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Determination of dioxin concentrations in fish and seafood samples using a highly sensitive reporter cell line, DR-EcoScreen cells

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ABSTRACT

There is a strong need for the development of relatively rapid and low-cost bioassays for the determination of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), and dioxin-like polychlorinated biphenyls (dl-PCBs) in environmental and food samples. In this study, we applied a reporter gene assay using DR-EcoScreen cells (DR-cell assay), which is highly sensitive to dioxins, to the determination of PCDD/Fs and dl-PCBs in fish and seafood samples. The PCDD/Fs and dl-PCBs were extracted from homogenated samples (10 g) of 30 fish and shellfish, purified by clean-up procedure using a multilayered silica gel column and an alumina column, and applied to DR-cell assay. Interestingly, the bioanalytical equivalent (BEQ) values obtained from the DR-cell assay [<0.1 ~ 5.4 pg BEQ g^{-1} wet weight (ww)] were closely correlated with the toxicity equivalent (TEQ) values from conventional high-resolution gas chromatography/high-resolution mass spectrometry (HRGC–HRMS) analysis (r^2 = 0.912), and the slope of regression line was 0.913. Therefore, we multiplied the BEQ values from the DR-cell assay by a conversion coefficient (1.095, the reciprocal of 0.913) to approximate the TEQ values from the HRGC–HRMS analysis. Furthermore, we used this DR-cell assay to perform a prescreening test of PCDD/Fs and dl-PCBs in 16 fish and seafood samples purchased from a supermarket, revealing that a sample from the fatty flesh of a bluefin tuna exceeded 8 pg TEQ g^{-1} ww (the European Union-tolerance limit). Taken together, these results suggest that the DR-cell assay might be applicable as a rapid and low-cost prescreening method to determine dioxin levels in fish and seafood samples.

1. Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and dioxin-like polychlorinated biphenyls (dl-PCBs), are widely distributed contaminants that can induce various toxic responses including immunotoxicity, carcinogenicity, and demonstrate adverse effects on reproduction, development, and endocrine functions via aryl hydrocarbone receptor (AhR) (Poland and Knutson, 1982; Safe, 1986; Fernandez-Salguero et al., 1996; Mimura et al., 1997). It is estimated that more than 90% of PCDD/Fs and dl-PCBs consumed by humans come from foods derived from animals as PCDD/Fs and dl-PCBs bioaccumulate and biomagnify in marine and terrestrial livestock. Based on a total diet study in Japan, Tsutsumi et al. (2001) reported that the mean daily intake of PCDD/Fs and dl-PCBs was highest from fish and seafood, including shellfish and shrimp (76.9%). Recently, the European Union (EU) decided many different actions and applied appropriate tolerance limits; e.g., the maximum level for fish and fishery products is 8 pg toxicity equivalent (TEQ) g^{-1} wet weight (ww) for the sum of PCDD/Fs and DL-PCBs (Commission Regulation (EC) 199/2006), and that for fish liver is 25 pg TEQ g^{-1} ww for the sum of PCDD/Fs and dl-PCBs (Commission Regulation (EC) 565/2008).

The conventional determination of PCDD/Fs and dl-PCBs in seafood samples is based on the individual measurement of 29 PCDD/Fs and dl-PCBs by high-resolution gas chromatography/high-resolution mass spectrometry (HRGC–HRMS), with the measured values multiplied by a toxicity equivalency factor (TEF) (Van den Berg et al., 2006) and totaled to give TEQ values. This method provides reliable data including the concentration of each of the 29 congeners in the test samples. However, it also requires expensive equipment and highly trained analysts, whilst the sample preparation procedures are often time-consuming and costly. In particular, this method might be less than useful when rapid data on PCDD/Fs and dl-PCBs from a large set of test samples is required. For this

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reason, the development of a rapid and inexpensive screening method for PCDD/Fs and dl-PCBs remains a high priority.

Reporter gene assays using hepatocarcinoma cells, which express the *AhR* gene and luciferase reporter gene containing the dioxin-responsive element (DRE), are applicable to the detection of dioxin-like compounds based on their activation of AhR (Garrison et al., 1996; Murk et al., 1996). A number of studies have reported the utility of reporter gene assay systems as possible alternatives for screening PCDD/Fs and dl-PCBs in retail fish (Tsutsumi et al., 2003a), human blood plasma (van Wouwe et al., 2004), bovine milk (Bovee et al., 1998), human breast milk (Laier et al., 2003), fly ash (Till et al., 1997), and river sediments (Vondracek et al., 2001). Recently, we have also developed a new, sensitive and rapid reporter gene assay (DR-cell assay) using a genetically engineered stable cell line, designated DR-EcoScreen cells (Takeuchi et al., 2008). The minimal detection limit (MDL) and 50% effective concentration (EC_{50}) of 2,3,7,8-TetraCDD (TCDD) in this DR-cell assay were 0.1 pM and 2.8 pM, respectively, with little variance in data (within CV 10%) (Takeuchi et al., 2008). These results suggest that the DR-cell assay might be more sensitive to PCDD/Fs and dl-PCBs than other reporter gene assays (Hepa1c1c7- and H4IIE-based CALUX assays), of which the MDL of 2,3,7,8-TCDD were reported to be 1 and 0.3 pM, and the EC_{50} of 2,3,7,8-TCDD were reported to be 10 and 10 pM, respectively (Behnisch et al., 2002; Han et al., 2004). Besides high sensitivity, the DR-cell assay has certain unique advantages compared to other bioassays. As the DR-EcoScreen cells have very strong luminescence intensity and can be measured using a long-lived luciferase substrate, a bioassay using these cells does not require well-washing and medium changes during the procedure. Thus, the DR-cell assay is compatible with high-throughput automation and can reduce the overall workload in a laboratory.

Most recently, based on a comparative study with HRGC–HRMS analysis, we have reported that the DR-cell assay was helpful in determining low levels of PCDD/Fs and dl-PCBs in ambient air samples (Anezaki et al., 2009). In the present study, we investigated the applicability of the DR-cell assay to the determination of PCDD/Fs and dl-PCBs in fish and seafood samples as a prescreening step to the HRGC–HRMS method. Following the extraction and clean-up of 30 samples, the bioanalytical equivalent (BEQ) values from the DR-cell assay were compared with the TEQ values from the HRGC–HRMS analysis. As a result, we found that the values from both methods showed a close correlation. Furthermore, in this study, a prescreening test for the determination of PCDD/Fs and dl-PCBs in 16 randomly selected fish and seafood samples was carried out using the DR-cell assay based on an indication (cut off value) of 8 pg TEQ g^{-1} ww and 25 pg TEQ g^{-1} ww (fish liver) for the sum of PCDD/Fs and dl-PCBs proposed by the EU, since no dioxin-limit value in food has yet been fixed in Japan. Here, we provide evidence that the DR-cell assay might be a promising method for the rapid and low-cost screening of PCDD/Fs and dl-PCBs in fish and seafood samples.

2. Materials and methods

2.1. Chemicals and cell culture materials

Acetone, hexane, toluene, dichloromethane, concentrated sulfuric acid, dimethyl sulfoxide (DMSO) and some kinds of silica gel for multi-layer column chromatographics were obtained from Wako Pure Chemicals Inc. Ltd. (Osaka, Japan). Alumina B-Super I was obtained from ICN Pharmaceuticals Inc. (Costa Mesa, CA, USA). The PCDD/F and dl-PCB standards were obtained from Wellington Laboratories (Canada) and Cambridge Isotope Laboratories (CIL; USA). The PCDD/Fs mixture and 3,3′,4,4′,5-pentachlorobiphenyl (PeCB) for the recovery test were purchased from CIL.

Fetal bovine serum (FBS) was obtained from JRH Bioscience, Inc. (Lenexa, KS, USA). Charcoal-dextran-treated FBS (CD-FBS) was obtained from Hyclone (Logan, UT, USA). Alpha-modified Eagle's minimum essential medium α-MEM) was obtained from MP Biomedicals (Solon, OH, USA). Hygromycin was obtained from Invitrogen (San Diego, CA, USA). Glutamine and penicillin–streptomycin (antibiotics) solutions were obtained from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). A 0.25% trypsin/0.02% ethylenediamine tetra-acetic acid (EDTA) disodium salt solution was obtained from Life Technologies (Paisley, UK). The luciferase substrate, Steady-Glo™ reagent, was purchased from Promega (Madison, WI, USA).

2.2. Collection, extraction, and cleanup of fish and seafood samples for DR-EcoScreen cell assay

Fish and seafood samples were purchased in 2002–2003 and 2007 from supermarkets in Tokyo and Sapporo, Japan, respectively. The samples were homogenized using a food cutter (GRINDOMIX GM 200, Retsch; Haan, Germany) and stored at −20 °C until analysis.

As shown in Fig. 1a, 10-g homogenized samples for the DR-cell assay were freeze-dried for 18 h. The sample was then extracted with 100 mL of 50%/50% *n*-hexane/acetone by soxhelation for 6 h. The solvent was reduced to around 30 mL in a rotary evaporator in order to remove acetone, and *n*-hexane was added to the extract to a final volume of approximately 100 mL. The *n*-hexane solution was treated with 20 mL of concentrated sulfuric acid any number of times until the *n*-hexane layer became colorless. After washing the extract two times with 50 mL of 2% NaCl solution, it was evaporated to a few mL in a rotary evaporator. The extract was then loaded onto a multilayered silica gel column filled from bottom to top with 3 g of 2% KOH silica gel, 4.5 g of 44% H_2SO_4 silica gel, 6 g of 22% H_2SO_4 silica gel, and 3 g of 10% $AgNO_3$ silica gel. The dioxins in the sample were eluted with 220 mL of *n*-hexane and further purified on an alumina column. On an alumina column filled with 15 g of alumina, mono-*ortho* PCBs were eluted with 200 mL of 2% (v/v) dichloromethane/*n*-hexane, and then non-*ortho* PCBs and PCDD/Fs were eluted with 200 mL of 60% (v/v) dichloromethane/*n*-hexane. For the DR-cell assay, each of these eluted fractions was evaporated and dissolved in 50 μL of DMSO.

2.3. DR-EcoScreen cell bioassay

A highly sensitive AhR-mediated reporter cell line, DR-EcoScreen cells, developed from a mouse hepatoma Hepa1c1c7 cell line, was stably transfected with a reporter plasmid containing seven copies of DRE fused to a luciferase gene (Takeuchi et al., 2008). The DR-EcoScreen cells were maintained in α-MEM supplemented with 5% FBS, antibiotics, glutamine, and 150 μg mL^{-1} of hygromycin at 37 °C in an atmosphere of 5% CO_2/95% air under saturating humidity, and passaged twice every week by trypsinization with 0.25% trypsin/0.02% EDTA. For the screening assay, cells were trypsinized and suspended at a density of 1.0×10^5 cells mL^{-1} in α-MEM containing 5% of CD-FBS. Ninety micro litre of the cell suspension was seeded in each well of a 96-well flat bottom plate (#136102 Nunclon™, Nalge Nunc, Denmark) at a final density of 9000 cells $well^{-1}$. After cultivation for 24 h at 37 °C, 10 μL of each of the various concentrations of fish and seafood samples dissolved in 10% DMSO was added to each well (final concentration of DMSO was 1%). Following cultivation for a further 24 h, 100 μL of Steady-Glo™ reagent was added to each well. The plate was then shaken at room temperature for 5 min, and the luminescence was measured with a microplate-luminometer (Wallac 1420 ARVO™ SX, Perkin–Elmer). The dioxin concentrations in the samples were calculated as cell-based BEQ values using the quantitative regression line of the standard curve for 2,3,7,8-TCDD (Fig. 1b). The limit of quantification (LOQ) was 0.1 pg BEQ g^{-1} ww,