Population toxicokinetics of ethylene: Calibration and preceding investigations

B. Quinke[1], S. Selinski[2], K. Golka[1], and M. Blaszkewicz[1]

[1] Institut für Arbeitsphysiologie an der Universität Dortmund, Ardeystr. 67, 44139 Dortmund,
[2] Fachbereich Statistik, Vogelpothsweg 87, Universität Dortmund, 44221 Dortmund,

Abstract

A basic step in the risk assessment of potential carcinogens is the determination of toxicokinetic parameters. The present approach is part of a strategy to determine the processes of uptake, elimination, and metabolism of the gas ethylene, an important industrial chemical, which is classified in category 3 of carcinogenic substances in the German list of MAK- and BAT-values.

This paper deals with the calibration, which is indispensable to determine the decline of atmospheric concentrations of ethylene within a broad range of initial concentrations applied in an inhalation experiment.

Key Words: Ethylene, ethylene oxide, calibration, population toxicokinetics, risk assessment

Introduction

The determination of toxicokinetic parameters is a basic part in the risk assessment of potential harmful chemicals. The partition of the xenobiotic in the body of the animal is a first step of the biochemical pathway of the formation of DNA adducts which might lead to the development of cancer.

The research depends on experiments with animals *in vivo* and *in vitro*, so that a critical step is the extrapolation from animal experiment to the human organism.

The aim of this investigation is to determine the kinetic parameters of uptake, elimination, and metabolism of the gas ethylene, an important industrial bulk chemical which is endogenously produced, too. We are interested in the intra- and interindividual differences as well as in differences in the kinetic data due to different initial concentrations. As doses used in our experiments ranged from 20 to 500 ppm ethylene our calibration procedure has to take into account that the aim is to compare observations due to these different initial concentrations. This is not necessarily the case for 1- and 2-point calibrations which are routinely provided by the integrator used. Thus, we decided to run a 3-point calibration every day before starting the experiment and at the end, so that we were able to compare results of different doses more precisely.

Project

The aim of this investigation on Sprague-Dawley rats is to determine the population mean kinetic parameters of uptake, elimination, and metabolism of ethylene and to quantify the variability due to interindividual and interoccasion differences.

Ethylene is an important industrial bulk chemical, which is present in the environment. Ethylene is also a plant hormone involved in the process of ripening and, moreover, it is formed endogenously at low concentrations in mammalian organisms. Preceding studies reveal, that ethylene is metabolised to ethylene oxide, which is directly alkylating different macromolecules. The major DNA adduct of ethylene oxide is 7-(2-

hydroxyethyl)-guanine and typical targets in proteins are the N-terminal aminoacids cysteine, histidine, and valine (Bolt et al., 1988).
Ethylene oxide is carcinogenic in animal studies; the carcinogenicity in humans is still discussed controversially (Bolt, 1998).
Former inhalation studies on animals indicate, that the metabolism of ethylene can be well approximated by first order kinetics at concentrations below 800 ppm. At higher concentrations the metabolism becomes more and more saturated (Bolt & Filser, 1987).

Materials and Methods

We applied a gas chromatograph ”Dany AS” connected to an integrator ”Shimadzu C-R6A Chromatopac”. The integrator was able to reproduce a standard peak of 1 mV height and 20 seconds in half width with a C.V. value of 0.1% or better on a day.
We used desiccators of a volume of 6.4 l.
The applied doses of ethylene corresponded to the following gas volumes:

20 ppm	0.128 ml
50 ppm	0.32 ml
100 ppm	0.64 ml
200 ppm	1.28 ml
500 ppm	3.2 ml

In pilot tests the use of one single injection per concentration turned out to yield more precise concentrations than adding 2 different volumes, e. g. using a 5 ml syringe to obtain 3.2 ml ethylene instead of using a 2 ml syringe and adding 2 ml and 1.2 ml. We decided to apply gastight Hamilton® syringes of the following volumes:

1 ml (resp. 100 µl)	for concentrations of 100 ppm (0.64 ml) or less
2 ml	for concentrations of 200 ppm (1.28 ml)
5 ml	for concentrations of 500 ppm (3.2 ml)

We used a gas collecting tube and a valved cannula system, since this method yielded much more precise concentrations than taking the respective volumes directly from the gas cylinder by a valved cannula system.

To obtain a pure ethylene atmosphere in the gas collecting tube, its volume was replaced every day before starting the experiment. We used 20 male Sprague-Dawley rats (Harlan Winkelmann, Borchen) of an average weight of 300 g.

Experimental design

Two different experiments were performed, each of which with 10 Sprague-Dawley rats. Both groups of experiments were carried out using the "closed chamber technique" as reviewed by Filser (1992), which allows investigations of kinetics of volatile chemicals *in vivo*. This technique is based on a closed inhalation chamber, a modified desiccator.

In each inhalation chamber one rat is exposed to the gas or vapour of interest. The exhaled CO_2 is absorbed by soda lime, and its volume is replaced by pure oxygen (cf. Fig. 1).

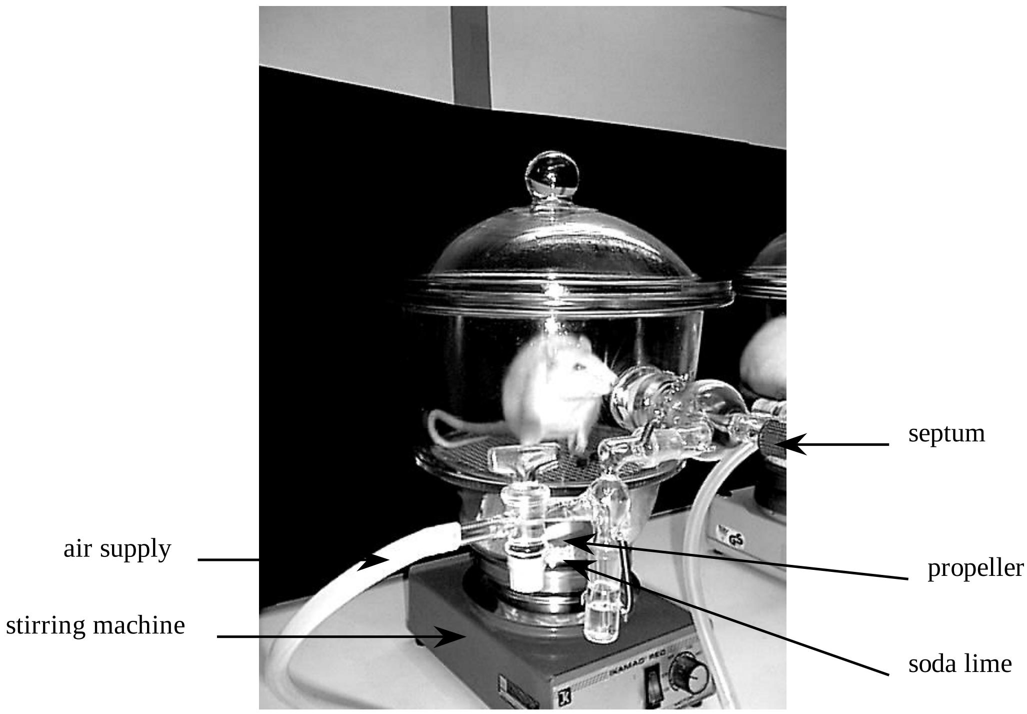

Figure 1: *Experimental design for investigating the kinetics of volatile compounds in vivo according to Filser.*

At the beginning of each experiment the calculated amount of ethylene gas for the applied concentration is injected into the chamber by a gastight syringe. In the course of time, the decline of the ethylene concentration within the chamber is monitored by gas chromatography.

To investigate inter- and intraindividual variability in kinetic data, in a first experiment five rats were separately exposed to a concentration of about 100 ppm ethylene for a time period of about 8 hours at five consecutive days. This experiment was repeated with another five rats. During the exposure the concentration of ethylene in each desiccator was measured every 25 minutes. Thus five inhalation experiments per animal were received under the same conditions (cf. Table 1).

Table 1: *Experimental design of the first group (group A1 and A2).*

	dose	1st week, group A1					2nd week, group A2				
day	(ppm)	rat No.					rat No.				
1	100	1	2	3	4	5	6	7	8	9	10
2	100	1	2	3	4	5	6	7	8	9	10
3	100	1	2	3	4	5	6	7	8	9	10
4	100	1	2	3	4	5	6	7	8	9	10
5	100	1	2	3	4	5	6	7	8	9	10

The experimental design of the second group (group B1 and B2) was similar to the first, except for the application of different initial concentrations in the desiccator. Observing another 10 rats, we obtain five concentration-time curves per animal at five different initial concentrations of 20 ppm, 50 ppm, 100 ppm, 200 ppm and 500 ppm ethylene (cf. Table 2).

__Table 2:__ *Experimental design of the second group (group B1 and B2).*

day	dose (ppm)	3^{rd} week, group B1 rat No.					4^{th} week, group B2 rat No.				
1	20	11	12	13	14	15	16	17	18	19	20
2	50	11	12	13	14	15	16	17	18	19	20
3	100	11	12	13	14	15	16	17	18	19	20
4	200	11	12	13	14	15	16	17	18	19	20
5	500	11	12	13	14	15	16	17	18	19	20

Five single animals were exposed at a time on five successive days. The doses were given each with a delay of 5 minutes. The gas chromatographic measurements were made in intervals of every 5 minutes, so that the samples of each inhalation chamber were taken every 25 minutes.

The complete investigation took 2 weeks for calibration and training and 4 weeks for the exposure experiment.

The applied ethylene doses were below the concentration of saturation of ethylene metabolism of about 800 ppm. Hence the data can be analysed approximating the real kinetic processes by first order kinetics using a two-compartment model (cf. Selinski & Urfer, 1998; Selinski et al, 1999; Selinski, 2000).

Preceding investigations

Before starting the inhalation experiments two weeks of training and calibration were necessary. During this time we also tried to detect and eliminate potential sources of errors as well as to collect prior information about the measurement error and about the precision of the reproducibility in generating the different ethylene concentrations in the exposure chambers.

In the course of the preceding investigations a daily 3-point-calibration turned out to be the adequate way to obtain a reliable calibration.

Preparing the inhalation experiments three main problems arised:

i. the appropriate choice and use of the materials to generate correct and reproducible ethylene concentrations,
ii. the constancy of the experimental conditions e.g. room temperature, and
iii. precise measurement for a time period of about 10 hours a day, 5 days a week and 4 weeks for the entire experiment while sampling every 5 minutes for about 8 hours, controlling, and recording the data.

During the preceding investigation we injected equal volumes into two to four desiccators and determined the ethylene concentrations in the inhalation chambers by gas chromatography. Moreover, we compared the measurements between desiccators of the same target concentration and determined the differences due to the way of application of the doses. We used the provided 1- and 2-point calibrations for the determination of the concentrations.

1- and 2-point calibration

The used integrator "Shimadzu C-R6A Chromatopac" offered 1- and 2-point calibrations and calculated the concentrations corresponding to the measured peak areas (cf. Chromatopac C-R6a Instruction Manual, for further details).

The 1-point calibration was performed by an automatic calculation of a linear calibration curve which passed the origin. We considered at least 4 calibration standards of the same concentration, 100 ppm, for instance, and at least 4 measurements per calibration standard.

The 2-point calibration was run alike but with two different concentrations, 50 and 100 ppm, for instance, and two calibration standards per concentration. The calibration curve did not necessarily pass the origin.

The conversion factors were stored in computer files and were used to convert the peak areas into ppm. Peak areas and calculated concentrations were registered in the printed chromatogram. Thus, we were able to compare the concentrations in the different desiccators.

The idea was to perform a calibration which we could use for the entire experiment. We planned to use for the different ranges of concentrations (e. g. 100 to 50, 50 to 20 ppm) the respective 1- or 2-point calibration of the preceding investigation.

In the course of the preliminary training it was obvious that the measurements contained variations from day to day which resulted from differences in room temperature and air condition. Consequently, we considered a manual 3-point calibration seriously as the method of choice.

3-point calibration

On each day before starting the experiment a 3-point calibration was performed and repeated at the end of the experiment. Every day we prepared two calibration standards per concentration and chose the one, which showed the least deviations to the calibration standards of the two other concentrations and to the standards of the preceding days.

The three concentrations for calibration were chosen considering the following aspects:

i. the administered dose,
ii. at least one concentration used as calibration standard on the preceding day, and
iii. at least one concentration below the actual dose, due to the supposed decrease of the concentration of ethylene in the inhalation chamber.

Therefore the following concentrations were used for calibration (Table 3).

Table 3: *Ethylene concentrations used in the exposure experiments and the 3-point calibration.*

exposure (in ppm)	3-point calibration concentrations (in ppm)		
20	10	20	100
50	20	50	75
100	50	75	100
200	100	150	200
500	100	200	500

Calculation of the conversion factors

In order to convert the peak areas registered by the integrator into ppm, we fitted a linear calibration curve, which passed the origin.

We included the measurements of 3 calibration standards of different concentrations before and after the experiments.

The curve was fitted using the method of least squares. So we obtained the gradient by minimising

$$Q(\beta_i) = \sum_{j=1}^{3} \sum_{k=1}^{K_j} \left(y_{ijk} - \hat{y}_{ijk}\right)^2 \text{, where} \qquad (1)$$

$$\hat{y}_{ijk} = \beta_i \cdot x_{ij} \text{, with} \qquad (2)$$

x_{ij} denoting the *j*th respective prescribed concentration on the *i*th day, $i = 1, ..., 20$, for example $x_{11,1} = 50$, $x_{11,2} = 75$, and $x_{11,3} = 100$. The registered peak area of the *k*th sample of the *j*th concentration is denoted by y_{ijk}, where K_j is the number of samples taken from the respective desiccator. Hence the conversion factor $\alpha_i = 1/\hat{\beta}_i$ is given by

$$\hat{\beta}_i = \frac{\sum_{j=1}^{3} \sum_{k=1}^{K_j} y_{ijk} \cdot x_{ij}}{\sum_{j=1}^{3} K_j \cdot x_{ij}^2} . \qquad (3)$$

The coefficient of determining R^2, as given by (4) was calculated to check the goodness of fit of the calibration curve.

$$R^2 = 1 - SSE / SST \text{ , where} \tag{4}$$

$$SSE = \sum_{j=1}^{3} \sum_{k=1}^{K_j} \left(y_{ijk} - \hat{y}_{ijk} \right)^2 \text{ and}$$

$$SST = \sum_{j=1}^{3} \sum_{k=1}^{K_j} \left(y_{ijk} - \bar{y}_{i..} \right)^2 \text{ , with } \bar{y}_{i..} = \frac{\sum_{j=1}^{3} \sum_{k=1}^{K_j} y_{ijk}}{3 \cdot K_j} .$$

Results

Considering the last five days of the preceding investigation, we found that the C.V. for each desiccator ranged from 0.66% to 3.27% for all concentrations (c.f. Appendix, Table 5). Samples were taken 3 to 26 times. There was no tendency towards higher values for higher or lower concentrations. Furthermore, the C.V. did not decline in the course of these days.

The concentrations were generated with the following C.V.:

Table 4: *C.V. of the mean peak areas per desiccator of the last five training days.*

concentration (in ppm)	C.V. (in % of total mean)
20	11.8
50	6.5
75	5.1
100	5.4
200	6.2
500	4.1

The daily 3-point calibration on the experimental days yielded gradients $\hat{\beta}_i$ ranged from 1282.3 to 1935.3. So, in general the conversion factor $\alpha_i = 1/\hat{\beta}_i$ was about 0.0007. There were five exceptions for 500 ppm (0.0005 on both days), 200 ppm (0.0006 on one day), 50 and 100 ppm (0.0008 on one day each).

For the maximum dose of 500 ppm we obtained a factor of about 0.0005. The other calibrations did not show any relationship between α_i and the applied concentrations. Thus, we obtained a linear calibration curve for concentrations of about 200 ppm.

R^2 ranged from 0.906 to 0.998, so that we received a reliable calibration curve on every experimental day.

Conclusions

The proposed method of a daily 3-point calibration was able to detect and eliminate possible sources of errors furthermore paying regard to potential variability of air conditioning and room temperature. Hence it was possible to calculate a day-dependent factor, to convert the peak areas into concentrations (ppm) and to compare the results on different days and with different administered doses. As the fit of all calibration curves was good and the conversion factors were almost all the same, we obtained a reliable calibration. Moreover, we gained useful prior information about the precision with which we were able to generate the required initial concentrations and about the measurement error in the course of the preceding investigation. This information can be included in the analysis of the data.

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References

Bolt, H. M. (1998). The carcinogenic risk of ethene (ethylene). *Toxicol. Pathol. 26*, 454-456.

Bolt, H. M., Filser, J. G. (1987). Kinetics and disposition in toxicology. Example: Carcinogenic risk estimate for ethylene. *Arch. Toxicol. 60*, 73-76.

Bolt, H. M., Peter, H., Föst, U. (1988). Analysis of macromolecular ethylene oxide adducts. *Int. Arch. Occup. Environ. Health 60*, 141-144.

Chromatopac C-R6A Instruction Manual. Shimadzu Corporation, Kyoto, Japan.

Filser, J. G. (1992). The closed chamber technique - uptake, endogenous production, excretion, steady- state kinetics and rates of metabolism of gases and vapours. *Arch. Toxicol. 66*, 1- 10.

Selinski, S., Golka, K., Bolt, H. M., Urfer, W. (1999). Estimation of toxicokinetic parameters in population models for inhalation studies with ethylene. *Environmetrics* (subm.).

Selinski, S., Urfer, W. (1998). Interindividual and interoccasion variability of toxicokinetic parameters in population models. *Technical Report 38/1998*, University of Dortmund.

Selinski, S. (2000). Estimation of toxicokinetic population parameters in a four-stage hierarchical model. *Technical Report 1/2000*, University of Dortmund.

Appendix

Table 5: *C.V. , concentration and number of samples (n) of each desiccator on the last five days of the preceding training.*

day	concentration (ppm)	C. V.	n
12 Aug.	50	0.70	7
	50	1.22	7
	50	1.50	7
	50	1.01	7
	75	1.78	8
	75	0.75	8
	75	2.34	8
	100	2.20	7
	100	2.52	8
	100	2.12	8
	100	0.86	8
13 Aug.	20	2.67	4
	20	2.21	4
	50	2.07	8
	50	1.99	8
	75	2.61	3
	100	1.84	8
	100	3.27	6
	100	1.83	8
	100	1.81	8
	200	1.45	4
	200	1.01	4
	500	1.53	4
	500	1.25	4
17 Aug.	100	2.57	26
	200	0.68	4
	200	2.86	5
	200	2.34	5
	500	2.94	5
	500	2.96	5
19 Aug.	20	2.85	6
	20	2.13	6
	50	1.59	6
	50	1.23	6
	100	1.96	10
20 Aug.	20	2.36	6
	20	2.03	4
	50	1.08	5
	50	0.66	4