

Comparison of Empirical and Mechanistic Models to Identify Relevant Toxicodynamic Interactions between Chemicals in a Mixture

Andreas P. Freidig¹, Diana Jonker¹, Jan J.P. Bogaards¹, Peter Hendriksen¹, Rob H. Stierum¹, Moiz Mumtaz² and John P. Groten¹. ¹TNO Quality of Life, Utrechtseweg 48, Utrechtseweg 48 3704-HE Zeist, The Netherlands. ²ATSDR, CDC, Atlanta, GA.

The goal of this research project was to improve risk assessment of chemical mixtures using mechanistic models to describe interactions between mixture components. In a four-year project, mechanistic models were established for both toxicokinetic and toxicodynamic interactions within mixture of lead, methyl mercury, benzene, and trichloroethylene (TCE), four chemicals which were expected to act by different modes of action in diverse target organs. In a pilot phase (Phase 1), new bioinformatics methods for toxicogenomics fingerprinting and multivariate statistics were developed to address the effects of toxicological mixture studies. In addition, kinetic modeling and *in vitro* tests in hepatocytes were evaluated as possible tools to predict the presence of toxicokinetic interactions. The lessons learned from Phase 1 were used to design a new mixture toxicity study focusing on toxicogenomics analysis and including a concomitant toxicokinetic study (Phase 2).

Conclusions from Phase 1

Although the developed toxicogenomic methodology was found to be suitable to analyze mixture experiments and provide mechanistic explanations, the pilot study was hampered by the high technical variability observed within the dual-label cRNA microarray platform. Therefore, for the repeated toxicity study in Phase 2, a more robust microarray platform was chosen (Affymetrix), and the maximum number of arrays was combined with a reduced two-level fractional factorial mixture design for three compounds. The three compounds for the Phase 2 study were methyl mercury, benzene and TCE. Based on the outcomes of the *in vitro* experiment and physiologically-based pharmacokinetic (PBPK)-model analysis, it was concluded that toxicokinetic interactions would most likely manifest by affecting the levels of relevant metabolites of the other compounds. Therefore, the concomitant toxicokinetic study was performed for the two organic compounds using radiolabelled benzene and TCE, respectively, to be able to assess changes in metabolite levels.

Phase 2

In a repeated-dose (14-day) oral toxicity study in male F344 rats, the effects of low and high lowest observed adverse effect level (LOAEL) dose levels of methyl mercury, benzene, TCE, and mixtures of these compounds (all possible binary mixtures plus the ternary mixture) were examined on conventional toxicity endpoints. The endpoints examined included clinical observations; growth, food, and water consumption; haematology; clinical chemistry; gross examination at sacrifice; and microscopic examination of the kidneys. The conventional endpoints affected upon exposure to the binary or ternary mixtures generally matched those affected by the individual compounds, but the severity of the effects was not always the same.

In a concomitant two-week, repeated-dose toxicokinetic study, toxicologically relevant doses of [¹⁴C] TCE and [¹⁴C] benzene were given to rats alone and in binary combinations with each other and with methyl mercury. Results showed that kinetics of the parent compounds TCE and benzene were only affected in benzene-TCE mixture group. In this group, the [¹⁴C] benzene blood levels were about twofold less and mass balance revealed an increased exhalation and a decreased urinary excretion of [¹⁴C] benzene. The other tested binary mixtures did not reveal any changes in plasma levels or urinary excretion compared to single compound dosing.

For each dose group in the toxicity study, RNA of three potential target organs (liver, kidney and lymphocytes) was collected. Affymetrix rat array (Rat array 230A) containing approximately 16,000 annotated genes were used for the gene expression analysis. On the resulting toxicogenomics dataset, several data analysis approaches were developed and applied to address the specific needs of a mixture experiment. The main goal was to discover mechanistic changes induced by each of the compounds and mixtures, and to determine the overlap in these between the different treatments:

1. Robust selection of differentially expressed genes according to predefined filtering criteria with false positive control.
2. Identification of compound, dose, and organ specific gene expression fingerprints.
3. Assessment of effect additivity, antagonism, or synergism based on fingerprint analysis.
4. Assessment of additivity of gene expression, based on multivariate statistics (principle component analysis).
5. Non-parametric gene set enrichment analysis (GSEA) with pathway and mode of action specific gene sets.

Gene expression analysis showed that interactions occurred in numerous biological processes, including metabolizing pathways, energy metabolism, and cell cycle. Although benzene, methyl mercury, and TCE had been selected on expected different modes of actions a number of genes and gene ontology processes were affected by more than one compound, indicating that these compounds will likely not act completely independent from each other. The study showed that target organ gene expression after mixture exposure differed qualitatively from the response observed after single compound exposure, a finding not observed in the analysis of the classical toxicity endpoints. The data obtained in this study did not change the LOAEL for the tested mixture, but it has revealed new joint effects on target organs at the LOAEL that must be taken into account when defining the hazard of the mixture.

To assess the presence of toxicokinetic interactions, we analyzed genes of Phase 1 and Phase 2 metabolizing enzymes. Numerous genes were affected upon exposure of any of the three compounds. Generally, affected enzymes were very tissue specific. Based on these gene expression changes, benzene, methyl mercury and TCE very likely affect each other's metabolism pathways in the investigated target tissues. The relevance of these changes, however, could not be confirmed by toxicokinetic analysis in plasma and urine.

Target organs responded differently to single compounds than they do to mixtures. This means that parameters characteristic for single compound exposure, such as peroxisome proliferation or PPAR α target-gene induction for TCE, are not *a priori* suitable as biological effect markers of TCE exposure in a mixture. Focusing on TCE derived biomarkers would lead to the erroneous conclusion that toxicity of TCE seems to decrease in mixtures. A genome-wide approach, however, reveals that gene expression of cell damage control (apoptosis) and cell regeneration pathways, which were not present after single compound exposure, are the predominant organ responses induced by the tested mixture. These are the most critical effects which need to be monitored in order to better establish the risk of combined exposure.

Conclusions from Phase 2

Detailed biological analysis of the actual joint effect of mixtures will be crucial to understand the potential additional hazard and risk from mixture exposure. In this study, a bottom-up, mechanistic approach going from single compound to the mixture has not proven fully feasible. With the present techniques, prediction of mixture effects based on single compounds data alone, seems not yet possible for compounds with different modes of action. This study, however, has demonstrated that toxicogenomics can provide a detailed, comprehensive analysis of joint effects. The identified gene expression patterns of mixtures proved to be quantitatively different from the patterns of individual

compounds. Based on our results, we recommend further investigation of whether there are joint effect patterns which are common to different mixtures. Furthermore, the relation between mixture gene expression patterns and long term adverse effects should be characterized. Improved understanding of commonly induced mixture effects will help to better assess whether observed or expected health effects are causally related to mixture exposure scenarios.

Implications: Benzene, methyl mercury and trichloroethylene are three contaminants which are frequently found at industrial waste sites. Due to their different toxicological properties, it has been difficult to understand their potential for joint toxic effects (mixture toxicity). The toxicogenomics approach for mixtures developed in this project resulted in an improved understanding of compound interactions and showed which toxic responses need to be taken into account when dealing with risk assessment of combined exposure to these compounds. In addition, the approach provides a generic framework on how to use toxicogenomics data for identification of relevant modes of action in a mixture exposure scenario.

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Presentations:

Presentation at Annual Meeting of the Society of Toxicology, Baltimore, Maryland, March 21-25, 2004.

Freidig A.P., Heijne W.H.M., Stierum R.H., Wortelboer H.M., Schut, M.W., El-Masri, H.A., Moffett, D., and Groten, J.P. (2004). Comparison of empirical and mechanistic models to identify relevant toxicodynamic and toxicokinetic interactions between chemicals in a mixture. Presentation at LRI Annual Science Meeting, Miami, Florida, May 5-6, 2004.

Presentation at Annual Meeting of the Society of Toxicology, New Orleans, LA, March 6-10, 2005.

Freidig, A., Hendriksen, P.J.M., Jonker, D., Thissen, U., Bogaards, J.J.P., Mumtaz, M.M., Groten, J.M., and Stierum, R.H. (2007). Analysis of gene expression experiments in combination toxicology. Presentation at International Congress of Toxicology, Montreal, Canada, July 15-19, 2007.

Peer-reviewed publications:

Hendriksen, P.J., Freidig, A.P., Jonker, D., Thissen, U., Bogaards, J.J., Mumtaz, M.M., Groten, J.P., and Stierum, R.H. (2007). Transcriptomics analysis of interactive effects of benzene, trichloroethylene and methyl mercury within binary and ternary mixtures on the liver and kidney following subchronic exposure in the rat. *Toxicol. Appl. Pharmacol.* 225(2): 171-188.

Other publications:

TNO Report 5317_02: Dose-effect relationship of four chemicals (benzene, trichloroethylene, lead, methyl mercury), individually on enzymatic activities in primary rat hepatocytes.

TNO Report 5317_03: In vivo interaction study between trichloroethylene, benzene and methyl mercury in rats.

TNO Report 6227: Repeated-dose (2 week) oral toxicity study with benzene, trichloroethylene, methyl mercury and their mixtures in F344 rats.

TNO Report 5317_04: Results from gene expression analysis studies on liver, kidney and lymphocytes in F344 rats exposed to mixtures of benzene, trichloroethylene and methyl mercury.

Heijne, W.H.M. (2004). Toxicogenomics: Applications of new functional genomics technologies in toxicology, PhD Thesis, Wageningen University, The Netherlands.

Data sets (E-TOXM-30) of 90 Microarrays in Array Express. ([http://www.ebi.ac.uk/microarray-as/aer/index.html#ae-main\[0\]](http://www.ebi.ac.uk/microarray-as/aer/index.html#ae-main[0]))

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