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MORE EFFICIENT USE OF ANIMALS IN ACUTE INHALATION TOXICITY TESTING

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Summary

By means of Monte Carlo simulation of NH_3 exposure of male and female rats at four different concentrations and using 20 different exposure periods the influence of the number of animals per sex per trial on the confidence limits of the LC-50 value was studied. It was found that using one animal per sex, 20–25 trials suffice to establish the concentration–time–mortality relationship. From this relationship any LC-50 can be determined over the range of exposure periods with confidence limits similar to those obtained when determining one LC-50 with five animals per sex per trial.

The advantage of the presented design in comparison to an acute inhalation toxicity study as described in the OECD-guidelines is that the LC-50 can be determined for a range of exposure periods while a similar number of animals is needed as in the study design according to the OECD guidelines which, however, yields only one LC-50 value.

The results of this investigation indicate the need for reconsideration of the OECD guideline 403 (LC-50) on acute inhalation toxicity.

Introduction

Product legislation such as the Toxic Substance Control Act (TOSCA) in the U.S.A. and the sixth amendment in the European Community (directive 831, 1979) require information about the acute toxicity of new chemical products. In case of volatile compounds the OECD** guidelines prefer a 4-hour LC-50 study, requiring about 40 to 50 rats. However, a 4-hour LC-50 test only yields one single figure containing hardly any information about lethal responses for shorter periods of ten minutes to one hour.

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**OECD stands for Organisation of Economic Cooperation and Development.

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In industrial and environmental hazard analysis it is important to be able to predict mortality responses of short exposures from five minutes to one hour. Furthermore, in recent years it has been recognized that extrapolation by means of Haber's rule of the 4-hour LC-50 is not allowed for most substances. Relevant to this, Appelman et al. [1] studied the acute inhalation toxicity of ammonia in rats at different exposure periods. They exposed five male and five female rats each to 20 different exposure conditions, thus using 200 animals in total.

The purpose of the present investigation was to establish the minimum number of rats per trial in the NH_3 study [1] necessary to obtain LC-50 values with confidence limits compatible to those obtained using the OECD guidelines. Using the statistical properties of the concentration–time–mortality relation found in the NH_3 study, we repeated the NH_3 study 500 times for one to ten animals per sex per trial applying a Monte Carlo simulation. To obtain the same information with *in vivo* experiments would have required 1.1 million of rats.

Methods

The NH_3 study

The NH_3 study [1] consisted of 20 trials with three independent variables: (a) time of exposure at four different time intervals, (b) concentration at five levels for each time interval, and (c) sex. From the experimental data of this study the probit equation according to Finney [2] was derived:

$$Y = -25.0 + 6.5 \ln C + 3.2 \ln T + 1.8 S \qquad (1)$$

with: Y=probit, C=concentration in g/m^3, T=time of exposure in minutes, and S=sex (1=male, 0=female).

The mortality response (P) can be calculated from the following equation:

$$P = (2\pi)^{-\frac{1}{2}} \int_{-\infty}^{y-5} \exp(-\tfrac{1}{2} u^2)\, du \qquad (2)$$

These equations fitted well with the experimentally observed mortality response because the chi-square, a measure for the goodness of fit, was 45.7 at 36 degrees of freedom (P=0.13).

Simulated exposure conditions

For the Monte Carlo simulation four concentrations and five corresponding exposure periods in about geometric progression were chosen and are given in Table 1. These 20 exposure conditions were studied in male and female rats. This resulted into 40 combinations of the independent variables C, T, and S controlling the mortality response.

TABLE 1

Simulated exposure conditions

Concentration (g/m^3)	Exposure periods (min)
30	5, 7, 10, 14, 20
20	10, 14, 20, 28, 40
15	18, 25, 35, 50, 70
12	30, 42, 60, 85, 120

Monte Carlo simulation

A specific combination of independent variables is substituted in the probit eqn. (1). The resulting probit, Y, is then used to calculate the mortality response, P (eqn. 2), for this combination of independent variables. Next, numbers between 0 and 1 are generated with a random number generator. If the random number is smaller than the above calculated mortality response, this is considered as one death. If the random number is greater than the above calculated mortality response, this is considered one survivor. As many random numbers between 0 and 1 were generated as the number of rats supposed to be in a trial. In this way the mortality response in a group of rats can be simply simulated for a specific set of independent variables.

This simulation was carried out for all 40 combinations of independent variables with a specific number of rats between one and ten. A probit equation such as eqn. (1) was derived based on all combinations of independent variables and the corresponding simulated mortality responses. Then the LC-50 at 10, 35, and 60 minutes was calculated for male and female rats. This was repeated 500 times for each number of animals in a trial between one and ten. Finally the geometric mean and standard deviation of the 500 simulated LC-50's and the 95% confidence limits were calculated.

LC-50 according to OECD guidelines

The mortality data of the NH_3 study for each time period were treated according to the OECD guidelines. Using the mortality data for 10 and 60 minutes we derived the LC-50 values according to the maximum likelihood method [2]. Confidence limits were calculated on the basis of Fieller's [3] theorem without correction for heterogeneity. A second way to determine the confidence limits was to repeat the Monte Carlo simulations based on the probit equations obtained either from the 10-minute or 60-minute mortality data, i.e.

$$Y_{10} = -27.2 + 9.2 \ln C + 2.8 S \quad (3)$$

$$Y_{60} = -10.7 + 5.9 \ln C + 2.4 S \quad (4)$$

Hardware and software

All calculations were performed on an IBM-AT personal computer with specifically designed software. Listings and programs will be available on request.

Results and discussion

Mortality data of NH_3 exposure by inhalation were taken from Appelman's study [1] in which five male and five female rats were each exposed for a given time to a given concentration. The resulting data set was considered to be representative for the "true" mortality response of rats to exposure of NH_3. The Monte Carlo simulation produced 500 probit equations for each number of animals per sex per trial. On the basis of these probit equations the 500 LC-50 values at 10, 35, and 60 minutes were calculated separately for male and female rats. The graphs of the mean and the 5% and 95% limits based on these 500 LC-50 estimates plotted against the number of animals per sex per trial, are presented as Figs. 1, 2, and 3. Similarly, the graph of the variation coefficient is presented as Fig. 4.

Figures 1, 2, and 3 show that with the simulated study design (four concentrations, 20 exposure periods) a good estimate of the LC-50 over the entire range of exposure times can be obtained independent of the number of animals in each trial. As would be expected, we observed an increase in the 95% confidence level when decreasing the number of animals involved in each trial. In case of one animal the coefficient of variation (Fig. 4) is about 9%, in case of five animals about 4%, and in the case of ten animals about 3%. A coefficient of variation of 9% results in 95% confidence limits of about 80 and 120% of the mean, The inhalation toxicity of NH_3 shows a considerable sex dependency (male > female). For nearly all numbers of animals in one trial the 95% confidence limits of the male and female estimates are not overlapping. Only the simulations with 1 animal per sex per trial show a small overlap.

How do these results of LC-50 estimates compare with LC-50 values as obtained according to OECD guidelines? From the probit equations (3) and (4) the following LC-50 and 95% confidence limits [3] were calculated for 10 and 60 minutes:

10-minute LC-50, males: 23.9 (22.0, 25.8) g/m^3,
10-minute LC-50, females: 32.3 (28.7, 37.5) g/m^3,
60-minute LC-50, males: 9.2 (4.5, 10.3) g/m^3,
60-minute LC-50, females: 13.8 (12.4, 27.0) g/m^3.

The estimated LC-50 values are in good agreement with the results of the Monte Carlo simulation all of them falling well within the 95% confidence limits. The 95% confidence limits for the 10-minute exposure based on Fieller's theorem were of the same range as obtained from the Monte Carlo simulations. However, for the 60-minute exposure the lower confidence limit of the males and the upper limit of the females deviated more than a factor two from the LC-

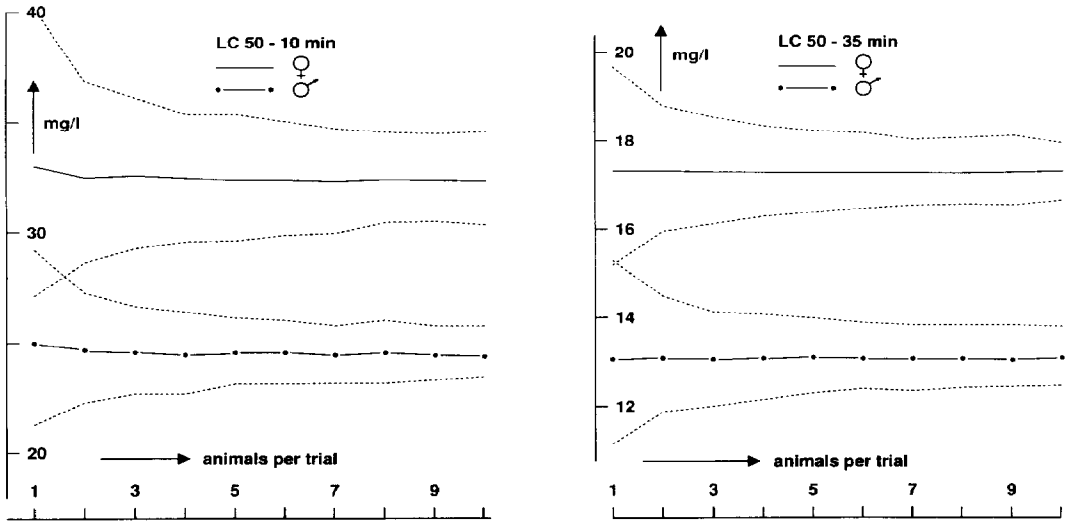

Fig. 1. Graph of the mean of the LC-50's of 10 min against the number of animals per trial. Dashed lines give 95% confidence limits.

Fig. 2. Graph of the mean of the LC-50's of 35 min against the number of animals per trial. Dashed lines give the 95% confidence limits.

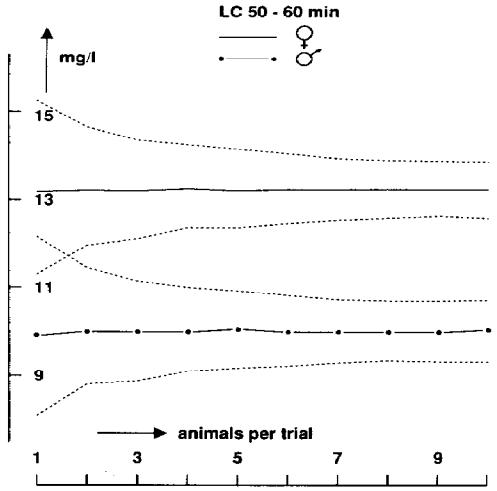

Fig. 3. Graph of the mean of the LC-50's of 60 min against the number of animals per trial. Dashed lines give the 95% confidence limits.

50. In practice, when determining one LC-50 value at least one additional exposure would be necessary.

We also estimated the 95% confidence limits by considering the probit equa-

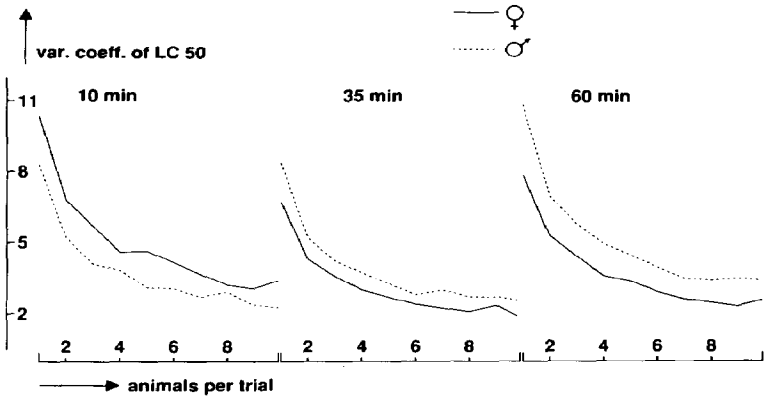

Fig. 4. graph of the coefficient of variation of the LC-50 at 10, 35, and 60 minutes against the number of animals per trial.

tions (3) and (4) as truly representative and applying the Monte Carlo technique described above, now to the probit functions obtained for exposure for either 10 or 60 minutes with five animals per sex per trial. The results were as follows:

10-minute LC-50, males: 24.0 (22.3, 25.6) g/m^3,
10-minute LC-50, females: 32.8 (29.3, 36.9) g/m^3,
60-minute LC-50, males: 8.9 (5.6, 10.2) g/m^3,
60-minute LC-50, females: 14.4 (12.5, 20.7) g/m^3.

The obtained 95% confidence limits are in good agreement with the 95% confidence limits obtained in the full study with one animal per sex per trial with the same problem in the confidence limits in the values for 60 minutes as when estimating the 95% confidence limits with Fieller's theorem. The scheme applied to the Monte Carlo simulation of the full study is basically a set up for the determination of LT-50 values. Applying one concentration of agent and removing one or two animals per sex after five concentrations require the same amount of work and the same number of animals as the determination of one LC-50 value according to the OECD guidelines. A well constructed nose-only set-up which allows intermediate removal of animals without affecting the concentration to which the other animals in the study are exposed and without polluting the workplace would be optimal. In such an experiment concentration–time–mortality relationships can be determined with confidence limits of the LC-50 which are of the same order as in the conventional LC-50 determination. General acceptance of the proposed scheme would thus provide accurate and highly valuable information for industrial and environmental risk analysis.

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