Using 3 and 6 dilutions respectively no full sample curve was intended. But instead of choosing only one of the dilutions we use as many as possible. In addition to strict values, the individual working range for a sample depends on its procedure blank[12] and the relation of the single dilution results to each other. Sample results were compared to the respective 2,3,7,8-TCDD standard curve (optimised by 4-parameter fit) and expressed in bioequivalents (BEQ).

To allow a data point into the analysis following premises must be fulfilled (s. figure 1):
- $CV_{Response}$ of solution triplicates <15% (from four wells one is dismissible)
- Standard curve with ≥ 5 valid concentrations and R^2 >95%
- ≥ 80% of the upper asymptote must be reached by one of the two high standards
- Sample points > Procedure Blank +3s

Starting at the lowest permitted data point sample dilutions are considered if their concentration lays within ±10% relative to the start concentration (10%-criterion). All permitted data points of a sample are coupled by their dilution relative to the X-axis. Now the least square of residues is calculated for the sample to fit the point(s) to the standard curve (s. figure 2). The resulting data of the original sub sample was corrected by the recovery of the spiking solution.

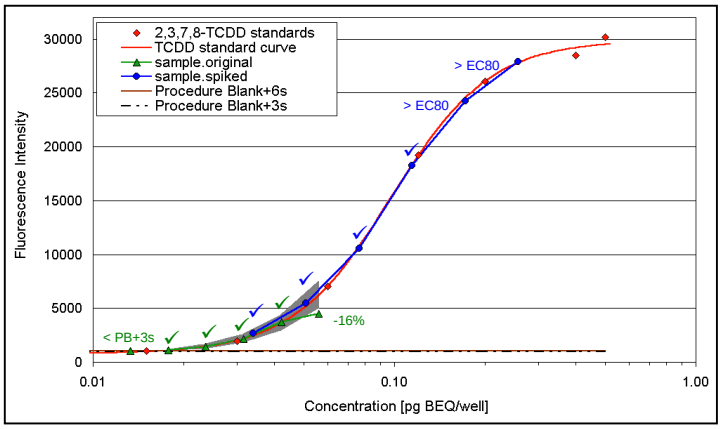

Figure 1: Data plot to specify evaluation criteria. All triplicates show $CV_{Response}$ < 8%, the standard curve meets all criteria. Four dilutions per sub sample are within the working range. Hooks indicate accepted dilutions of the original (✓) and spiked (✓) sub sample. For the original sub sample the 10%-criterion is shown as a grey band. One point is <Procedure Blank +3s (PB +3s), another does not meet the 10%-criterion (-16%). In this example all dilutions of the spiked sub sample meet the 10%-criterion, thus points above EC80 (>EC80) did not change the final result.

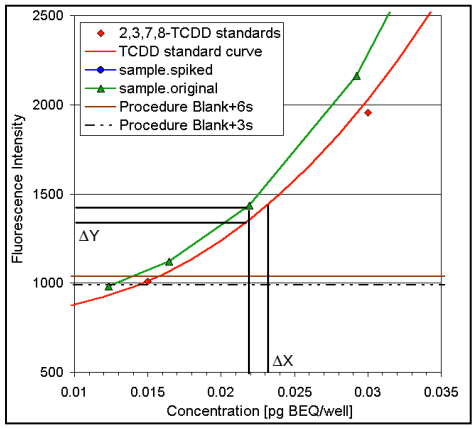

Figure 2: Two concepts for fitting the sample points onto the standard curve. Least squares of residues can either be calculated to minimize the distance at the X-axis (ΔX) or that at the Y-axis (ΔY). All accepted points are included with their data are coupled by the sample dilutions. This means that the ratio between the points regarding the X-axis stays constant.

Results and discussion:
The analysis of samples with 3 test points showed that most samples produced at least one data point inside the working range. Sample points within the upper or lower asymptote of the standard curve showed GC/MS results clearly above or below the decision limits respectively.
In a further approach a sample result should be represented by more than one concentration. A good coverage of possible concentrations was achieved by diluting the stem to 3/4 (original sub sample) and 2/3 (spiked sub sample) of the preceding concentration. No significant difference could be detected using least square deviation in X-direction or in Y-direction to fit the coupled data to the standard curve (see figure 3).

Sample points with a high response (e.g. >EC80) often show higher CVs in concentration results due to their asymptotic position while the CV of the fluorescence response is <15% and/or they do not fulfil the 10%-criterion relative to the lowest permitted sample concentration. The mean number of analysed dilutions was 2.31 and 2.94 for original and spiked sub samples respectively (from 48 and 50 sub samples with six dilutions each). 31% of the original samples but 18% of the spiked sub samples were represented by only one dilution.

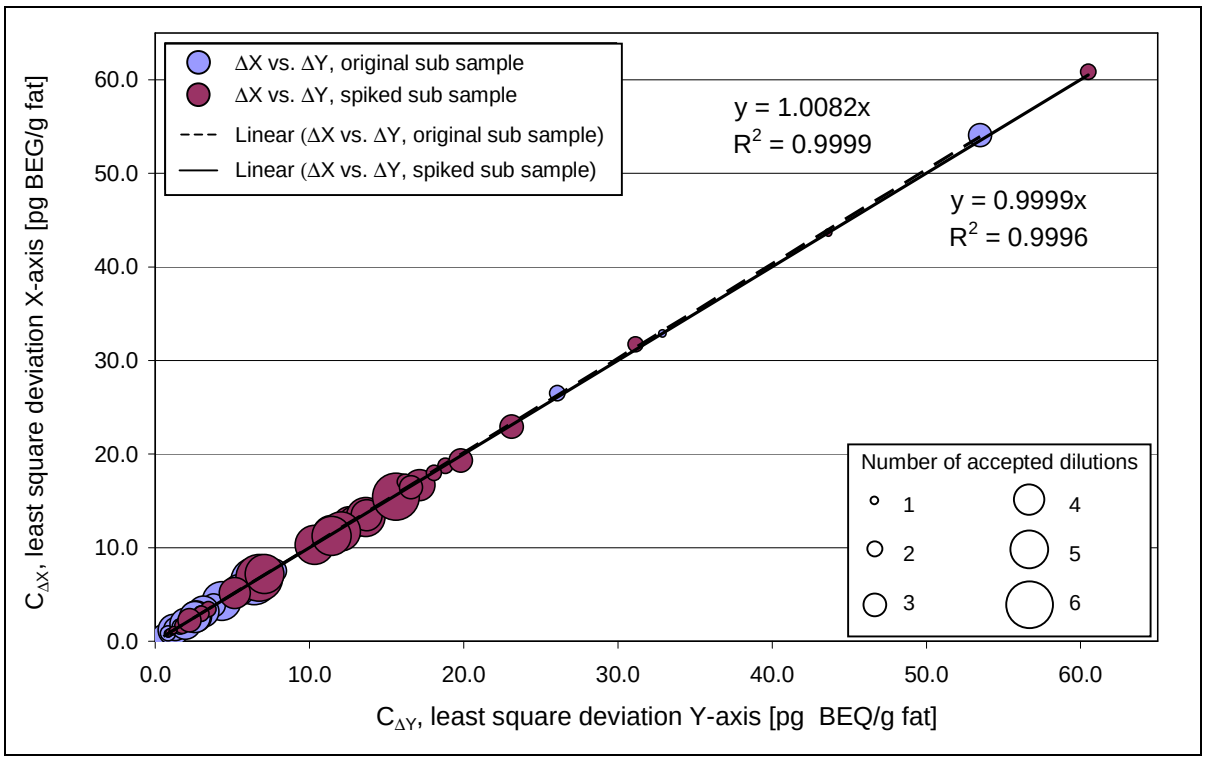

Figure 3: Comparison of the concepts for fitting sample data to the standard curve. The number of accepted dilutions per sample is indicated by the diameter of the data point. No bias could be detected. Calculation of the least square for the X- or Y-axis delivered almost identical results for all data within the working area. No relevant differences between spiked and original sub samples were observed.

The chosen dilutions combined the advantage of a broad range of possible results (as it was successfully used for three concentrations per sample before) and the charm of more data points around the interesting concentration. Linking data points leads to more robust results if required The 10%-criterion relative to the lowest permitted concentration is a useful tool to eliminate data points of sample constituents which result in curves with lower asymptotes and different slopes relative to 2,3,7,8-TCDD. As shown during the first test[5] no asymptote related bias between results from least square deviations of X- and Y-axis respectively was observed. Within the lower asymptote data were often reduced to a single point by using the Procedure Blank +3s as cut off. Sample points in the upper asymptote are usually eliminated by the 10%-criterion or an irregular standard deviation.

With only one point left the result depends merely on the parameters of the standard curve. In many cases points beyond Procedure Blank +3s or EC80 would fit the 10% criterion. For the latter case the inclusion of the data may be acceptable. In the other a risk of false positive artefacts would occur.

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References:

1. Hilscherowa K, Machala M, Kannan K, Blankenship A L, Giesy J P (2000); *Environ. Sci. Pollut. Res.*, 7 (3): 159-171
2. Whyte J J, Schmitt C J, Tillitt D E (2004); *Critical Reviews in Toxicology*, 34 (1): 1-83
3. Engwall M, Lindström G, Bavel B v (2003); *Man Technology Environment Research Centre, Dept. of Natural Sciences, Örebro University*; ISBN 91-7668-339-7: Appendix C
4. Commission Regulation (EC) No 1883/2006
5. Thiem I, Böhmler G (2010); *5th Symposium GRK 1427: New Insights in Ah-Receptor Function and Modulation by Food Constituents*; Oct. 7-8 Düsseldorf
6. Gizzi G, Hoogenboom LAP, von Holst C, Rose M, Anklam E (2005); *Food Additives and Contaminants* 22(5): 472-481
7. Thiem I, Böhmler G, Borowski U (2006); *J. Verbr. Lebensm.* 1: 310–316
8. Schwirzer S (1998); Dissertation, Technische Universität München, Germany
9. Schwirzer S M G, Hofmaier AM., Kettrup A, Nerdinger PE, Schramm K-W, Thoma H, Wegenke M, Wiebel FJ (1998); *Ecotox. & Environ. Safety* (41): 77-82.
10. Doods K (2003); Dissertation, Technische Universität München, Germany
11. Hanberg A, Ståhlberg M, Georgellis A, Wit Cd, Ahlborg A G (1991); *Pharmacology & Toxycology*, 69: 442-449
12. Haedrich J, Eppe G, Goeyens L, Hoogenboom R, Malagocki P, Scippo M, Vanderperren H, Windal I, Denison M, Stumpf C, Kotz A, Malisch R (2010); *30th International Symposium on Halogenated Persistent Organic Pollutants (POPs)*; Sept 12-17 San Antonio, TXs