STUDY DESIGNS

IN BIOMEDICAL RESEARCH

INDIRECT BIOASSAYS

DIRECT BIOASSAYS

- In direct assays, the doses of the standard and test preparations are "directly measured" for (or until) an "event of interest". Response is fixed (binary), dose is random.
- When an event of interest occurs, e.g.. the death of the subject, and the variable of interest is the dose required to produce that response/event for each subject. The value is called "individual effect dose" (IED).
- For example, we can increase the dose until the heart beat (of an animal) ceases to get IED.

INDIRECT BIOASSAYS

- In indirect assays, the doses of the standard and test preparations are applied and we observe the "response" that each dose produces; for example, we measure the tension in a tissue or the hormone level or the blood sugar content. For each subject, the dose is fixed in advance, the variable of interest is not the dose but the response it produces in each subject; The response could be binary or continuous.
- Statistically, indirect assays are more interesting (and, of course, also more difficult).

AN EXAMPLE: LUNG TUMORIGENESIS

- A group of mice were injected with NNK (a toxin from tobacco products) dissolved in saline when mice are 6 weeks old.
- About 16-20 weeks after treated by NNK, most mice have lung tumors; there will be an average of 10 surface tumors per lung, an average total tumor volume per lung = 400 mm^3 +/- 100 (SD)

A DOSE-RANGING EXPERIMENT

- Among a group of NNK-treated mice (with tumors after 16 weeks), say n=50, 10 mice are selected and sacrificed to measure tumor volumes – serve as baseline (or data for controls)
- The other 40 mice are randomized into 10 groups of 4 mice each treated by 10 different doses of a cancer agent/drug; the doses are spread over a very wide range from very low to very high
- Aim is to calculate the dose for 50% reduction of tumor volume (ED50) , the “median effective dose” which characterizes the agent’s potency.

DATA SUMMARIES

- Let “d” be one of the doses; x = log (d)
- v_0 = average tumor volume of control group
- v_x = average tumor volume of group treated with dose “d”; and
- $p_x = (v_0-v_x)/v_0$ the per cent of tumor reduction to treatment with dose d.

A REGRESSION MODEL

$$\ln \frac{p_x}{1-p_x} = \beta_0 + \beta_1 x$$

After estimating intercept and slope, β_0 by "b_0" and β_1 "b_1", we can calculate the median effective dose by setting $p_x = .5$:

$$ED_{50} = \exp(-b_0/b_1)$$

Where Does This Model, a Logistic Regression model, Come From?

$$\ln \frac{p_x}{1-p_x} = \beta_0 + \beta_1 x$$

MEASUREMENT SCALE

Depending on the “measurement scale” for the response (of indirect assays), we have:

(1) Quantal assays where the response is binary: whether or not an event (like the death of the subject) occurs,

(2) Quantitative assays where measurements for the response are on a continuous scale.

Anyway, “Dose Ranging Experiment” is a “Quantal Bioassay”; it allows us to obtain a measure of potency. However, the result (ED50) is not relevant in product/drug development … yet because potency measures such as ED50 are dependent on the biological system use. For example ED50 for cats is not the same as ED50 for mice. Eventually, we want to know or to measure the strength of the agent for human use.

To complete the process, one needs to conduct a similar dose ranging experiment for a standard agent, if not yet done; then take the ratio of the two ED50's to obtain the Relative Potency which is system independent.

The common indirect assay is usually one in which the ratio of equipotent doses is estimated from curves relating quantitative responses and doses for the two preparations. The shape of these "curves" further divides quantitative indirect assays into:

(1) Parallel-line assays are those in which the response is linearly related to the log dose,

(2) Slope-ratio assays are those in which the response is linearly related to the dose itself.

PARALLEL-LINE ASSAYS

- Parallel-line assays are those in which the response is linearly related to the log dose.
- From the definition of "relative potency" ρ, the two doses are related by $D_S = \rho D_T$.
- The model: $E[Y_S|X_S=\log(D_S)] = \alpha + \beta X_S$, for Standard and, for the same dose we have $E[Y_T| X_S=\log(D_S= \rho D_T)]= (\alpha + \beta \log\rho) + \beta X_T$
- We have 2 parallel lines with a common slope and different intercept.

The primary aim of a statistical analysis is to estimate the "relative potency" ρ of an agent or stimulus ; a point estimate as well as confidence limits (i.e. 95% confidence interval). We can estimate $\log\rho$, called M, by subtracting the intercepts and divided by the common slope :

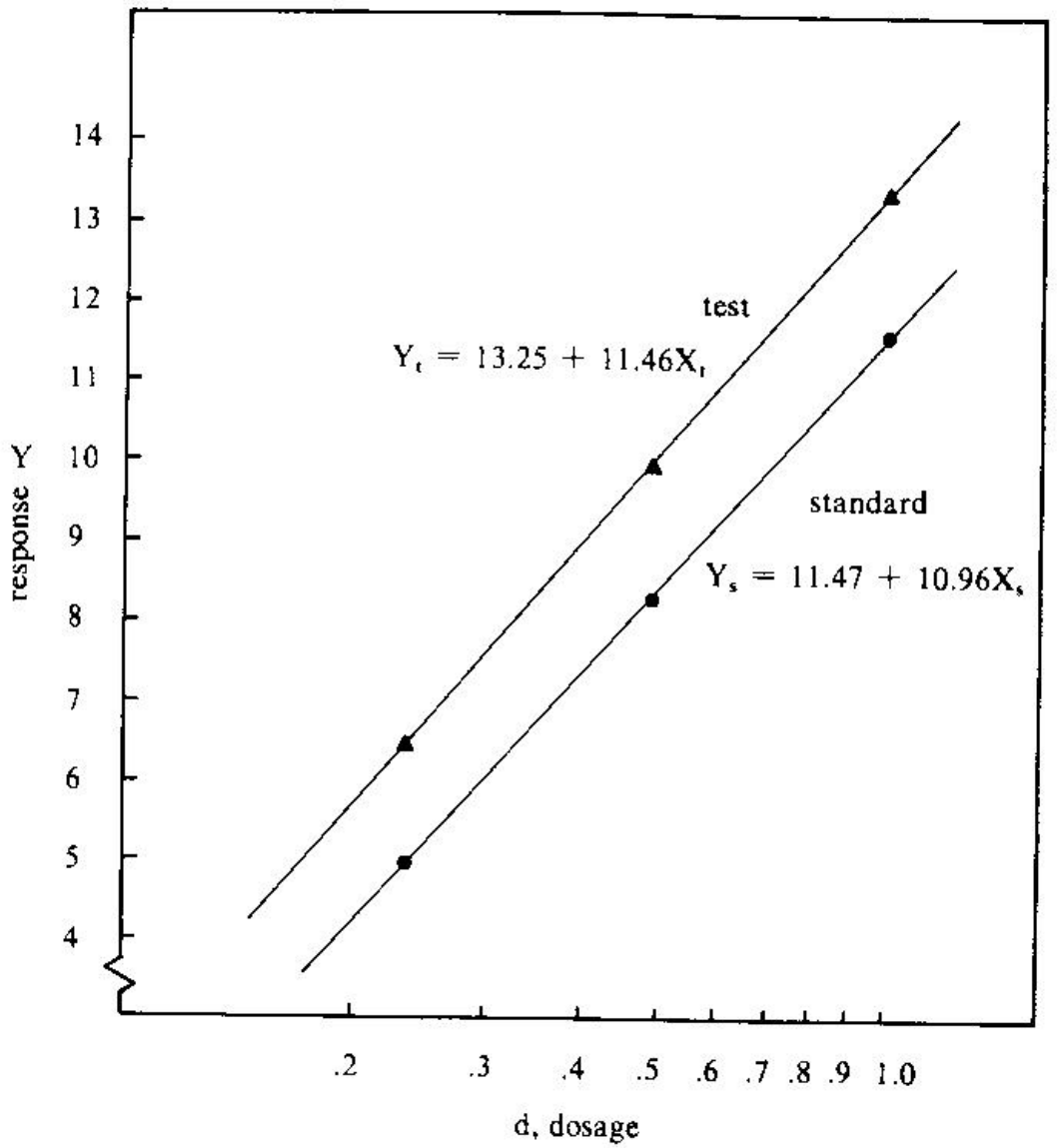
$$E[Y_S|X_S] = \alpha + \beta X_S$$
$$E[Y_T| X_S] = (\alpha + \beta \log\rho) + \beta X_T$$

EXAMPLE

- In this example, test and standard preparations of the agent are tested at the same three dose levels (.25, .50, and 1.0 mg/cc); and there are 8 replications at each dose of each preparation.
- It is designed with 8 dishes/plates, each contains 6 identical bacterial cultures - one in a "well" (randomized complete block design), also called "6-point assay"; the response was the amount of decrease in growth.

	Preparation					
	Standard Preparation			Test Preparation		
Dose (D; mmgcc)	**0.25**	**0.50**	**1.00**	**0.25**	**0.50**	**1.00**
X = log10(Dose)	-0.602	-0.301	0.000	-0.602	-0.301	0.000
Response (Y; mm)	4.9	8.2	11.0	6.0	9.4	12.8
	4.8	8.1	11.5	6.8	8.8	13.6
	4.9	8.1	11.4	6.2	9.4	13.4
	4.8	8.2	11.8	6.6	9.6	13.8
	5.3	7.6	11.8	6.4	9.8	12.8
	5.1	8.3	11.4	6.0	9.2	14.0
	4.9	8.2	11.7	6.9	10.8	13.2
	4.7	8.1	11.4	6.3	10.6	12.8

The Indirect Quantitative Assays



Quantitative parallel line assay responses against log doses.

MULTIPLE REGRESSION

- The simple approach is pooling data from both preparations and using “Multiple Regression”;
- Dependent Variable: Y = Response;
  Two Independent Variables are:
  X = log(Dose) &
  P = Preparation (a “dummy variable” coded as P = 1 for “Test” and P = 0 for “Standard”)

Multiple Regression Model :

$$E(Y) = \beta_0 + \beta_1 X + \beta_2 P$$

β_1 is the common slope and

β_2 is the "difference of intercepts ";

$$M = \log\rho = \frac{\beta_2}{\beta_1}$$

X = log(Dose) & P = Preparation

DOSE-RESPONSE RELATIONSHIP

- We view “dose-response curve” simply as a description of experimental results; necessary but mysterious!.
- There is a dosage D and a biological response Y, and we assume that experimental results are described by a well-behaved dose-response curve of the form y=f(D).
- There may be some physical or chemical principle behind this process, but in traditional bioassay, determination of this “f” is entirely “empirical”.
- Given the data one can go to search for a relationship between response variable Y to the fixed dosage D. One could plot Y versus X=D, Y versus X=log(D), or Y=X=(1/D) etc…That’s how we found parallel lines!

To compensate for a lack of theory behind the dose-response relationship, we make some efforts to check for the model's validity. In the Multiple Regression approach, we set Dependent Variable: Y = Response; two Independent Variables are:

X = log(Dose) &
P = Preparation (a binary "dummy variable" coded as P = 1 for "Test" & P = 0 for "Standard")

One can simply include an interaction term X*P to check for parallelism, or quadratic terms to check for linearity of each of the two lines.

SLOPE RATIO ASSAYS

- Slope-ratio assays are those in which the response is linearly related to the dose itself.
- From the definition of "relative potency" ρ, the two doses are related by $D_S = \rho D_T$.
- The model: $E[Y_S|X_S=D_S)] = \alpha + \beta X_S$, for same dose $E[Y_T| X_S=D_S]= \alpha + \beta\rho X_T$; the lines have the same intercepts - the mean response at zero dose.
- We have 2 straight lines with a common intercept and different slopes.

The primary aim of a statistical analysis is to estimate the "relative potency" ρ of an agent or stimulus ; a point estimate as well as confidence limits (i.e. 95% confidence interval). In this model, we have straight lines with a common intercept but different slopes; We can obtain ρ, the relative potency, as the ratio of two slopes.

MULTIPLE REGRESSION

- The simple approach is pooling data from both preparations and using “Multiple Regression”;
- Dependent Variable: Y = Response;
  Two Independent Variables are:
  X = Dose &
  P = Preparation (a “dummy variable” coded as P = 1 for “Test” and P = 0 for “Standard”)

Multiple Regression Model #1 :

$$E(Y) = \beta_0 + \beta_1 X + \beta_2 PX$$

β_0 is the common intercept and

$$\beta_T = \beta_1 + \beta_2$$

$$\beta_S = \beta_1$$

$$\rho = \frac{\beta_1 + \beta_2}{\beta_1}$$

$$= 1 + \frac{\beta_2}{\beta_1}$$

X = Dose & P = Preparation

Multiple Regression Model #1:

$$E(Y) = \beta_0 + \beta_1 X + \beta_2 PX$$

This model is a bit "unconventi onal";

it includes the interactio n term P * X

but only one main effect (X only, no P)

MULTIPLE REGRESSION #2

Let Y be the response, X_S and X_T the doses. Consider the following model in which for any observation on S, set X_T=0, for any observation on T, set X_S=0; the model may include control observations for which we set X_S= X_T= 0:

$$E(Y) = \beta_0 + \beta_S X_S + \beta_T X_T;$$

$$\beta_0 = \text{Common Intercept}$$

$$\rho = \beta_T / \beta_S$$

GOODNESS-OF-FIT

In the Multiple Regression approach, we set
Dependent Variable: Y = Response;
Two Independent Variables are: X = log(Dose) &
P = Preparation (a binary "dummy variable" coded as P = 1 for "Test" & P = 0 for "Standard").

Two issues of goodness-of-fit: (1) Two straight lines with equal slopes & (2) Two lines with equal intercepts.

Full Multiple Regression Model #1:

$$E(Y) = \beta_0 + \beta_1 X + \beta_2 PX + \beta_3 P$$

$$\alpha_S = \beta_0$$

$$\beta_S = \beta_1$$

$$\alpha_T = \beta_0 + \beta_3$$

$$\beta_T = \beta_1 + \beta_2$$

Lines have same intercept if $\beta_3 = 0$

& One can simply include quadratic terms to check for linearity of each of the two lines, if there are 'enough" data.

QUANTAL ASSAYS

Quantal response assays belong to the class of qualitative indirect assays. They are characterized by experiments in which each of a number of pre-determined levels of a stimulus (e.g. dose of a drug) is applied to n experimental units; r of them respond and (n - r) do not response. That is “binary” response (yes/no). The group size “n” may vary from dose to dose; in theory, some n could be 1 (so that r = 0 or 1).

DIRECT ASSAYS

- In direct assays, the doses of the standard and test preparations are "measured" for an "event of interest"; intra patient adjustment is needed.
- When an (pre-determined) event of interest occurs, e.g.. the death of the subject, and the variable of interest is the dose required to produce that response/event for each subject.
- That is, the dose is measured right at the time the event occurs; it is not possible to do it if the dose is fixed in advance (indirect assays).

QUANTAL ASSAYS VS. DIRECT ASAYS

It is assumed that each subject has its own tolerance to a particular preparation. In a direct assay, the amount of stimulus needed to produce the response in each individual subject can be measured, called IED. In quantal bioassays, we cannot measure IEDs because only one fixed dose is given to a group of n subjects; (1) if that dose is below some particular IED, the response does not occur; (2) Subjects who response are those with IEDs below the given fixed dose.

QUANTAL ASSAYS VS QUANTITATIVE ASSAYS

- Quantal bioassays are qualitative; we observe occurrences of an event - not obtain measurements on continuous scale.
- Because the event is well-defined, we can estimate agent's potency. The most popular parameter is the level of the stimulus which result in a response by 50% of individuals in a population. It is often denoted by LD50 for median lethal dose, or ED50 for median effective dose, or EC50 for median effective concentration.
- However, measures of potency depend on the biological system used; the estimates of LD50's for preparations of the same system can be used to form the relative potency – which would be more likely independent from the system.

The most popular parameter LD50 (for median lethal dose), or ED50 (for median effective dose), or EC50 (for median effective concentration) is the level of the stimulus which result in a response by 50% of individuals in a population.

(1) It is a measure of the agent's potency, which could be used to form relative potency.

(2) It is chosen by a statistical reason; for any fixed number of subjects, one would attain greater precision as compared to estimating, say, LD90 or LD10 or any other percentiles.

THE ASSAY DESIGN

- The usual design consists of a series of dose levels with subjects completely randomized among/to the dose levels. The experiment may include a standard and a test preparations; or maybe just the test.
- The dose levels chosen should range from “very low” (few or no subjects would respond) to “rather high” (most or all subjects would respond).
- The objective is often to estimate the LD50; the number of observations per preparation depends on the desired level of precision of its estimate – sample size estimation is a very difficult topic.

Data :

$$\{n_i, r_i; x_i = \log(dose_i)\} \Rightarrow \{p_i = \frac{r_i}{n_i}; x_i\}$$

The proportion p_i is the estimate of some (unknown) probability P_i. This dependent variable is a proportion, a number bounded between 0 and 1. In order to perform "regression analysis", we first need a transformation to turn that proportion into a number, unbounded, on the continuous scale.

Four-step Process:

(1) A transformation from P_i to Y_i which is unbounded and on a linear scale,

(2) Put in a linear regression model relating Y_i to x_i, say $E(Y_i) = \alpha + \beta x_i$,

(3) Estimating the parameters α and β,

(4) Estimating LD50 or ED50 from the results for α and β in step #3.

Let P be the probability of response at a particular dose - where the log dose is X, it is estimated by p = r/n. The "first step" in the analysis process is to obtain "the equivalent deviate of P" using the following transformation (P $\rightarrow$ Y):

$$P = C + \int_{-\infty}^{Y} f(\theta)\, d\theta$$

$f(\theta)$ is some probability density function; $0 \le C \le 1$.

C represents background response (noise); but, for simplicity we often set C=0.

A value of P or of Y determines the other uniquely; both are results of input x (which is log(dose)).

In theory, any probability density function can be used. We can choose one either by its simplicity and/or its extensive scientific supports. And we can check to see if the data fit the model (however, it's practically hard because we need lots of data to tell).

A VERY SIMPLE CHOICE

A possibilit y is "Unit Exponentia l Distributi on" with density :

$$f(z) = e^{-z};\ z \geq 0$$

Result (for one covariate X) is:

$$\pi = \int_{-\beta_0-\beta_1 x}^{\infty} e^{-z}\,dz$$

$$= e^{\beta_0+\beta_1 x};\ \text{or}$$

$$\ln \pi = \beta_0 + \beta_1 x$$

That is to model the "log" of the probability as a "linear function" of covariates.

The advantage of the approach of modeling the "log" of the probability as a "linear function" of covariates, is easy interpretation of model parameters, the probability is changed by a multiple constant (i.e. "multiplicative model" which is usually plausible)

A HISTORICAL CHOICE

Besides the Unit Exponential probability density, one can also use of the Standard Normal density in the transformation of π:

$$\pi = \int_0^{y^*} f(\theta)\, d\theta$$

"f" is the Standard Normal density :

$$f(\theta) = \frac{1}{\sqrt{2\pi}} \exp\left(-\frac{\theta^2}{2}\right)$$

This “Probit Transformation” leads to the “Probit Model”; Y* is called the “probit” of π. The word “probit” is a shorten form of the phrase “PROBability unIT” (but it is not a probability), it is a standard normal variate.

The Probit Model was popular in years past and had been used almost exclusively to analyze "bioassays" for many decades. However, there is no closed-form formula for Y* (it's not possible to derive an equation relating π to x without using an integral sign):

$$\pi = \int_0^{\beta_0+\beta_1 x} \frac{1}{\sqrt{2\pi}} \exp\left(-\frac{\theta^2}{2}\right) d\theta$$

Since it's not possible to derive an equation relating π to x without using an integral sign, the computation is much more complicated.

There is a SAS program (It's PROC PROBIT) but the use of the Probit Model has been faded.

LOGISTIC TRANSFORMATION

(Standard) **Logistic Distributi on** with density :

$$f(\theta) = \frac{\exp(\theta)}{[1+\exp(\theta)]^2}$$

Result is:

$$\pi = \int_{-\infty}^{Y^* = \beta_0 + \beta x} \frac{e^{\theta}}{[1 + e^{\theta}]^2} d\theta$$

$$= \frac{\exp(\beta_0 + \beta_1 x)}{1 + \exp(\beta_0 + \beta_1 x)}$$

$$= \frac{1}{1 + \exp(-\beta_0 - \beta_1 x)}$$

$$\pi = \frac{e^{\beta_0 + \beta_1 x}}{1 + e^{\beta_0 + \beta_1 x}}$$

$$1 - \pi = \frac{1}{1 + e^{\beta_0 + \beta_1 x}}$$

$$\frac{\pi}{1 - \pi} = e^{\beta_0 + \beta_1 x}$$

$$\log \frac{\pi}{1 - \pi} = \beta_0 + \beta_1 x$$

We refer to this as “Logistic Regression”

Advantages:

(1) Also very simple data transformation:
$Y = \log\{p/(1-p)\}$

(2) The logistic density, with thicker tails as compared to normal curve, may be a better representation of real-life processes (compared to Probit Model which is based on the normal density).

A POPULAR MODEL

- Although one can use the Standard Normal density in the regression modeling process (or any density function for that purpose),
- The Logistic Regression, as a result of choosing Logistic Density remains the most popular choice for a number of reasons: closed form formula for π, easy computing (Proc LOGISTIC)
- The most important reasons: interpretation of model parameter and empirical supports!

REGRESSION COEFFICIENTS

$$\pi = \frac{\exp(\beta_0 + \beta_1 x)}{1 + \exp(\alpha + \beta x)}$$

$$\ln \frac{P}{1 - P} = \beta_0 + \beta_1 x$$

β_1 represents the log of the odds ratio associated with X, if X is binary, or with "an unit increase" in X if X is on continuous scale; β_0 only depends on "event prevalence"- just like any intercept.

SUPPORTS F OR LOGISTIC MODEL

The fit and the origin of the linear logistic model could be easily traced as follows. When a dose D of an agent is applied to a pharmacological system, the fractions f_a and f_u of the system affected and unaffected satisfy the so-called "median effect principle" (Chou, 1976):

$$\frac{f_a}{f_u} = \left\{ \frac{d}{ED_{50}} \right\}^m$$

where ED_{50} is the "median effective dose" and "m" is a Hill-type coefficient; m = 1 for first-degree or Michaelis-Menten system. The median effect principle has been investigated much very thoroughly in pharmacology. If we set " $\pi = f_a$", the median effect principle and the logistic regression model are completely identical with a slope $\beta_1 = m$.

$$\frac{f_a}{f_u} = \left\{ \frac{d}{ED_{50}} \right\}^m$$

$$= \frac{f_a}{1 - f_a}$$

$$\ln \frac{f_a}{1 - f_a} = \ln \frac{p}{1 - p}$$

$$= m\ln(ED_{50}) + m\ln(d)$$

$$= \beta_0 + \beta_1 x$$

There are several possible ways to transform the proportion p into some measurement Y on the continuous scale with an unbounded range. The most solid and popular one is the logistic transformation leading to the logistic regression model, mostly because of its strong empirical supports from the “median effect principle”:

USE OF “SAS”

- PROC PROBIT
- General model, C≠0; can include control group.
- Can choose transformation for drug dose, including common choice: $x = \log_{10}(\text{dose})$
- May include other covariates
- Three choices of density function: logistic, standard normal, and “extreme value”

PROC PROBIT covers three transformation – including the popular Probit (standard normal density) and Logistic (logistic density) transformations. If you only prefer the logistic transformation, can use PROC LOGISTIC but it does not have a few options which are specific for bioassays; you can easily complete the job by hand.

Suggested Exercise:

Use the following data set, fit the two parallel lines and calculate the Relative Potency – including its Standard Error, if you can.

	Preparation					
	Standard Preparation			Test Preparation		
Dose (D; mmgcc)	**0.25**	**0.50**	**1.00**	**0.25**	**0.50**	**1.00**
X = log10(Dose)	-0.602	-0.301	0.000	-0.602	-0.301	0.000
Response (Y; mm)	4.9	8.2	11.0	6.0	9.4	12.8
	4.8	8.1	11.5	6.8	8.8	13.6
	4.9	8.1	11.4	6.2	9.4	13.4
	4.8	8.2	11.8	6.6	9.6	13.8
	5.3	7.6	11.8	6.4	9.8	12.8
	5.1	8.3	11.4	6.0	9.2	14.0
	4.9	8.2	11.7	6.9	10.8	13.2
	4.7	8.1	11.4	6.3	10.6	12.8