

Research Article

## Development of a web-based tool for probit analysis to compute $LC_{50}/LD_{50}/GR_{50}$ for its use in toxicology studies

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### Abstract

The toxicity bioassays are essential to detect and estimate the potential toxicological effects of chemicals on an organism.  $LC_{50}/LD_{50}/GR_{50}$  is the estimation of dose/concentration necessary to kill 50 per cent of a population of the test species. Experimentally this is done by administering a chemical at different doses to a group of organism and then observing the resulting mortalities in a set of the time period. A web-based module for a statistical analysis tool to calculate and compare the median lethal dose has been developed in ASP scripting language based on Server-Client Architecture. The module produces the final probit line, dose-response curve,  $LC_{50}/LD_{50}$  with 95 % confidence interval. The Chi-square statistic has been obtained for testing the adequacy of fit. A user-friendly interface for entering/pasting the data and various parameters such as number of variables, number of observations etc. has also been provided. Additionally, a complete procedure to perform probit analysis has also been provided in the help file.

**Keywords:** Concentration,  $LC_{50}/LD_{50}$ , Probit Analysis, Server-Client Architecture

## INTRODUCTION

Probit analysis is widely used in research areas dealing with the binomial response. Its main application is observed in the field of toxicological studies, where it transforms the sigmoidal dose-response curve to a straight line that can further be easily analyzed by regression through either least squares or maximum likelihood. The methodology transforms the complex percentage affected vs. dose-response into a simple linear relation of probit vs. dose-response. The probit is then be translated into percentages. This method is appropriate because of the typical shape found in the dose-response curve and although approximate but allows quantification of the consequence due to exposure. Lei and Sun (2018) compared lethal dose ratios using probit regression with arbitrary slopes using bioassay data of chrysanthemum aphids, three populations of the pest bug "Wicked Witch of the West" and two popula-

tions, of Godfather larvae. Singh and Zahara (2017) studied  $LC_{50}$  assessment of cypermethrin in *Heteropneustes fossilis* using probit analysis.

The term "Probit" is an abbreviation of the term "probability unit" and it was the first developed and studied to treat data such as the percentage of pest killed by a pesticide. Bliss (1934) proposed the transformation of the percentage killed into "probit". He defined arbitrarily 0 for 0.0001 and 10 for 0.9999 and included a table to aid researchers to convert kill-percentage to probit, which then is plotted against the logarithm of dose. The table introduced by Bliss was further carried forward in the toxicological application by Finney (1952). Values tabulated by Bliss can be derived from probit by adding 5. Using Bliss's idea of probit analysis, Leslie *et al.* (1945) were able to discuss the distribution of body-weight of female rats where they reach maturity.

Probit analysis is mainly used to analyze data from

bioassays like the assessment of vitamins, hormones, toxicants and drugs by means of organism's response such as the proportion of insects killed by insecticide at different concentrations time intervals (Throne *et al.*, 1995). One such type of assay which has been found valuable in toxicological studies is dependent upon quantal or all-or-none response. Though quantitative measurement of response is almost always preferred when practicable, yet there are certain responses which do not permit graduation and can only be expressed as 'occurring' or 'not-occurring'. The most common example is mortality in many insecticidal studies where the interest lies in whether or not a test insect is dead, or whether the insect has reached a certain degree of inactivation. In fungicidal investigations, failure of a spore to germinate is a quantal response of similar importance. Keeping in view the importance of probit analysis in toxicological studies an attempt has been made to develop a web based probit analysis tool to compute  $LC_{50}/LD_{50}/GR_{50}$  to facilitate the researcher for their data analysis.

**MATERIALS AND METHODS**

A web-based module for probit analysis using scripting language Active Server Pages (ASP) module based on the techniques of Finney (1952) and Lei and Sun (2018) described below: Let n be the number of subjects treated with various doses 'i' and 'r' of them showed a response to each dose. Then the empirical proportion of responder  $p^*$  can be given by

$$p_i^* = \frac{r_i}{n_i} \tag{1}$$

Where, i = 1 to n and n is the number of doses. In cases of a control group in the experiment and response occurs in it with proportion C, then Abbott equation (2) is applied for correction of proportions of responders for each treatment dose

$$p_i = \frac{p_i^* - c}{1 - c} \tag{2}$$

The corrected proportion are then converted into a probit value ( $y_i$ ) using following equation

$$y_i = \Phi^{-1}(p_i) \tag{3}$$

where  $\Phi^{-1}$  is inverse of Normal distribution. A regression line between  $y_i$  and  $\log(\text{dose}(x_i))$  is worked out (4) for computing expected probits  $Y_i$  as given in equation (5)

$$y_i = \alpha_0 + \beta_0 x_i \tag{4}$$

$$Y_i = \alpha_0 + \beta_0 x_i \tag{5}$$

The expected response proportion  $P_i$  are computed and given in equation (6)

$$P_i = \Phi(Y_i) * (1 - C) + C_i \tag{6}$$

Where,  $\Phi(Y_i)$  is the cumulative probability of the standard normal distribution corresponding to ( $Y_i$ ), and C is the natural response proportion, if exist. The working probit ( $y_i$ ) is calculating as:

$$y_i = Y_i - \left(\frac{P_i}{Z_i}\right) - \left(\frac{P_i}{Z_i}\right) \tag{7}$$

where

$$Z_i = \frac{1}{\sqrt{2\pi}} e^{-\frac{1}{2}(y_i - s)^2} \tag{8}$$

The expected probits are then computed using weighted regression of working probits weighted on  $x_i$ , with each  $y_i$ . A weight,  $n_i w_i$ , is assigned to each  $x_i$  and  $y_i$  where  $w_i$  is the weighting coefficient (9).

$$w_i = \frac{Z_i^2}{\left(P_i + \frac{C}{1 - C}\right)(1 - P_i)} \tag{9}$$

where C is the natural response of control group. The slope  $\beta$  and intercept  $\alpha$  of the working probit regression are obtained from equations(10) and (11), respectively.

$$\beta = \frac{\sum_{i=1}^n n_i w_i (x_i - \bar{x})(y_i - \bar{y})}{\sum_{i=1}^n n_i w_i (x_i - \bar{x})^2} \tag{10}$$

$$\alpha = \bar{y} - \beta \bar{x}, \tag{11}$$

Where,

$$\bar{y} = \frac{\sum_{i=1}^n n_i w_i y_i}{\sum_{i=1}^n n_i w_i}, \bar{x} = \frac{\sum_{i=1}^n n_i w_i x_i}{\sum_{i=1}^n n_i w_i}$$

The standard error of  $\beta$  and  $\alpha$  are computed using equations (12) and (13), respectively.

$$\sigma(\hat{\beta}) = \sqrt{\frac{1}{\sum_{i=1}^n n_i (x_i - \bar{x})^2}} \tag{12}$$

$$\sigma(\hat{\alpha}) = \sqrt{\frac{1}{\sum_{i=1}^n n_i w_i} + \bar{x}^2 \sigma(\hat{\beta})^2} \tag{13}$$

To test the adequacy of goodness of fit, the  $\chi^2$  statistic is computed by equation (14)

$$\chi^2 = \sum_{i=1}^n \frac{n_i (P_i - P_i)^2}{P_i(1-P_i)} \quad (14)$$

The right-tailed probability of the chi-squared distribution with  $n - 2$  degrees of freedom (*d.f.*) is used to compute the significance level 'p' of  $\chi^2$  statistic. A significant  $\chi^2$  statistic ( $p < 0.05$ ) shows that the population does not respond independently or that the fitted probit -log(dose) regression line did not adequately describe the dose-response relationship in the test samples.

The maximum likelihood estimation method is used to obtain an optimal fit. In this method,  $\alpha_0$  and  $\beta_0$  are taken as initial values and new values of  $\alpha$  and  $\beta$  are obtained using equations (5) to (14). The procedure is repeated until a stable  $\chi^2$  appears. The significance of the slope is assessed using equation (15)

$$h = \frac{\chi^2}{n-2} \quad (15)$$

For computation of lethal dose of toxicants on populations and their 95% CLs, first logarithms of the doses ( $\theta_\pi$ ) at a particular level of interest ( $\pi$ ) is calculated and then expected response proportion is computed using equation (16)

$$\theta_\pi = \frac{y_\pi - \alpha}{\beta} \quad (16)$$

Where,  $y_\pi$  is the  $\pi^{\text{th}}$  percentile of the probit distribution curve calculated in inverse of normal distribution for the probit distribution. The  $\pi^{\text{th}}$  lethal dose is computed as  $LD_\pi = 10^{\theta_\pi}$  and the standard error of  $\theta_\pi$ ,  $\sigma(\theta_\pi)$  can be calculated from equation (17) and the 95% CL of the  $LD_\pi$  is computing using equation(18)

$$\sigma(\theta_\pi) = \frac{1}{\beta} \sqrt{\frac{1}{\sum_{i=1}^n n_i w_i} + \frac{(\theta_\pi - \bar{x})^2}{\sum_{i=1}^n n_i w_i (x_i - \bar{x})^2}} \quad (17)$$

$$10^{\theta_\pi \pm t_{0.05, k-2} \sigma(\theta_\pi)} \quad (18)$$

## RESULTS AND DISCUSSION

Initially, probit analysis was used and published in science by Bliss (1934) with the primary focus on finding an effective dose of pesticide to control insects that fed on grape leaves using a graphical method. In this method, the response of the insects to various concentrations of pesticides was plotted and the effect of each pesticide on insect was inspected visually. This method did not have a sound statistical base to compare the differences. Keeping in view of the above fact, an attempt had been made to develop an online statistical analysis tool for probit regression using the scripting language ASP.

**Development of web-based tool:** After preparing the data, the corrected proportion ( $p_i$ ) was converted to a probit value ( $y_i$ ) by using the function *NormalCdfInv*( $p_i$ , *mean=0*, *variance=1*). A regression line was then fitted with *log(mortality)*( $c_i$ ) as independent variable and *empirical probit*( $y_i$ ) as dependent variable using sub procedure [linreg(log( $c_i$ ), *empricalprobit*, *noofdoses*, *intercept*, *beta*)] to obtain an initial estimate of intercept and slope (fig.1). Iterative maximum likelihood estimation procedure was then applied for computing the final probit line by taking these initial estimates of intercept and slope. The module computed expected probits ( $Y_i$ ), by function *NormalCdfInv*(*cmort*( $i$ ), *0*, *1*) here *cmort*( $i$ ) was the input argument which was corrected mortality and return expected probit. Expected response proportion ( $P_i$ ) was computed by calling sub program *NDTR*( $D(i)$ ,  $P(i)$ ,  $z(i)$ ) that takes  $D(i)$  empirical probit as input argument and return expected response proportion ( $P_i$ ) and  $Z(i)$  as output. Working probit, ( $y_i$ ) was worked out as per formula given in Lei and Sun (2018). These expected probits were estimated from weighted regression of working probits weighted on  $x_i$  with each  $y_i$ . Intercept and slope along with their standard errors and their significance and finally Chi-square statistic for adequacy of fit was computed. The above steps were repeated until an optimum solution was achieved. The output of the module includes

Complete computational steps for better understanding of the procedure

The Final Probit Line

The Dose-response curve

Computation of  $LC_{50}/LD_{50}$  with 95 % confidence intervals

A Chi-square statistic for testing the adequacy of fit.

**Testing the validity of results:** To test the validity of results obtained from module, the data taken from Lei and Sun (2018) and outputs were compared. The output produced by the module was in agreement with Lei and Sun, 2018.

**Procedure for analysis of data:** The web-based tool consisted of data entry interface written in HTML to facilitate the user in entering/pasting raw data in the text area provided. Results were obtained by following these steps:

**Step 1:** Data was arranged in increasing order of dose, i.e. the lowest dose to the highest dose. The control was at the end. Data of dose-response was entered/pasted in the text area provided in the web page of the module. The data was arranged as [dose][total no. of subject] [No. of Subjects] in a single line corresponding to the first dose. In similar manner, data was entered for other doses in separate lines. The data for the control group was entered in the last line as given below (Do not enter Conc No Killed as heading only data). For illustration, a sample data of rotenone had been

taken from Lei and Sun (2018) as given below

**Table 1.** A sample data of Rotenone (Lei and Sun, 2018).

Dose(ml.)	Total No	Dead
2.6	50	6
3.8	48	16
5.1	46	24
7.7	49	42
10.2	50	44
0	50	0

The entered data in the data entry page looks as under

**Probit Analysis (Computing LC50, LD50 or GR50)**

Please Enter or paste data tab or space delimited in text area below. Please Enter each concentration data in separate line i.e. ([Conce] [total no] [dead]). After entering the concentration data please enter the data for control in similar manner

2.6	50	6
3.8	48	16
5.1	46	24
7.7	49	42
10.2	50	44
0	50	0

Submit    Reset

**Probit Analysis**

Probit Analysis is a specialized regression model of binomial response variables (variable with only two outcomes). It transforms the sigmoid dose-response curve to a straight line that can then be analyzed by regression either through least squares or maximum likelihood.

**Applications of probit analysis**

Probit Analysis is commonly used in toxicology to determine the relative toxicity of chemicals to living organisms. This is done by testing the response of an organism under various concentrations of each of the chemicals in question and then comparing the concentrations at which one encounters a response. Here, the response is always binomial (e.g. death/no death) and the relationship between the response and the various concentrations is always sigmoid. Probit analysis transforms the sigmoid to linear and performs regression on the relationship. There are many endpoints used

**Step 2:** After entering the data press “Submit” button.

**Step 3:** Enter the number of doses excluding control group (e.g. if there are 6 doses including control group then enter 5) in next page and press “Analyze” button.

**Probit Analysis (Computing LC50 LD50 or GR50)**

Number of Concentrations (without control)

Analyse

**Probit Analysis**

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**Applications of probit analysis**

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**The Estimation of the Median Effective Dose:**

In Probit analysis successive batches of insects are exposed to different concentrations of the poison/chemical for a constant time and, after a suitable interval, the numbers dead and alive are recorded.

The probit analysis transforms the experimental results in order to estimate  $\mu$  and  $\sigma^2$  from the following equation.

$$dP = \frac{1}{\sigma \sqrt{2\pi}} e^{-\frac{1}{2\sigma^2}(x-\mu)^2} dx \quad (1)$$

The results are displayed on a separate webpage which can be printed or saved. The output of the module is given below

**Table 2.** Various computations for probit line.

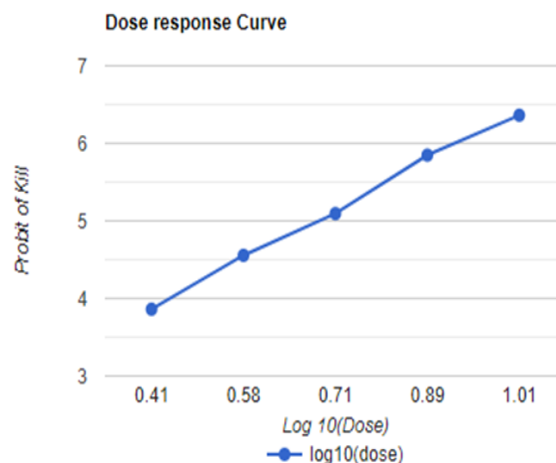
Conc.	Tot No.	No. Kills	% Mort.	Log (Dose)	Exp. Prop	Emp Probit	Exp Probit	Work. probit	Weight	wx	wy	wx2	wy2	wxy	Y'
2.60	50.00	6.00	0.12	0.41	0.12	-1.17	-1.13	-1.17	19.56	8.12	-22.97	3.37	26.97	-9.53	-1.14
3.80	48.00	16.00	0.33	0.58	0.32	-0.43	-0.44	-0.43	28.42	16.48	-12.24	9.55	5.27	-7.09	-0.44
5.10	46.00	24.00	0.52	0.70	0.53	0.05	0.09	0.05	29.19	20.65	1.59	14.61	0.08	1.12	0.09
7.70	49.00	42.00	0.85	0.88	0.80	1.06	0.84	1.04	23.91	21.20	25.03	18.79	26.20	22.19	0.84
10.20	50.00	44.00	0.88	1.01	0.91	1.17	1.36	1.15	15.74	15.87	18.10	16.01	20.83	18.26	1.36

**Table 3.** Maximum likelihood estimation of LC<sub>50</sub>/LD<sub>50</sub>.

Parameter	Estimate	Std. Error	t-cal	p-value
Intercept	-2.887	0.351	-8.225	0.001
Beta	4.213	0.481	8.767	0.001

**Table 4.** Estimation of Probit Line, LC<sub>50</sub>/LD<sub>50</sub> and fit statistics.

Component	Estimate
Mean X	5.066
Mean Y	0.081
Intercept	-2.887
Beta	4.213
<b>LC<sub>50</sub></b>	<b>4.845</b>
<b>LL</b>	<b>4.125</b>
<b>UL</b>	<b>5.691</b>
Log(LC <sub>50</sub> )	0.685
Log LL	0.615
Log UL	0.755
Chi-Square ML	1.729
Sign (Chi-Square)	0.631



**Fig. 1.** Working regression line.

**Table 5.** Estimation of Probit with a confidence interval for 1 to 99 per cent.

Percent	Probit	95 % Confidence Limit for Conc			Variance	95 % Confidence Limit for log(Conc)		
		LC	LL	UL		Log LC	Log LL	Log UL
1.000	-2.326	1.359	1.157	1.596	0.005	0.133	0.063	0.203
2.000	-2.054	1.577	1.343	1.852	0.004	0.198	0.128	0.268
3.000	-1.881	1.734	1.476	2.036	0.003	0.239	0.169	0.309
.	.	.	.	.	.	.	.	.
<b>50.000</b>	<b>0.000</b>	<b>4.845</b>	<b>4.125</b>	<b>5.691</b>	<b>0.000</b>	<b>0.685</b>	<b>0.615</b>	<b>0.755</b>
<b>90.000</b>	<b>1.282</b>	<b>9.761</b>	<b>8.311</b>	<b>11.465</b>	<b>0.002</b>	<b>0.990</b>	<b>0.920</b>	<b>1.059</b>
91.000	1.341	10.082	8.584	11.842	0.002	1.004	0.934	1.073
92.000	1.405	10.443	8.891	12.266	0.002	1.019	0.949	1.089
93.000	1.476	10.855	9.242	12.749	0.002	1.036	0.966	1.105
94.000	1.555	11.333	9.649	13.312	0.002	1.054	0.984	1.124
<b>95.000</b>	<b>1.645</b>	<b>11.905</b>	<b>10.136</b>	<b>13.983</b>	<b>0.002</b>	<b>1.076</b>	<b>1.006</b>	<b>1.146</b>
96.000	1.751	12.614	10.740	14.816	0.003	1.101	1.031	1.171
97.000	1.881	13.544	11.531	15.908	0.003	1.132	1.062	1.202
98.000	2.054	14.887	12.674	17.485	0.003	1.173	1.103	1.243
<b>99.000</b>	<b>2.326</b>	<b>17.278</b>	<b>14.710</b>	<b>20.294</b>	<b>0.004</b>	<b>1.237</b>	<b>1.168</b>	<b>1.307</b>

There are many standalone software such as SPSS, POLO-PC, Minitab, SAS and TRAP etc are available which are commercial and need installation also. This web-based tool is freely available and does not need any installation as it is based on Client-Server architecture and can be used over the internet with a web browser. User doesn't have to install anything, download any software, or worry about upgrades. The only requirement to use this tool is the net connectivity. Also, the submitted data will be erased after analysis resulting in data security.

### Conclusion

The developed tool will be extremely helpful to researchers as compared of other existing tools because of its capability to compute  $LC_{50}/LD_{50}$  with 95 % confidence interval and Chi-square statistic for testing the adequacy of fit. This freely available online tool will meet the growing needs of researchers to perform probit analysis. It will save time automatically doing complex calculations and generating results in understandable format. The tool is available online at <http://14.139.232.166/probit/probitanalysis.html>

### Conflict of interests

The authors declare that they have no conflict of interests.

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