BIOANALYTICAL APPROACHES FOR THE DETECTION AND RELATIVE QUANTIATION OF HALOGENATED DIBENZO-P-DIOXINS AND RELATED CHEMICALS

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Abstract

Proper epidemiological, risk assessment and exposure analysis of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and related chemicals requires accurate measurement of these chemicals both in species and tissues of interest and in various exposure matrices. While high-resolution instrumental analysis methods are established for these chemicals, these procedures are costly and time-consuming and as such, are impractical for large-scale sampling and screening analysis. Numerous bioanalytical methods (immunoassays and bioassays) have been developed for the detection of these chemicals in extracts from a wide variety of matrices. Immunoassays utilize the high specificity of antibodies generated against dioxin-like polychlorinated dioxin/furans or polychlorinated biphenyls for detection and the bioassays take advantage of the ability of these same target chemicals to activate the Ah (dioxin) receptor (AhR) and AhR signal transduction. Comparison of the relative sensitivities of the various cell bioassays, applications and limitations and examples of their use in screening and analysis of environmental, biological and food/feed samples are presented. Validation studies have demonstrated the utility of these bioanalytical methods as relatively rapid, accurate and cost effective screens for the detection and relative quantitation TCDD and related chemicals in various matrices and have led to the regulatory acceptance/certification of several as validated screening methods for dioxin-like chemicals.

Overview

Halogenated aromatic hydrocarbons (HAHs), such as polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs), represent a large group of compounds that can produce adverse effects at environmentally relevant concentrations. HAHs have been identified worldwide in tissues from wildlife, humans and domestic animals, in food, water, and soil samples and in many other materials. Because of their ubiquitous distribution, resistance to biological and chemical degradation, high toxicity and potential for bioaccumulation/biomagnification, HAHs can have a significant impact on the health and well being of human and animals. Accordingly, proper epidemiological, risk characterization and exposure analysis requires accurate measurements of these chemicals both in the species of interest and in various exposure matrices (i.e. biological, environmental, food and other sources). Given that HAHs are found not as individual congeners, but as complex mixtures of HAHs, of which the relative and absolute concentrations of individual congeners can vary dramatically, the identification and quantitation of these toxic/bioactive HAH congeners in environmental, biological and food samples and prediction of their overall toxic potential has been problematic. Sophisticated cleanup procedures followed by high-resolution analytical methodologies (gas chromatography-high resolution mass spectrometry

(GC/HRMS)) allows separation, identification and quantitation of individual PCDD, PCDF and PCB congeners. At present, this methodology is considered the “gold standard" for quantitative determination of these toxic HAHs in sample extracts. The relative toxicological/biological potency of a complex mixture of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin)-like HAHs is estimated from GC/HRMS results using the toxic equivalent factor (TEF) approach. In this approach, the concentration of individual toxic HAHs present in the mixture (determined by GC/HRMS) are multiplied by their specific TEF value (an in vivo toxicity value for each toxic HAH expressed relative to that of TCDD) to yield toxic equivalency (TEQ) values for each active chemical. Summation of the individual TEQs for all active congeners in a sample extract yields the total TEQ for that extract. While the GC/HRMS-TEF approach currently provides the most accurate measurement of the level of TCDD-like HAHs in a given sample and a reasonable estimate of the relative toxic potency of the sample extract (in TEQs), this methodology is not amenable or cost-effective for high throughput routine screening analysis needed for large scale sample studies (i.e., epidemiological studies, site contamination assessment and safety monitoring of food/feed contamination). Accordingly, numerous rapid and relatively inexpensive bioanalytical methods for use in the detection and relative quantitation of TCDD-like HAHs present in complex mixtures of HAHs extracted from a variety of matrices have been developed.[1-5]

Research efforts directed toward the development, validation, application and utilization of bioanalytical methods for detection of TCDD and TCDD-like HAHs have rapidly expanded since the first cell-based system (the rat hepatoma cell ethoxyresorufin O-deethylase (EROD) bioassay) for screening was described in the late 1970's.[6] A cursory examination of the PubMed online database identified more than 300 publications related to dioxin immuno/bioassays since 1980, more than 100 of which have been published since January of 2005. Bioanalytical methods for detection and relative quantitation of TCDD-like HAHs are divided into two basic categories, immunoassays and bioassays. While numerous assays have been described over the years, few have gained widespread use as screening assays and even fewer have received official certification or acceptance.

Immunoassays are perhaps the most specific and simplest of all the bioanalytical methods and they take advantage of the high affinity and specificity of a unique sensor (antibody) that has been generated against a specific target chemical(s).[1-5] Numerous antibodies specifically directed against toxic TCDD-like PCDD/Fs or PCBs have been described over the years and although these antibodies can cross-react with structurally related congeners (the cross-reacting congeners are typically known), a positive signal obtained in these assays does confirm the presence of the specific target chemical or closely-related congener in the sample extract. This is something that none of the currently available bioassays can determine. Other advantages of immunoassay approaches include less extensive clean-up of crude sample extracts, faster processing and analysis than most bioassays and availability in commercial kit form for ease of use. The limitations of immunoassay approaches include significantly lower sensitivity and cross-reactivity with non-target chemicals that can result in false positive results or overestimation of target chemical

concentrations (similar to that of bioassays). Numerous immunoassays have received regulatory certification as acceptable screening methods for PCDD/Fs and PCBs (i.e. USEPA Method 4025 and 4020, respectively) and have been used to screen a wide variety of environmental and biological matrices and food products.

Many bioassay systems for TCDD-like HAHs have been developed over the years and all are based on the molecular mechanism by which these chemicals produce their toxic/biological effects and each takes advantage of one or more aspect of the ability of these chemicals to specifically bind to and activate the Ah receptor (AhR) signal transduction pathway.[1-5] AhR-dependent bioassays can be subdivided into: 1) assays that require the use of cell/tissue extracts and measure the ability of chemicals in a sample extract to stimulate AhR DNA binding and 2) those that require the use of living cells in culture and measure the ability of chemicals in a sample extract to stimulate AhR-dependent gene expression.

All of the currently available in vitro (cell-free) AhR-based bioassays use AhR-containing tissue extracts (typically liver cytosol) and are dependent upon the ability of TCDD-like HAHs to bind to the AhR, transform it into its DNA binding form (an event which requires it to dimerize with the Arnt protein) and to bind to a specific DNA sequence (the dioxin-responsive element). Detection of HAH-dependent transformation of the AhR into its DNA binding form in these assays is accomplished by direct measurement of the amount of AhR:Arnt complex bound to radio- or fluorescently-labeled DRE (GRAB and FITC-DRE assays, respectively), detection of the AhR:Arnt:DRE complex with an anti-Arnt antibody (Ah Immunoassay) or quantitation of the amount of DRE bound to the transformed AhR complex after isolation and amplification by PCR (AhRC-PCR and EPM-PCR assays).[1-4] Formation of the AhR:Arnt:DRE complex occurs in a chemical-, dose- and AhR-dependent manner and it has been used to demonstrate that a specific chemical or a chemical(s) present in crude or purified extracts has the ability to stimulate AhR transformation and DNA binding. These assays have an advantage of being rapid, inexpensive, easy to carry out and very sensitive (although somewhat less sensitive than cell-based bioassays) and several are commercially available. However, in contrast to the cell based assays, the in vitro DNA binding assay methods suffer from one major disadvantage with regards to their utility as screening bioassays for TCDD-like HAHs and that derives from the well documented ability of the AhR to bind and be transformed in vitro by a very large number of structurally diverse chemicals with no similarity to TCDD or other HAHs.[9-11] Contributing to this, we have also found that many solvents and chromatographic matrices used for the extraction and cleanup of samples for HAH analysis can contain AhR agonists that are active in the in vitro DNA binding assays. Thus, in vitro AhR-based assays will be prone to having high levels of false positives when examining unknown sample extracts unless extreme care is taken in sample preparation and cleanup to eliminate these chemicals. These cell-free AhR-based assays have been developed relatively recently and have not been used as extensively as the immunoassays or cell-based assays. Accordingly, testing and validation studies of these assays with real world sample extracts are in progress.

Perhaps the most extensively used of all bioanalytical approaches for the detection and relative quantitation of TCDD-like HAHs are the in vitro cell-based bioassays.[1-4] In these systems, incubation of cells in culture for varying lengths of time with extracts containing TCDD-like HAHs leads to the induction of AhR-dependent gene expression that occurs in a chemical-, dose- and AhR-dependent manner and the magnitude of gene induction is directly proportional to the concentration of bioactive HAH in the original extract. Measurement of AhR-dependent induction of cytochrome P4501A1-dependent EROD activity in rat hepatoma (H4IIe) cells was the first dioxin cell bioassay and it has been the most extensively used of all bioassays for detection of TCDD-like HAHs in a wide variety of matrices.[6,12] Induction of EROD activity as a bioassay in primary cells and continuous cell lines from a variety of other species has also been reported. In this assay, CYP1A1-dependent metabolism of 7-ethoxyresorufin results in a highly fluorescent product (resorufin) that can be easily quantitated. Analysis using this assay have not only revealed a strong correlation between the induction of EROD activity and the toxicity of various HAHs and HAH-containing mixtures, but also a good correlation with HAH levels as determined by GC/HRMS. The H4IIe EROD bioassay is rapid, simple and relatively inexpensive, it can be carried out in whole or lysed cells in 96-well microplate formats and there are no limitations or restrictions for free use of these cells for sample analysis. However, the EROD bioassay does have lower sensitivity when compared to other cell based assays (i.e. the chemically-activated luciferase expression (CALUX)) and EROD activity is susceptible to inhibition by high concentrations of HAHs and PAHs, which could result in underestimation of total activity if full concentration-response curves are not obtained. The remaining cell bioassay systems utilize recombinant cells (yeast or mammalian cell lines) that have been engineered to contain a stably transfected AhR-responsive reporter gene that responds to TCDD-like HAHs and other AhR agonists.[3] Reporter genes in these bioassay include luciferase (contained in the XDS-CALUX, DR-CALUX, RGS-101L and DRE-CALUX bioassays), secreted alkaline phosphatase (contained in the DRESSA bioassay), beta-galactosidase (contained in AhR/Arnt transfected YCM3 yeast cells), and green fluorescent protein (contained in the CAFLUX bioassays).[1,3,13] These recombinant cell bioassays are for the most part very rapid, simple, sensitive and relatively inexpensive and many have been used for analysis of a variety of chemicals and sample matrices. Some of these cell based systems (CALUX and CAFLUX) and their respective AhR-responsive plasmid vectors are freely available for academic research purposes from out laboratory. A major advantage of recombinant approaches in bioassay development is that it is relatively simple to increase the sensitivity and specificity of these assays by manipulation of the reporter gene construct, so it is expected that these systems will continue to develop and improve. Cell-based assays, like the cell-free AhR-based bioassays, could be activated by other AhR agonists present in sample extracts, potentially leading to false positives or over estimation of activity. However, the advantage of the cell-based bioassays is that they contain metabolic enzymes that can degrade nonHAH AhR ligands (agonists) resulting in a dramatic reduction or elimination of these confounding activities (unless they are present in extremely high concentrations). Additionally, various modifications have also been incorporated in extraction and clean-up procedures that reduce these undesired agonist

activities. Acceptance of these cell-based bioassays by regulatory agencies for routine screening and monitoring of TCDD-like HAHs has been slow, understandably so, given the lack of sufficient numbers of appropriate blinded validation results. To date, the majority of the analysis of real world environmental, biological and food samples has been carried out using the EROD and XDS- and DR-CALUX bioassay systems. Comparisons of results obtained with a wide variety of sample extracts using these bioassays has shown a high correlation between TEQs determined by the GC/HRMS-TEF approach and equivalents determined by the EROD and CALUX assays, contributing to the increased use of these bioassays. Recent double-blind analysis of a large number of biological and environmental matrices was carried out using the XDS-CALUX bioassay and a high degree of correlation with of the bioassay results with TEQs determined by GC/HRMS. Not only do these double blinded study results provided strong validation for this method and the XDS-CALUX bioassay, but they contributed to the recent certification of the XDS-CALUX assay as USEPA Method 4435. Validation studies and submission of numerous other AhR cell-based bioassays have resulted in several receiving method certification by organizations and governmental agencies for screening and monitoring purposes (XDS-CALUX (BELTEST Certification; Approved Method of the Japan Ministry of the Environment), DR-CALUX (ISO1725 Accreditation; GMP+ Certification) and the RGS-101L bioassay (USEPA Method 4425-1; Method E1953-96 ASTM)). With these recent certifications, the application and utilization of these and other cell based bioassay systems for screening and monitoring of TCDD-like HAHs are certain to continue to expand.

While bioanalytical methods, when coupled to an appropriate sample extraction and clean-up method, can provide assay endpoint results that are strongly correlated to those obtained using GC/HRMS-TEF approaches (as a result of mechanistic considerations), the results obtained from these assays are not identical. Data analyzed by GC/HRMS are expressed in WHO-TEQs and use TEF values that are derived from direct measures of the relative toxicity of individual TCDD-like HAHs from animal studies. In contrast, while the bioassay methods do provide a measure of the overall relative biological potency of an extract (based on the specific mechanistic assay endpoint being examined) data resulting from these bioassays methods do not provide any direct measurement of the toxicity or toxic potency of a sample extract. The endpoints being examined in all AhR-based bioassays and immunoassays are not the results of toxicity. Given that the exact chemical components contained within a given "cleaned-up" sample are typically unknown and assumed to contain only HAHs, their ability to produce toxicity can not directly be assessed by these bioassays and relative toxicity is simply inferred based on AhR mechanistic considerations. Accordingly, the use of the term TEQ, which is defined as Toxic Equivalents, is inappropriate for expression of overall results obtained using bioassay methods. Given the rapidly expanding use and development of new bioassays and use of the TEQ term for results obtained in all AhR bioassays regardless of the endpoint examined, a reassessment of bioassay terminology should be considered. As it stands, there is considerable confusion in the literature and among investigators using the assays and regulators examining bioassay results. One possibility is to

consider the use of the term "TEQ" only for those results determined using GC/HRMS-TEF approaches and the use of different terminology, such as bioanalytical equivalents (BEQs) or something similar, for results obtained using bioanalytical approaches. While a change in terminology will not affect interpretation of the results, correlations of the data or the utility of these assays, they will present the results in a manner consistent with the results obtained from the methodologies employed.

Over the past decade, our increased understanding of the molecular mechanisms of action of dioxins and related HAHs has allowed the continued development of relatively rapid, inexpensive and sensitive bioanalytical methods for the detection and relative quantitation of these chemicals. While many of these assays have numerous advantages over the more costly instrumental analysis for rapid screening applications, they also have limitations. However many of these methodologies are still in early stages of development and given the rapid progress in biotechnology, it is certain that additional improvements in sensitivity, specificity and detection and chemical clean-up methods will be available in the very near future. Several of these immunoassays and bioassays are now commercially available. In addition to technical improvements in the assays themselves, before these bioassays can be accepted for regulatory use, they must be subjected to full method validation studies and must meet widely accepted performance criteria of national and international standards organizations and federal agencies (European Commission, USEPA, ASTM, APHA, NATA, STER, BELTEST, ISO 17025 and others). The availability of fully validated and standardized AhR-based bioassay methodologies for detection and relative quantitation of TCDD-like HAHs will not only facilitate large scale screening studies for these chemicals, but they provide relatively inexpensive screening methods to complement and enhance instrumental analysis of unknown samples by allowing these laboratories to prioritize samples and focus their efforts on analysis of positive samples. Validation and standardization of several AhR bioassay methodologies are currently in progress, some have either received or are currently pending regulatory approval and certification.

Acknowledgements
Support for our continued development of dioxin bioanalytical methods has been obtained from an US NIEHS Superfund Basic Research Grant (ES04699).

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