QUANTIFYING DIOXIN-LIKE ACTIVITY IN FLY ASH USING H4IIE-LUCIFERASE BIOASSAY

Shigeki Masunaga[1], Chieko Morinaka[1], Junko Nakanishi[1,2]

[1]Graduate School of Environment and Information Sciences, Yokohama National University, 79-7, Tokiwadai, Hodogaya, Yokohama 240-8501, Japan
[2]Research Center for Chemical Risk Management, National Institute of Advanced Industrial Science and Technology, 16-1 Onogawa, Tsukuba, Ibaraki 305-8569, Japan

Introduction

Considerable efforts have been amassed in several developed countries both in terms of construction of dioxin emission inventory and its oriented reduction. Mmonitoring and emission control of dioxins under these efforts have been focused on polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (PCBs). However, it has been known that the dioxin-like toxicity can also be found in other class of compounds such as brominated dibenzo-*p*-dioxins and dibenzofurans (PBDD/DFs)[1)], polychlorinated naphthalenes (PCNs)[2)], polycyclicaromatic hydrocarbons (PAHs)[3)], etc. Therefore, it is necessary to examine whether the classes of compounds that we are focusing on cover the major portion of dioxin-like toxicity present in emissions or in environmental media. Considering those gaps, in this study, we measured dioxin-like activity through bioassay by H4IIE cells in incinerator fly ash. In addition, PCDD/DF individual congener concentrations were determined by ordinary instrumental analysis in order to understand the variation of dioxin-like toxicity between H4IIE cells and instrumental analysis.

Materials and Methods

Chemicals

PCDD/DF individual congener standards were purchased from AccuStandard, Inc.

Fly ash samples

Totally, six fly ash samples were employed in this study. Samples A, B and C was provided by the dioxin intercalibration study sponsored by Uméa University. Rest of the samples (fly ash D, E and F) were obtained from three different municipal solid waste incinerators located in Yokohama, Japan.

H4IIE-luciferase cell line

H4IIE-luciferase cell line is a rat liver hepatoma cell stably transfected with a luciferase reporter gene under control of dioxin response elements (DREs)[4)]. When this cell line is exposed to chemicals that bind to arylhydrocarbon receptor (AhR), the cell produce luciferase, an enzyme that is responsible for luminescence in fireflies. With the addition of luciferin to the cell culture, rate of luminescence was measured to quantify the presence of AhR responsible chemicals or dioxin-like activity. This cell line was provided from Prof. J. P. Giesy, Michigan State University.

Sample preparation

Fly ash sample was treated with 2N hydrochloric acid and separated into solid and liquid phases by a filter. Further, solid phase fraction was extracted with toluene by Soxhlet/Dean-Stark extractor for 16 hours. The liquid phase was liquid-liquid extracted with dichloromethane and both of the extracts were combined (crude extract). Aliquot of the crude extract was cleaned-up with sulfuric acid/silica gel

column (H_2SO_4-silica extract). Then aliquot of the H_2SO_4-silica extract was further cleaned up with activated carbon column into two fractions (AC pre fraction and AC post fraction). The brief analytical flowchart has been shown in Figure 1.

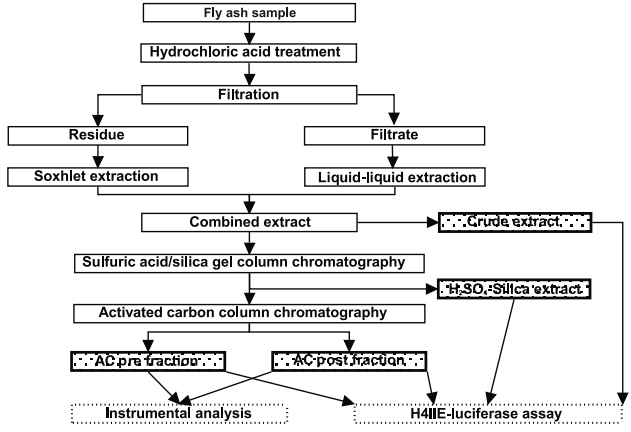

Figure 1. Pretreatment procedure of fly ash samples

Instrumental analysis

Only the cleaned-up samples (AC pre and post fractions) were analyzed for 2378-PCDD/DF determination. The identification and quantification analysis was conducted using HRGC/HRMS (HP6890, Hewlett-Packard and Autospec-Ultima, Micromass) fitted with DB-5 and DB-17 columns (60 m, J&W Scientific).

H4IIE-luciferase bioassay

H4IIE-luciferase cells for bioassay were plated into the 60 interior wells of 96-well microplate (Packard 96-Well View PlatesTM). Two hundred fifty ml of culture fluid was dispensed into each well at a density of approximately 75,000 cells/ml. The 36 exterior wells of each plate were filled with 250 ml of culture media. Cells were incubated overnight in a CO_2 incubator at 37 °C prior to dosing. Test wells were dosed with 2.5 ml of sample extracts or standard solution. Solvent control wells were dosed with the similar solvent used for sample extracts except blank wells, which received no dose. For standard calibration, six different concentrations of 2378-tetrachlorodibenzo-*p*-dioxin (2378-TCDD) solutions were dosed triplicate. Luminescence was measured by microplate luminometer (LumiCount, Packard) three days after exposure with the addition of luciferine.

Results and Discussion

Dose-response curves and relative potencies for seventeen 2378-PCDD/DFs by H4IIE-luciferase assay

In order to confirm the reliability of H4IIE-luciferase cell for determination of dioxin-like activity, dose-response curves for seventeen 2378-PCDD/DFs were obtained (Figure 2**)**. The inclinations of the curves were similar for the seventeen 2378-PCDD/DFs. In addition, maximum responses were more or less similar for most of 2378-PCDD/DFs. These results indicated that this assay method was suitable for dioxin-like activity measurement for variety of 2378-PCDD/DF congeners. Besides, relative potencies of individual 2378-PCDD/DFs against 2378-TCDD were calculated at 50 % maximum response of 2378-TCDD (50 % TCDD*max*). The obtained relative potencies are shown in Table 1. Some PCDFs (as for an example: 2378-TCDF, 23478-PeCDF and 123478-HxCDF) had smaller relative potencies compared with WHO-TEF values.

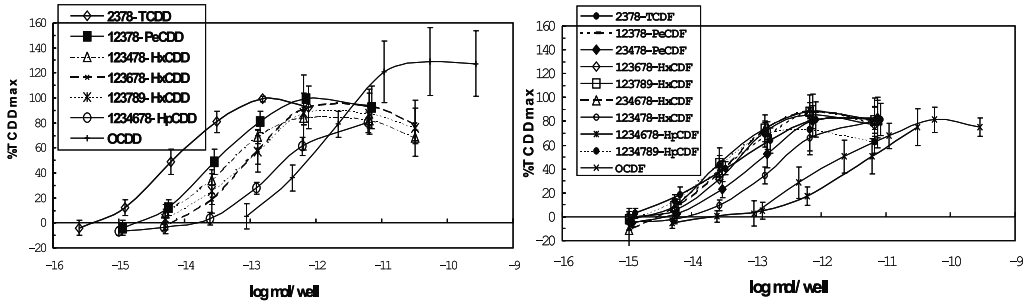

Figure 2. Dose-response curves for 2378-PCDD/DFs by H4IIE-luciferase assay

Table 1. Relative potencies for 2378-PCDD/DFs by H4IIE-luciferase assay

Congener	Relative Potency	Congener	Relative Potency	Congener	Relative Potency
2378-TCDD	1	OCDD	0.0064	123678-HxCDF	0.077
12378-PeCDD	0.68			123789-HxCDF	0.12
123478-HxCDD	0.082	2378-TCDF	0.093	234678-HxCDF	0.10
123678-HxCDD	0.060	12378-PeCDF	0.107	1234678-HpCDF	0.0023
123789-HxCDD	0.061	23478-PeCDF	0.044	1234789-HpCDF	0.080
1234678-HpCDD	0.0081	123478-HxCDF	0.019	OCDF	0.0013

Additive response by H4IIE-luciferase assay

Dosing of several 2378-PCDD/DF standard mixtures that simulated the 2378-PCDD/DF compositions in fly ash was performed to test additivity of response in the assay. The dose-response curve for the mixture was plotted against 2378-TCDD equivalent [calculated by the equation of $TCDD_{eq\text{-}calc}=\Sigma C_i \times RP_i$] where; i = individual PCDD/DF congener, C_i = Concentration of PCDD/DF congener i, RP_i = Relative potency for PCDD/DF congener i]. The result provided that the response of the mixture was coincided with the response given solely by 2378-TCDD congener, showing additivity of response by the mixture.

Dioxin-like activity in fly ash

For each fly ash sample, four kinds of extracts (crude extract, H_2SO_4-silica extract, AC pre fraction and AC post fraction) were prepared and their dioxin-like activities were measured by H4IIE-luciferase assay. No significant activity was found in AC pre fractions from all the six fly ash samples. As these fractions might contain PCB congeners other than non-*ortho*-PCBs, the contributions of mono- and di-*ortho* chlorinated PCBs to the measured dioxin-like activity were considered to be negligible. Activities found in the H_2SO_4-silica extracts were nearly equal to that found in AC post fraction except for one sample. This result was consistent with the former one of no significant activities in AC pre fractions. The results obtained for the crude extracts and AC post fractions are shown in Figure 3. In addition, the TCDD equivalents in AC post fractions calculated from the results of instrumental analysis of individual 2378-PCDD/DF congeners and their relative potency values ($TCDD_{eq\text{-}calc}$) are shown in Figure 3. The results comprehended that TCDD equivalent measured by H4IIE assay ($TCDD_{eq\text{-}assay}$) are larger than $TCDD_{eq\text{-}calc}$ except for one case (fly ash C) and indicated that non-*ortho* PCBs and/or other compounds with dioxin-

like activity were present in the AC post fraction. The differences between $TCDD_{eq\text{-}assay}$ and $TCDD_{eq\text{-}calc}$ were significant for fly ash E and F. The $TCDD_{eq\text{-}assay}$ in crude extracts were larger than $TCDD_{eq\text{-}assay}$ in AC post fractions except for one case (fly ash A); especially the difference was significant for fly ash B and F. The possible explanation for these fly ashes is that chemicals with AhR responsible activity but other than halogenated aromatics might have existed in the fractions. For instance, polyaromatic hydrocarbons (PAHs) may be possible compounds responsible for the activity[5].

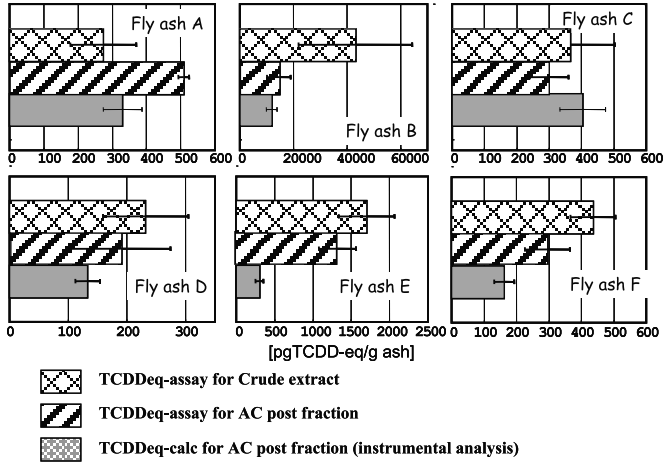

Figure 3. Comparison of dioxin-like activity in fly ash extract determined by H4IIE assay ($TCDD_{eq\text{-}asssay}$) and calculated from instrumental analysis ($TCDD_{eq\text{-}calc}$)

On the whole, the present study showed that H4IIE-luciferase assay could be used to measure the dioxin-like activity in fly ash samples. Combination of $TCDD_{eq\text{-}assay}$ and $TCDD_{eq\text{-}calc}$ determinations would help to find the existence of dioxin-like activity due to unknown compounds and to identify its origin.

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