Methods for Assessing the Effects of Mixtures of Chemicals
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Evaluation of Mixtures: Interpretation of Results and Prediction of Effects in Humans

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ABSTRACT

The prediction of a biological effect of a mixture of chemicals in man, from animal experiments, is much facilitated when the biochemical basis of interactions is known. Several examples of materials encountered in the workplace and of drugs are given which support this thesis. It is also known that species extrapolation, in general, is less accurate for a mixture than for a single chemical; in some (rare) cases it is even impossible.

1 INTRODUCTION

In the human environment simultaneous exposure to chemicals is a common event. In addition, chemical and physical environmental factors are known to interact (WHO, 1981). Thus, synergistic effects of noise and of carbon monoxide exposure on cochlear function have been established. This means that, in many cases, conditions of realistic human exposure are much more complex than those in animal experiments. This is one of the difficulties in extrapolating animal data to man.

Moreover, it might be difficult to interpret a combined effect of which the underlying mechanism is not known. Finally, differences in physiology between man and the particular animal species in question may entirely exclude a valid extrapolation. This chapter provides examples highlighting these points.

2 INTERPRETATION OF RESULTS FROM ANIMAL EXPERIMENTS

In a descriptive way, a combination of chemicals may result in independent action, synergism (potentiation), or antagonism (see section 5, this volume).

2.1 Additivity and Potentiation

In the absence of, or in addition to, other specific effects, a critical toxicological manifestation of high-level exposures to many compounds (e.g. organic gases and solvent vapours) is narcosis or prenarcotic sedation. Usual combinations of different anaesthetic agents in clinical practice are based on additivity of the narcotic effects of different compounds when given simultaneously. Superadditivity (potentiation) was observed in the case of the combination of ethanol with chloral hydrate when human volunteers, on exposure to these compounds, were subjected to vigilance performance tests (Sellers *et al.*, 1972b). The most likely explanation, in addition to the simple combination of effects at the target site, is a pharmacokinetic interference: ethanol, given subsequent to a chloral hydrate dose, enhances the levels of the ultimately effective metabolite, trichloroethanol. Conversely, chloral hydrate ingestion affects ethanol metabolism and causes higher peak blood ethanol contractions than observed after ethanol alone (Sellers *et al.*, 1972a). This example demonstrates the importance of metabolic interactions.

Interactions occur within various organ systems. A special kind of interaction of inhaled chemicals in the lung has been described by Witschi and Hakkinen (1982): sulphur dioxide produces acute bronchoconstriction in guinea pigs. If such animals are simultaneously exposed to sulphur dioxide and to aerosols of soluble sodium, manganese, iron or potassium salts, the irritating properties of sulphur dioxide are greatly potentiated. Presumably more sulphur dioxide, adsorbed to aerosol particles, is carried deep into the lung.

2.2 Different Compounds Acting via the Same Toxic Metabolite

Not uncommon are combinations of compounds which act via the same metabolite. In this case, determination of that metabolite provides a clue for predicting combined toxicity. Two characteristic examples may be given.

(1) *Methylene chloride* is biotransformed to *carbon monoxide*. Hence, the most relevant toxic effect of methylene chloride is impairment of oxygen transport by COHb (carboxyhaemoglobin) formation. The same effect is produced directly by exposure to carbon monoxide. Most countries accept levels up to 5% COHb as a 'biological standard' for occupational exposure to both compounds (DFG, 1983; NIOSH, 1976). The additive effect of combined exposures to carbon monoxide and methylene chloride in terms of COHb formation is well established in experimental animals and in man.

(2) Many recent publications conclusively show that the ultimately neurotoxic metabolite of *n-hexane* is 2,5-hexanedione. Microsomal monooxygenases and cytosolic alcohol dehydrogenases are involved in the formation of this metabolite. One of the metabolic intermediates is *methylbutylketone* (2-hexanone). In accordance with this concept, the neurotoxicity of

methylbutylketone in experimental animals is consistently higher than that of *n*-hexane (Abou-Donia *et al.*, 1982). Determination of metabolites, especially 2,5-hexanedione, in blood or urine has been recommended for monitoring toxicity of the mixture (Perbellini *et al.*, 1981).

2.3 Metabolic Interactions

The importance of pharmacokinetic effects resulting from induction and inhibition processes has been outlined in other chapters of this volume. Interpretation of results from animal experiments must take this properly into account. Thus, in accordance with the above concept of *n*-hexane toxicity, it has been demonstrated that mixtures of *n*-hexane and toluene are less toxic to peripheral nerves of rats than is *n*-hexane alone (Takeuchi *et al.*, 1981). Subsequent experiments in rats *in vivo* and with rat liver microsomes *in vitro* (Perbellini *et al.*, 1982) revealed a marked inhibition by toluene of oxidative metabolism of *n*-hexane, whereas *n*-hexane did not modify the biotransformation of toluene *in vivo*. *In vitro*, the inhibition constant (K_i) of toluene on *n*-hexane biotransformation was 7.5 μM and that of *n*-hexane on toluene was 30 μM, which explains the observed toxicological behaviour of *n*-hexane–toluene mixtures. Other authors have described pharmacokinetic interactions between toluene and trichloroethylene (see Bolt, 1981).

2.4 Effects of Sequential versus Simultaneous Applications

It is well established that the time sequence in application of chemicals is critical for the interpretation of experimental results; the time course of induction and inhibition processes is especially important.

Outstanding examples of the diverse effects of sequential versus simultaneous applications of the same chemicals can be drawn from the field of chemical carcinogenesis.

When preneoplastic enzyme-altered foci in rat liver are induced by a hepatocarcinogen, e.g. diethylnitrosamine, different effects of phenobarbital are observed. (1) If phenobarbital is given concurrently with diethylnitrosamine, fewer preneoplastic foci are observed than are seen after diethylnitrosamine alone (Rickart *et al.*, 1980). The lower the diethylnitrosamine dose given, the greater is the decreasing effect of phenobarbital. Most likely, the carcinogen is detoxified by a monooxygenase species of high affinity and low capacity which is induced by phenobarbital (Rickart *et al.*, 1980). (2) If phenobarbital is administered after a first dose of diethylnitrosamine (30 hours later), preneoplastic foci rapidly enlarge (Schulte-Hermann *et al.*, 1981). This effect of phenobarbital is thought to be caused by a stimulation of cell replication.

The mechanisms of inhibition and enhancement of the action of chemical carcinogens have recently been reviewed. Inhibition (Wattenberg, 1980) may

result from direct blocking of enzymatic carcinogen activation, by stimulation of coordinated detoxification response, or by direct scavenging of a reactive carcinogenic species. Enhancement (Clayson, 1980) may occur by influence on metabolic activation, the 'interaction phase' or the 'tumour developmental phase'.

Another example of the importance of the time factor in administration of chemicals will be mentioned (Witschi and Hakkinen, 1982). The severity of lung lesions following treatment of experimental animals with butylated hydroxytoluene and oxygen is dependent on the concentration of oxygen, the duration of oxygen exposure, the time at which oxygen exposure follows the initial lesion by butylated hydroxytoluene, and the extent of initial lung damage.

2.5 Effects of Complex Mixtures

Often it is nearly impossible to predict quantitatively an effect of a very complex mixture, even when effects of individual components are known. Classical support for this statement may be derived from carcinogenicity data on polycyclic hydrocarbons in such a mixture, but not as a measure of its overall cancer-generating potential.

It will not be possible to establish connections on a firmer, quantitative foundation between exposure and cancer risk until more precise knowledge of the compositions of specific mixtures and of their cancer-producing effect is available (DFG, 1982).

3 PREDICTION OF EFFECTS IN HUMANS

From the few examples given above it follows that recognition of synergistic/antagonistic effects from a mechanistic consideration is the most relevant basis for a valid prediction of the effect of chemical combinations in man. In addition, species differences in the biological response must be considered. As there is a large body of experimental data on relevant drug combinations in a variety of animal species and in man, this is best supported by examples from that field.

3.1 Effects of Fixed Combinations in Different Species: Example of Contraceptive Formulations

Rules that regulate fixed drug combinations in different countries require safety testing of the individual components and of the mixture, as administered to man. Multiples of the 'human dose' are usually administered for long periods, based on calculations of mg/kg body weight. In this case, the balance of effects may vary from species to species.

A well-recognized example is represented by contraceptive oestrogen–gestagen mixtures (Neumann and Elger, 1971; Neumann *et al.*, 1973). A contraceptive formulation of ethinyloestradiol and norethisterone which is 'balanced' for human therapeutic use and, in women, exhibits oestrogenic as well as progestational effects, behaves like a nearly pure oestrogen in rats: the gestagen norethisterone is predominantly oestrogenic in this species. Prolonged high-dose oestrogen administration in rats, because of species specificities, results in formation of pituitary and mammary tumours. By contrast, in the rabbit norethisterone is a strong gestagen.

On the other hand, the induction of canine mammary gland nodules by synthetic 17α-hydroxyprogesterone gestagens and mixtures of these compounds with oestrogens is now considered to be species specific. Nodule induction is related to the extraordinarily high progestational activity of 17α-hydroxyprogesterone gestagens in the dog and their stimulatory effect on the highly mammotropic canine growth hormone (El Etreby and Graf, 1979).

Such species differences in response to a distinct chemical and to mixtures containing that chemical require a detailed knowledge of mechanism before valid predictions of effects in man can be made. Furthermore, some effects in man cannot be extrapolated from animal experiments. Again, oral contraceptives provide a prominent example.

Much interest has focused on changes in serum lipoproteins due to long-term intake of steroid contraceptives, especially after publication of the 'Framingham Study' on risks of cardiovascular disease. This study (Gordon *et al.*, 1977) favoured the view that high-density lipoproteins (HDL) are a protective factor against coronary heart disease. Hence, considerable efforts have been made toward developing oestrogen–gestagen combinations that do not affect this particular metabolic variable. In general, gestagens lower HDL-cholesterol levels (Kremer *et al.*, 1980). This effect is partly antagonized by oestrogens, depending on the oestrogen dose (Larsson-Cohn *et al.*, 1981). Comparative evaluations in women of four different combinations of ethinyloestradiol with levonorgestrel have revealed that their effect on lowering concentrations of HDL-cholesterol is minimized by properly timing the gestagen dose ('three-phasic') throughout the menstrual cycle. Differences between humans and experimental animals in reproductive physiology, in lipid metabolism and in pharmacokinetics of synthetic steroids render the choice of a suitable animal model for such investigations extremely difficult, if not impossible.

3.2 Quantitative Evaluation of Mixtures Acting on Humans

A quantitative solution of the toxicological problem of mixtures of chemicals has been proposed for the threshold limit values, related to chemical compounds in air at the workplace, by the American Conference of Governmental Industrial Hygienists (ACGIH, 1983). The proposed formula is justified by the statement,

'When two or more hazardous substances, *which act upon the same organ system*, are present, their combined effect, rather than that of either individually, should be given primary consideration. In the absence of information to the contrary, the effects of the different hazards should be *considered as additive*.' Thus, the prerequisites for such a procedure are: (a) the same target for the individual compounds, and (b) simple additivity of the effects of the pure chemicals. When these two assumptions in fact hold true, the prediction of a combined effect in man from animal data can be made with ease and considerable accuracy. The target, however, which here is presupposed to be the same for the chemicals in question, should be the same not only in the organ but also at the cellular level.

If the situation is more difficult, i.e. when there is no simple additivity of the partial effects, an understanding of the mechanisms of interaction of the compounds, and probably of the species differences of that interaction, must be attempted. There may be cases where valid species extrapolation is impossible.

When effects of different chemicals are obviously completely unrelated to each other and when these are directed toward different target systems in the organism, it may be justified to base the evaluation of the mixture entirely on the effects of each individual chemical. In practice, this may be a relatively rare situation.

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