We assume a logistic regression model as an approximation of the time-response curve (Shiraishi et al., 2005, Shiraishi et al., 2006). The estimation method using a logistic regression model is essentially the same as the one proposed by Omori et al. (1998). Omori proposed, in his article, a method for estimating ED50 (50% effective dose) in the validation study to evaluate the feasibility of cytotoxicity assay as an alternative method for the Draize eye irritation test. According to Omori's method, as a suitable model for the dose-response curve for a test substance, the logistic regression model for absorbance instead of cell viability is defined by

$$y_{ij} = \frac{\beta_3}{1 + \exp(\beta_1 + \beta_2 \log_{10} d_i)} + \beta_0 + e_{ij}, \tag{2}$$

where y_{ij} is the j-th measurement of a test substance at dose d_i, β_0 is the effect of a blank, β_1 is a location parameter, β_2 is a scale parameter, β_3 is the effect of a negative control and e_{ij} is a random error term peculiar to y_{ij}. In addition to the model for a test substance, the models for a negative control and a blank are, respectively, assumed as

$$y_{Cj} = \beta_3 + \beta_0 + e_{cj}, \tag{3}$$

$$y_{Bj} = \beta_0 + e_{bj}, \tag{4}$$

where, y_{Cj} and y_{Bj} are j-th measurements of a negative control and a blank, respectively, e_{Cj} and e_{Bj} are random error terms peculiar to y_{Cj} and y_{Bj}, respectively.

We modify model (2) to estimate ET50 from the time-response curve since Omori's method assumes the dose-response curve. First, we substitute $\log_2 t_i$ for $\log_{10} d_i$, on the

grounds that the time point for measurement, t_i, is generally allocated at a common ratio of 2. Next, we apply a logistic regression model for a test substance to the variable $y'_{ij} = y_{ij} - \bar{y}_{B\cdot}$, since the variation in the absorbance of a blank would be negligible. Similarly, we apply a model for a negative control to the variable $y'_{Cj} = y_{Cj} - \bar{y}_{B\cdot}$. The models for a test substance and a negative control are redefined by

$$\begin{cases} y'_{ij} &= \dfrac{\beta_3}{1+\exp(\beta_1+\beta_2\log_2 t_i)} + e_{ij}, \\ y'_{Cj} &= \beta_3 + e_{Cj}, \end{cases} \tag{5}$$

Although an underestimate of the precision may become a problem by the above formulation, it is possible to estimate the parameters stably by decreasing the number of parameter to be estimated. Since the absorbance corresponding to ET50 is $\beta_3/2$, the $\log_2$ET50 $= \log_2 \theta_{logit}$ is defined by

$$\log_2 \theta_{logit} = -\frac{\beta_1}{\beta_2}, \tag{6}$$

which yields

$$\theta_{logit} = 2^{-\beta_1/\beta_2}. \tag{7}$$

There are various sources of measurement error that are thought to be additive and continuous in nature. We use the non-linear least squares method to estimate parameters, β_1, β_2 and β_3, in which Q_{logit} defined as follows is minimized:

$$Q_{logit} = \sum_i \sum_j \left(y'_{ij} - \frac{\beta_3}{1+\exp(\beta_1+\beta_2\log_2 t_i)} \right)^2 + \sum_j \left(y'_{Cj} - \beta_3 \right)^2. \tag{8}$$

ET50 estimate, $\hat{\theta}_{logit}$, is obtained by substituting acquired estimates, $\hat{\beta}_1$ and $\hat{\beta}_2$, into the definition of ET50 given by (7).

We evaluate the precision of $\hat{\theta}_{logit}$ using a confidence interval based on the delta method. Using a first order Taylor series approximation, the $\log_2 \hat{\theta}_{logit}$ can approximately be expanded as

$$\log_2 \hat{\theta}_{logit} = -\frac{\hat{\beta}_1}{\hat{\beta}_2} \approx -\frac{\beta_1}{\beta_2} - \frac{(\hat{\beta}_1 - \beta_1)}{\beta_2} + \frac{\beta_1(\hat{\beta}_2 - \beta_2)}{\beta_2^2}, \tag{9}$$

which yields an approximate variance of $\log_2 \hat{\theta}_{logit}$ as

$$\sigma^2(\log_2 \hat{\theta}_{logit}) \approx \frac{\mathrm{Var}(\hat{\beta}_1)}{\beta_2^2} + \frac{\beta_1^2 \mathrm{Var}(\hat{\beta}_2)}{\beta_2^4} - \frac{2\beta_1 \mathrm{Cov}(\hat{\beta}_1, \hat{\beta}_2)}{\beta_2^3}. \tag{10}$$

By obtaining estimates of the variance and covariance of $\hat{\beta}_1$ and $\hat{\beta}_2$ through linear approximation in the non-linear least squares method, an estimate of $\sigma^2(\log_2 \hat{\theta}_{logit})$ can be calculated by substituting $\hat{\beta}_1$ and $\hat{\beta}_2$ into (10) (Cox, 1990). We can obtain an approximate $1-\alpha$ confidence interval for $\log_2 \theta_{logit}$ as

$$\log_2 \hat{\theta}_{logit} \pm z_{1-\alpha/2}\ \hat{\sigma}(\log_2 \hat{\theta}_{logit}), \tag{11}$$

which yields the confidence interval for θ_{logit} as

$$\exp(\log_2 \hat{\theta}_{logit} \pm z_{1-\alpha/2}\ \hat{\sigma}(\log_2 \hat{\theta}_{logit})), \tag{12}$$

where $z_{1-\alpha/2}$ is the $(1-\alpha/2)$ quantile of the standard normal distribution.

A log-time regression method and a linear regression method

The models for a test substance and a negative control using a log-time regression method and a linear regression method are, respectively, described as

$$\begin{cases} y'_{ij} &= \beta_4 + \beta_5 \log_2 t_i + e_{ij}, \\ y'_{Cj} &= \beta_3 + e_{Cj}, \end{cases} \tag{13}$$

$$\begin{cases} y'_{ij} &= \beta_6 + \beta_7 t_i + e_{ij}, \\ y'_{Cj} &= \beta_3 + e_{Cj}. \end{cases} \tag{14}$$

Since the absorbance corresponding to ET50 is $\beta_3/2$, the $\log_2$ ET50 obtained from a log-time regression method, $\log_2 \theta_{log}$, and ET50 obtained from each method, θ_{log} and θ_{lin}, are defined by

$$\log_2 \theta_{log} = \frac{1}{\beta_5}\left(\frac{\beta_3}{2} - \beta_4\right), \tag{15}$$

$$\theta_{log} = 2^{(\beta_3/2-\beta_4)/\beta_5}, \tag{16}$$

$$\theta_{lin} = \frac{1}{\beta_7}\left(\frac{\beta_3}{2} - \beta_6\right). \tag{17}$$

We use the ordinary least squares method to estimate parameters, β_3, β_4, β_5, β_6 and β_7, in which Q_{log} and Q_{lin} defined as follows are minimized:

$$Q_{log} = \sum_i \sum_j \left(y'_{ij} - (\beta_4 + \beta_5 \log_2 t_i)\right)^2 + \sum_j \left(y'_{Cj} - \beta_3\right)^2, \tag{18}$$

$$Q_{lin} = \sum_i \sum_j \left(y'_{ij} - (\beta_6 + \beta_7 t_i)\right)^2 + \sum_j \left(y'_{Cj} - \beta_3\right)^2. \tag{19}$$

ET50 estimate are obtained by substituting acquired estimates, $\hat{\beta}_3$, $\hat{\beta}_4$, $\hat{\beta}_5$, $\hat{\beta}_6$ and $\hat{\beta}_7$, into the definition of ET50 given by (16) and (17), respectively. Using a first order Taylor series approximation, the $\log_2 \hat{\theta}_{log}$ and $\hat{\theta}_{lin}$ can approximately be expanded as

$$\begin{aligned} \log_2 \hat{\theta}_{log} &= \frac{1}{\hat{\beta}_5}\left(\frac{\hat{\beta}_3}{2} - \hat{\beta}_4\right) \\ &\approx \frac{1}{\beta_5}\left(\frac{\beta_3}{2} - \beta_4\right) + \frac{(\hat{\beta}_3 - \beta_3)}{2\beta_5} - \frac{(\hat{\beta}_4 - \beta_4)}{\beta_5} - \frac{1}{\beta_5^2}\left(\frac{\beta_3}{2} - \beta_4\right)(\hat{\beta}_5 - \beta_5), \\ \hat{\theta}_{lin} &= \frac{1}{\hat{\beta}_7}\left(\frac{\hat{\beta}_3}{2} - \hat{\beta}_6\right) \end{aligned} \tag{20}$$

$$\approx \frac{1}{\beta_7}\left(\frac{\beta_3}{2}-\beta_6\right)+\frac{(\hat{\beta}_3-\beta_3)}{2\beta_7}-\frac{(\hat{\beta}_6-\beta_6)}{\beta_7}-\frac{1}{\beta_7^2}\left(\frac{\beta_3}{2}-\beta_6\right)(\hat{\beta}_7-\beta_7). \quad (21)$$

Then approximate variances of $\log_2\hat{\theta}_{log}$ and $\hat{\theta}_{lin}$ are given by

$$\begin{aligned}\sigma^2(\log_2\hat{\theta}_{log}) &\approx \frac{\mathrm{Var}(\hat{\beta}_3)}{4\beta_5^2}+\frac{\mathrm{Var}(\hat{\beta}_4)}{\beta_5^2}+\frac{\mathrm{Var}(\hat{\beta}_5)}{\beta_5^4}\left(\frac{\beta_3}{2}-\beta_4\right)^2 \\ &\quad +\frac{2\mathrm{Cov}(\hat{\beta}_4,\hat{\beta}_5)}{\beta_5^3}\left(\frac{\beta_3}{2}-\beta_4\right), \quad (22)\\ \sigma^2(\hat{\theta}_{lin}) &\approx \frac{\mathrm{Var}(\hat{\beta}_3)}{4\beta_7^2}+\frac{\mathrm{Var}(\hat{\beta}_6)}{\beta_7^2}+\frac{\mathrm{Var}(\hat{\beta}_7)}{\beta_7^4}\left(\frac{\beta_3}{2}-\beta_6\right)^2 \\ &\quad +\frac{2\mathrm{Cov}(\hat{\beta}_6,\hat{\beta}_7)}{\beta_7^3}\left(\frac{\beta_3}{2}-\beta_6\right). \quad (23)\end{aligned}$$

Estimates of $\sigma^2(\log_2\hat{\theta}_{log})$ and $\sigma^2(\hat{\theta}_{lin})$ can be calculated by substituting $\hat{\beta}_3$, $\hat{\beta}_4$, $\hat{\beta}_5$, $\hat{\beta}_6$ and $\hat{\beta}_7$, into (22) and (23). We can obtain an approximate $1-\alpha$ confidence interval for $\log_2\theta_{log}$ as

$$\log_2\hat{\theta}_{log}\pm z_{1-\alpha/2}\;\hat{\sigma}(\log_2\hat{\theta}_{log}), \quad (24)$$

which yields the confidence interval for θ_{log} as

$$\exp(\log_2\hat{\theta}_{log}\pm z_{1-\alpha/2}\;\hat{\sigma}(\log_2\hat{\theta}_{log})). \quad (25)$$

Similarly, we can obtain an approximate $1-\alpha$ confidence interval for θ_{lin} as

$$\hat{\theta}_{lin}\pm z_{1-\alpha/2}\;\hat{\sigma}(\hat{\theta}_{lin}). \quad (26)$$

A two-stage method

Parameter estimates and/or variance covariance matrices occasionally cannot be obtained from the logistic regression method due to the small sample sizes. We consider a two-

stage method in which the log-time regression method is alternatively applied if the logistic regression method cannot construct a confidence interval for ET50.

Design of the simulation study

We evaluate the performance of each estimation method through a Monte-Carlo simulation involving the following steps under the similar conditions to the validation study for TESTSKIN^{TM} (2002) and Vitrolife-Skin^{TM} (2004).

Step1. Specify true ET50 value between 4 and 18 hours assuming a mild test substance. The time point for measurement is allocated as $(t_1, t_2, t_3, t_4) = (2, 4, 8, 16)$ in 4-point design and is allocated as $(t_1, t_2, t_3, t_4, t_5) = (2, 4, 8, 16, 24)$ in 5-point design.

Step2. Generate virtual data for a test substance from the logistic curve on the time-response defined by

$$y'_{ij} = \frac{\beta_3}{1 + \exp(\beta_1 + \beta_2 \log_2 t_i)} + e_{ij}, \tag{27}$$

where $\beta_2 = 2.0$, $\beta_3 = 1.0$ and e_{ij} is mutually distributed as a normal distribution $N(0,\ 0.1^2)$. Since the ET50 value, θ_{logit}, is a function of β_1 and β_2, β_1 is determined from β_2 and θ_{logit}. Figure 1 shows the assumed time-response curves of model (27).

Step3. Generate virtual data for a negative control from $y'_{Cj} = \beta_3 + e_{Cj}$, where $\beta_3 = 1.0$ and e_{Cj} is mutually distributed as a normal distribution $N(0,\ 0.1^2)$.

Step4. Estimate ET50 and construct a confidence interval for ET50 using each estimation method.

Step5. Iterate 10,000 times Step 2 through Step 4, and calculate a proportion of estimable cases, a bias in estimates, and a coverage probability in which each interval contains the true ET50 values. A bias in estimates is defined as the median of the difference of the estimate of ET50 and the true ET50 value.

The reason we assume a mild test substance in Step 1 is that estimating ET50 for clearly strong or weak substances is not essential.

Results

We report the results of the Monte-Carlo simulation study in Tables 1 through 3 and present the corresponding scatter plots in Figures 2 through 4 to compare the performance of each estimation method. In these tables and figures, the left side shows the results in 4-point design and the right side shows those in 5-point design.

The characteristics of each estimation method are summarized below.

A logistic regression method

- The proportion of estimable cases decreases as low as 85% with the increase of true ET50 values in 4-point design, whereas it is almost 100% in 5-point design.
- The bias in estimates is negligible in both 4- and 5-point designs.

- The coverage probability is always below the nominal confidence level of 95% and as low as 88% in some cases.

According to the above mentioned results, the logistic regression method is appropriate in 5-point design, whereas another method should complementarily be used in addition to the logistic regression method in 4-point design.

Examples of simulated data that yielded feasible and unfeasible estimates, when the true ET50 value is 14 hours, are shown in Figure 5 together with the true and estimated time-response curves. This figure suggests that we tend to encounter difficulty in obtaining confidence intervals when we do not have measurements on time points around ET50.

A log-time regression method

- The proportion of estimable cases is almost 100% in both 4- and 5-point designs.
- ET50 estimates tend to be greater than the true ET50 value with the increase of true ET50 values in 4-point design.
- The bias in estimates is within 2 hours and, therefore, negligible in 5-point design.
- Although the coverage probability tends to be greater with the increase of true ET50 values in both 4- and 5-point designs, the discrepancy from the nominal confidence level of 95% is within $\pm 5\%$.

These results suggest caution in rarely adopting 4-point design because estimates tend to be great when the true ET50 value is great, although no remarkable defects appear in 5-point design.

A linear regression method

- The proportion of estimable cases is almost 100% in both 4- and 5-point designs.
- The estimates of ET50 tend to be great in both 4- and 5-point designs, when the true ET50 value is small.
- The coverage probability is seriously low since it is as low as 50% in 4-point design or 40% in 5-point design in the worst cases.

These results suggest that the linear regression method should not be adopted due to low coverage probabilities irrespective of design. The 5-point design is more disadvantageous than the 4-point design because the time points for obtaining measurements in 5-point design included $T_5 = 24$ hour in our simulation setting. Actually, the measurement at the 24 hour point leads to a smaller value of the gradient than the expected value.

Two-stage method

- The proportion of estimable cases is almost 100% in both 4- and 5-point designs.
- The estimates of ET50 are on average almost the same as the true ET50 value in both 4 and 5-point designs.
- Although the coverage probability tends to be greater with the increase of true ET50 values in both 4- and 5-point designs, the discrepancy from the nominal confidence level of 95% is within $\pm 5\%$.

These results indicate that the two-stage method is reasonable for obtaining a confidence interval for ET50, although it should be slightly adjusted so as to keep the coverage probability near the nominal confidence level.

Discussion

We recommend using the two-stage method for obtaining a confidence interval for ET50. However, further investigations are necessary to extend the conclusion to any case of the design and analysis of experiments using 3D skin models, since the adopted simulation conditions are adaptable only for the real validation studies of TESTSKIN and Vitrolife-Skin. When the use of refined statistical software such as SAS or R is difficult, we recommend using the log-time regression method with 5-point design although the biased estimates within 2 hours are occasionally obtained.

The condition where the proportion of estimable cases in the application of logistic regression method in 4-point design realizes values below 100% depends on the number of time points, the positioning of time points, and the scale of measurement errors (Sozu et al., 2005, Sozu et al., 2006). Properly setting these conditions considering the convenience of workers is important and further studies are necessary to address this issue.

The results of this research would promote the use of 3D skin models through the achievement of adequate and quantitative evaluations of skin irritation of test substances.

Acknowledgment

The authors would like to thank the editor and two anonymous referees for helpful comments that greatly improved this article. This research was supported in part by a grant from the Japanese Society of Alternative to Animal Experiments.

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Tables and Figures

Table 1: Results of proportion of estimable cases (%) for each estimation method.

	4-point design				5-point design			
	Estimation method				Estimation method			
True ET50 values	Logistic	Log-time	Linear	Two-stage	Logistic	Log-time	Linear	Two-stage
4	99.2	100.0	100.0	100.0	99.1	100.0	100.0	100.0
5	100.0	100.0	100.0	100.0	99.9	100.0	100.0	100.0
6	99.8	100.0	100.0	100.0	99.7	100.0	100.0	100.0
7	99.0	100.0	100.0	100.0	99.0	100.0	100.0	100.0
8	99.1	100.0	100.0	100.0	99.3	100.0	100.0	100.0
9	99.8	100.0	100.0	100.0	99.8	100.0	100.0	100.0
10	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
11	99.9	100.0	100.0	100.0	100.0	100.0	100.0	100.0
12	99.6	100.0	100.0	100.0	100.0	100.0	100.0	100.0
13	98.9	100.0	100.0	100.0	100.0	100.0	100.0	100.0
14	97.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
15	94.9	100.0	100.0	100.0	100.0	100.0	100.0	100.0
16	92.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
17	88.6	100.0	100.0	100.0	100.0	100.0	100.0	100.0
18	85.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Table 2: Results of bias in estimates for each estimation method.

	4-point design				5-point design			
	Estimation method				Estimation method			
True ET50		Log-		Two-		Log-		Two-
values	Logistic	time	Linear	stage	Logistic	time	Linear	stage
4	−0.01	0.32	1.41	0.00	−0.01	0.46	1.16	0.00
5	−0.02	0.20	1.88	−0.02	−0.01	0.51	2.55	−0.01
6	−0.01	−0.02	1.90	−0.01	0.00	0.34	3.06	0.00
7	−0.01	−0.27	1.71	−0.01	0.00	0.06	3.15	0.01
8	−0.02	−0.48	1.44	−0.02	−0.01	−0.26	3.03	−0.01
9	−0.05	−0.59	1.15	−0.05	−0.02	−0.60	2.78	−0.02
10	−0.06	−0.54	0.88	−0.06	−0.02	−0.90	2.47	−0.02
11	−0.05	−0.27	0.67	−0.05	−0.02	−1.15	2.15	−0.02
12	−0.04	0.31	0.48	−0.03	−0.02	−1.32	1.82	−0.02
13	−0.02	1.30	0.39	−0.01	−0.01	−1.39	1.50	−0.01
14	−0.04	2.84	0.39	0.01	−0.01	−1.31	1.22	−0.01
15	−0.08	5.18	0.49	0.02	−0.02	−1.07	0.97	−0.02
16	−0.15	8.67	0.71	0.02	−0.02	−0.57	0.78	−0.02
17	−0.22	13.80	1.05	0.08	−0.03	0.20	0.64	−0.03
18	−0.22	21.38	1.55	0.31	−0.05	1.32	0.55	−0.05

Table 3: Results of coverage probability for each estimation method.

	4-point design				5-point design			
	Estimation method				Estimation method			
True ET50 values	Logistic	Log-time	Linear	Two-stage	Logistic	Log-time	Linear	Two-stage
4	88.1	90.6	93.2	88.1	86.5	91.3	98.7	86.6
5	88.4	91.3	71.6	88.4	87.0	88.0	86.1	87.0
6	89.0	92.5	56.2	89.0	87.9	90.6	63.7	87.9
7	88.9	91.7	53.1	89.0	88.0	93.3	46.8	88.1
8	90.1	91.6	59.6	90.2	89.5	92.4	40.1	89.6
9	90.0	92.9	70.7	90.0	90.1	90.6	40.1	90.1
10	89.9	95.1	81.7	89.9	90.4	89.4	45.8	90.4
11	89.7	96.9	88.2	89.7	90.5	89.3	54.2	90.5
12	90.0	98.2	91.6	90.0	90.4	90.2	64.9	90.4
13	90.3	98.6	93.2	90.4	89.8	91.9	74.3	89.8
14	91.4	98.8	93.9	91.7	89.1	94.0	81.7	89.1
15	93.2	98.4	94.2	93.6	89.6	96.1	87.5	89.6
16	94.1	97.8	94.9	94.5	90.6	97.7	90.4	90.6
17	93.2	96.7	95.2	94.0	91.2	98.5	92.1	91.3
18	91.5	95.4	95.7	92.7	92.0	99.2	93.2	92.0

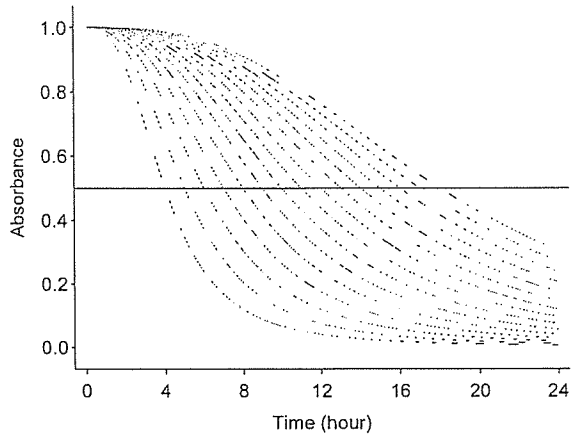
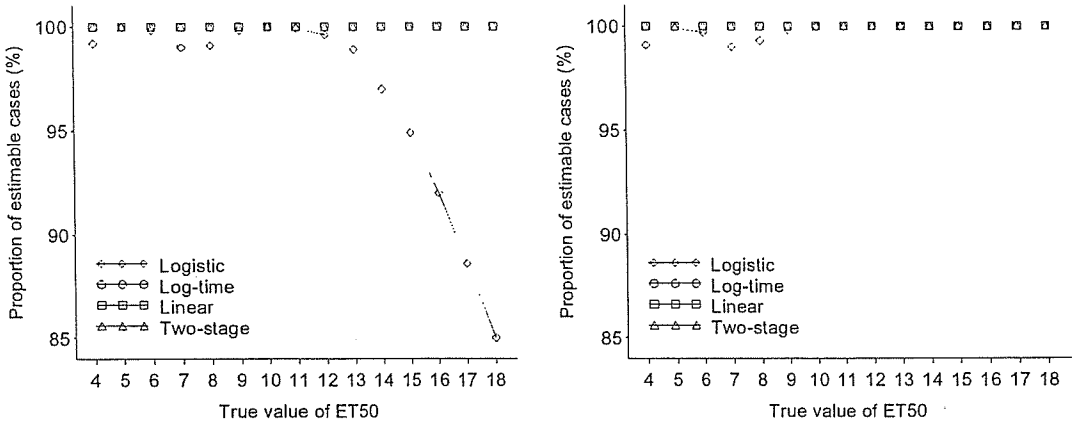
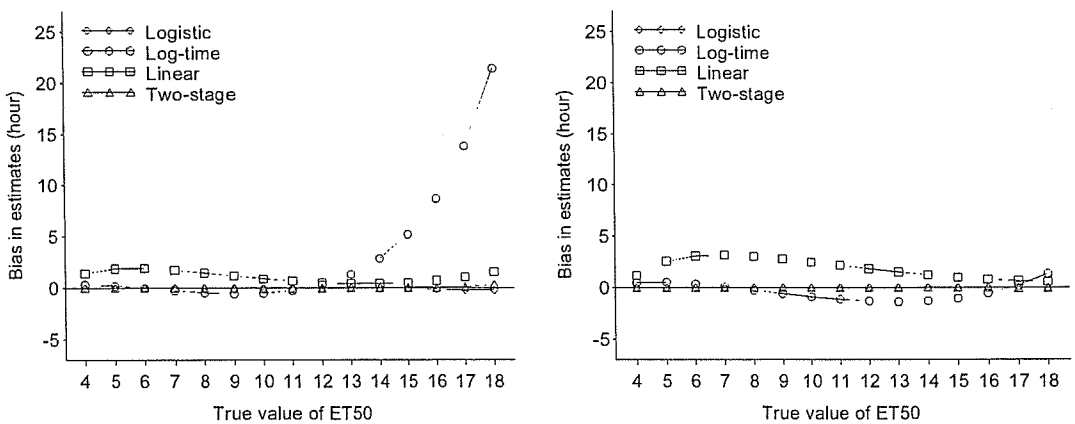


Fig. 1: Assumed time-response curves based on the logistic regression model.



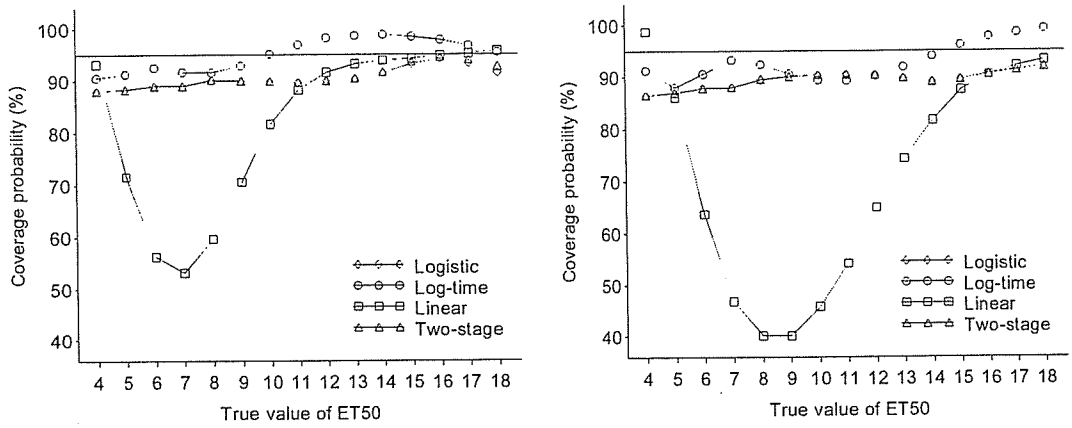
4-point design　　5-point design

Fig. 2: Results of proportion of estimable cases (%) for each estimation method.



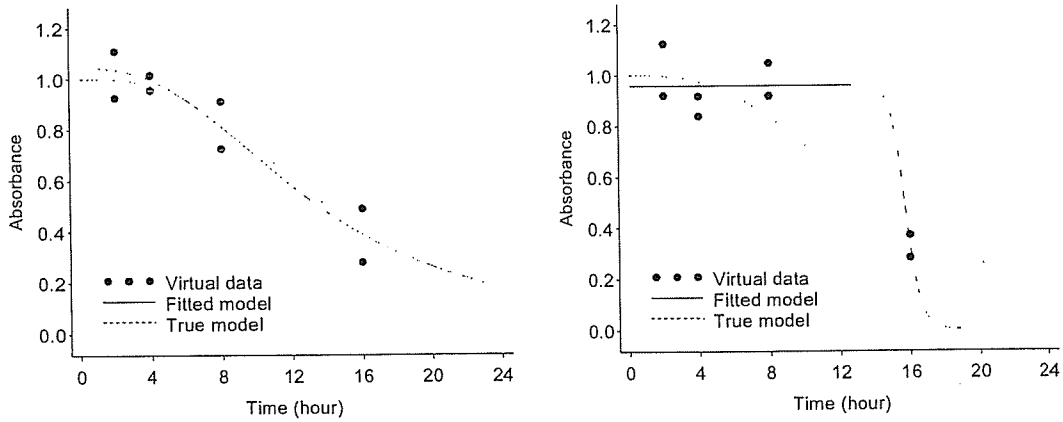
4-point design　　5-point design

Fig. 3: Results of bias in estimates for each estimation method.



4-point design　　5-point design

Fig. 4: Results of coverage probability for each estimation method.



Example of estimable case　　Example of not estimable case

Fig. 5: Examples of simulated data for a test substance with the true and estimated time response curves.

ORIGINAL ARTICLE

A Measure Evaluating Relevance of a Validation Study of Alternatives to Animal Testing

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Abstract

Sensitivity, specificity and accuracy are well known measures for evaluating the relevance of an inter-laboratory validation study for alternative tests. It is not generally discussed that the measures are dependent on two determining factors: a set of chemicals and the number of laboratories. Furthermore, some alternative tests such as these for the phototoxicity test have an "Equivocal" category for judging the toxicity of chemicals. These facts have made it difficult to interpret the value of the measures.

Therefore, in this paper we propose new measures to evaluate the alternatives, which depend on a set of chemicals rather than on both factors, and can treat data which have "Equivocal" category. We also propose their confidence intervals, which are measures of their precision.

Key words: *relevance, inter-laboratory validation study, sensitivity, specificity, accuracy, confidence interval*

Introduction

Recently, due to an increasing social concern for animal welfare, a lot of alternative animal tests have been proposed, and in order to examine their feasibility and practicality various inter-laboratory validation studies have been conducted (e.g. Ray et al., 1994; Spielmann et al., 1998). Generally, the primary purpose of the validation study is to evaluate both the relevance and reliability of a proposed alternative test from the results of experiments using the alternative test (Balls et al., 1999). Sensitivity, specificity and accuracy are measures to determine the effectiveness of the alternative test when both the alternative and the animal tests have a binary classification for judging toxicity of chemicals, as "Positive" and "Negative". These are well known measures which have been widely used to evaluate the relevance of the alternative test in many validation studies (e.g. Balls et al., 1990; Roy et al., 1994; Spielmann et al., 1998).

However, two points should be taken into consideration concerning the interpretation of the summarized data from validation studies. The first point is that the values in the 2 by 2 table, which summarizes data, depend not only on a selected set of chemicals in the study but also on the number of participant laboratories. The other point is that a category for "Equivocal" produced from some alternative tests such as these for the photoxicity test, which is neither a "Positive" nor "Negative" category, is often provided. For instance, the test guideline of the in vitro 3T3 NRU phototoxicity test states that *'a test substance with a PIF < 2 or an MPE < 0.1 predicts: "no phototoxicity". A PIF >2 and < 5 or an MPE > 0.1 and < 0.15 predicts: "probable phototoxicity" and a PIF > 5 or an MPE > 0.15 predicts: "phototoxicity".'* where the PIF and the MPE are measurements of phototoxicity for the test (OECD, 2004). In this case, since there was a range suggesting similar performance when several cut-off points were examined, the