Application of an Ah receptor-based competitive binding assay to the analysis of dioxin-like compounds in environmental matrices

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Introduction

The cost and labour of determining the TCDD Equivalent Concentration (TEQ) of dioxin-like compounds in environmental matrices makes it impractical to mount extensive screening or environmental monitoring programs for these substances. After spiking with an isotopically labelled dioxin surrogate such as [$^{13}C_{12}$]-TCDD as a recovery standard, conventional analysis involves solvent extraction, multiple chromatographic procedures to isolate the PCDD/PCDF fraction, and quantitation of the individual PCDD and PCDF congeners using capillary GC/high resolution MS. The TEQ of the sample is obtained by applying Toxic Equivalency Factors (TEFs)[1] for the 17 "toxic" PCDD and PCDF congeners which are chlorinated in the 2,3,7 and 8 positions. The TEQ might be underestimated because the I-TEF scheme omits other substances which exhibit dioxin-like activity, for example polychlorinated azobenzenes and coplanar PCBs[2], whose concentrations in animal tissues may be orders of magnitude higher than those of PCDD/PCDFs[3].

An ideal screening assay would afford a TEQ value inclusive of all dioxin-like components of the sample. Several years ago, Hutzinger *et al*[4] proposed competitive binding of the analyte and a reference radioligand to the intracellular Ah (aryl hydrocarbon) receptor protein as the basis of a dioxin assay. Bradfield and Poland[5] applied this idea to the analysis of individual halodibenzo-*p*-dioxins. The concept is attractive because the toxicity of dioxin-like compounds is mediated through the Ah receptor[6,7], and so the extent of binding to the receptor might serve as a surrogate for toxicity. Correlations between the strength of binding to the Ah receptor *in vitro*, enzyme induction *in vitro*, and toxic potency *in vivo* have been established for numerous classes of halogenated aromatic compounds[2].

Objectives

This overview focusses on two aspects of recent research in our laboratory: (i) the extension of the competition assay to dioxin-like compounds of widely varying structure, and the

demonstration of additive binding for a variety of mixtures of these dioxin-like compounds to mouse hepatic Ah receptor; (ii) the application of the assay to the determination of the TEQ of matrices such as fish, soil, and pulp mill sludge.

Methodology

Hepatic cytosol was obtained from immature male C57BL/6N mice, whose Ah receptor has been shown to be more stable *in vitro* than the Ah receptors of other rodents[8]. After sacrifice, the livers were immediately perfused *in situ* via the hepatic portal vein with fresh buffer (ice-cold 23 mM N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid + 1 mM tetrasodium ethylenediaminetetraacetate + 10% v/v glycerol + 1 mM dithioerythritol, pH 7.6). The excised livers were rinsed once with 15 mL of fresh buffer, finely minced, rinsed with buffer and homogenized in buffer. The homogenates were centrifuged at 10,000 x g for 20 minutes at 4° C, and the supernatant was centrifuged at 100,000 x g for 60 minutes at 4° C. The cytosol fraction (supernatant) was collected by aspiration, and its protein concentration determined[9]. Aliquots were stored in small volumes at -70° C until used.

Receptor assay protocol: EC_{50} curve: [^{3}H]-2,3,7,8-TCDD (1.0 nM) and 15 competitor concentrations ranging two decades on either side of the expected EC_{50}-value were incubated with a fixed aliquot of hepatic cytosol. The cytosol solution was always added to the premixed ligands, rather than adding the ligands sequentially to the cytosol, because we have previously shown[10] that the first ligand to interact with the Ah receptor occupies a diproportionate number of specific binding sites. The %SB values (Specific Binding in the presence of a concentration of competitor as a percent of the maximum Specific Binding) were transformed into normits (log((100 - %SB) / %SB)). Linear regression between the normits and log(concentration) gave the slope and Y-intercept, from which the EC_{50} value (the competitor concentration at which %SB was reduced to half of its maximum value) was calculated.

Receptor assay for environmental samples: The samples after chromatography were evaporated to dryness, and taken up in 100 μL of DMSO. Four 10 μL samples were withdrawn; two were used in duplicate assays at their original concentrations; the other two were serially diluted x 10 with DMSO to provide a dilution series ranging in concentration from 10^{-1} to 10^{-4} x the original concentration. Each dilution was analysed by the hydroxylapatite assay[11] using a 10 μL aliquot of Ah receptor preparation and a reference radioligand concentration of 1 nM. From a plot of log (concentration) *vs* %SB we could estimate the approximate dilution needed to afford a solution whose TEQ corresponded to the EC_{50} (1 nM). A second dilution series was then carried out over a narrower concentration range to locate the dilution factor more precisely. The TEQ of each sample was compared with that obtained by GC/MS.

Results and Discussion

Table 1 lists EC_{50} values for the competition of selected dioxin-like compounds with 1.0 nM [^{3}H]-TCDD for a fixed aliquot of mouse hepatic Ah receptor. In the competitions between mixtures of the substances in Table 1 and 1.0 nM [^{3}H]-TCDD for a fixed aliquot of Ah receptor,

log(TEQ) was calculated on the basis of the results from Table 1. Plots of log(TEQ) *vs* %SB consistently afforded EC_{50} values, based on these TEQ concentrations, of 1 nM within experimental error (Table 2). This demonstrated that the various dioxin-like ligands compete in an additive fashion for the receptor, and hence that a receptor-based assay for environmental samples would include all substances which interact with the receptor, whether or not they have I-TEFs assigned. The total TEQ of the sample is obtained in a single step, without pre-separation of the mixture, although the chemical identities of the specific dioxin-like compounds present are not determined. After testing this hypothesis with synthetic mixtures of dioxin-like compounds, the assay was applied to several environmental samples (Table 3); the results were compared with those obtained by GC/MS (by Wellington Laboratories, using US EPA Method 1613).

Fly Ash: Fly Ash A samples were taken from the Commissioners Street municipal solid waste incinerator, Toronto, Ontario, now closed on account of concerns about its high emission levels; Fly Ash B samples were taken from the more modern Hamilton (Ontario) Solid Waste Reduction Unit (SWARU). Fly ash A had TEQ values determined by GC/MS ~ 110 ppb, within a factor of ≈ 2 of those obtained by bioassay (240 ppb). Congener specific analysis by GC/MS indicated that the most highly chlorinated PCDDs and PCDFs predominated, typical of combustion sources[12] (data not shown). PAHs were below the level of detection. For fly ash B the TEQ obtained by bioassay was approximately an order of magnitude greater than that obtained by GC/MS. Further analysis by GC/MS showed the presence of other PCDDs and PCDFs, from tetra- to hepta-chlorinated subunits (totalling 9.9; 13.1 ppb), and PAHs (3.6 ppb; 6.9 ppb respectively).

Petrochemical Catalyst Wash Water: PCDFs are formed during the reactivation of the catalysts used in catalytic reforming of petroleum[13], and pass out with the caustic wash water. The TEQ values obtained by bioassay were 2-3 times greater than those found by GC/MS. Further analysis by GC/MS revealed up to 50 other PCDD/PCDF congeners in absolute amounts >10 ppb; in agreement with the results of Beard *et al.*[13], these were mostly PCDFs rather than PCDDs. Other substances detected included hexachlorobenzene, anthraquinone, dichloroanthracene, (or dichlorophenanthrene), hexachloronaphthalene, trichlorofluoranthene (or trichloropyrene), nona- and deca-chlorobiphenyls, and low levels of PAHs.

Fish Tissue : The dioxin-like activity of the Lake Ontario lake trout samples was mostly due to coplanar PCBs, consistent with other work with aquatic organisms from the Great Lakes[14]. The total TEQ of the Lake Huron carp samples obtained by bioassay was also much greater than that contributed by the 17 named PCDDs and PCDFs, but in the case of these bottom-feeding fish the additional dioxin-like activity was contributed by hundreds of organochlorines, including nearly 4,000 ppb of total PCB congeners, several chlorinated diphenyl ethers, and various organochlorine pesticides: *a*-chlordane (6 ppb), *t*-nonachlor (6 ppb), *p,p'*-DDE (200 ppb), *p,p'*-DDD (40 ppb).

Soil: Three PCB-contaminated soil samples were obtained from different locations at a site that had been used for 20 years for salvaging copper from PCB-contaminated transformers. Soil C had the lowest levels of contamination (TEQ = 22 ppb), most of which comprised PCDDs and PCDFs (TEQ = 1 ppb) and coplanar PCBs (TEQ = 18 ppb), together with 172 ppb of total PAH. Congener specific analysis by GC/MS identified 41 other PCDD/PCDF congeners in addition to the

17 named PCDDs and PCDFs (3 of which were not detected), for a total PCDD/PCDF concentration of 20 ppb. Soil B was heavily contaminated, with a TEQ of approximately 1 ppm (10^3 ppb), of which only 1 ppb was accounted for by PCDDs and PCDFs, and 2 ppb by coplanar PCBs. Further analysis by GC/MS showed 37 ppb of total PCDDs and PCDFs (53 congeners) and heavy contamination by numerous PAHs (total 1.2 ppm). The contamination pattern of Soil A was similar; the 17 named PCDDs and PCDFs and coplanar PCBs contributed 16 ppb of TEQ, the total concentration of PCDDs and PCDFs was 55 ppb and that of PAHs was 316 ppb. Much of the unidentified material was removed when Soil A was initially extracted with acid, in that the TEQ by bioassay fell to 48 ppb, only 3-fold greater than the total TEQ due to PCDDs, PCDFs and coplanar PCBs (16 ppb).

Pulp Mill Sludge: These samples came from a waste treatment lagoon of a Canadian chlorine-bleaching mill, prior to the replacement of chlorine bleaching by ClO_2. They therefore represent "historical" levels of PCDD/PCDF contamination. The bioassay TEQs was 500 ppb, very little of which could be accounted for by PCDDs and PCDFs; this is consistent with recent work[15] showing that MFO induction in fish down-stream of pulp mills does not correlate with the concentrations of PCDDs and PCDFs in the effluent.

For all samples the receptor-based assay gave TEQ values greater than those obtained by GC/MS, because other dioxin-like substances such as coplanar PCBs, other PCDD/PCDF congeners, and PAHs are substrates for the Ah receptor and can co-elute with the 17 named PCDDs/PCDFs throughout the chromatographic sequence. Therefore the TEQ obtained by GC/MS could underestimate the toxicity of an environmental sample, although it is an oversimplification to assume that the bioassay's TEQ is a true reflection of the toxic potential of a sample, in that not all substances which bind to the Ah receptor form receptor-ligand complexes of equal biological activity[10].

With the present protocols, in which [^{3}H]-TCDD is used as the reference radioligand, the bioassay has a detection limit of approximately 64 pg of TEQ per individual assay point. These translate to detection limits of about 0.3 ppb for the fish, soil, fly ash, and pulp mill sludge samples, where about 2.5 g of sample provided the dioxin-like analytes for the final assay. For the caustic wash water samples, a lower detection limit of 4 ppt was achieved because a larger sample ($\approx$ 1 kg) was used. Lower detection limits could be achieved by employing a radiolabelled dioxin analog with higher specific activity than is possible with tritium, *e.g.*, ^{125}I, as used by Bradfield and Poland[5]. All the techniques discussed in this paper could be directly applied to any radioligand.

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Table 1: Selected EC_{50} values for the competitive hydroxylapatite assay with single compounds.

Compound (No. of runs)		EC_{50}, nM	95% CI, nM	EC_{50} Ratio[c]	I-TEF
2,3,7,8-TCDD	(14)	1.22	0.97 - 1.52	1.0	1.0
1,2,3,4,6,7,8-HpCDD	(6)	4.67	3.58 - 6.07	0.26	0.01
2-nitro-3,4,7,8-TCDD	(2)	7.02	4.29 - 11.5	0.17	N/A
2,3,7,8-TCDF	(6)	2.07	1.07 - 4.00	0.59	0.1
1,2,3,7,8-PeCDF	(4)	2.67	2.38 - 3.00	0.46	0.05
1,2,3,6,7,8-HxCDF	(4)	3.50	2.33 - 5.26	0.35	0.1
1,2,3,4,6,7,8-HpCDF	(6)	5.01	3.62 - 6.93	0.24	0.1/0.01[a]
3,3',4,4'-TCBP	(6)	60.5	47.8 - 76.7	0.02	0.01[a]
2,2',4,4',5,5'-HCBP	(6)	2.50×10^4	$(1.82 - 3.45) \times 10^4$	5×10^{-5}	0.001[a]
3,3',4,4'-TCAzoxyB	(11)	8.78	6.75 - 11.4	0.14	N/A
3,3',4,4'-TCAzoB	(6)	6.03	4.06 - 8.96	0.20	N/A
2,2',4,4'-TCAzoB	(6)	150	105 - 212	8×10^{-3}	N/A

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Table 2: Selected TCDD-Equivalence EC_{50}-values for the competitive hydroxylapatite assay with mixtures containing several ligands.

Mixture Components (No. of runs)	Ratio	EC_{50}, nM (95% CI)
2378-TCDD/2378-TCDF (2)	1:1	1.24 (0.39 - 3.96)
2378-TCDD/12378-PeCDF (2)	1:2	1.45 (0.72 - 2.92)
2378-TCDD/1234678-HpCDF (2)	1:4	1.59 (1.17 - 2.18)
2378-TCDD/22'44'55'-HxCBP (3)	1:1	2.33 (0.65 - 1.45)
2378-TCDD/22'44'55'-HxCBP (3)	1:1,000	0.97 (0.64 - 1.45)
2378-TCDD/22'44'55'-HxCBP (2)	1:10,000	0.76 (0.06 - 10.3)
2378-TCDD/33'44'TCBP (3)	1:1	0.54 (0.29 - 0.99)
2378-TCDD/33'44'-TCBP (3)	1:1,000	0.88 (0.28 - 2.78)
2378-TCDD/33'44'-TCBP (2)	1:10,000	1.06 (0.28 - 4.05)
2378-TCDD/33'44'-TCAzoB (2)	1:1	1.37 (0.53 - 3.57)
2378-TCDD/22'44'-TCAzoB (2)	1:200	1.42 (1.23 - 1.65)
PeCDF/HxCDF/HpCDF (4)	1:1:1	1.07 (0.79 - 1.45)
TCDD/HpCDD/PeCDF/HpCDF/TCBP (4)	1:1:1:1:100	1.54 (1.32 - 1.80)
TCDD/HpCDD/HxCDF/HpCDF/TCBP (3)	1:1:1:1:100	1.77 (1.27 - 2.46)
HpCDD/PeCDF/HxCDF/HpCDF/TCBP (4)	1:1:1:1:100	0.92 (0.74 - 1.14)
TCDD/HpCDD/PeCDF/HxCDF/HpCDF (3)	1:1:1:1:1	1.04 (0.39 - 2.81)

Table 3: Summary of bioassay and GC/MS analysis of environmental samples after the last stage of chromatographic clean up

Sample	Bioassay	GC/MS named PCDD/F	GC/MS TEQ (coplanar PCB)	GC/MS Total PCDD/F	GC/MS Other
Fly ash A	240 ppb	110 ppb	--	530 ppb	PAH < 1 ppb
Fly ash B	3 ppb	0.4 ppb	--	11 ppb	PAH, 5 ppb
Petrochem. wash	640 ppt	250 ppt	--	13 ppb	PAH, 2 ppt
Lake trout A	720 ppt	72 ppt	720 ppt	—	—
Lake trout B	760 ppt	76 ppt	150 ppt	—	—
Carp A	1100 ppt	19 ppt	40 ppt	--	OC, >250 ppb
Carp B	< 380 ppt	19 ppt	40 ppt	--	OC, >250 ppb
Soil A	800 ppb	2 ppb	14 ppb	54 ppb	PAH, 320 ppb
Soil B	1100 ppb	1 ppb	2 ppb	37 ppb	PAH, 1200 ppb
Soil C	22 ppb	1 ppb	18 ppb	20 ppb	PAH, 170 ppb
Pulp mill sludge	500 ppb	7.5 ppb	—	—	—