Evaluation of Mixtures: Laboratory Tests

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ABSTRACT
Simultaneous exposure of humans to a number of chemicals may produce toxicity which is different than that seen if the exposure to these compounds were sequential. If there is more than one toxic compound in the mixture, their toxicity may be greater than additive, additive, or less than additive. Which of these alternatives occurs depends on a complex set of factors among which are the ability of one or more of these toxic (and non-toxic) compounds present in the mixture to change the metabolism, excretion, or binding to receptors of others in the mixture. One way of determining the potential toxic effects in humans of exposure to the mixture is to carry out laboratory tests in experimental animals using the chemical mixture to which humans are exposed. In a number of instances, humans will be exposed to a highly complex mixture of chemicals, some of which will be present in very low concentrations. Also, some of the compounds in the mixture may have physical or chemical properties which suggest they may pose less risk to human health than others in the mixture. In these cases, consideration should be given to restricting the laboratory tests to compounds present above some minimal concentration, or possessing chemical or physical properties which suggest they pose a greater human risk. This approach could be used in assessing the potential toxicity of chemical mixtures to which humans were exposed by ingestion, inhalation, or dermal contact.

1 INTRODUCTION
Toxicologists have only recently begun to actively consider the design, conduct, and interpretation of laboratory tests of the potential toxicity of chemical mixtures. The assessment of the potential toxicity of complex mixtures of chemicals is considered by many scientists and regulators to be a nearly impossible task. However, in determining the toxicity of single compounds, we are often assessing the toxicity of a mixture of chemicals. This is because the forms of some compounds which are tested are commercial formulations (e.g. pesticides) or technical grades. With few exceptions these compounds contain chemical impurities ranging from trace amounts to a percentage by weight which is greater than that of the compound of interest. In spite of the fact that a
commercial formulation or the technical grade of a compound has been tested, the toxic effects seen may be attributed to the main or active ingredient in the mixture. The modifying effects which the other chemicals present in the mixture may have had on the toxicity of the main ingredient may not have been considered. Occasionally, toxicity attributed to the main or active ingredient is actually caused by some impurity. For example, the teratogenic effects in rats which were originally attributed to 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Courtney et al., 1970) were subsequently shown to be the result of the presence of the contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Courtney and Moore, 1971).

Recently, there has been an increase in the testing of mixtures for potential toxicity. The most notable examples are the testing of various petroleum products for subacute and chronic toxicity in laboratory animals. Also, concentrates of chemical mixtures from drinking water have been tested for genotoxic activity and reproductive toxicity in a variety of in vitro and in vivo tests (Bull et al., 1982; Gruener, 1979; Lang et al., 1980). Another example of the testing of mixtures is studies of commercial formulations of polychlorinated biphenyls (PCBs). For example, the PCB identified as Aroclor 1254 consists of over 50 compounds differing from each other in the degree of chlorination (1-10 chlorine atoms) or the positions on the biphenyl ring system which are chlorinated (e.g. 2,3,5,2',3',5'-hexachlorobiphenyl, 2,3,4,2',3'-pentachlorobiphenyl, etc.).

Although the examination of chemicals for potential toxic effects in humans has focused almost exclusively on individual compounds, it is obvious that humans are frequently exposed to mixtures of potentially toxic chemicals. Thus, human exposure to potential toxic compounds in the workplace, as a result of improper disposal of chemical wastes, or due to contamination of food, air, or water, often involves an exposure to chemical mixtures. On exposure to a mixture, the toxicity of a single compound in the mixture may remain the same or may be increased or decreased by the presence of other compounds in the mixture. In addition, if more than one toxic compound is present in the mixture, their effects may be additive, greater than additive (potentiation or synergism), or less than additive (antagonism).

2 METHODS FOR ASSESSING TOXICITY

Three methodologies can be used in examining for potential adverse health effects in humans resulting from exposure to a mixture of chemicals. These are epidemiology, 'short-term' tests for potential carcinogens or mutagens, and tests in experimental animals.

2.1 Epidemiology

Since epidemiological methodologies and limitations are discussed in detail in other papers, the subject will not be examined here.
2.2 Short-term Tests

Substantial evidence indicates that one step in carcinogenesis is damage to or mutation of the DNA. Many bacterial and mammalian cell culture assays can detect such chemically induced alterations. Thus, these short-term test assays can be useful in qualitatively predicting the potential carcinogenicity of a mixture of chemicals to humans. The major advantage of these tests is that they are relatively rapid and inexpensive as compared with animal tests or epidemiological studies. A major shortcoming of the short-term tests currently available is that they do not provide reliable data on the potential potency of carcinogenic chemicals in man. It is apparent that, for all carcinogenic chemicals, there is a level of human exposure at which the risk is considered acceptable. Thus, information on the 'carcinogenic potency' of chemicals is necessary in regulating human exposure. Carcinogenic potency data are currently provided only by epidemiology studies or lifetime assays in animals.

2.3 Animal Bioassays

The results of animal bioassays, conducted largely in rodents, have been and will continue to be the major source of data for estimating human risk from exposure to potentially toxic chemicals or mixtures of chemicals. However, it is important to remember that the rodent bioassays, as currently designed, are only approximately predictive of the potential of a chemical to produce disease in humans. In a recent publication, Purchase (1980) examined data on 250 chemicals which had been tested for carcinogenicity in both rats and mice. These data permitted the determination of how accurately the mouse can predict cancer in the rat and vice versa. The rat as a predictor of cancer in the mouse was shown to have a specificity of about 85%. The mouse as a predictor of cancer in the rat was shown to have a specificity of about 82%. It is reasonable to assume that the ability of the rat or mouse to predict the potential or lack of potential of a chemical to cause cancer in man is no better than their ability to predict these same effects in each other. We might also expect that, in general, the rat and mouse may not predict with any greater specificity the ability of a chemical or mixture of chemicals to cause toxic effects in humans other than cancer. Another limitation to animal studies is that they are expensive.

However, their advantages outweigh their limitations as a predictor of the potential toxicity of chemicals to humans. Many of the neoplasms and other disease states seen in rats and mice exposed to chemicals have similar morphological and behavioural characteristics to those in man. Mice and rats are also metabolically and anatomically similar to man and, since the production of cancer and other diseases resulting from exposure to chemicals is often related to the metabolism of the chemicals to reactive intermediates, the metabolic similarity is of extreme importance.
3 GENERAL CONSIDERATIONS IN EXAMINING THE TOXICITY OF A MIXTURE OF CHEMICALS

In examining for potential adverse health effects resulting from human exposure to a mixture of chemicals, the first step should be to determine, to the degree possible, the chemical composition of the mixture. For those compounds in the mixture which have an adequate toxicology data base, the potential for adverse health effects in humans can be determined and interim maximum concentration limits (MCLs) established. In this way, control over human exposure to known toxic chemicals can be quickly established. An interim MCL could also be set for those compounds in the mixture for which some, although inadequate, toxicology data exist which suggest a potential for toxic effects. Quantitative and qualitative information on industrial, agricultural, and household chemicals which may be present in a specific mixture, because of local practices, is essential to the analytical evaluation of the compounds in the mixture which may pose a potential adverse health risk.

Toxicological evaluation of each of the chemicals in the mixture for which there is not an adequate toxicological data base will usually not be economically feasible. In addition, humans will be exposed to the mixture of chemicals and not to individual compounds. Therefore, the use of a mixture of the chemicals in laboratory tests is a toxicologically sound approach. This approach will automatically take into account the possible antagonistic, additive, and synergistic effects which may occur between the various compounds present in the mixture. Therefore, in evaluating its potential to produce adverse health effects, the mixture or a facsimile of the mixture of chemicals to which humans are exposed should be administered to experimental animals by the most appropriate route and examined for evidence of toxic effects using classical toxicological procedures.

The major toxic end-points which may occur in animals as a result of exposure to a mixture of chemicals include acute toxicity, organ system toxicity, cancer, somatic or germ cell mutations, teratogenicity, embryotoxicity, reproductive toxicity, and immunotoxicity. In conducting animal tests for the potential toxicity of the chemical mixture to humans, it is desirable to examine for each of these toxic end-points using doses much larger than those to which humans are or may be exposed. The route of exposure of the experimental animals to the mixture should be, ideally, the same route by which humans are exposed. Thus, if humans are exposed to the mixture predominantly by inhalation, this should be the preferred route of exposure of the animals.

If the mixture of chemicals which is of concern as regards human health effects consists of a few chemicals present at relatively high concentrations, an attempt should be made to test that mixture in experimental animals. The mixture to be tested could be artificially created by mixing together the individual compounds to which humans will be exposed in the same proportions in which they exist in
the home, environmental, or industrial setting. However, if the mixture consists of a large number of compounds, some of which are present in very low concentrations, some procedure must be considered which will reduce the number of compounds in the mixture to be tested without, hopefully, materially affecting the predictability of the laboratory tests for potential human toxicity. An example of the difficulties which may be encountered in evaluating the potential toxicity of a complex chemical mixture is the issue of organic chemical contamination of drinking water. A procedure for simplifying the evaluation of the potential toxicity to humans of organic chemical contaminants in drinking water has recently been proposed (Neal, 1983).

Hundreds of organic compounds have been identified as contaminants of drinking water. Those compounds which have been identified are thought to represent only about 10% of the organic matter present in drinking water. The majority of those compounds which have been identified are present in very low concentrations (< 1 μg/litre). The cost of analysing for and evaluating the potential toxicity to humans of each individual organic compound present in finished drinking water would be prohibitive. The task of formulating an artificial mixture of the compounds present in the drinking water for use in laboratory tests would also be formidable. An alternative to formulating an artificial mixture of the compounds present would be the concentration of the mixture of chemicals present in the water, using techniques such as lyophilization or reversed osmosis, and the use of this concentrate in the laboratory tests. These techniques are feasible for the concentration of non-volatile compounds present in water. However, a large percentage of the potentially more toxic volatile organic compounds would be lost using these techniques.

A procedure which would simplify the assessment of potential toxicity using an artificial mixture would be to limit the number of chemical contaminants present in the artificial mixture to those which pose the greatest health risk. Those compounds with less potential for toxicity could be examined later if resources permit. It has been proposed that chemical analyses for the compounds present in the drinking water should be given priority, concentrating first on identifying those volatile compounds present in finished drinking water at concentrations > 1 μg/litre (Neal, 1983). For those volatile compounds found in drinking water which have the potential to cause acute or chronic toxicity in man, the concentrations at which there is concern almost invariably exceed 1 μg/litre. For example, the pesticide aldicarb which, of all the compounds so far identified in drinking water, has the greatest potential for acute toxicity, apparently does not pose a significant human health risk at a concentration of 3 μg/litre or, perhaps, higher. For those compounds in drinking water which cause chronic toxicity, the concentrations which are judged to pose a significant risk to humans are almost always > 1 μg/litre. For example, the World Health Organization has recently noted that the concentrations of carbon tetrachloride, tetrachloroethylene, and trichloroethylene in drinking water which pose a significant risk are considerably
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in excess of 1 μg/litre based on a multistage mathematical extrapolation to a $10^{-5}$ risk level (WHO, 1983).

Thus, in preparing a suitable mixture of the organic chemicals present in drinking water for animal testing, it is proposed that an artificial mixture of the volatile organic compounds detected analytically at concentrations > 1 μg/litre should be combined with a non-volatile organic fraction concentrated from the water. The majority of the non-volatile organic compounds present in drinking water cannot be structurally identified by currently available analytical techniques. Therefore, these compounds must be obtained by concentration from the finished drinking water. As noted above, techniques such as lyophilization followed by removal of the inorganic chemicals from the residue, reversed osmosis, and the use of resins can be used to prepare the sample of non-volatile organic compounds from the drinking water supply to be tested.

The potential human toxicity of the mixture of the volatile and non-volatile organic chemicals prepared from finished drinking water should be evaluated by administering to animals levels of the mixture considerably in excess (up to 1000 times) of the levels found in the drinking water. Those volatile compounds added to the mixture should be present in the same relative concentration in which they occur in the water supply.

Although this approach is suggested for the evaluation of mixtures of organic chemicals in water, it should also be generally applicable to mixtures of inorganic chemicals or mixtures of inorganic and organic chemicals to which humans are exposed by inhalation, dermal absorption, or through contamination of food or drinking water.

All compounds detected in air, water, or food which are of toxicological concern may not be available commercially. This would necessitate a decision as to whether to synthesize the compound and include it in the mixture.

4 LIMITATIONS TO THE ASSESSMENT OF THE TOXICITY OF MULTIPLE CHEMICAL CONTAMINANTS

There are a number of limitations in any procedure which uses laboratory studies to assess the potential toxicity to humans of multiple chemical contaminants. For example, it will not be possible to prepare a totally representative mixture of the chemical contaminants present in, for example, drinking water for use in laboratory studies. This is because there are technical limitations both in the preparation of an artificial mixture of the volatile chemicals and in preparing a suitable concentrate of the non-volatile chemicals which are present in the water.

The artificial mixture used in the laboratory studies may be reasonably representative of the chemical contaminants present at any one time. However, there is no guarantee that the chemical contaminants will not vary both qualitatively and quantitatively with time and that some variation in toxicity may result.
Another limitation to the design of studies to test the toxicity of a mixture is the availability of a suitable control group. Theoretically, a suitable control for the study of chemical contaminants in water would be animals administered elevated levels of a representative chemical mixture from an ‘uncontaminated’ water source. For inhalation studies, the control group should be one exposed to an atmosphere essentially free of chemical contaminants. However, the determination of what represents a ‘contaminated’ as compared with an ‘uncontaminated’ water or air supply may not be straightforward.

4.1 Specific Laboratory Tests for the Toxicity of Chemical Mixtures in Drinking Water

In proposing a series of specific laboratory tests to be used in examining the potential toxicity of human exposure to a mixture of chemicals, chemical contamination of drinking water will again be used as the primary example. This is because this route of exposure has been studied to a greater extent than human exposure to mixtures from other sources.

A number of studies have been conducted of the genotoxic potential of mixtures of chemicals present in finished drinking water supplies. For example, Lang et al. (1980) examined for the malignant transformation of BALB/3T3 cells using mixtures of chemicals isolated from the drinking water supplies of five US cities using reversed osmosis and ion-exchange resins. Positive results were obtained with some of these chemical mixtures. In another study, Gruener and Lockwood (1980) examined the mutagenicity to Chinese hamster embryo cells of chemical mixtures obtained by lyophilization of drinking water. In these studies mutagenicity was seen when the chemical mixtures were incubated with the cells in the presence of a mammalian metabolic activation system.

The genotoxicity of chemical mixtures isolated from drinking water has also been studied in vivo. Bull et al. (1982) examined concentrates of chemical mixtures from the drinking water of five US cities for promotional activity in a mouse skin initiation/promotion assay. Promotional activity was detected using some of these mixtures. Mutagenic activity was also detected using a bacterial assay system.

The subchronic toxicity in mice of a chemical mixture isolated from drinking water has also been examined (Gruener, 1979). The concentrations of the chemicals in the mixture administered to the mice were about 1000 times those to which humans drinking the water would have been exposed. No effects were noted on blood chemistry, motor activity, body weight, or reproductive efficiency.

4.1.1 Laboratory Tests

Two comparative laboratory protocols for examining the potential toxicity to humans of multiple chemical contaminants in drinking water have been
proposed. One of these (Neal, 1982a) recommends only the use of animal studies in vivo. Another (NAS/NRC, 1982) recommends the use of both in vitro and in vivo tests. The latter protocol (NAS/NRC, 1982) recommends the use of a three-tiered approach to toxicity testing using a mixture of chemicals concentrated from the water supply to be examined. The first phase consists of in vitro testing for mutagenicity and mammalian cell transformation. In addition, the chemical concentrate would also be examined for acute toxicity, teratogenicity, and short-term (14-day) repeated dose toxicity including a cytogenetics assay. In phase 2, a subchronic (90-day) study would be carried out in at least one, and preferably two, rodent species. Also during phase 2, the chemical mixture would be examined for its ability to produce reproductive toxicity in one rodent species. In phase 3, a chronic toxicity test would be carried out in one rodent species.

Because of the difficulties inherent in the interpretation of the results, the present author does not recommend the use of in vitro tests for assessing the potential toxicity to humans of exposure to a mixture of chemical contaminants present in drinking water or other sources. Rather the recommendation is that the assessment be made primarily on the result of animal studies using the results of short-term tests for genotoxicity as an aid in interpreting the results of the animal studies. Short-term tests may also be useful in making a preliminary determination whether a particular control measure may alter the composition of the mixture and thus any possible human carcinogenic risk which may be inherent in exposure to the mixture. The animal testing procedures recommended by this author (Neal, 1982a,b), which are detailed below, and by the National Research Council Committee (NAS/NRC, 1982) are in general agreement.

In carrying out the animal studies (other than acute toxicity), the highest dose of the artificial mixture of the chemicals present in the drinking water, food, the inspired air, or applied dermally should be considerably higher (500–1000 times) than the expected human dose. In any case, the maximum tolerated dose, in the context of the use of this term in animal testing, should be the upper limit. It is recommended that two additional doses be administered in order to maximize the potential for obtaining a dose response for any toxic effect which is seen.

4.1.2 Acute Toxicity

To measure the potential acute toxic effects of chemicals present in, for example, a drinking water supply will require a combination of monitoring for known acutely toxic organic chemicals which may be present, plus the testing of a representative mixture of the contaminants in experimental animals. The acute oral (dermal or inhalation) toxicity of the chemical mixture should be examined in young adult male and female rats. The mixture, in the case of drinking water, should be administered by gavage or contained in a gelatin capsule. Detailed
procedures for measuring acute toxicity in rodents have been described elsewhere (NAS/NRC, 1977; OECD, 1981).

4.1.3 Subchronic Toxicity

Subchronic testing in experimental animals can provide extremely useful information about the potential toxic effects in humans of exposure to a mixture of the chemical contaminants present in finished drinking water or other sources. Results of this test will give valuable information about the ability of the combination of organic chemicals to produce mortality and adverse effects on various organ systems including the haematopoietic system, the nervous system, and the immune system. This test is usually conducted in two species: one test in a rodent and another in a non-rodent species. The usual non-rodent species is the dog. However, the difficulty in obtaining sufficient amounts of the various chemicals which will likely comprise the artificial mixture to be administered in these studies precludes the use of dog. Therefore, it is recommended that two rodent species be used. Detailed procedures for the conduct of a subchronic study are described elsewhere (NAS/NRC, 1977; OECD, 1981).

4.1.4 Chronic Toxicity

The induction of cancer as a result of human exposure to mixtures of chemicals is, perhaps, the major concern of the public and government agencies. However, it must be kept in mind that chronic effects other than cancer can occur on exposure of humans to mixtures of chemicals, and the studies designed to examine for chronic health effects should consider all possible effects. These include, in addition to cancer, organ system effects, neurotoxicity, and effects on the haematopoietic and immune systems.

Chronic toxicity tests using artificial chemical mixtures should be carried out in two rodent species, the laboratory rat and mouse. The animals should be exposed to the concentrated mixture of organic chemicals on a daily basis for approximately 24 months or the normal lifespan of the strain used. At least three dose levels of the concentrated organic chemical mixture should be tested. The highest dose level should preferably be one which demonstrates some toxicological/pharmacological effects or, in the absence of biological effects, it should be considerably higher (1000 times) than the anticipated human exposure level. However, this dose should be selected so as not to cause excess mortality in the progress of the test. The lowest dose level should be one which, ideally, does not produce any evidence of toxicity. In the case of chemical contaminants of drinking water, the chemical mixture should, if possible, be administered to the experimental animals in the drinking water. Otherwise, the organic concentrate should be administered by gavage on a daily basis. Clinical laboratory testing should be conducted periodically throughout the study such as carrying out haematology, blood chemistry and urine analyses, and any other tests indicated
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by the symptoms shown by the animals. At the termination of the experiments, all animals should be subjected to gross necropsy and histopathological examination for organ system effects and for evidence of an increased incidence of neoplasia. Observation of the animals for behavioural anomalies should also be included as part of this experiment. Additional details on conducting chronic rodent studies have been described elsewhere (NAS/NRC, 1977; OECD, 1981).

4.1.5 Teratology

The chemical mixture should be examined for teratogenicity. In these studies the mixture should be administered daily to a rodent species. In the case of chemical contaminants of drinking water, the mixture should also be administered to the experimental animals in the drinking water or, if this is not possible, by gavage or in a gelatin capsule beginning at or before the time of implantation and continuing through the period of major organogenesis. The study should be carried out with at least three dosage levels of the mixture. The highest dosage level should preferably be one which induces some foetal or maternal toxicity, as demonstrated by body weight reduction or other toxic signs, but not cause more than 10% maternal fatalities. However, a dose of the chemical mixture which is 500–1000 times higher than the daily intake that is anticipated in humans probably need not be exceeded. Additional details for the conduct of teratogenicity studies are described elsewhere (NAS/NRC, 1977; OECD, 1981).

4.1.6 Reproductive Toxicity

An assessment should be made of the potential for adverse health effects on the rodent male and female reproductive systems caused by a representative mixture of the chemicals to which humans are exposed. This can be accomplished using the standard two-generation reproductive test. At least three dose levels should be administered. The highest level should, preferably, produce an observable toxicological/pharmacological effect in the test animals but not cause more than 10% fatalities. It is probable that a dose representing a 500- to 1000-fold increase in the daily intake anticipated in humans consuming the finished drinking water need not be exceeded. The lowest dose level should produce no adverse effects and, ideally, the chemical mixture should be administered by the route by which humans are exposed, if the animal will tolerate it. Otherwise, it should be given by gavage. Additional details for conducting reproductive studies are described elsewhere (NAS/NRC, 1977; OECD, 1981).

5 REFERENCES


