

Experimental Designs for Mixtures of Chemicals along Fixed Ratio Rays

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Experimental design is important when studying mixtures/combinations of chemicals. The traditional approach for studying mixtures/combinations of multiple chemicals involves response surface methodology, often supported by factorial designs. Although such an approach permits the investigation of both the effects of individual chemicals and their interactions, the number of design points needed to study the chemical mixtures becomes prohibitive when the number of compounds increases. Fixed ratio ray designs have been developed to reduce the amount of experimental effort when interest can be restricted to a specific ray. We focus on the design and analysis issues involved in studying mixtures/combinations of compounds along fixed ratio rays of the compounds. To obtain the inference regarding the interactions among the compounds, we show that the only data required are those along the fixed ratio ray. **Key words:** additivity, interaction index, isobologram. *Environ Health Perspect* 110(suppl 6):979–983 (2002).

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Determining and characterizing the nature of interactions among components of a combination of c drugs or chemicals is a problem of current interest (where c is the number of drugs/chemicals in a mixture). Although assessments based on single-drug/chemical exposure enable us to acquire fundamental knowledge about individual drugs or chemicals under carefully controlled conditions, they do not reflect real-world exposures. Thus, it is often of interest to study the effects of exposure to multiple drugs/chemicals. Of ultimate interest in such studies is the determination and characterization of interactions among the components in a mixture. For example, Gennings et al. (1) report on a study of the nature of the interaction involving the mixture of four metals. The four metals chosen for the study were arsenic (As), cadmium (Cd), chromium (Cr), and lead (Pb), which are among the top contaminants in site frequency count by the Agency for Toxic Substances and Disease Registry (ATSDR) Completed Exposure Pathway Site Count Report (2). In addition, human health risk assessment associated with exposure to disinfection byproducts in drinking water is of concern because of the widespread exposure of persons who receive disinfected water. Other examples of human exposure to combinations of agents can be found in the treatment of numerous diseases including cancer, AIDS, diabetes, and asthma. These examples illustrate the importance of studying mixtures/combinations of drugs or chemicals. Determining departures from additivity for a combination of drugs or chemicals is a problem that has been considered by many authors (3–7).

Classic Methodology for Detecting and Characterizing Departures from Additivity

Isobolograms. The classic method for detecting and characterizing departures from additivity between combinations of drugs or chemicals is the isobologram. The isobologram, introduced as a graphical tool by Fraser (8,9), is a plot of a contour of constant response of the dose–response surface associated with the combination superimposed on a plot of the same contour under the assumption of additivity. Its use was extended by Loewe and Muischnek (10), Loewe (11), and Berenbaum (12), and reviewed by Gessner (13), Wessinger (14), and Berenbaum (15). For a two-component mixture, the analysis of an isobologram compares the observed isobol (e.g., combination dose/concentration associated with 50% response [ED₅₀]) to the line of additivity. The line of additivity is formed by joining the ED₅₀ associated with each of the individual components calculated from the dose–response data for the individual components. Figure 1 presents illustrations of possible isobolograms for a combination of two drugs/chemicals. As indicated, if the isobol is below the line of additivity, a synergism is claimed. On the other hand, if the isobol is above the line of additivity, an antagonism is claimed. However, there are shortcomings associated with the use of isobolograms. For instance, the method used in the construction of an isobologram typically does not take data variability into account. Additionally, because it is a graphical method, isobolograms effectively are limited to the study of combinations of two or three drugs or chemicals.

Interaction index. The interaction index, introduced by Berenbaum (12), provides a convenient method to determine and characterize departures from additivity for a combination of $c > 2$ or 3 components. The interaction index, II , is defined by

$$II = \frac{X_1}{ED_{100\mu}(CHEM_1)} + \frac{X_2}{ED_{100\mu}(CHEM_2)} + \dots + \frac{X_c}{ED_{100\mu}(CHEM_c)}, \quad [1]$$

where c is the number of components, X_1, X_2, \dots, X_c are the doses in combination associated with a desired effect, and $ED_{100\mu}(CHEM_i), i = 1, \dots, c$ is the dose of the i th component that, when administered alone, produces the same effect. When the interaction index, defined in Equation 1, is equal to 1, the c components interact additively. When II is greater than 1, the components interact antagonistically; when II is less than 1, the components interact synergistically. Again, it should be noted that the individual component dose–response information is required to calculate the interaction index. As described by Berenbaum (12), the interaction index is directly related to the isobologram, i.e., when $II = 1$, the isobol is coincident with the line of additivity; when $II > 1$, the isobol bows above the line of additivity; and when $II < 1$, the isobol bows below the line of additivity. An advantage of using the interaction index over the isobologram is that the interaction index is not limited to combinations/mixtures of just two or three components. However, as developed by Berenbaum (12), the biological variability associated with the data is not taken into account by the interaction index.

Statistical models. Statisticians frequently use models of the form

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$$Y = \beta_0 + \sum_{i=1}^c \beta_i x_i + \sum_{\substack{i=1 \\ i < j}}^c \sum_{j=1}^c \beta_{ij} x_i x_j + \sum_{\substack{i=1 \\ i < j < k}}^c \sum_{j=1}^c \sum_{k=1}^c \beta_{ijk} x_i x_j x_k + \dots + \beta_{12\dots c} x_1 x_2 \dots x_c$$

to approximate the relationship between a response of interest, Y , and concentrations of c chemicals (x_1, x_2, \dots, x_c).

Carter et al. (4) showed that a relationship exists between the interaction index proposed by Berenbaum (12) and the parameter in a statistical model associated with the interaction of the components of the combination. Without loss of generality, consider that the combination/mixture of interest involves two chemicals and that the response is continuous. Therefore, following the logic of Carter et al. (4), for the linear models case, the relationship between the response and the doses or concentrations of the components in combination can be expressed as

$$\mu = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_{12}, \quad [2]$$

where

μ is the mean response, $E(Y)$,

β_0 is the unknown intercept,

β_1 is the unknown slope parameter associated with the first component,

β_2 is the unknown slope parameter associated with the second component,

β_{12} is the unknown parameter associated with the interaction of the two components, and

x_1 and x_2 are the doses of the respective chemicals.

From the model defined in Equation 2, the $ED_{100\mu}$ ($CHEM_i$) for the respective components can be derived to be

$$ED_{100\mu}(CHEM_1) = \frac{\mu - \beta_0}{\beta_1}$$

$$ED_{100\mu}(CHEM_2) = \frac{\mu - \beta_0}{\beta_2}$$

Thus, after algebraic manipulation, the model defined in Equation 2 becomes

$$\frac{\beta_1 x_1}{\mu - \beta_0} + \frac{\beta_2 x_2}{\mu - \beta_0} + \frac{\beta_{12} x_{12}}{\mu - \beta_0} = 1$$

or

$$1 - \frac{\beta_{12} x_{12}}{\mu - \beta_0} = \frac{x_1}{(\mu - \beta_0)/\beta_1} + \frac{x_2}{(\mu - \beta_0)/\beta_2}$$

Therefore, it follows that when $\beta_{12} = 0$, the combination of components 1 and 2 is additive, i.e., the isobologram is coincident with the line of additivity and the interaction

index equals 1. Similarly, when $\beta_{12} > 0$, the combination of components 1 and 2 is synergistic, i.e., the isobologram bows below the line of additivity and the interaction index is < 1 ; and when $\beta_{12} < 0$, an antagonism is present, i.e., the isobologram bows above the line of additivity and the interaction index is > 1 . This demonstrates the algebraic equivalence between the statistical model and the interaction index. Gennings et al (16) demonstrated the experimental convergence of the statistical modeling approach and the interaction index. The number of components that can be considered in the statistical model can be generalized to c , and data variability is appropriately accounted for in the resulting inference.

The various methods used to determine and classify departures from additivity described above use both single-compound data as well as combination data. Consider the situation in which the single-compound dose–response data are not available. The approach discussed in the following sections of this article allows one to test the null hypothesis of additivity using only combination/mixture data collected along a fixed ratio ray. Thus, single-compound dose–response data are not needed.

New Methodology

Problems with statistical modeling are associated with the size of the experiment required to generate data to support the model. Factorial experiments, e.g., 2^c or 3^c , are often considered. When c is large such experiments may not be feasible, so in a sense, this approach is limited to combinations of relatively few drugs or chemicals. An alternative to the traditional factorial design for studying interactions, and the design to be considered here, is the ray design. Ray designs, described by Martin (17), Mantel (18), Finney (19), Bruden and Vidmar (20), and others, are used to study mixtures of c drugs or chemicals at a fixed mixing ratio, $[a_1:a_2:\dots:a_c]$, where $\sum_{i=1}^c$

with the total dose, t , varying. The fraction of the total dose represented by the i th drug or chemical is a_i , and the amount of the i th drug or chemical in the mixture is $a_i t$. This approach is appealing because the dimensionality of the study is reduced along each ray, i.e., each ray can be considered as an individual drug or chemical, with only the total dose varying. For example, in a study involving c drugs or chemicals, the fitted model based on a response surface approach is a $(c + 1)$ -dimensional surface. In contrast, the fitted model based on a ray design defines a set of two-dimensional dose–response curves.

What can be stated about departures from additivity in the mixture? Meadows (21) showed that when mixture data are collected along a fixed ratio ray, the additivity model

reduces to a simple linear regression model. In addition, the interaction model reduces to a higher-order polynomial model. Thus, the test for additivity is equivalent to the test of the adequacy of the simple linear regression model. Consider that the combination/mixture of interest involves c drugs or chemicals and that the response of interest is continuous. The underlying additivity model, i.e., the model with no cross-product terms is defined by

$$Y = \beta_0 + \beta_1 x_1 + \dots + \beta_c x_c, \quad [3]$$

where

Y is the observed response,

x_i is the dose of the i th drug or chemical,

β_0 is an unknown parameter associated with the intercept, and

β_i is an unknown parameter associated with the slope of the i th drug or chemical.

When the mixing ratios are invoked, the dose of the i th drug or chemical is $x_i = a_i t$, where a_i is the mixture fraction for the i th drug or chemical and t is the total dose. As a result, the additivity model becomes

$$\begin{aligned} Y &= \beta_0 + \beta_1 a_1 t + \beta_2 a_2 t + \dots + \beta_c a_c t \\ &= \beta_0 + (\beta_1 a_1 + \beta_2 a_2 + \dots + \beta_c a_c) t \\ &= \beta_0 + \beta_1^* t, \end{aligned} \quad [4]$$

where

$$\beta_1^* = \sum_{i=1}^c \beta_i a_i$$

For convenience, we assume the experimental region along the fixed ratio ray in terms of total dose is transformed to the region $-1 \leq t \leq 1$. Thus, under additivity, the dose–response relationship along the ray can be described with a simple linear regression on total dose.

It also follows that when the slope of the regression line for total dose is $\beta_1 a_1 + \beta_2 a_2 + \dots + \beta_c a_c$, the interaction index, defined in Equation 1 equals 1. This would suggest that single-component dose–response data would

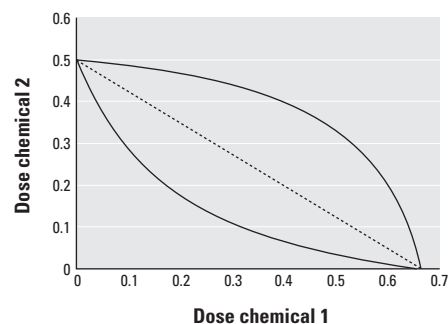


Figure 1. Illustrations of isobolograms for a combination of two drugs/chemicals. The dashed line is the line of additivity. When the isobol bows below the line of additivity, a synergism is claimed; when the isobol bows above the line of additivity, an antagonism is demonstrated.

be required. In the absence of single-chemical data, the slope β_i for each drug/chemical alone is unknown, so that the hypothesis $H_0: \beta_1^* = \sum_{i=1}^c \beta_i a_i$ cannot be directly tested.

However, consider the following model:

$$Y = \beta_0 + \sum_{i=1}^c \beta_i x_i + \sum_{\substack{i=1 \\ i < j}}^c \sum_{j=1}^c \beta_{ij} x_i x_j \\ + \sum_{\substack{i=1 \\ i < j < k}}^c \sum_{j=1}^c \sum_{k=1}^c \beta_{ijk} x_i x_j x_k \\ + \dots + \beta_{12\dots c} x_1 x_2 \dots x_c.$$

Notice that this model is the model that would be supported by a factorial experiment, and the $\beta_{ij}, \beta_{ijk}, \dots, \beta_{12\dots c}$ terms are coefficients associated with the various two-factor, three-factor, and higher-order interactions. The ray design will not support this model; however, invoking the mixing ratio associated with the ray design results in

$$Y = \beta_0 + \left(\sum_{i=1}^c a_i \beta_i \right) t + \left(\sum_{\substack{i=1 \\ i < j}}^c a_i a_j \beta_{ij} \right) t^2 \\ + \left(\sum_{\substack{i=1 \\ i < j < k}}^c a_i a_j a_k \beta_{ijk} \right) t^3 \\ + \dots + (a_1 a_2 \dots a_c \beta_{12\dots c}) t^c \\ = \beta_0 + \beta_1^* t + \beta_2^* t^2 + \beta_3^* t^3 + \dots + \beta_c^* t^c. \quad [5]$$

It follows that interactions among pairs of chemical components are associated with second-degree terms, interactions among three chemicals are associated with third degree terms, and so on.

Of ultimate interest is the determination of departure from additivity among a particular combination/mixture of drugs or chemicals. When comparing the model under additivity to the interaction model, defined in Equations 4 and 5, respectively, evidence of curvature indicates departure from additivity, i.e., there is interaction among the compounds if at least one $\beta_i^* \neq 0$, $i = 1, \dots, c$. Thus, any polynomial lack of fit associated with the additive model $Y = \beta_0 + \beta_1^* t$ would be associated with a lack of additivity. Meadows (21) showed that the test statistic for the null hypothesis of additivity,

$$H_0: \begin{bmatrix} \beta_2^* \\ \beta_3^* \\ \vdots \\ \beta_c^* \end{bmatrix} = 0, \text{ is given by} \\ F = \frac{R(\theta_2 | \theta_1) / (c-1)}{s^2}. \quad [6]$$

Experimental Design

Experimental design implications for studying a c component mixture include the following:

- Place a minimum of $c + 1$ points on the ray of interest to maximize the power of the test for lack of fit of the additivity model, i.e., $Y = \beta_0 + \beta_1^* t$.
- Replicate the experiment at these points to make the lack of fit test possible.

When the response variable is continuous and the method of least squares has been used to estimate the model parameters, Meadows (21) showed that we can incorporate the statistical results of Jones and Mitchell (22) to determine values of total dose that maximize the design's ability to detect lack of fit or departure from additivity.

The overall lack of fit answers the question of whether there is a departure from additivity. Rejection of his hypothesis that simultaneously tests that the interaction parameters are equal to zero, i.e., $H_0: \beta_2 = \beta_3 = \dots = \beta_c = 0$, implies that interaction is present among the chemicals globally. Thus, if the overall test for additivity is rejected, tests of the form

$$H_0: \beta_j^* = 0 \\ H_0: \beta_j^* \neq 0$$

using Hochberg's (23) correction for multiple testing, can be used to answer the question of whether a j -factor interaction exists. If such an interaction is detected, recall that

$$\beta_2^* = \sum_{\substack{i=1 \\ i < j}}^c \sum_{j=1}^c a_i a_j \beta_{ij}, \\ \beta_3^* = \sum_{i=1}^c \sum_{j=1}^c \sum_{k=1}^c a_i a_j a_k \beta_{ijk}, \text{ etc.}$$

Here, interest will be focused on which of the j -factor interactions are present. This can be determined by performing $\binom{c}{j}$ additional ray experiments at the same ratio as were present on the original ray.

Illustration

The methodology introduced in this article is illustrated with cytotoxicity data obtained from assessing interactions among As, Cd, Cr, and

Pb in human keratinocytes. The experimental data were obtained from R. Yang and colleagues at Colorado State University (Fort Collins, Colorado). The end point of interest is the percent viability of treated NHEK (normal human epidermal keratinocytes) cells using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. The mixture point of interest for As, Cr, Cd, and Pb contained the estimated dose/concentrations associated with 50% lethality (LD_{50} s) of 7.7 μ M, 4.9 μ M, 6.1 μ M, and 100 μ M, respectively. This 1X solution was serially diluted at a 1:3 ratio to get 0.333, 0.111, 0.037, 0.0123, 0.004, and 0.0014 dilution groups. Double deionized water was used as the vehicle control in all cases.

After exposure to individual metals or metal exposure, cells were re-fed with fresh metal-free KGM medium and incubated for 3 days prior to viability analysis by the MTT assay. Details of the experimental protocol and methods are described elsewhere (1,24) and are not included here. The summary statistics for the LD_{50} mixture data presented in Table 1 are linearized cytotoxicity response data from Gennings et al. (1).

The nonlinear additivity model selected for fitting the single-compound data by Gennings et al. (1) was based on a Gompertz function where the mean viability was modeled as

$$\mu = \alpha + \gamma \exp\left(-\exp\left(-\left(\beta_0 + \sum_{i=1}^4 \beta_i x_i\right)\right)\right).$$

From this model, α is the parameter associated with the minimum mean response, and γ is the range of mean response values. Therefore, for this example, it is reasonable to assume that a transformation on the response, conditioning on the values of $\alpha = 8.76$ and $\gamma = 109$ obtained by Gennings et al. (1), will induce linearity in the additivity model. As a result, the additivity model becomes

$$-\log\left(-\log\left(\frac{\mu - \alpha}{\gamma}\right)\right) \\ = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4.$$

Table 1. Summary statistics for the LD_{50} mixture data based on linearized cytotoxicity response data from Gennings et al.^a—NHEK cells.

Mixture dilution	Total dose ^b (μ M)	Sample mean	Sample variance	Sample size
0	0	1.81	0.23	9
0.0014	0.2	2.24	0.20	6 ^c
0.004	0.5	1.65	0.55	9
0.0123	1.5	1.58	1.07	8 ^c
0.037	4.4	0.77	0.11	9
0.111	13.2	0.40	0.01	9
0.333	39.6	0.02	0.18	9
1	118.7	-0.55	0.27	9

^aData from Gennings et al. (1). ^b LD_{50} mixing ratio (7.7 μ M, 4.9 μ M, 6.1 μ M, and 100 μ M) for As, Cr, Cd, and Pb, respectively. ^cOverall, four data values are missing because of transformation on the response.

Table 2. Estimated model parameters for the additivity model given in Equation 7 and the interaction model given in Equation 8.

Parameter	Estimate	SE	p-Value
Additivity model			
β_0	1.36	0.12	< 0.001
$\beta_1^*(t)$	-0.02	2.34×10^{-3}	< 0.001
$SS_{RES} = 38.04, df_{RES} = 66$			
Interaction model			
β_0	1.96	0.12	< 0.001
$\beta_1(t)$	-0.36	0.09	< 0.001
$\beta_2(t^2)$	0.03	0.01	0.010
$\beta_3(t^3)$	-0.0006	2.44×10^{-4}	0.024
$\beta_4(t^4)$	0.00000319	1.43×10^{-6}	0.030
$SS_{RES} = 20.49, df_{RES} = 63$			

Abbreviations: df_{RES} , degrees of freedom associated with SS_{RES} ; SS_{RES} , residual sum of squares.

As shown in “New Methodology,” because the mixture data were collected along a fixed ratio ray, the additivity model can be rewritten as

$$\begin{aligned} \mu_{m, add} &= -\log\left(-\log\left(\frac{y-\alpha}{\gamma}\right)\right) \\ &= \beta_0 + \beta_1^*t. \end{aligned} \quad [7]$$

Additionally, the interaction model along the same fixed ratio ray becomes

$$\begin{aligned} \mu_{m, interaction} &= -\log\left(-\log\left(\frac{y-\alpha}{\gamma}\right)\right) \\ &= \beta_0 + \beta_1^*t + \beta_2^*t^2 + \beta_3^*t^3 + \beta_4^*t^4. \end{aligned} \quad [8]$$

Therefore, conditioning on the values of $\alpha = 8.76$ and $\gamma = 109$, the transformation

$$-\log\left(-\log\left(\frac{y-8.76}{109}\right)\right)$$

on the observed responses for the mixture data was performed. The additivity model given in Equation 7 and the interaction model given in Equation 8 were fit to the mixture data using the method of least squares. The GLM (general linear model) procedure of SAS (25) was used to estimate the unknown parameters in Equations 7 and 8. Parameter estimates and their p values are provided in Table 2. Figure 2 presents the fitted concentration effect curve under additivity for total concentrations of the four metals along the ray associated with the LD₅₀ mixing ratio. Asterisks (*) indicate the observed transformed responses at the seven dilution points. From this figure, there is some question as to whether the data fall along the line of additivity. In comparison, Figure 3 presents the observed mixture data and the fitted interaction (higher-order polynomial) model. Dots (•) indicate the design locations of the total dose values selected by the Λ_1 -optimal

design, which are presented by total doses of 0, 16, 59.4, 102.7, and 118.7 μM . Notice that the values selected as the Λ_1 -optimal design are symmetrically spread throughout the total dose region, whereas the majority of the points used in the current study are directed toward the lower total dose region. An enlarged version of the lower total dose region of the plot of the fitted interaction model is presented in Figure 4. The test statistic for the null hypothesis of additivity,

$$H_0: \begin{bmatrix} \beta_2 \\ \beta_3 \\ \beta_4 \end{bmatrix} = 0,$$

is given by

$$\begin{aligned} F &= \frac{R(\theta_2|\theta_1)/(c-1)}{s^2} \\ &= \frac{(SS_{Res, reduced} - SS_{Res, full})/3}{s^2} \\ &= \frac{(38.04 - 20.49)/3}{0.3252} \\ &= 17.99. \end{aligned}$$

Table 3 presents the overall test for departure from additivity given in Equation 6. Based on this test, we reject the null hypothesis of additivity (p value < 0.001) and conclude that at least one of the j -factor interactions exists, $j = 2, \dots, 4$. Because the overall test for additivity is rejected, it is of interest to determine whether two-, three-, or four-factor interactions are present. Therefore, we want to test the following hypotheses using Hochberg’s correction (23) for multiple testing:

$$H_{0A}: \beta_2^* = 0 \text{ vs. } H_{1A}: \beta_2^* \neq 0$$

$$H_{0B}: \beta_3^* = 0 \text{ vs. } H_{1B}: \beta_3^* \neq 0$$

$$H_{0C}: \beta_4^* = 0 \text{ vs. } H_{1C}: \beta_4^* \neq 0.$$

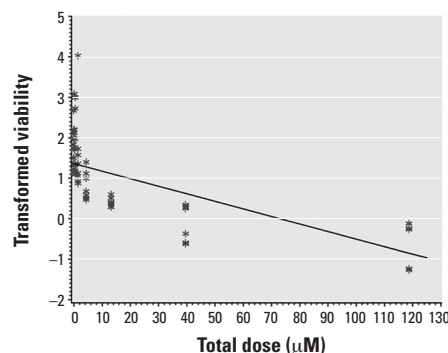


Figure 2. Observed responses and fitted curve under the additivity model for the fit of the mixture data using the NHEK cells.

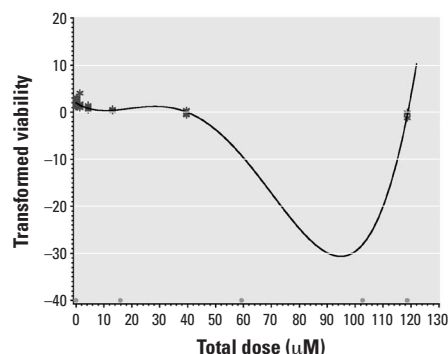


Figure 3. Observed and predicted responses for the higher-order polynomial model for the fit of the mixture data using the NHEK cells. Dots (•) indicate the design locations of the total dose values selected by the Λ_1 -optimal design.

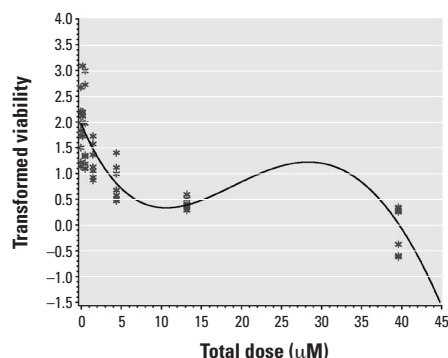


Figure 4. Observed and predicted responses for the higher-order polynomial model for the fit of the mixture data using the NHEK cells (enlarged).

Table 3. Test results for testing the hypothesis of additivity, as well as the hypotheses that the j -factor interactions do not exist, $j = 2, \dots, 4$.

Hypothesis	F	p-Value
Overall test for additivity		
$H_0: \beta_2 = \beta_3 = \beta_4 = 0$	17.99 (3, 63)	< 0.001
Individual tests		
$H_{0A}: \beta_2 = 0$	23.95 (1, 65)	< 0.001**
$H_{0B}: \beta_3 = 0$	16.54 (1, 64)	< 0.001**
$H_{0C}: \beta_4 = 0$	4.92 (1, 63)	0.0302*

*Using Hochberg’s correction (23) for multiple comparisons, these tests are associated with a significant j -factor interaction using an overall 5% test. **Significant with an overall significance level of 1%.

Table 4. Ratios (%) of compounds to be used for the four additional experiments, which are based on the LD₅₀ mixing ratio (7.7 μM, 4.9 μM, 6.1 μM, and 100 μM).

	As	Cr	Cd	Pb	Total dose
Four-factor combination					
Original experiment	6.5	4.1	5.1	84.3	118.7 μM
Three-factor combination					
Experiment #1	41.2	26.2	32.6	—	18.7 μM
Experiment #2	6.8	4.4	—	88.8	112.6 μM
Experiment #3	6.8	—	5.3	87.9	113.8 μM
Experiment #4	—	4.4	5.5	90.1	111.0 μM

—, indicates the metal is not used in the mixture experiment.

Table 3 also presents the single-parameter tests associated with each of the j -factor interactions, $j = 2, \dots, 4$. Using Hochberg's (23) correction for multiple comparisons, all three parameters are significantly different from zero when the overall significance level is set at 5%. However, if we consider the case where an overall significance level of 1% is used, only the two smallest p values are significant using Hochberg's correction (23). Therefore, we conclude that a three-factor interaction exists, implying that the two-factor interactions are not constant. Now it is of interest to determine which three metals are interacting with one another. This can be accomplished by performing $\binom{4}{3} = 4$ additional experiments at the same ratios of metals that were used in the original ray. Table 4 gives the ratios of compounds along with the corresponding total dose to be used for the four additional experiments. This approach limits the inferences of the original experiment, as well as the four additional experiments, to be made about the particular mixing ratio used in the experiment.

Conclusion

It was shown that the classic methodology used in evaluating an interaction requires single-drug/chemical data. In "New Methodology" it was shown that the evaluation of interactions could be accomplished with a ray design that did not generate single-drug and single-chemical data. When a ray design is used,

departure from additivity is associated with higher-order polynomial terms in a linear model. Additivity, or absence of interaction, is described by a simple linear model in terms of total dose. As a result, we have shown that we can obtain information about departures from additivity from data collected along a fixed ratio ray. This result is important in that it permits a reduction in the total experimental effort for studying a combination when compared with that associated with a traditional factorial design. Additionally, by incorporating the approach taken by Jones and Mitchell (22), we have presented methodology for determining optimal levels along the fixed ratio ray (total dose) to be considered in the experiment for detecting model inadequacy.

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