Hazard and Risk Assessment of Chemical Mixtures Using the Toxic Equivalency Factor Approach

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There is considerable public, regulatory, and scientific concern regarding human exposure to endocrine-disrupting chemicals, which include compounds that directly modulate steroid hormone receptor pathways (estrogens, antiestrogens, androgens, and antiandrogens) and aryl hydrocarbon receptor (AhR) agonists, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds. Based on quantitative structure–activity relationships for both AhR and estrogen receptor (ER) agonists, the relative potency (RP) of individual compounds relative to a standard (e.g., TCDD and 17β-estradiol) have been determined for several receptor-mediated responses. Therefore, the TCDD or estrogenic equivalent (TEQ or EQ, respectively) of a mixture is defined as TEQ = Σ(Ti/Ri) or EQ = Σ(Ei/Ei), where Ti and Ei are concentrations of individual AhR or ER agonists in any mixture. This approach for risk assessment of endocrine-disrupting mixtures assumes that for each endocrine response pathway, the effects of individual compounds are essentially additive. This paper will critically examine the utility of the TEQ/EQ approach for risk assessment, the validity of the assumptions used for this approach, and the problems associated with comparing low dose exposures to xeno and natural (dietary) endocrine disruptors. — Environ Health Perspect 106(Suppl 4):1051-1058 (1998).

Key words: endocrine disruptors, TCDD, TEFs, hazard, limitations, interactions

Introduction

The potential adverse impacts of chemicals are dependent on a number of factors, including levels and duration of exposure, chemical potency, timing of exposure, mechanism of action, and interactions between chemicals in a mixture. Hazard and risk assessment of chemicals carried out by regulatory agencies have focused primarily on the toxicities of individual compounds, whereas wildlife and humans are exposed to complex mixtures of man-made compounds that act through multiple pathways. Moreover, the human diet contains many natural products and cooking-derived compounds that exhibit many of the same toxic, mutagenic, and carcinogenic properties of industrial-derived contaminants (1–3). In most cases, humans are exposed to significantly higher levels of natural products than the man-made chemicals that act through the same pathway. For example, early development of the Ames test for detecting bacterial mutagens generated considerable scientific, regulatory, and public concern over human exposure to the many different industrial chemicals that exhibited mutagenic activity in one or more of the highly sensitive bacterial tester strains (4,5). Subsequent studies demonstrated that some of the most mutagenic compounds in the human diet are not industrial-derived contaminants, but natural compounds that include a complex series of heterocyclic aromatic amines derived from cooking proteinaceous foods (e.g., fish, beef, poultry) (6–8). Thus, the public health concern regarding human exposure to mutagens must take into account intake and potency of both natural and man-made chemicals that act through various pathways.

Hazard and risk assessment of human exposures to chemicals must also take into account scenarios where chemical interactions may significantly influence toxic outcomes. For example, despite relatively high levels of human exposure to natural carcinogens in the diet, there are several other classes of natural products (e.g., flavones, antioxi-
dants) that inhibit P450-mediated metabolic activation or induce detoxifying enzymes, and these compounds may provide protection against natural or man-made toxins (9–11). In contrast, workplace or environmental exposures to nontoxic levels of organochlorine solvents such as chloroform may lead to hepatotoxic effects if there is concurrent exposure to ketones because of their nonadditive (synergistic) interactions (12,13). Thus, chemical interactions are important determinants in evaluating the potential hazards and risks of exposure to chemical mixtures. This manuscript will outline the development, validation, and pitfalls associated with the toxic equivalency factor (TEF) approach for risk assessment of complex mixtures.

Toxic Equivalency Factors: An Approach for Hazard and Risk Assessment

The TEF approach has been extensively used for hazard assessment of different classes of toxic chemical mixtures. The overall toxicity or toxic equivalents (TEQs) of a mixture are defined by the concentration of individual compounds (Ci) in a mixture times their relative potencies or TEFs.

\[
TEQ = \Sigma (C_i \times TEF_i)
\]

The assumptions implicit in utilizing the TEF approach include: the individual compounds all act through the same biologic or toxic pathway; the effects of individual chemicals in a mixture are essentially additive at submaximal levels of exposure; the dose–response curves for different congeners should be parallel; and the organotropic manifestations of all congeners must be identical over the relevant range of doses (14,15). TEF values are either derived for a
species-specific response or are a composite value obtained from TEFs for several responses, and individual TEFs are usually determined relative to the activity of a standard or reference compound. The TEF approach has been applied to different structural classes of compounds, including polynuclear aromatic hydrocarbons (PAHs), halogenated aromatic hydrocarbons (HAHs), and endocrine disruptors. The utility and problems associated with TEFs and TEQs will be discussed.

**Toxic Equivalency Factor Approach for Polynuclear Aromatic Hydrocarbons**

Individual PAHs such as benzo[a]pyrene (B[a]P) have been extensively investigated as carcinogens and as ligands for the aryl hydrocarbon receptor (AhR). The carcinogenic activity of PAHs is dependent on the oxidative metabolic activation of these compounds into genotoxic metabolites, which subsequently interact with DNA to initiate carcinogenesis. The carcinogenic potencies of individual PAHs have been determined in different bioassays and TEF values proposed for various PAHs are summarized in Table 1 (16–19). The utility of this approach was demonstrated in studies using relatively simple reconstituted PAH mixtures in rodent carcinogenicity models (20,21). However, Warshawsky and co-workers (22) recently demonstrated that there are a number of important factors that can significantly modulate the genotoxicity of PAH mixtures, including the presence or absence of B[a]P, the dose, and the solvents used in carcinogetic administration. This variability of carcinogenic potency suggests that the TEF approach for PAHs may not be appropriate for some animal models. Studies in several laboratories have investigated the biochemical, toxic, and genotoxic activities of manufactured gas plant (MGP) residues, which contain complex mixtures of PAHs (23–27). Comparisons of the effects of MGP PAHs with B[a]P or a reconstituted mixture of PAH hydrocarbons suggested that the mixture induced synergistic responses or that other factors were important. Based on results of recent studies (24,25,28), the high activity/genotoxicity of the MGP–PAH mixture may be due to unidentified alkyl PAHs. For example, a reconstituted mixture of the 17 major PAHs in an MGP sample (24) did not induce liver tumor formation in the B6C3F1 male juvenile mouse model at a dose of 1071 mg/kg; in contrast, the field-derived sample induced a 45% incidence of liver tumors at the same dose (25,28). These results demonstrate that applications of TEFs for PAHs require a more detailed knowledge of the complete composition of these mixtures and the TEFs of all active components. Thus, the approach may be useful for defined PAH mixtures containing only parent hydrocarbons; however, for mixtures containing alkyl PAHs, the TEF approach is not valid because of the minimal data available on the identities and relative potencies of these compounds.

**Toxic Equivalency Factor Approach for Halogenated Aromatic Hydrocarbons**

Polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), polychlorinated naphthalenes (PCNs), and polychlorinated biphenyls (PCBs) are HAHs that are industrial compounds or industrial combustion by-products (Figure 1). These compounds are chemically and environmentally stable and have been identified in almost every component of global ecosystems, including fish and wildlife and human tissues, adipose tissue, and milk (29,30). HAHs are also routinely detected as residues in diverse food products, and the diet is the major source of human exposure to HAHs (30,32).

The mechanism of action of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related HAHs has been extensively investigated and the results support a pathway that involves initial ligand (HAH) binding to the intracellular AhR, which is widely expressed in mammalian tissues (33). The mechanism of AhR-mediated CYP1A1 induction has been extensively investigated; the results show that the AhR is a ligand-induced nuclear transcription factor in which transactivation is associated with interaction of the heterodimeric nuclear AhR complex with dioxin-responsive elements located in the 5′-promoter region of the Ah-responsive gene (34,35). The mechanisms of AhR-mediated toxicity are

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### Table 1. Different toxic equivalency factor values proposed for individual polycyclic aromatic hydrocarbon congeners.

| Compound     | Thorslund et al. (18) | Chu and Chen (17) | U.S. EPA (19) | Nisbet and LaGoy (16) |
|--------------|-----------------------|-------------------|---------------|-----------------------|
| Benzo[a]pyrene| 1                     | 1                 | 1             | 1                     |
| Dibenzo[a]anthracene | 1.1                 | 0.69              | 1             | 5                     |
| Benzo[a]anthracene | 0.145               | 0.013             | 0.1           | 0.1                   |
| Benzo[ghi]fluoranthene | 0.140              | 0.08              | 0.1           | 0.1                   |
| Benzo[k]fluoranthene | 0.066               | 0.004             | 0.01          | 0.1                   |
| Indeno(1,2,3-c,d)pyrene | 0.232              | 0.017             | 0.1           | 0.1                   |
| Acenaphthene  | ND                    | ND                | 0             | 0.001                 |
| Acenaphthylene| ND                    | ND                | 0             | 0.001                 |
| Anthracene    | 0.32                  | ND                | 0             | 0.01                  |
| Benzo[a]pyrene| 0.022                 | ND                | 0             | 0.01                  |
| Chrysene      | 0.0044                | 0.001             | 0.001         | 0.01                  |
| Fluoranthene  | ND                    | ND                | 0             | 0.001                 |
| Fluorene      | ND                    | ND                | 0             | 0.001                 |
| 2-Methylnaphthalene | ND                   | ND                | 0             | 0.001                 |
| Naphthalene   | ND                    | ND                | 0             | 0.001                 |
| Phenanthrene  | ND                    | ND                | 0             | 0.001                 |
| Pyrene        | 0.001                 | ND                | 0             | 0.001                 |

Abbreviations: ND, not determined; U.S. EPA, U.S. Environmental Protection Agency.

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**Figure 1.** Halogenated aromatic hydrocarbons.
unknown; however, it is assumed that many of the responses are because of altered gene expression.

Hazard and risk assessment of PCDDs and PCDFs initially focused on quantitation of TCDD in various environmental samples; however, with development of high-resolution analytical techniques coupled with studies on structure–toxicity relationships, it was apparent that the bulk of the toxicity induced by most PCDD/PCDF mixtures is not due to TCDD alone. Based on the well-characterized structure–activity relationships established for PCDDs and PCDFs (36–39), a TEF approach has been developed for these compounds:

\[
\text{TEQ} = \sum [\text{PCDF}] \times \text{TEF}\text{; and} \sum [\text{PCDD}] \times \text{TEF},
\]

where \([\text{PCDD}]\) and \([\text{PCDF}]\) represent the concentrations of the individual compounds (40–46). Individual TEFs have been assigned to all the 2,3,7,8-substituted PCDD and PCDF congeners (Table 2) because these are the compounds that have primarily been detected in environmental samples and are among the most potent congeners. The TEF for each 2,3,7,8-substituted congener compared to TCDD is variable among cell types, laboratory animal species, target organs, and responses. Research in our laboratory has extensively investigated the immunotoxicity-derived TEFs for several HAHs in mouse models (47–50); TEFs for inhibition of the plaque-forming cell response to trinitro-phenyl–lipopolysaccharide by 2,3,4,7,8-pentachlorodibenzofuran in C57BL/6, DBA/2, and B6C3F1 mice varied by approximately 7-fold, and in some assays this congener was more potent than TCDD. Over a broader spectrum of responses, TEFs for individual PCDD/PCDF congeners can vary by over 100-fold. The broad range of TEF values for a specific congener compromises the use of a single TEF for this congener and may over- or underestimate the calculated TEQ for a mixture. Variable TEFs are due to many factors including differential pharmacokinetics and metabolism of HAHs in various in vivo and in vitro bioassays.

Validation of the TEF approach can be investigated by determining the in vitro or in vivo toxicities of reconstituted mixtures of PCDDs and PCDFs and comparing their observed versus calculated potencies. Eadon and co-workers (51) utilized a PCDF/PCDD mixture (primarily PCDFs) resulting from a PCB fire and compared the calculated versus observed effects for several end points in the guinea pig, including decreased body and thymus weights, increased serum triglycerides, decreased serum alanine aminotransferase levels, and formation of hepatocellular cytoplasmic inclusion bodies. Their results showed that the experimentally observed TEQs per kilogram ranged from 2 to 21 ppm depending on end point; the calculated value using a set of provisional TEFs was 14.5 ppm. These results demonstrated a good correspondence between the observed and calculated values. Other reports using multiple end points in both in vivo and in vitro models demonstrated that for several PCDD/PCDF mixtures, there is a reasonable correspondence between calculated and experimentally determined TEQs (52–60). For more complex mixtures containing compounds that act through multiple pathways to give both similar and different toxic responses, the TEF/TEQ approach may not be appropriate. Moreover, it should also be noted that even for PCDDs/PCDFs, there is some evidence that TEFs do not always predict relative congener potency in different rat strains (61).

Several studies have also demonstrated that the coplanar PCBs (i.e., 3,3',4,4'-tetra-, 3,3',4,4',5-penta, and 3,3',4,4',5,5'-hexachlorobiphenyl [CB]) (Figure 2) bind to the AhR and induce a broad spectrum of AhR-mediated biochemical and toxic responses (62,63). Tanabe and co-workers (64,65) first utilized CYP1A1 induction-derived TEFs (66) for coplanar PCBs and their mono-ortho-substituted analogs to show that these compounds contributed substantially to the TEQs of diverse industrial/environmental extracts. Provisional TEFs have been proposed for coplanar and mono-ortho coplanar PCBs and these values are used routinely for determining total TEQs (i.e., PCDDs, PCDFs, and PCBs) in various extracts (67). The relative TEQ contributions of different classes of HAHs are variable; however, there are numerous examples that demonstrate that PCB TEQs contribute >50% of total TEQs. For example, a recent study on HAHs in a human milk sample (2 weeks after birth) in the Netherlands showed that TEQ values for PCDDs/PCDFs and PCBs were 30.2 and 35.5 ppt (fat weight basis), respectively (68).

The major problems associated with the TEF approach for HAHs is primarily associated with nonadditive antagonistic interactions between AhR agonists (PCDDs/PCDFs and PCBs) and PCB congeners that exhibit response and cell/species-specific
antagonistic activity (69–84) (Table 3). Research in our laboratory has clearly demonstrated that several PCB congeners and some commercial mixtures exhibit AhR antagonist activity. For example, 2,2’,4,4’,5,5’-hexaCB (PCB 153), a major persistent congener in environmental samples, inhibits the following TCDD or 3,3’,4,4’,5-pentachlorobiphenyl-induced responses: induction of ethoxyresorufin O-deethylation activity in chick embryo hepatocytes; inhibition of the plaque-forming cell response to sheep red blood cells in mice; inhibition of the plaque-forming cell response to trinitrophenyl lipopolysaccharide in mice; inhibition of serum IgM units in mice; induction of fetal cleft palate in mice; induction of chick embryo malformations; induction of chick embryo edema; induction of chick embryo liver lesions; and induction of fetal hydrocephalus in mice. These nonadditive interactions, coupled with the unusually broad range of TEF values observed for some PCB congeners (e.g., 3,3’,4,4’-tetraCB), compromises the utility of the TEF approach for hazard and risk assessment of HAHs that contain PCBs. Therefore, TEFs/TEQs for HAHs must be used very selectively, and more research on the utility, applications, and limitations of this method should be conducted.

### Toxic Equivalency Factor Approach for Endocrine Disruptors

It has been hypothesized that industrial-derived estrogenic compounds (xenoestrogens) and possibly other naturally occurring estrogens may be responsible for a global decrease in male reproductive capacity (e.g., sperm counts) and increased incidence of breast cancer in women (85,86). The validity of these hypotheses has been questioned (1,87), and resolution of the role of hormonally active compounds in human disease requires further study.

Like AhR agonists, hormonelike compounds that act through specific cellular receptors should be good candidates for using a TEF approach. Verdeil and Ryan (88) previously compared human exposures to man-made and naturally occurring estrogenic compounds using a TEF/TEQ approach and diethylstilbestrol equivalents. The recent discovery of a second form of the estrogen receptor (ER), ERβ (89), further complicates development of an estrogen equivalent (EQ) approach for estrogenic compounds. The specific responses that are mediated via ERα or ERβ have not been delineated, and relative potency factors for these responses by different structural classes of natural and man-made estrogenic compounds have not been determined. Several groups have reported TEFs for both natural ligands for the ER (e.g., flavonoids, lignans) and for xenoestrogens, which are industrial-derived chemicals and their bi-products (90–93). Although individual TEFs have been assigned for each compound, most assay systems indicate that with few exceptions, both natural and xenoestrogens are >1000 times less potent than 17β-estradiol (E2). A close inspection of the data obtained for estrogenic compounds reveals that there are many problems in development of a TEF approach for these compounds and some of these problems are similar to those observed for HAHs and PAHs.
compared to that observed for the compounds alone. This type of nonadditive interaction would negate a TEF/EQ approach for hazard assessment of these mixtures. However, studies from other laboratories (97,98) using the same compounds have not observed synergism. The paper on synergism was recently withdrawn (99); however, this does not preclude the possibility of other synergistic interactions.

**Interactions between Endocrine Response Pathways**

The TEF/EQ approach is most applicable for hazard and risk assessment of a specific class of endocrine active compounds that act through a common receptor. However, as noted previously, there are a number of factors that complicate this approach and the problems are magnified with xenoenocrine active agents that act through steroid hormone receptor or thyroid hormone receptor-mediated pathways. For example, assessment of xenoestrogen exposure and potency (EQs) is complicated by tissue-specific agonist/antagonist activities, lack of data on intake and serum levels, and their relative contribution to total estrogen equivalents compared to much higher intakes of natural estrogenic chemicals in the diet (1). In addition, many compounds may interact with more than one hormone receptor and modulate multiple endocrine response pathways. For example, $2',3',4',5'$-tetrachloro-2-biphenylol binds to the ER and exhibits ER-agonist activities (100,101). The same compound also binds to the androgen receptor in a yeast-based assay but inhibits dihydrotestosterone-induced reporter gene activity in human Hep G2 liver cancer cells transiently transfected with the human androgen receptor and an androgen-responsive construct (102). Although $2',3',4',5'$-tetrachloro-4-biphenylol did not bind the progesterone receptor, in a progesterone-responsive yeast assay this hydroxy-PCB inhibited progesterone receptor-mediated transactivation (103). These data illustrate how one endocrine-active compound can modulate multiple endocrine response pathways.

Another major problem for hazard assessment of xenoestrogens is associated with tissue-specific cross talk between different receptor-mediated pathways, which can lead to significant modulation of estrogen-induced responses. It has been pointed out that in human breast cancer cells, cross talk between the ER- and AhR-signaling pathway results in inhibition of estrogen-induced responses (1). Although this interaction is likely to be cell specific, it is possible that the estrogenic activity associated with xenestrustrogen in the mammary gland will be inhibited by both xeno AhR and natural AhR agonists in the diet. Research in this laboratory has also focused on cross talk between the ER and other receptors that bind natural dietary constituents. Vitamin A, retinoids, phytol, and phytanic acid are vitamins or plant degradation products and are also important dietary constituents that act through the retinoic acid and retinoid X receptors, and there is cross talk between these receptors and the ER. For example, all-trans-retinoic acid, 9-cis-retinoic acid, and phytol inhibit estrogen-induced responses in breast cancer cells, and any effects of xenoestrogens in these cells would be opposed by retinoic acid and retinoid X receptor ligands. The importance of this type of counteractive cross talk in other tissues and organs has not been determined. These are only some examples of cross talk between endocrine-signaling pathways that must be considered in an overall risk assessment of dietary exposure to xenoestrogens and other xenodocrine active compounds, as well as natural compounds in food that act through the same signaling pathways.

**Summary**

Humans and wildlife are exposed in the diet to complex mixtures of natural and man-made chemicals. Hazard and risk assessment of these mixtures is a difficult process and the TEF approach has been utilized for several different classes of chemicals, including HAHs (AhR agonists), PAHs (carcinogens), and xenoestrogens (ER agonists). This review has pointed out both the utility and problems associated with the TEF approach for all three classes of chemicals. For example, although TEFs may be useful for regulating HAH emissions and cleanup levels, application of this concept for determining dietary TEQs is complicated by the unknown contributions of naturally occurring AhR agonists, which exhibit both AhR agonist and antagonist activities (2). Moreover, the issue of cross talk between multiple endocrine pathways would further compromise the validity of the TEF approach when applied to dietary intakes of different classes of man-made and natural chemicals. Based on these uncertainties, the TEF approach should be used for limited applications and only after validation in animal models.

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