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Review Article

Risk assessment of chemical mixtures

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Abstract: Humans are exposed daily to numerous mixtures of chemicals present in their environment instead of just a single chemical. The classical health risk assessment for regulatory decisions uses the chemical toxicity of a single test agent one at a time. Comparison of the toxicity of even a simple chemical mixture with that of its parts often shows significant differences. Therefore, the classical chemical risk assessment paradigm does not represent real-world human exposure, making a risk assessment of chemical mixtures an even more complex process. *In vitro*, *in silico*, organs-on-a-chip, and 3D cell culture models are examples of alternative approaches that have been used for toxicity screening. The developing genomic and epigenomic technologies also show promise for human health risk assessment. However, all these execting experimental models and tools must be validated before they can be used to support hazard and risk assessments of chemical mixtures for regulatory approval.

Keywords: Mixtures, chemical mixtures, health risk assessments, NAMs

Introduction

The regulation of industrial and household chemicals, food additives, pesticides, and drugs, many of which are complex mixtures, can vary from one regulatory agency to another; however, in addition to the currently regulatory-driven in vivo animal studies required for a typical health risk assessment, the value of New Approach Methods (NAMs), especially in understand mode and/or mechanisms of action, are being given greater consideration [1, 2]. Such a health assessment process consists of four steps: (a) hazard identification, (b) exposure assessment, (c) dose-response relationship, and (d) risk characterization [3], typically undertaken using *in vivo* animal studies for hazard identification and dose-response assessment.

Guidelines for the risk assessment of chemical mixtures were published by U.S. Environmental Protection Agency (EPA) in 1986 [4]. It allowed the risk assessment of chemical mixtures to be determined by the toxic or carcinogenic properties of the components in the mixture. This dose additive model predicted reasonably well the toxicities of mixtures composed of a substantial variety of both similar and dissimilar compounds [5]. In addition, in 2014, the Joint Research Centre of the European Union issued its 136-page report on the Assessment of Mixtures-Review of Regulatory Requirements and Guidance [https://publications.jrc.ec.europa.eu/repository/handle/JRC90601]. Notwithstanding the usefulness of these two documents, resolving the toxicity of mixtures, much less assessing the risk of a mixture, is a problem that needs investigation.

In classical health risk assessments, the dose-response relationship is typically determined using high and often unrealistic doses of the test material administered frequently by a single route of exposure compared to the real-world human exposure that often includes multiple routes of exposure. Humans are generally exposed not only to the oral route but also to dermal contact and inhalation. Extrapolations of experimental animal results from animal to human, from high dose to low dose, from the experimental route of exposure to real-world exposure are major challenges that currently are resolved by employing uncertainty factors [6].

To reduce the cost and time of classical animal studies, the U.S. National Academy of Sciences published its landmark report "Toxicity Testing in the 21st Century: A Vision and a Strategy" in 2007 [7]. This report suggested in vitro studies in human cells as alternatives to classical animal studies to determine the dose of the test material required for health hazard assessment for regulatory decisions. However, Tice and his colleagues [8] cautioned in 2013 that the task of converting completely to such approaches has several difficulties including 1] perfect assays do not exist; 2] coverage of all chemicals of interest is incomplete (i.e., volatiles), 3] a high throughput system for measuring the free concentration of a compound in vitro is not yet available; 4] the lack of xenobiotic metabolism in virtually all in vitro assays, interactions between cells are poorly captured; 5] distinguishing between statistical and biological significance is difficult; 6] extrapolating from in vitro concentration to in vivo dose or blood levels is not straightforward; 7] assessing the effects of chronic exposure conditions in vitro is not possible; 8] identifying when a perturbation to a gene/pathway would lead to an adverse effect in animals or humans remains a challenge, and; 9] achieving routine regulatory acceptance of the developed prediction models is years away. Several of their cautions still exist in 2022. Felter and her colleagues recently published the outcome of a Toxicology Forum Workshop on Assessing Chemical Carcinogenicity in which emphasis on the use of NAMs was encouraged [9].

Systems toxicology leverages the tools of systems biology to characterize the physiological and molecular perturbations associated with biologically active substances or their metabolites [10]. Systems toxicology provides a holistic view of biological processes by using systems-wide molecular measurements, commonly termed "omics", such as genomics, proteomics, lipidomics, and metabolomics. Computing omics data from different biological systems and experimental conditions allow a better characterization of the molecular interactions and networks and their roles in cellular processes. The genome maintains the genetic code and the epigenome controls when, where, and how genes are expressed. The objective of genetic and epigenetic toxicology is to determine and predict potential hazards that directly interact with the genome or can adversely affect gene expression by regulating the epigenome, respectively, using computational predictions, biological assays, state-of-the-art "-omics" technologies, and quantitative risk assessment tools.

The classical health risk assessment paradigm relies on developing the toxicity profile for each chemical in a mixture even in the case of complex mixtures such as superfund sites, air pollution, or botanicals. However, humans are rarely if ever exposed to a single chemical by a single pathway but are exposed to mixtures, daily and to different mixtures often by multiple routes of exposure. Therefore, the classical chemical risk assessment, based on individual chemicals, does not represent the real-world human exposure to mixtures of chemicals occurring at the same or different times [11].

Furthermore, it has been demonstrated that the combination of toxicities of individual components of a chemical mixture is not always additive and can result in variations of toxicity depending on the interactions of the individual component of the mixture and routes of exposure [12]. One must keep in mind that when assessing the toxicity of a mixture it is important to test the null hypothesis of no interactions. Only upon its rejection should the possibility of synergistic interactions be considered. An assessment of chemical mixtures should represent all the available integrated scientific evidence on their potential individual toxicities [13] as well as the combined toxicity of the mixture.

The toxicity of a chemical mixture may not always be additive of the toxicity of the individual components of the mixture but may elicit synergistic toxicity. For example, Hayes and colleagues [14] studied the effects of nine pesticides individually and in combination on the time to foreleg emergence and complete tail resorption in Rana pipiens and concluded that the mixture had a greater than additive effect than that of the individual chemicals contained in the mixture.

Risk assessment of botanicals

Archeological studies have shown that medicinal or herbal plants have been used since antiquity (http://baike.baidu.com/view/14642-63.htm). Extracts of natural products will vary but will consist of a mixture of individual com-

ponents, ranging from a few chemicals to several hundred chemicals. Plants, although promising sources of new therapeutic agents or new dietary ingredients are extremely challenging from the standpoint of regulatory evaluation because of their chemical diversity and complexity. Identification of the active ingredient is another major but separate issue.

Risk assessment of botanicals is a challenging process because of the chemical complexity of plants. Natural ingredients are complex mixtures that vary in composition due to variation from differing growing conditions and geographical locations, different processing methods, and extractions from different parts of the plant (e.g., leaves, roots, flowers, fruits, and seeds).

Clemens et al [15] have discussed the uncertainty of hazard identification and risk assessments of palm oil and threats to a critically important food source. Constable et al [16] have presented an integrated approach to the safety assessment of food additives. Hayes et al [17] have discussed various approaches to risk assessment of complex chemical mixtures using new emerging technologies. Booth, Kruger, Hayes, and Clemons [18] proposed an innovative approach to the safety evaluation of natural products using a Vaccinium macrocarpon Aiton leaf aqueous extract as a case study by quantitating the individual chemicals in the extract, evaluating each chemical against its know toxicity, and establishing strict production specifications.

An international roundtable meeting brought together scientists to discuss the needs, available tools, and ongoing data gaps in the botanical safety risk assessment process [19]. The identified critical areas and data gaps include better context on the history of use, systematic assessment of the weight of evidence, use of *in silico* approaches, the inclusion of threshold of toxicological concern considerations, individual substances/matrix interactions of plant constituents, assessing botanical-drug interactions and adaptations needed to apply to *in vitro* and *in vivo* pharmacokinetic modeling of botanical constituents.

Alternate animal models for risk assessment of chemical mixtures

High cost and time-consuming evaluation of animal testing for human health risk assessment have propelled the use of alternative animal models and emerging new technologies for risk assessment. Zebrafish and the worm, *Caenorhabditis elegans,* are just two examples. Zebrafish have been a useful alternate animal model for toxicity testing [20]. Zebrafish embryos have been used as an alternate animal model for risk assessment of chemical mixtures [21]. *Caenorhabditis elegans* (*C. Elegans*) is another alternate model that is being used for the health risk assessment of chemical mixtures [22].

In vitro and *in silico* models for health risk assessment of chemical mixtures

The OECD [23] supports the use of *in vitro* data for risk assessment of chemical mixtures. New *in vitro*, *in silico*, organs-on-a-chip, and 3D cell culture models are being developed, validated, and used as predictive toxicity screening [6, 24]. Quantitative modeling that uses systems toxicology approaches can identify exposure-induced cellular and molecular alterations that would not be detected by standard toxicology assays [17]. All these tools, however, must be validated or at least be shown to be fit for purpose for the indicated endpoint before they can be successfully used for the risk assessment of chemical mixtures [25, 26].

Estimation of combined toxicities of chemical mixtures

A risk probability-based method for evaluation of combined health risks of a chemical mixture of aflatoxin B1 and microcystin LR was developed by Li et al [27]. This approach may be useful for estimating the combined effects of chemical mixtures for human health by dietary exposure. Other models have indicated that the observed synergistic effects are due to response addition or response multiplication joint actions and that most synergistic joint actions are non-interactive and are governed by the dose-response relationship of the individual toxicants [28].

The Online Chemical Modeling Environment (OCHEM, http://ochem.eu) is a web-based platform that provides tools for the automation of typical steps necessary to create a predictive QSAR/QSPR model. Until recently, the OCHEM was limited to the processing of individual compounds; however, the OCHEM has been extended with a new ability to store and model proper-

ties of binary non-additive mixtures. Liess and colleagues have provided an additional approach for the evaluation of the combined toxicant effect as an R package and/or as an Indicate model (http://www.systemecology.eu/indicate/).

Safety assessment of natural complex substances

While many consumers assume that 'natural' ingredients are inherently safe, this is not necessarily the case. Various reports have demonstrated the adverse effects of exposure to numerous natural ingredients [29]. These effects include irritation, dermal sensitization, photosensitization, and allergic reactions [1, 29].

The safe use of botanicals or other natural ingredients is dependent on chemically characterizing the composition of its constituents. Various analytical methods are available to identify constituents of complex mixtures including high-performance liquid chromatography, high-resolution mass spectrometry, ultraviolet detection, and charged aerosol detection [29]. The constituents and levels present need to be identified and documented so that reasonable product specifications can be established.

Once the chemical composition of the product is available, the safety assessment process is similar to that described for synthetic ingredients [29]. This includes identifying hazards (including those discussed above for synthetic ingredients, as well as the potential for Type I allergy at the site of contact), and defining consumer exposure. Since many natural ingredients are complex mixtures and there are often data gaps in the safety/toxicity information, the Threshold of Toxicological Concern (TTC) [30] has been used as part of the safety assessment. A TTC of 10 μg per person per day of botanical plant material, based on dry weight, has been suggested to be sufficiently protective, but it should not be applied to concentrated extracts such as essential oils [31].

If there is not sufficient data to complete a safety assessment on the whole extract, and the estimated exposure exceeds the TTC, then additional safety data needs to be collected. Non-animal testing approaches can be conducted to fill many such data gaps. This testing can be done on the extract or the individual constituents.

Humans are exposed daily to numerous chemical mixtures. Risk assessments for regulatory decisions typically evaluate the toxicity of the individual chemicals in a mixture. Comparison of the toxicity of even a simple chemical mixture with that of its individual parts often shows significant differences. *In vitro*, *in silico*, organs-on-a-chip, and 3D cell culture models are examples of alternative approaches that have been used to evaluate individual chemicals within a mixture or the mixture.

Conclusion

The current chemical risk assessment paradigm does not represent real-world human exposure, making a risk assessment of chemical mixtures an even more complex process. Recent advances in 'omics' technologies continue to provide useful data for hazard and risk assessment of chemical mixtures. The new developing genomic and epigenomic technologies also show promise for human health risk assessment. However, all these exciting experimental models and tools must be validated, or fit for the endpoint of interest, before they can be used to support hazard and risk assessments of chemical mixtures for regulatory approval.

Disclosure of conflict of interest

None.

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References

[1] Hayes AW, Wang T and Dixon D. Loomis's Essentials of Toxicology. 5th Edition (ISBN: 9780128159217). Academic Press/Elsevier; 2019. The Netherlands.

[2] Hilton GM, Adcock C, Akerman G, Baldassari J, Battalora M, Casey W, Clippinger AJ, Cope R, Goetz A, Hayes AW, Papineni S, Peffer RC, Ramsingh D, Williamson Riffle B, Sanches da Rocha M, Ryan N, Scollon E, Visconti N, Wolf DC, Yan Z and Lowit A. Rethinking chronic toxicity and carcinogenicity assessment for agrochemicals project (ReCAAP): a reporting frame-

work to support a weight of evidence safety assessment without long-term rodent bioassays. Regul Toxicol Pharmacol 2022; 131: 105160.
[3] National Research Council (US) Committee on the Institutional Means for Assessment of Risks to Public Health. Risk Management in the Federal Government: Managing the Process. Washington (DC): National Academies Press (US); 1983.
[4] EPA. Guidelines for the Health Risk Assessment of Chemical Mixtures Published on September 24, 1986, Federal Register 51(185): 34014-34025; https://www.epa.gov/sites/production/files/2014-11/documents/chem_mix_1986.pdf.
[5] Murphy SD. 1980. Assessment of the potential for toxic interactions among environmental pollutants. In: Galli CL, Murphy SD and Paoletti R, eds. The principles and methods in modern toxicology. Amsterdam, The Netherlands: Elsevier/North Holland Biomedical Press.
[6] Dourson M, Ewart L, Fitzpatrick SC, Barros SBM, Mahadevan B and Hayes AW. The future of uncertainty factors with *in vitro* studies using human cells. Toxicol Sci 2022; 186: 12-17.
[7] National Research Council, Toxicity testing in the 21st century: a vision and a strategy. 2007. National Academies Press, Washington, DC. SBN: 9780309151733, 0309151732.
[8] Tice RR, Austin CP, Kavlock RJ and Bucher JR. Improving the Human hazard characterization of chemicals: a Tox21 update. Environ Health Perspect 2013; 121: 756-65.
[9] Felter SP, Bhat VS, Botham PA, Bussard DA, Casey W, Hayes AW, Hilton GM, Magurany KA, Sauer UG and Ohanian EV. Assessing chemical carcinogenicity: hazard identification, classification, and risk assessment. insight from a toxicology forum state-of-the-science workshop. Crit Rev Toxicol 2021; 51: 653-694.
[10] Sturla SJ, Boobis AR, FitzGerald RE, Hoeng J, Kavlock RJ, Schirmer K, Whelan M, Wilks MF and Peitsch MC. Systems toxicology: from basic research to risk assessment. Chem Res Toxicol 2014; 27: 314-29.
[11] Boberg J, Dybdahl M, Petersen A, Hass U, Svingen T and Vinggaard AM. A pragmatic approach for human risk assessment of chemical mixtures. Curr Opin Toxicol 2019; 15: 1-7.
[12] Kar S and Leszczynski J. Exploration of computational approaches to predict the toxicity of chemical mixtures. Toxics 2019; 7: 15.
[13] Drakvik E, Altenburger R, Aoki Y, Backhaus T, Bahadori T, Barouki R, Brack W, Cronin MTD, Demeneix B, Hougaard Bennekou S, van Klaveren J, Kneuer C, Kolossa-Gehring M, Lebret E, Posthuma L, Reiber L, Rider C, Rüegg J, Testa G, van der Burg B, van der Voet H, Warhurst AM, van de Water B, Yamazaki K, Öberg M and Bergman Å. Statement on advancing the assessment of chemical mixtures and their risks for human health and the environment. Environ Int 2020; 134: 105267.
[14] Hayes TB, Case P, Chui S, Chung D, Haeffele C, Haston K, Lee M, Mai VP, Marjuoa Y, Parker J and Tsui M. Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? Environ Health Perspect 2006;114 Suppl 1: 40-50.
[15] Clemens R, Hayes AW, Sundram K, and Peter Pressman Peter. Palm oil and threats to a critically important food source: the chloropropanols-caution, controversy, and correction. Toxicol Res Appl 2017; 1: 1-9.
[16] Constable A, Mahadevan B, Pressman P, Garthoff JA, Meunier L, Schrenk D, Speijers G, O'Sullivan A and Hayes AW. An integrated approach to the safety assessment of food additives in early life. Toxicol Res Appl 2017; 1: 1-26.
[17] Hayes AW, Li R, Hoeng J, Iskandar A, Peistch MC and Dourson ML. New approaches to risk assessment of chemical mixtures. Toxicol Res Appl 2019; 3: 1-10.
[18] Booth N, Kruger CL, Hayes AW and Clemons R. An innovative approach to the safety evaluation of natural products: cranberry (vaccinium macrocarpon aiton) leaf aqueous extract as a case study. Food Chem Toxicol 2012; 50: 3150-65.
[19] Galli CL, Walker NJ, Oberlies NH, Roe AL, Edwards J, Fitzpatrick S, Griffiths JC, Hayes AW, Mahony C, Marsman DS and O'Keeffe L. Development of a consensus approach for botanical safety evaluation - a roundtable report. Toxicol Lett 2019; 314: 10-17.
[20] Nishimura Y, Inoue A, Sasagawa S, Koiwa J, Kawaguchi K, Kawase R, Maruyama T, Kim S and Tanaka T. Using zebrafish in systems toxicology for developmental toxicity testing. Congenit Anom (Kyoto) 2016; 56: 18-27.
[21] Jakobs G, Krüger J, Schüttler A, Altenburger R and Busch W. Mixture toxicity analysis in zebrafish embryo: a time and concentration resolved study on mixture effect predictivity. Environ Sci Eur 2020; 32: 143.
[22] Queirós L, Pereira JL, Gonçalves FJM, Pacheco M, Aschner M and Pereira P. Caenorhabditis elegans as a tool for environmental risk assessment: emerging and promising applications for a "nobelized worm". Crit Rev Toxicol 2019; 49: 411-429.
[23] OECD. 2018. Considerations for Assessing the Risks of Combined Exposure to Multiple Chemicals, Series on Testing and Assessment No. 296, Environment, Health and Safety Division, Environment Directorate.

[24] Hernandez AF, Buha A, Constantin C, Wallace DR, Sarigiannis D, Neagu M, Antonijevic B, Hayes AW, Wilks MF and Tsatsakis A. Critical assessment and integration of separate lines of evidence for risk assessment of chemical mixtures. Arch Toxicol 2019; 93: 2741-2757.

[25] Sauer UG, Deferme L, Gribaldo L, Hackermüller J, Tralau T, van Ravenzwaay B, Yauk C, Poole A, Tong W and Gant TW. The challenge of the application of 'omics' technologies in chemicals risk assessment: background and outlook. Regul Toxicol Pharmacol 2017; 91 Suppl 1: S14-S26.

[26] Beronius A, Zilliacus J, Hanberg A, Luijten M, van der Voet H and van Klaveren J. Methodology for health risk assessment of combined exposures to multiple chemicals. Food Chem Toxicol 2020; 143: 111520.

[27] Li Y, Wang J, Li J and Sun X. An improved overall risk probability-based method for assessing the combined health risks of chemical mixtures: an example about a mixture of aflatoxin B1 and microcystin LR by dietary intake. Food Chem Toxicol 2020; 146: 111815.

[28] Chen CY and Lu CL. An analysis of the combined effects of organic toxicants. Sci Total Environ 2002; 289: 123-32.

[29] Hayes AW and Claire LK. 2014. Hayes' principles and methods of toxicology, 6th Edition, London: CRC Press.

[30] Kroes R, Kleiner J and Renwick A. The threshold of toxicological concern concept in risk assessment. Toxicol Sci 2005; 86: 226-30.

[31] Mahony C, Bowtell P, Huber M, Kosemund K, Pfuhler S, Zhu T, Barlow S and McMillan DA. Threshold of toxicological concern (TTC) for botanicals - concentration data analysis of potentially genotoxic constituents to substantiate and extend the TTC approach to botanicals. Food Chem Toxicol 2020; 138: 111182.