

# Estimation of Toxicity of Chemical Mixtures through Modeling of Chemical Interactions

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The Agency for Toxic Substances and Disease Registry (ATSDR), in collaboration with the Dutch Organization for Applied Scientific Research (TNO) Nutrition and Food Research Institute, is conducting studies to evaluate the role of chemical interactions in the expression of toxicity from low-level exposure to combinations of chemicals. The goal of this collaborative effort is to use a weight-of-evidence (WOE) approach to estimate joint toxicity of some simple chemical mixtures and to compare the estimations with test results from animal toxicity studies. The WOE approach uses individual chemical dose-response assessments and algorithms that incorporate various assumptions regarding potential chemical interactions. Qualitative evaluations were prepared for binary combinations of chemicals for the effect of butyl hydroxyanisole on di(2-ethylhexyl) phthalate, the effect of stannous chloride on Cd chloride (CdCl<sub>2</sub>), and the effect of CdCl<sub>2</sub> on loperamide. Analyses of these evaluations and their comparison with the conclusions of laboratory animal experiments indicate that the WOE approach can be used to estimate qualitatively the joint toxicity of such simple mixtures. To further test the utility of the WOE approach, qualitative and semiquantitative evaluations were prepared for two chemical mixtures—one with similarly acting halogenated aliphatics (trichloroethylene, tetrachloroethylene, hexachloro-1,3-butadiene [HCBD], and 1,1,2-trichloro-3,3,3-trifluoropropene [TCTFP]) and the other with dissimilarly acting nephrotoxic components (mercuric chloride, lysinolanine, D-limonene, and HCBD). These two sets of data were used to estimate the overall toxicities of the mixtures using the WOE algorithm for the mixture. The comparison of the results of the estimated toxicity with experimentally determined toxicity of the mixture of similarly acting nephrotoxicants demonstrated that the WOE approach correctly adjusted for the observed interactions in experimental animal studies. However, this was not true for the mixture of dissimilarly acting nephrotoxicants. This could be attributed to the fact that WOE evaluations are based on dose additivity that postulates that all chemicals in a given mixture act in the same way—by the same mechanism—and differ only in their potencies. In these cases the WOE approach evaluations, based on consideration of common mechanisms for simple chemical mixtures, can lead to better estimates of joint toxicity of chemical mixtures than the default assumption of dose additivity. The results also show that the WOE evaluations should be target-organ specific because none of the models tested could approximate the observed responses in organs other than the target organs in the laboratory animal studies. — *Environ Health Perspect* 106(Suppl 6):1353–1360 (1998). <http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-6/1353-1360mumtaz/abstract.html>

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Abbreviations used: ASAT, aspartate aminotransferase; ATSDR, Agency for Toxic Substances and Disease Registry; BHA, butyl hydroxyanisole; BINWOE, binary weight of evidence; CdCl<sub>2</sub>, Cd chloride; CNS, central nervous system; DA, dose addition; DEHP, di(2-ethylhexyl)phthalate; HCBD, hexachloro-1,3-butadiene; HgCl<sub>2</sub>, mercuric chloride; HI, hazard index; LALA, lysinolanine; LIMO, D-limonene; Lop, loperamide; MRLs, minimal risk levels; OBV, observed values; PalmCoA, palmitoyl-Co A oxidase; RA, response addition; RfDs, reference doses; SnCl<sub>2</sub>, stannous chloride; TCE, trichloroethylene; TCTFP, 1,1,2-trichloro-3,3,3-trifluoropropene; TETRA, tetrachloroethylene; TNO, Organization for Applied Scientific Research; UF, uncertainty factor for interactions; WOE, weight of evidence.

Assessing the toxicity and health risk of environmental chemicals is a complex process. Major data gaps exist in the hazard-, dose-response-, and exposure-assessment components of the risk assessment process that leads to the risk characterization step as defined by the National Academy of Sciences (1). Although complete information is often not available, decisions must be made; thus, the practice of using empirically derived uncertainty factors has emerged out of necessity and has been readily assimilated in the health risk assessment process (2,3). Because so many chemicals and their mixtures are found in our environment, the challenges presented for risk assessment are immense. The focus of health risk assessment should be on chemical mixtures of actual concern to the public health, i.e., those that are found in completed exposure pathways rather than all those found in the environment. To identify chemicals of actual concern at hazardous waste sites the Agency for Toxic Substances and Disease Registry (ATSDR) has defined a multistep process that systematically evaluates the chemicals found at a site. The first step in identifying chemicals found at a site is to identify the contaminated media and the chemicals found in the media. Next, the route of exposure and a receptive population are identified. The focus of health risk assessments is those chemicals and their mixtures that may pose health risk to a human population through past, current, or future exposure (4–6).

Once the composition of a chemical mixture of actual concern has been determined, the toxicity assessment is performed, most often through the use of the hazard index (HI) approach. The HI approach allows the toxicity of the mixture to be estimated through potency-weighted dose addition (DA) of each component of the mixture, thus allowing exposure levels and toxicologic consequences of the exposure to be combined into a single value. This method allows the approximation of the toxicity of a mixture that has not been experimentally tested. Because of the preponderance of toxicity data on single chemicals, this method is the most often used of the approaches available for risk assessment of chemical mixtures (7). The major shortcoming of this approach is the potential for interactions of the components in a biologic system that could influence the overall toxicity of a given mixture.

A weight-of-evidence (WOE) methodology for using information on binary interactions to modify risk assessments for chemical mixtures has been published (8,9). In the first of the two parts of this procedure, a WOE determination for interactions of the binary components of the mixture (BINWOE) is performed. Due consideration is given to the pharmacokinetic and pharmacodynamic behavior of the chemical components to obtain a composite representation of all the pertinent toxicologic evidence. In the second part of this procedure the BINWOEs are integrated into the overall risk assessment process for the mixture using the HI approach. This involves the use of dose-response assessments on individual chemical minimal risk levels ([MRLs] or comparable values), estimated levels of exposure, inferences on how binary combinations of the chemicals will interact, and algorithms that incorporate various assumptions on how chemical interactions will influence the joint action of the chemicals. The method is based on classical concepts of joint action, i.e., DA and its simple deviations. To a large extent the usefulness of such a method depends on two characteristics: consistency of application and accuracy. The application of this methodology was deemed consistent by expert toxicologists who reviewed the same body of data and determined the WOE for specific pairs of chemicals (10). The current work was undertaken to evaluate the accuracy of this method and to determine if the WOE approach evaluations can better estimate and predict the toxicity of complex mixtures than the default assumption of dose additivity.

A series of animal studies for subchronic exposure to chemical mixtures has been conducted (11–13). A study that employed a nine-component mixture was used to evaluate the consistency of the BINWOE determinations that were considered significant in the experimental studies (11). Although the data for toxicity of the single components of this mixture were not available, the study used a fractional factorial design that permitted the identification of binary interactions among the chemicals. Described in detail among the several interactions suggested were those between three binary pairs of chemicals, namely, butyl hydroxyanisole (BHA) and di(2-ethylhexyl)phthalate (DEHP), Cd chloride ( $\text{CdCl}_2$ ) and loperamide (Lop), and stannous chloride ( $\text{SnCl}_2$ ) and  $\text{CdCl}_2$ . The second study tested a mixture that consisted of similarly acting renal toxicants

trichloroethylene (TCE), tetrachloroethylene (TETRA), hexachloro-1,3-butadiene (HCBd), and 1,1,2-trichloro-3,3,3-trifluoropropene (TCTFP). The third study of this series of mixtures consisted of dissimilarly acting renal toxicants that included mercuric chloride ( $\text{HgCl}_2$ ), lysinolalanine (LALA), D-limonene (LIMO), and HCBd. Several effects in the target organ as well as nontarget organ effects—such as increase in kidney weight (nephrotoxicity) and increase in liver weight (hepatotoxicity)—have been studied. Data from these studies were used to test the accuracy of the WOE approach and to determine if the WOE approach can be used to account for observed chemical interactions in experimental animal studies. Individual animal data were made available for all the mixture doses tested in these two studies (12,13). Also available were toxicity data of the individual chemicals used in the mixtures and assayed at more than one dose level. Thus, these studies provided data to perform a joint test for both the WOE determinations and the algorithms used in the overall WOE approach.

## Methods

### Qualitative Assessment of the Influence of Chemical Interactions

The overall procedure for deriving the WOE has been published by Mumtaz and Durkin (8). As a first step qualitative BINWOEs were prepared for three binary combinations of chemicals to evaluate the effect of: BHA on DEHP,  $\text{SnCl}_2$  on  $\text{CdCl}_2$ , and  $\text{CdCl}_2$  on Lop. Also, BINWOEs were

prepared for all the possible binary combinations of the chemical components of two 4-component mixtures, namely, the mixture of TCE, TETRA, HCBd, and TCTFP and a second mixture of  $\text{HgCl}_2$ , LALA, LIMO, and HCBd. To accomplish this assessment, literature searches were performed on databases such as Medline and Toxline (14) to identify the primary sources of information for chemical interactions for these chemicals. Also, secondary sources such as the toxicologic profiles for these chemicals were used to identify pertinent studies for the chemicals. Relevant studies were identified and examined in detail, and draft qualitative BINWOEs were prepared for each pair of chemicals. These first drafts contained narrative explanations of the rationale including the available mechanistic and toxicologic information. These summaries also contained a discussion of the limitations and uncertainties that were associated with a given assessment. The draft summaries underwent an internal review, and interaction matrices (Tables 1, 2) were prepared for the four-component chemical mixtures before being sent to the ATSDR. ATSDR reviewers worked in groups to determine how well each evaluation reflected the information contained in the body of the summary assessment provided to them. If opinions regarding an assessment differed, a consensus was developed and appropriate modifications were made to the assessment. For the simple mixtures, the consensus BINWOEs were summarized by arraying them in descriptive

**Table 1.** Qualitative weight of evidence interaction matrix for similarly acting nephrotoxicants.

	Effect on			
	HCBd	TCE	TETRA	TCTFP
TCTFP	—	= IIC 0.0	= IIC 0.0	< IIC -0.23
TCE	< IIC -0.1	—	= IIB 0.0	< IIC -0.1
TETRA	< IIC -0.1	< IIB 0.0	—	< IIC -0.1
TCTFP	< IIC -0.23	= IIC 0.0	= IIC 0.0	—

The WOE of the influence of one chemical on another is determined by identifying the row for the first chemical and reading across to the column associated with the second chemical. For the WOE of the influence of TCE on TCTFP see row 2, column 5.

**Table 2.** Qualitative weight of evidence interaction matrix of dissimilarly acting nephrotoxicants.

	Effect on			
	HCBd	$\text{HgCl}_2$	LALA	LIMO
HCBd	—	> IIB2bii 0.25	= IIC2bii 0.0	= IIC2bii 0.0
$\text{HgCl}_2$	< IIB2bii -0.25	—	= IIC2bii 0.0	= IIC2bii 0.0
LALA	= IIC2bii 0.0	= IIC2bii 0.0	—	= IIC2b2 0.0
LIMO	= IIC2bii 0.0	= IIC2bii 0.0	= IIC2bii 0.0	—

The WOE of the influence of one chemical on another is determined by identifying the row for the first chemical and reading across to the column associated with the second chemical. For the WOE of the influence of HCBd on  $\text{HgCl}_2$  see row 1, column 3.

interaction matrices. Each matrix lists the potential binary classification along both axes and the column headings indicate the chemicals that are affected by the chemicals listed in the row headings.

Because the BINWOEs were used in this assessment to evaluate the WOE method with the experimental results, the BINWOEs were prepared in an unbiased and independent fashion. In both the original draft and the review, individuals involved in the preparation of BINWOEs were given all the pertinent published studies obtained from literature searches except literature published from the Organization for Applied Scientific Research (TNO) mixtures studies (11–13). To prepare the BINWOEs the ATSDR contracted with the Kevric Company (Silver Spring, Maryland). The initial BINWOEs thus obtained were reviewed by a group of ATSDR toxicologists who had not seen the results and findings of the TNO mixtures reports (11–13). Following this review the quantitative WOE analysis was performed at Syracuse Environmental Research Associates (Syracuse, New York).

### Weight of Evidence for the Joint Toxicity of Simple Mixtures

The assessments and the resulting companion interaction matrices obtained as previously stated were used in the second part of this study to determine if the overall WOE method, in conjunction with the HI approach as proposed by Mumtaz and Durkin (8), could be used to explain the observed experimental toxicity in animals. When experimental data for evaluating the WOE method were used, some adaptations to the published method were necessary. For the most part, these adaptations were relatively straightforward. The qualitative evaluations were converted to numerical scores using the data quality weighting factors described in Table 3.

For the experimental data on single chemicals, the continuous response variables—such as change in relative kidney weight—are fit to the exponential model:

$$R = e^{\alpha + \beta_i \times d_i} \quad [1]$$

$R$  is a continuous response variable,  $\alpha$  is the estimate of the control response—the response when dose is 0— $i$  is the potency parameter for the  $i$ th chemical, and  $d_i$  is the dose of the  $i$ th chemical. For each end point, this dose–response function was fit to

each of the chemicals in the mixture based on single-chemical data using a common background response, i.e.,  $\alpha$ .

In these analyses as well as regression analyses of the data on mixture exposures, maximum likelihood estimates were computed by the method of least squares. The estimate of the observed dose–response relationship, the 95% confidence limits of the regression, and the 95% prediction limits were also calculated. Model fit was assessed based the square of the correlation coefficient as well as the standard F-ratio of the mean square variance of the model to the mean square of the residual. In cases for which duplicate observations were available for a given dose, the F-ratio for the lack of fit against pure error was used to assess the assumptions of independence, normality, and uniform variance (15).

Because of the nature of the single-chemical data only the linear and exponential models were considered. The exponential model was selected because it provided a somewhat better fit to most of the experimental data sets than the simple linear model. This is not to suggest that either the simple linear or exponential models are plausible over a wide range of doses. For continuous variables such as organ weights, a more plausible model would incorporate an estimate of a maximum change and would probably be sigmoidal in shape. For the single-chemical bioassays, however, only two positive dose groups plus a control group were tested. In all cases the lower dose was selected to approximate the no observed effect level and the high dose to approximate the lowest observed adverse effect level. These data are not adequate for fitting more complex

sigmoidal models. The selection of the dose–response model has relatively little impact on this analysis because the range of doses used in the mixture studies is largely encompassed by the range of doses used in the single-chemical bioassays and little low-dose extrapolation and no high-dose extrapolation are required.

For the exponential dose–response model involving multiple chemicals, the dose–response model is expressed as

$$R = e^{\alpha + \sum_{i=1}^n \beta_i \times d_i} \quad [2]$$

where  $n$  is the number of compounds. This is essentially identical to the assumption of DA and is thus directly analogous to the HI.

The WOE method itself was modeled as:

$$R = e^{\alpha + \sum_{i=1}^n \beta_i \times d_i \times UF_i^{WOE_N}} \quad [3]$$

which is analogous to the modification of HI (8). As with the WOE method using the HI, Equation 3 multiplies the potency-weighted doses by an uncertainty factor taken to the power of  $WOE_N$ , which can range from  $-1$  to  $+1$ . Thus, if a factor of 10 is used for uncertainty factor for interactions ( $UF_i$ ) to illustrate the method, the above this equation alters the adjusted potency-weighted total dose by a factor that may range from 0.1 to 10—This is identical to the WOE method applied to the HI (8).

The maximum likelihood of the dose–response relationships based on the assumptions of DA, response addition (RA), and the WOE method for various

**Table 3.** Data quality weighting factors used to convert qualitative weight of evidence for binary mixtures to quantitative values in the application of the weight-of-evidence methodology.

Category	Designation	Description	Quantitative weight
Mechanistic information	I	Direct and unambiguous	1.0
	II	Related compounds	0.71
	III	Inadequate or ambiguous	0.32
Toxicologic significance	A	Direct	1.0
	B	Inferred	0.71
	C	Unclear	0.32
Modifiers	1	Duration and sequence of concern	1.0
	2	Different duration/sequence	0.79
	a	<i>In vivo</i>	1.0
	b	<i>In vitro</i>	0.79
	i	Route of exposure	1.0
	ii	Different route of exposure	0.79
		Maximum possible weight for each quality weighting factor	1.0
		Minimum possible weight for each quality weighting factor	0.05

effects using these equations and the estimated potency obtained from the single chemical data are shown in Figures 1 to 5, with the estimates based on the observed values (OBV).

## Results

### Qualitative Joint Toxicity Assessment Based on Pairs of Chemicals

**Effect of Butyl Hydroxyanisole on Di-(2-ethylhexyl)phthalate.** A review of the published literature showed no direct studies that experimentally determined the potential interactions or the influence of BHA on the toxicity of DEHP. When administered in rats and other rodents, DEHP causes a series of effects in the liver including hyperplasia and hypertrophy, proliferation of peroxisomes, and induction of peroxisomal enzymes leading to lipid peroxidation and oxidative damage of DNA. In some studies of chronic oral DEHP exposure in rats, an increase in renal weight was reported. Also, DEHP causes developmental effects including resorptions and malformations and reproductive effects including testicular atrophy in rats. BHA is an antioxidant food additive that acts as a free radical scavenger. Chronic BHA exposure caused forestomach lesions in rats. Uncertainties in this assessment arise because the toxic effects of DEHP in the liver and BHA in the rat forestomach are linked with carcinogenicity observed in the animals. Based on this limited toxicologic information, BHA was assessed to protect hepatic and renal lipid peroxidation as well as oxidative DNA damage due to peroxisome proliferation. Thus, the direction of influence was determined to be less than additive (<). Support for this summation is based on data from chemicals other than DEHP, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and endrin, that cause lipid peroxidation (16,17). Because the mechanistic information is based on related chemicals a classification of II was assigned for this category and because the toxicologic significance was inferred a classification of B was assigned. Thus, the overall alphanumeric representation for the influence of BHA on DEHP was <IIB, meaning that the joint toxicity of these two chemicals should be less than additive and the confidence in this assessment is medium because direct evidence is lacking and has been inferred from related chemicals.

**Effect of Cadmium Chloride on Loperamide.** A review of the published literature revealed no direct studies that experimentally determined the potential

interactions or the influence of CdCl<sub>2</sub> on the toxicity of Lop. Lop is an antidiarrheal drug that is well absorbed when administered orally. It can cause central nervous system (CNS) effects through opiate receptors including those receptors that mediate analgesia and respiratory depression. When administered orally, Cd causes irreversible renal proximal tubular damage in experimental animals and humans and also causes hepatic, hematologic, gastrointestinal, reproductive, and developmental effects. CdCl<sub>2</sub> damages the liver; if this damage is sufficient to impair liver function and disrupt enterohepatic circulation of Lop, this could cause an increased systemic circulation of Lop that in turn could lead to increased CNS effects. Additionally, both Lop and CdCl<sub>2</sub> at high levels can affect male fertility. The available information is limited because dispositional consequences from combined exposures have not been studied. Based on the available limited information, the direction of influence of CdCl<sub>2</sub> on Lop was determined to be more than additive (>). Because the mechanistic information was inadequate a classification of III was assigned and because the toxicologic significance was unclear a classification of C was given for this category. Thus, the overall alphanumeric representation for the influence of CdCl<sub>2</sub> on Lop was >IIIC, meaning that the joint toxicity of these two chemicals should be more than additive but the confidence in this assessment is low because direct evidence is lacking.

**Effect of Stannous Chloride on Cadmium Chloride.** The published literature did not contain any direct studies that experimentally determined the potential interactions or the influence of SnCl<sub>2</sub> on the toxicity of CdCl<sub>2</sub>. Cd causes renal toxicity through irreversible damage to renal proximal tubules and it does not undergo any metabolic conversion. At low oral doses SnCl<sub>2</sub> causes no adverse effects and is a substance generally recognized as safe. However, at high oral doses SnCl<sub>2</sub> causes gastrointestinal irritation, pancreatic atrophy, testicular degeneration, reduced hemoglobin concentration, and reduced growth in animals. From the available information it is not clear whether Sn is able to affect metallothionein levels with a subsequent effect on Cd toxicity. Because of its relatively low toxicity, Sn possibly has an antagonistic effect on Cd similar to protective effects found in Cd interactions with Zn—a potential source of uncertainty in the assessment. Hence, the direction of influence of SnCl<sub>2</sub> on CdCl<sub>2</sub> was assessed

as less than additive (<). Because the mechanistic information was inadequate a classification of III was assigned and because the toxicologic significance was unclear a classification of C was given for this category. Thus, the overall alphanumeric representation for the influence of SnCl<sub>2</sub> and CdCl<sub>2</sub> was <IIIC, meaning that the joint toxicity of these two chemicals should be less than additive but the confidence in this assessment is low because direct evidence is lacking.

**Joint Toxicity of the Similarly and Dissimilarly Acting Nephrotoxicant Mixtures.** A qualitative matrix of the binary interactions of the components of the similarly acting mixture is shown in Table 1. Additive toxicity can be anticipated for all the possible binary combinations of TCE (HCBD and TCE, TETRA and TCE, and TCTFP and TCE). The same is true for all the possible combinations of TETRA (HCBD and TETRA, TCE and TETRA, and TCTFP and TETRA). However, less than additive toxicity can be anticipated for all possible combinations of HCBD (TCE and HCBD, TETRA and HCBD, and TCTFP and HCBD) and also for all possible binary combinations of TCTFP (HCBD and TCTFP, TCE and TCTFP, and TETRA and TCTFP). Thus, the overall qualitative WOE points to less than additive kidney toxicity for this mixture.

Of the components of the dissimilarly acting mixture (Table 2), additive toxicity can be anticipated for all the combinations except for the toxicity of mercuric chloride and HCBD. Mercuric chloride was judged to antagonize the toxicity of HCBD, and HCBD was judged to synergize the toxicity of mercuric chloride. Because the qualitative component of the BINWOEs for these interactions is identical (i.e., IIB2bii), the quantitative scores differ only in direction (i.e., -0.25 vs +0.25). For equitoxic mixtures, therefore, the net effect of such interactions would be none, and the overall qualitative WOE points to additive kidney toxicity for this mixture.

**Weight of Evidence for the Joint Toxicity of Simple Mixtures.** The results of the analysis for similarly acting kidney toxicants were performed for the target organ kidney using relative kidney weights as an indicator (marker) of toxicity (Figure 1). In Figure 1, observed individual animal values for relative kidney weight are plotted against total potency-weighted dose (OBV). The dashed lines represent the maximum likelihood estimates of the dose-response relationships based on the

assumptions of DA, RA with a completely positive correlation of tolerances, and the WOE method. Substantial variability is apparent in the responses of the individual animals and the squared correlation coefficient is low ( $r^2=0.5$ ). Although the model fit is highly significant based on the F-ratio of the mean square variance of the model to the mean square of the residual ( $p<0.00001$ ), the F-ratio for the lack of fit against pure error is also statistically significant ( $p=0.0055$ ).

For effects on relative kidney weight, DA (Figure 1) substantially overestimates the observed responses. In other words the increase in kidney weight is less than predicted. Conversely, RA with a completely positive correlation of tolerances underestimates the response (i.e., the observed kidney weights are greater than expected). Both of the deviations are dose related. The WOE method, however, yields an estimated dose–response relationship that is highly consistent with the observed values. Essentially, Figure 1 indicates that DA overestimated the observed responses, RA with a completely positive correlation of tolerance underestimated the observed responses, and the WOE method closely approximated the observed responses.

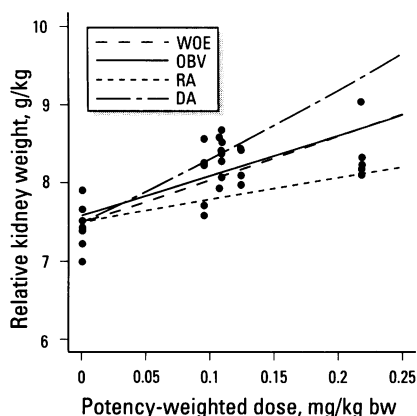
The corresponding plot for relative liver weight is given in Figure 2. As illustrated in this figure, there is much less scatter in the experimental data and the model provides a very good fit to the data ( $r^2=0.92$ ). As with the analysis of the kidney data, the model fit is highly significant based on the F-ratio of the mean square

variance of the model to the mean square of the residual ( $p<0.00001$ ). In addition the F-ratio for lack of fit against pure error is statistically insignificant ( $p=0.44$ ). None of the models for joint action, however, closely approximated the observed responses of the liver following exposure to this mixture. All models underestimated the observed relative liver weights. Unlike the effects on kidney weight, the WOE method provides a poorer approximation of the observed liver weights than does DA. This is because, as with the effects on the kidney, the estimates of liver weights are based on BINWOEs that suggest a less than additive interaction. For liver weight the interaction appears greater than additive regardless of the additivity model used.

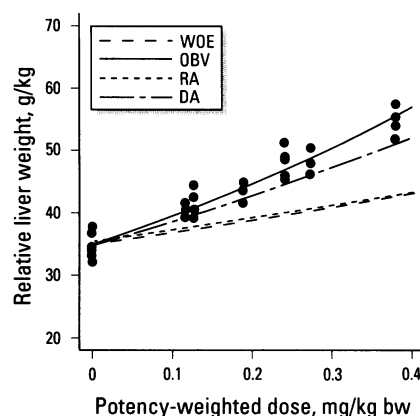
A comparison of the estimates of the joint action models for the changes in relative kidney weights in female rats after exposure to the mixture of dissimilarly acting chemicals is presented in Figure 3. As with this end point for the similarly acting chemicals (Figure 1), there is substantial scatter in the experimental data. The squared correlation coefficient is extremely low ( $r^2=0.07$ ) and the model fit to the observed responses based on the mean square variance of the model to the mean square of the residual is not statistically significant ( $p=0.09$ ). In addition all the interaction models overestimated the response. In other words, based on either DA or RA, the observed increases in relative kidney weight were less than expected. Mercuric chloride is the most potent agent for this end point in female rats, and the interaction

that most affects the estimate made by the WOE method is greater than additive BINWOE for the effect of HCB on the toxicity of mercuric chloride (Table 2). Consequently, the WOE method overestimates the responses to a greater extent than does dose additivity.

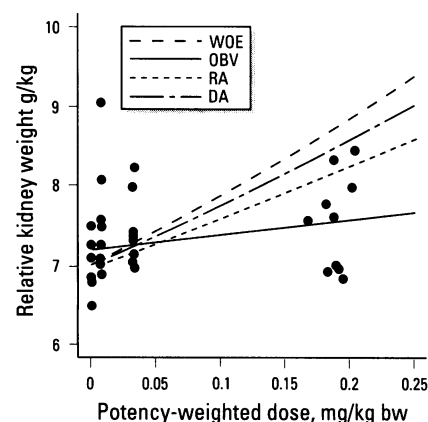
For male rats the estimated changes in relative kidney weights follow the same ordinal relationship: WOE method > dose additivity > response additivity (Figure 4). Compared with the data set in female rats, less scatter is apparent ( $r^2=0.64$ ). Unlike the data set on female rats, the model fit to the observed responses based on the mean



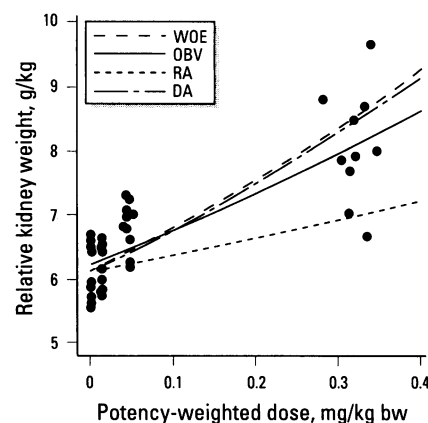
**Figure 1.** The maximum likelihood estimate of the observed dose–response relationship when relative kidney weight is plotted against total potency-weighted dose of female rats exposed to mixtures of similarly acting nephrotoxicants (OBV). The figure also shows responses estimated based on various joint toxicity assessment models viz., DA, RA, and the WOE method.



**Figure 2.** The maximum likelihood estimate of the observed dose–response relationship when relative liver weight is plotted against total potency-weighted dose of female rats exposed to mixtures of similarly acting nephrotoxicants (OBV). The figure also shows responses estimated based on various joint toxicity assessment models viz., DA, RA, and the WOE method.



**Figure 3.** The maximum likelihood estimate of the observed dose–response relationship when relative kidney weight is plotted against total potency-weighted dose of female rats exposed to mixtures of dissimilarly acting nephrotoxicants (OBV). The figure also shows responses estimated based on various joint toxicity assessment models viz., DA, RA, and the WOE method.



**Figure 4.** The maximum likelihood estimate of the observed dose–response relationship when relative kidney weight is plotted against total potency-weighted dose of male rats exposed to mixtures of dissimilarly acting nephrotoxicants (OBV). The figure also shows responses estimated based on various joint toxicity assessment models viz., DA, RA, and the WOE method.

square variance of the model to the mean square of the residual is statistically significant ( $p < 0.00001$ ) and the F-ratio for the lack of fit against pure error is not statistically significant ( $p = 0.07$ ). There is relatively little difference between the estimates based on the WOE method and dose additivity, however, because both mercuric chloride and HCBd are active and approximately equipotent agents in male rats and are present in approximately equitoxic amounts. Consequently, the interactions of HCBd and mercuric chloride are offsetting (Table 2).

## Discussion

The WOE method is intended to be used as a quantitative modifier to HIs in risk assessments involving multiple chemicals. The method implies that judgmental BINWOEs can be used to account quantitatively for uncertainties concerning the impact of toxicologic interactions in much the same way that uncertainty factors are used to account for uncertainties in species-to-species or high- to low-dose extrapolations in the derivation of MRLs or reference doses (RfDs). Although there is some experimental support for the use of uncertainty factors in deriving MRLs or RfDs, experimental data have not previously been used to assess the WOE method for chemical mixtures. The final BINWOEs determined for all the possible combinations of chemicals used in this study are summarized in Tables 1 and 2. There was no change in the direction of the interaction for any of these evaluations because the ATSDR reviewers agreed with the initial assessments and thus did not recommend any changes from one form of interaction to another. However, in some cases changes were recommended from additivity to antagonism.

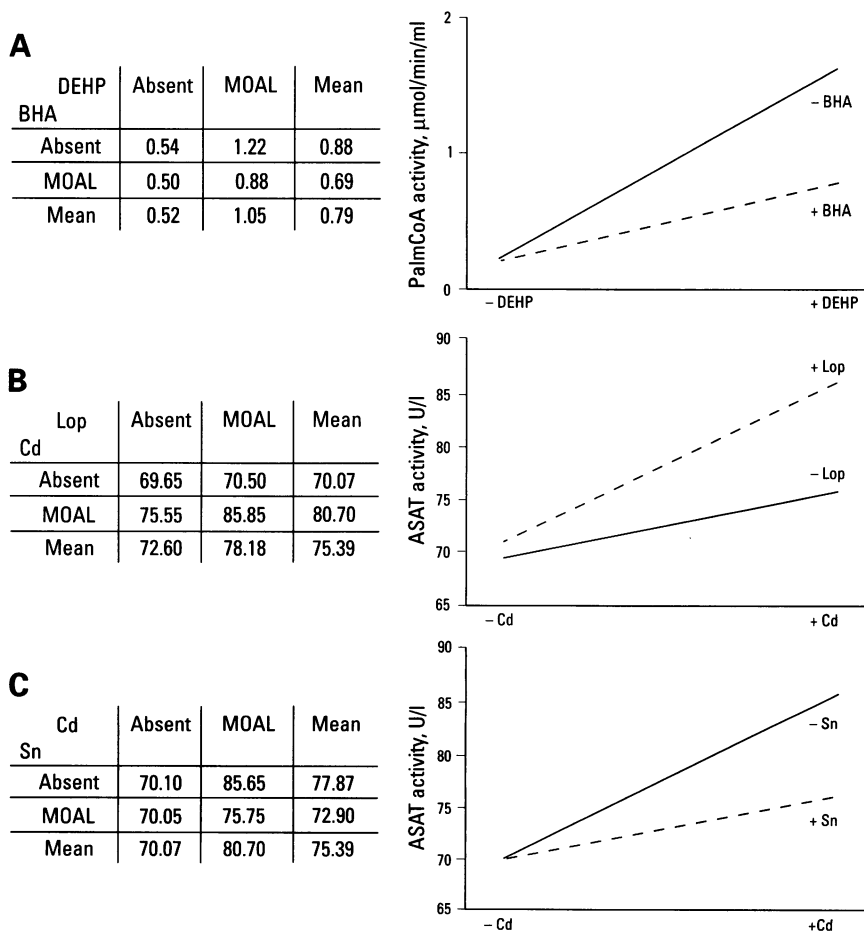
Detailed interactions between three binary pairs of chemicals (BHA and DEHP, CdCl<sub>2</sub> and Lop, and SnCl<sub>2</sub> and CdCl<sub>2</sub>) have been published by Groten et al. (11). The interaction between BHA and DEHP resulted in a decreased total palmitoyl-CoA oxidase (PalmCoA) activity as shown in Figure 5, which is a two-by-two plot of the effect of the individual chemicals. The interaction between CdCl<sub>2</sub> and Lop resulted in increased liver toxicity, indicated by a 4.74 U/liter higher aspartate aminotransferase (ASAT) than could be expected on the basis of summation of the effects of the two single chemicals. Finally, the interaction between SnCl<sub>2</sub> and CdCl<sub>2</sub> resulted in lower ASAT and hemoglobin levels than could be expected on the basis of

additivity of the two chemicals. The absence of parallel lines in Figure 5A to 5C indicates the interactive effect between these pairs of chemicals. The results of the assessment, performed at the ATSDR, for these binary interactions indicate that the joint toxicity of BHA on DEHP should be less than additive (< IIB) and that the confidence in this assessment is medium. The joint toxicity of CdCl<sub>2</sub> on Lop should be more than additive (> IIC) but the confidence in this assessment is low. Also, the overall joint toxicity of SnCl<sub>2</sub> and CdCl<sub>2</sub> should be less than additive (< IIC) and the confidence in this assessment is low. Thus, there are parallels in the qualitative assessments and the experimental findings of these combinations of chemicals. The qualitative evaluations for binary combinations of these chemicals showed that the WOE approach can be used to qualitatively estimate the joint toxicity of chemicals for certain end points. Such analysis and resulting

information can be used by health assessors to express their concern regarding the joint toxicity of chemical mixtures.

The four-component mixture studies (12,13) were considered suitable, to a limited extent, in determining whether the WOE method can be applied to studies on experimental animals. The strongest characteristic of these four component mixture studies was that the data were obtained in concurrent experiments conducted with individual components as well as their mixtures. These data were adequate for estimating dose-response relationships for individual chemicals as well as their mixtures. In addition, these studies focused on end points of kidney toxicity for which the mechanisms of action have been relatively well characterized.

The use of the HI in risk assessment involves the assumption of DA and the WOE method provides a modification to dose additivity (8,9). The WOE method



**Figure 5.** Binary interactions between (A) butyl hydroxyanisole and di(2-ethylhexyl)phthalate, (B) Cd chloride and loperamide, and (C) stannous chloride and Cd chloride. MOAL, minimum observed adverse effect level. Adapted from Groten et al. (11).

should work best for those compounds that appear to act by similar mechanisms. The current analysis supports this conclusion. For similarly acting kidney toxicants (Figure 1) the application of the WOE method appears to adjust correctly for the estimates of effects on relative kidney weight based on DA. The resulting estimate of the dose–response relationship based on the WOE method is almost identical to the estimated dose–response relationship derived from the experimental data. This close quantitative agreement of the WOE estimate to the estimate derived from experimental data is probably coincidental. As discussed in “Methods,” the application of the WOE method involves the use of an uncertainty factor of 10 to account for uncertainties associated with interactions. If a different factor were used the quantitative estimates from the WOE method would not be as close to those estimates derived from the experimental data. Nonetheless, regardless of the size of the factor used to modify the potency-weighted dose, the WOE correctly indicates that a less than dose-additive interaction is likely. The other two joint toxic action models, RA and DA, were either lower or higher in their estimates.

However, for the same mixture for effects on relative liver weight, the nature of the interaction appears to be suggesting a somewhat greater than dose-additive interaction (Figure 2). For this end point the WOE method performs in the same manner qualitatively as it did for relative kidney weight, i.e., the WOE method estimates a less than dose-additive effect. Consequently, the WOE method leads to an adjustment in expected responses that differs further from the experimental results than do the estimates based on dose additivity. In some respects this lack of agreement might be expected. The four compounds used in this study have the same mechanism of action in the kidney; however, no information is available about their mechanism of action in the liver. In fact, one of the compounds, HCB, was associated with a marginal decrease in relative liver weight whereas the other three compounds were associated with increases in relative liver weight. If the compounds do not have a similar mechanism of action for the end point being assessed, dose additivity is not a plausible model. Consequently, it is not reasonable to anticipate that a model based on a modification of dose additivity will account for any observed interactions. In addition,

during the preparation of the BINWOEs, emphasis was placed on interactions involving the kidney because much of the available information on these compounds involved kidney effects.

For the kidney toxicants acting with dissimilar mechanisms (12), the WOE method did not correctly account for any of the observed interaction patterns. In both male and female rats less than dose-additive interactions are apparent based on changes in relative kidney weight. For relative kidney weights in female rats, the WOE method estimates are somewhat greater than dose additivity because the predominant chemical in the mixture is mercuric chloride. HCB, the other component of the mixture, is judged to enhance the toxicity of mercuric chloride (Table 2). For male rats the potencies of HCB and mercuric chloride are essentially the same. Thus, based on the BINWOEs for these compounds (Table 2), the interactions are offsetting and the estimates for DA and the WOE method are essentially the same (Figure 4).

Based on the findings of this rather limited study, the data suggested that the WOE method can be applied to assess the toxicity of similarly acting toxicants. Because the WOE method is based on a modification of DA that is in turn based on the assumption of similar mechanism of action, this limitation seems reasonable. However, compared with DA, RA appears to be better for characterizing the observed responses for toxicants acting with dissimilar mechanism of action, as seen for increases in kidney weight in female rats (Figure 3). For relative kidney weights in male rats (Figure 4), the observed response is intermediate between DA and RA.

As we showed in this paper, most of the WOE evaluations indicated a direction of the interaction that was consistent with the animal data. For the similarly acting toxicants the WOE methodology closely approximated the dose–response relationship compared with the other available joint toxicity models (Figure 1). The results also show that the WOE evaluations must be target-organ-specific because none of the available models could closely approximate the observed responses in organs (such as liver) other than the primary (critical) target organs (Figure 2). Computational techniques based on structure–activity relationships, pattern recognition techniques, and mathematical methods should be applied to facilitate the development of target-organ-specific evaluations. Thus, to

understand the role of chemical interactions and the overall toxicity of a mixture at a whole-animal level, investigators should take a systems approach to toxicology studies with chemical mixtures; the focus should be on the integrated animal to evaluate multiple target organs and not to limit studies to a specific target organ(s).

For nephrotoxicants acting by similar or dissimilar mechanisms at potency-weighted doses of 0.00 to 0.05, there are no significant differences in the estimates among the joint toxicity models applied in this study, namely, DA, RA, or the WOE method (Figures 1–4). If this range of 0.00 to 0.05 potency-weighted dose represents the common environmental exposure levels to mixtures of chemicals, then using any of these joint toxicity models will serve the purposes of a public health assessor for evaluation of chemical mixtures. However, at a range between 0.05 and 0.1 potency-weighted dose, the differences between use of a particular model become apparent. In other words, unless the magnitude of the interaction is substantial there will be little quantitative difference among the different models for joint action in the low dose region, i.e., doses that individually would not be expected to cause substantial biologic responses.

The findings of this study can be verified and further clarified through a WOE analysis of data available from several recent animal studies that have used elaborate experimental designs to study potential chemical interactions (18,19). A full factorial experimental design was used to study developmental effects of a three-component mixture of TCE, DEHP, and heptachlor by gavage exposure (18). Norotsky et al. (18) have offered tabulated mean data and individual animal data in electronic form for further analysis. Several interesting observations were made in this study and a WOE method analysis would further enlighten the understanding of the complexities of chemical interactions. The potential interactive effects in the nasal epithelium of mixtures of formaldehyde, acetaldehyde, and acrolein at nontoxic as well as toxic levels have been studied (19). Animals were exposed to either single chemicals or combinations of the chemical components and individual animal data were collected. Thus, these data would also lend themselves to the WOE analysis, making this the first inhalation study for which such an analysis would be done. This methodology could be further evaluated through additional *in vitro* and *in vivo*



testing of carefully selected chemical mixtures (5). In a collaborative effort with the TNO, an *in vitro* study using assays of kidney slices is being designed to test the concept of dose dependency and the overall role of chemical interactions in the joint toxicity of chemical mixtures.

## Conclusion

In conclusion, the results we presented here suggest that the WOE method for chemical mixtures may be a useful tool for estimating chemical interactions and characterizing the overall joint toxicity of chemical mixtures. Also, the WOE evaluations, based on consideration of common mechanisms for simple chemical mixtures,

can lead to better estimates of the observed toxic responses than the default assumption of dose additivity. Such evaluations can then be used to estimate and predict the toxicity of higher combination mixtures based on the data of simple binary mixtures.

Efforts are currently underway to conduct additional experiments to specifically address the issues of when it might be reasonable to apply the WOE method and to what extent BINWOEs may need to be organ or end-point specific. In the interim it seems reasonable that the WOE method can be used as a tool that allows the risk assessor to express concern for potential chemical interactions. Until further data become

available to assess the applicability and, if needed, modifications of the method for different classes of compounds and ranges of effects, the WOE method can be used to modify HIs in site-specific risk assessments on a case by case basis.

To understand the role of chemical interactions and to estimate the overall toxicity of a mixture, specially designed experimental studies should be conducted. The focus of such studies should be the understanding of mechanisms involved and their influence on the expression of the overall toxicity of chemical mixtures in multitarget organs. Such research can help advance the methods for the toxicity assessment of chemical mixtures.

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