HIGH-THROUGHPUT MOLECULAR BIOASSAY FOR THE DETERMINATION OF DIOXINs AND PCBs IN MEAT AND ANIMAL FEEDS

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Introduction

Polyhalogenated aromatic hydrocarbons (PHAHs) such as dioxins and PCBs induce a wide variety of toxic effects including tumor promotion, immunotoxicity, birth defects and changes in hormone metabolism[1,2]. The trials for limiting human exposure to dioxins is more important than ever because 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been recently re-classified as a Class 1 human carcinogen by International Agency for Research on Cancer (IARC)[1]. Animal products including meat, milk and etc are major exposure sources of dioxin to human[3]. Such as, animal feeds are major exposure sources to livestock animals. Monitoring dioxin levels in food and animal feeds is a useful basis for identification of contamination events in animal products and provides a basis for assessing the level of current exposure to human through animal diet[4]. However, dioxin analysis through either conventional or modern analytical techniques involves exhaustive sample preparation and long time consuming for analysis. This study is performed for lowering costs and saving time for the determination of residual level of dioxins in meat and animal feeds by bioassay system using recombinant cell line that is very sensitively and specifically responding to dioxins and PCBs.

Methods and Materials

Chemicals

All chemicals used were of pesticide analysis or HPLC grade. 2,3,7,8-TCDD, 1,2,3,7,8-PnCDD, 1,2,3,4,7,8-HxCDD, 1,2,3,4,6,7,8-HpCDD, OCDD, 2,3,7,8-TCDF, OCDF, PCB126, PCB169, PCB105, PCB118, PCB156 were purchased from ChemService(Westchester, USA). 17 different 2,3,7,8-congeners of PCDD/F (500ng TEQ/ml of nonane) was purchased from Cambridge Isotope Laboratories Inc. (Massachusetts, USA)

Recombinant cell line and culture condition

Mouse hepatoma cell line (Hepa1c1c7) stably transfected with the pGudLuc1.1 plasmid containing luciferase reporter gene and four dioxin responsive elements was used[3]. The cells(HEPALUC1.1) were grown in 24-well cell culture plates in 0.5 ml minimal essential medium (α-MEM, Gibco) with 10% heat-inactivated fetal bovine serum, 50 IU/ml penicillin and 50 μg/ml streptomycin. The cells were grown in 24-well cell culture plates with 0.5ml growth medium. The cells were treated with test compounds or sample extracts solved in DMSO (1% of medium) for 4.5 hrs. Luciferase activity was measured with luminometer.

Preparation of spiked fat of meat or animal feed samples

Fat was prepared from beef containing large portion of fat or swine feed through n-pentane extraction. Clean fat passed through activated carbon was prepared. An aliquot of fat was dissolved in 4 times volume of *n*-hexane/diethylether(97:3, v/v) and mixed with 17 different 2,3,7,8-congeners of PCDD/F to final concentration of 100 pg TEQ/g fat. Standard samples for calibration curve are made by dilution of stock fat with clean fat at six different dioxin levels of 0, 0.2, 0.4, 2, 5 and 10 pgTEQ/0.5g fat.

Clean-up of meat fat or feed fat for bioassay

Meat fat or feed fat was extracted from ground meat (10g) or feedstuffs (150g), and then 0.5g fat solved in 2ml distilled *n*-hexane/diethylether (97:3, v/v) was added to 8g of 33% acidified silicagel colume. The final extract was solved in 5μl DMSO and treated to the recombinant cells for 4.5 hrs. Luciferase synthesized in the exposed cells was measured. All procedures are described in Fig. 1.

Fat collection from samples (10g meat or 100g feed)

Grind thoroughly in mortar
Extract fat with 2 fold volume of *n*-pentane
Repeat this extraction step until the pentane remained colorless
Filter the extract over glass mineral wool with anhydrous sodium sulfate
Wash the filter with *n*-pentane
Evaporate the combined filtrate to dryness under vacuum

Clean-up of fat

Load aliquot of 0.5g fat in 2ml distilled *n*-hexane/diethyl ether (97:3,v/v) to pre-rinsed 33% acid silicagel column
Elute dioxin with 18ml *n*-hexane/diethyl ether (97:3,v/v)
Dry the eluate by rotor-evaporation and then under a gentle flow of nitrogen
Add 5 $\mu\ell$ DMSO, mix with 0.5ml medium(1% vehicle/ml culture medium)

Treatment of extracts to cells for 4.5hrs

Determination of the Luciferase activity

Calculate the dioxin level against a dose-response curve generated from 17 mixed-dioxins standards included in each test

Fig. 1. Flow scheme of dioxin bioassay

Statistics

For determination of the TCDD equivalency factors (TEF) for PCDD, PCDF and PCBs in the bioassay, dose-response curves of each chemical in triplicate for every concentration were made. *EC50 of each chemical was obtained by fitting the dose-response curve using an sigmoidal fitting* method (Origin, version 6.0). It represented the concentration of agonist giving a half-maximal response of 2,3,7,8-TCDD. The TEF was calculated by dividing the EC50 value of 2,3,7,8-TCDD by EC50 value of the compound of interest. TEQs from HR-GC/MS data were calculated using

WHO TEF values (1998)[5]. Dioxin quantities obtained by bioassay were calculated by comparison of the luciferase activity induced by a sample extract against a calibration curve generated from the each concentration of 17 mixed-dioxin standards spiked to clean fat or elution solution followed same procedure to sample.

Results and Discussion

1. TCDD-inducible luciferase activity in recombinant cells

The dose dependence of luciferase induction in HEPALUC1.1 cells was determined by incubation of the cells with increasing concentrations of TCDD for 4.5 hrs. Induction of luciferase was dose dependent, with a maximal induction by 500pM TCDD (11 fold), EC50 of 10.29 pM and a minimal detection limit of about 0.1pM (Fig.2). 0.1pM was equal to 16fg per 0.5ml culture medium (24 well). With consideration of these results, the recombinant cell can be used for bioassay system of highly sensitive and throughput.

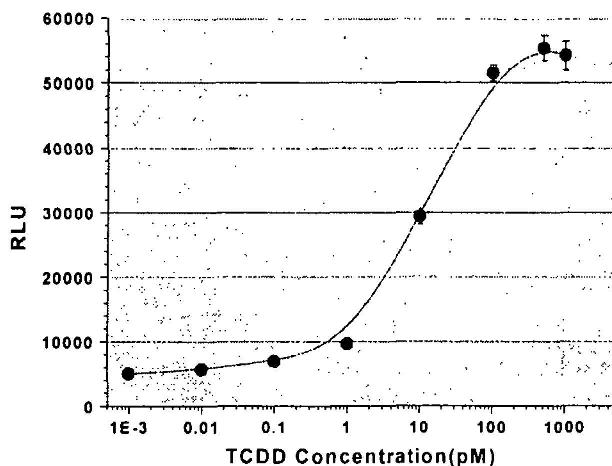


Fig.2. Dose-response curve for the induction of luciferase activity by 2,3,7,8-TCDD in HEPALUC1.1 cell

2. Dioxins- and PCBs-inducible luciferase activity in the recombinant cell line

The dose-responses for a number of other selected dioxins and some coplanar PCBs were also observed (Fig.2). The shape of the curves for these compounds was comparable to that of TCDD, but shifted to a higher dose range. The maximum responses of these chemicals were also similar, with the exception of PCB126(22% higher response), and PCBs 105 and 118(67-69% responses of that of TCDD). The EC50 values of dioxins and PCBs determined by sigmoidal fitting method (Origin program 6.0) with fix value of maximal response of 500pM TCDD (Table 1). There was a good correlation between WHO-TEFs('98) and this bioassay TEFs. Also, luciferase activity induced by TEQ dose of the 17 different 2,3,7,8-substituted PCDDs and PCDFs was comparable to that by same dose of TCDD (data are not shown)

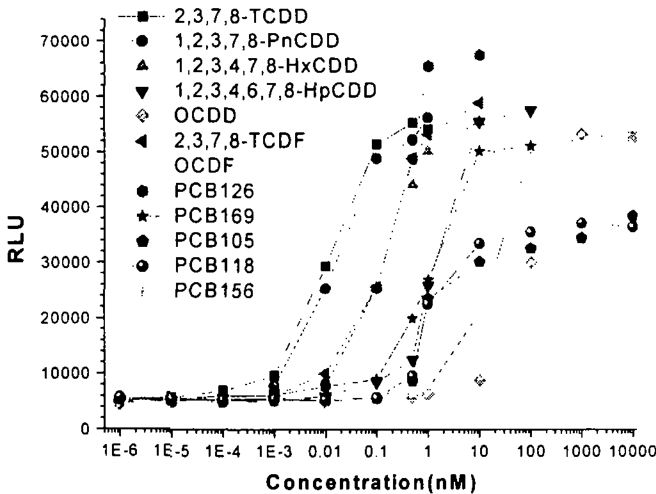


Fig. 3. Dose-responsive curves obtained in the bioassay with a number of different dioxins and PCBs.

Data points are means of the three independent measurements.

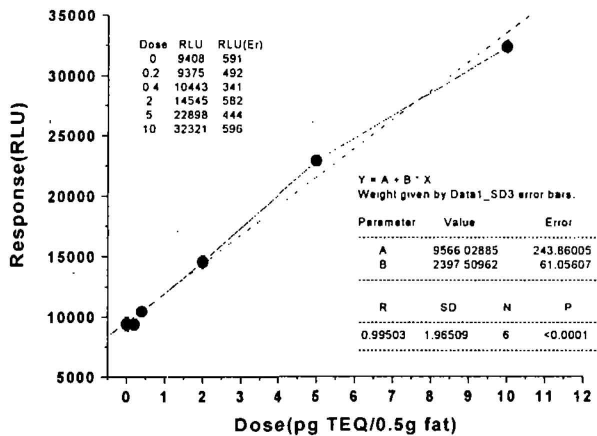
Table 1. Toxic Equivalency factors (bioassay-TEFs) for induction of luciferase in HEPA-LUC1.1 cells of a number of selected dioxins and coplanar PCBs

Compound	EC50 (pM)*	Bioassay-TEF	WHO-TEF('98)
2,3,7,8-TCDD	10.29	1	1
1,2,3,7,8-PnCDD	14.80	0.70	1
1,2,3,4,7,8-HxCDD	139.2	0.074	0.1
1,2,3,4,6,7,8-HpCDD	1,185	0.0087	0.01
OCDD	98,601	0.0001	0.0001
2,3,7,8-TCDF	123.8	0.083	0.1
OCDF	113,921	0.00009	0.0001
PCB126	120.8	0.085	0.1
PCB169	1,267	0.0081	0.01
PCB105	110,972	0.000093	0.0001
PCB118	64,245	0.00016	0.0001
PCB156	18,423	0.00056	0.0005

* EC50 values were calculated by sigmoidal fitting method

3. Limits of detection and quantitation in bioassay

Figure 4 shows the calibration curves for dioxins obtained from 8 independent experiments over a period of 2 months. 0, 0.2, 0.4, 2, 5, and 10 pgTEQ of 17 different 2,3,7,8-substituted PCDDs and PCDFs were added to 2ml solution of hexane/diethyl ether (97:3) and then processed same treatment to sample. The luciferase activities induced by spiked standards to 0.5g of clean fat (from beef or animal feed products) and then processed same treatment showed same luciferase activities to those of spiked standards to elution solution omitting clean fat (data not shown). With this reason, calibration curve was made from clean fat-omitted procedure. The within-laboratory coefficients of variation (CVs) ranged from 1.8 to 6.3% and mean recoveries of the spiked dioxins from the fat (or elution solution) ranged from 66.6 to 143.2%(Table 2). These values of CVs and recoveries are satisfied to the recommendations for validation of analytical methods by CODEX, EU EMEA and USA FDA. The detection limit(LOD) and quantitation limit(LOQ) were calculated from 8 different experiments(n=24) following to the EU VICH guideline on validation of analytical procedures(1999). LOD was 0.33 pgTEQ/0.5g fat and LOQ was 1.00 pgTEQ/0.5g fat. The tolerances or guidelines for meats are from 3 to 5 pgTEQ/g fat in European countries. So, this bioassay can be used for the determination of dioxins and PCBs in meat. In the case of animal feed, guidelines for dioxins in complete feeds is settled as 0.75



ng/kg product in Belgium. The bioassay system can be also adopted to determine dioxins in animal feed products.

Fig. 4. Calibration curve for 17 mixed-dioxins (n=24).

Table 2. Within-laboratory variation, recovery and limits of detection and quantitation

Dioxin content (pg TEQ/0.5g fat)	CV(%)	Recovery(%)	LOD[a] (pgTEQ/0.5gfat)	LOQ[b] (pgTEQ/0.5gfat)
0	6.3	143.2 ±7.5	0.33	1.00
0.2	5.2	122.3 ±6.1		
0.4	3.3	72.1 ±2.4		
2	4.0	61.2 ±1.9		
5	1.9	67.6 ±1.4		
10	1.8	66.4 ±1.2		

n=24, LOD: detection limit = 3.3σ/S(σ,the standard deviation of the y-intercept of regression line in calibration curve; S, the slope of the calibration curve), LOQ: quantitation limit = 3×LOD

4. Correlation between Bioassay- and GC/MS-determined TEQ contents in meats and animal feeds

The meats used in bioassay and HR-GC/MS analysis were collected during 1999 from imported or domestic beefs. Contents of the 17 mixed-2,3,7,8-substituted PCDD and PCDF congeners were determined by HR-GC/MS analysis and then TEQ concentrations were calculated using the WHO(1998)-TEFs. Correlation between bioassay- and GC/MS-determined dioxin levels was 0.85. The HR-GC/MS analysis revealed any samples that exceeded the tolerance of 5pgTEQ/g fat. Bioassay-determined dioxin levels were approximately 1.5 times higher than GC/MS-determined level (Fig. 5).

The animal feeds used in bioassay and GC/MS analysis were collected during 1998 from farmland growing cattle, pig and poultry. The correlation between bioassay- and GC/MS-determined dioxin levels was 0.75. Neither the GC/MS analysis, nor the bioassay revealed any samples that exceeded the Belgian tolerance of 0.75 pgTEQ/g feed. Bioassay-determined dioxin levels were approximately 2 times higher than GC/MS-determined level (Fig. 6). It can be concluded that bioassay is a very valuable tool, allowing the screening of relatively large number of meat or feed samples for the contamination of dioxins and dioxin-like PCBs.

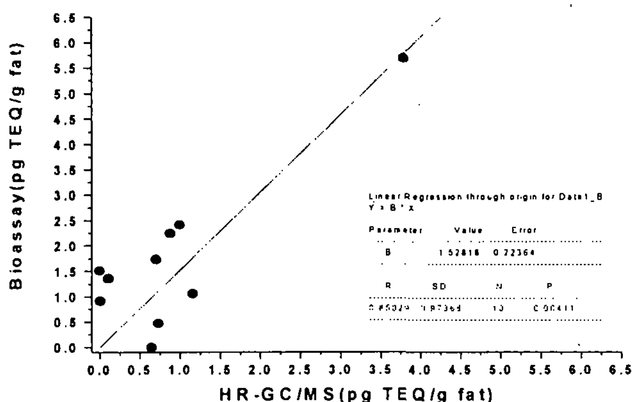


Fig. 5. A comparison of the dioxin TEQ-contents determined by bioassay with those from HR-GC/MS for 10 meat samples.

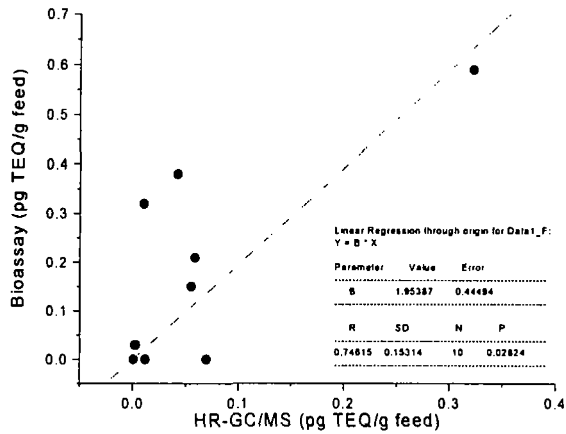


Fig. 6. A comparison of the dioxin TEQ-contents determined by bioassay with those from HR-GC/MS for
10 animal feed samples.

Acknowledgements

This study is performed in National Veterinary Research & Quarantine Service (NVRQS) and supported with budget. HR-GC/MS analysis in meat and animal feed products was performed in Department of environmental engineering in University of Seoul and Environment Toxicology Division of NVRQS, respectively.

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