PubH 7470: STATISTICS

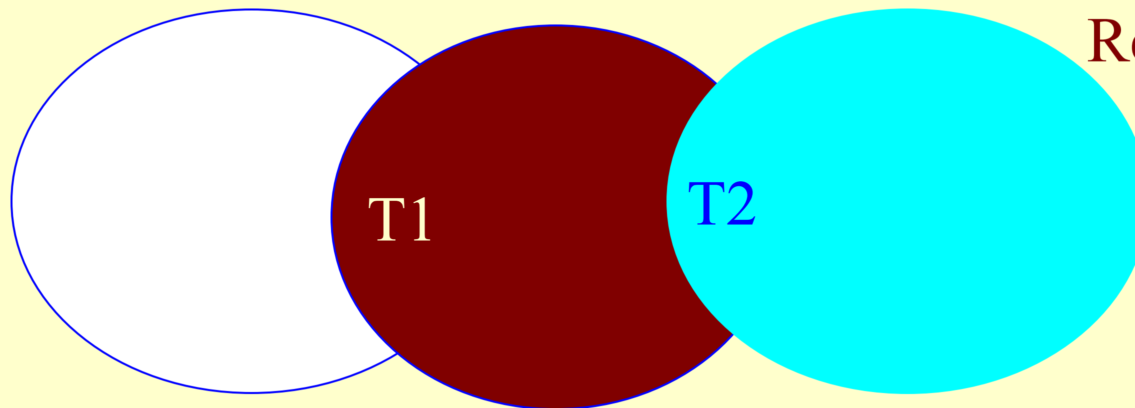
FOR TRANSLATIONAL & CLINICAL RESEARCH

From Basic to Translational:

DIRECT BIOASSAYS & Estimation of Ratios

Clinical Research

Population Research



Laboratory Research

Translational Research is the component of **basic science** that interacts with clinical research (T1) or with population research (T2).

We often emphasize more on the first area of translational research, T1; they are research efforts and activities needed to bring discoveries in the laboratories to the bed sides.

And it is hard to pinpoint precisely the starting point of “T1”; many believe that translational research starts with “biological assays” – or bioassays, but some could point to In Vitro or In Vivo which are pre-clinical.

DEFINITION

- “Biological assays” or “bioassays” are methods for estimating the potency or strength of an “agent” or “stimulus” by utilizing the “response” or “effect” or “reaction” caused by its application to biological material or experimental living “subjects”.
- Simple examples:
  (1) Six aspirin tablets can be fatal to a child;
  (2) Certain dose of a lethal drug can kill a cat.

From Webster International Dictionary:

"Biological Assay is the estimation of the strength of a drug by comparing its effect on biological material, as animals or animal tissue, with those of a standard product."

In other words, we (usually) can only estimate "relative potency" of an agent, not its "potency".

COMPONENTS OF A BIOASSAY

- The subject is usually an animal, a human tissue, or a bacteria culture,
- The agent is usually a drug, a chemical
- The response is usually a change in a particular characteristic or even the death of a subject; responses can be binary or measured on continuous scale.

(1) There are deterministic or non-stochastic assays; but they are not subjected to statistical analyses – so they are not our targets.

(2) An assay is stochastic if the relative potency is influenced by factors other than the preparations; i.e. extraneous factors which cannot be completely controlled or explained. In other words, the response is subjected to a random error; e.g. either the "dose" or the "response" is a "random variable" – depending on the design.

BASIC PROCESS

- For stochastic assays, our only targets, we refer to the relationship between stimulus level and the response it produces as "a regression model".
- A "test preparation" of the stimulus - having an unknown "potency" - is "assayed" to find the response.
- We find the dose of the standard preparation which produces the same response (as that by test preparation).

There are two types of bioassays:

(1) direct assays and
(2) indirect assays.

They are both stochastic

DIRECT ASSAYS

- 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.

Typical Experiment:

A group of subjects (e.g. animals) are randomly divided into two subgroups and then IED of a standard preparation is measured in each subject of group 1; the IED of the test or unknown preparation is measured in each subject of group 2. The aim is to estimate the “relative potency”, that is the “ratio of concentrations” of the test relative to standard to produce the same biological effect/event.

Keep in mind that the “concentration” and the “dose” are inversely proportional - when concentration is high, we need a smaller dose to reach the same response. In other words , we define the “relative potency” or the “ratio of concentrations” of the test to standard as the “ratio of doses” of the standard to test:

$$\rho = \frac{Dose_S}{Dose_T}$$

When the relative potency $\rho > 1$, the Test Preparation is stronger (we need a larger dose of the Test in order to produce the same response) – and vice versa. Pairs of doses that give the same response are termed "equipotent", meaning "same strength".

Data are very simple: two (2) independent samples, the type you usually have for two-sample t-test or Wilcoxon test; but we will not compare them using a test of significance. We want to **estimate** "Relative Potency":

Recall that we define the "relative potency" as the "ratio of concentrations" of the test to standard, or the "ratio of doses" of the standard to test, point estimate is easy.

The more difficult part is its precision and confidence interval; we would need a Statistical Model.

For students of statistics or biostatistics, the only new feature is learning to deal with the "**ratio** of sample means" – instead of the usual "difference of sample means". For example, what is "exact" variance? We usually approximate it by using "Delta Method"

INDIRECT ASSAYS

- In indirect assays, the doses of the standard and test preparations are 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 each subject; The response could be binary or continuous.
- Statistically, indirect assays are more interesting (and, of course, also more difficult).

In Indirect Assays, the dose is fixed and the response is random; and that response could be a measurement or the occurrence of an event (whereas the response in Direct Assays is always binary, the occurrence of an event).

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.

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.

CHEMICAL CONSTITUENTS

Indirect assays are also divided into "analytic dilution" or "comparative dilution".

(i) **Analytic dilution assays** are such that the test and standard preparations behaved as though they are identical (same constituents), except for the concentration,

(ii) In **Comparative assays**, the two preparations are not the same; For example, the concentration of one protein is estimated by using a different protein as standard.

For analytic dilution assays, the only difference is “concentration”; the constant relative potency is the reciprocal of the “dilution factor”. In other words, its existence/solution is global – that is, a solution always exists and is a constant.

For comparative dilution assays, the response-producing constituents in the two preparations are only qualitative similar; value of the relative potency may not be constant. In other words, its existence is “local”. **Statistical analyses are mostly the same**; however, the existence or solution for a relative potency may depend upon the particular experiment, material, or techniques.

Unless we know the chemical/biological system well, most of the times it is impossible to tell a analytic dilution assay from a comparative assay from the resulting data. The exception is perhaps Direct Assays.

A MODEL FOR DIRECT ASSAYS

It is commonly assumed that the test doses and the standard doses follow two normal distributions with the same variance:

$$\{x_{1T}, x_{2T}, \ldots, x_{n_T T}\} \text{ are i.i.d. } N(\mu_T, \sigma^2), \text{ and}$$

$$\{x_{1S}, x_{2S}, \ldots, x_{n_S S}\} \text{ are i.i.d. } N(\mu_S, \sigma^2);$$

$$\rho = \mu_S / \mu_T$$

RESULTS

The following **results** can be obtained approximately by Taylor's expansion:

$$\rho = \mu_S / \mu_T \text{ and } r = \overline{x_S} / \overline{x_T}$$

$$E(r) \cong \rho$$

$$Var(r) \cong \frac{\sigma^2}{\mu_T^2}\{\frac{1}{n_S} + \frac{\rho^2}{n_T}\}$$

$$n_S^{1/2}(r - \rho) \cong N[0, \frac{\sigma^2}{\mu_T^2}(1 + \frac{n_T}{n_S}\rho^2)]$$

SKETCH OF PROOF

$$r = \overline{x_S} / \overline{x_T}$$

$$Var(r) \cong \frac{\sigma^2}{\mu_T^2}\{\frac{1}{n_S} + \frac{\rho^2}{n_T}\}$$

by "Error Propagation" of "Delta Method"

$$Var(r) \cong \frac{\sigma^2}{\mu_T^2}\{\frac{1}{n_S}+\frac{\rho^2}{n_T}\}$$

$$SE(r) \cong \frac{s_p}{\overline{x_T}}\{\frac{1}{n_S}+\frac{r^2}{n_T}\}^{1/2}$$

$$s_p^2 = \frac{(n_S-1)s_S^2+(n_T-1)s_T^2}{n_S+n_T-2}$$

Two things should be noted here: (1) We do not have the "exact" variance, we approximate it using the **Delta method**; (2) The variance of the estimated relative potency r can be easily obtained, at least approximately, <u>but</u> the normal distribution for r, the ratio of sample means, may fit very poorly – especially when the sample sizes are often rather small.

HOMOGENEITY OF VARIANCES

$\{x_{1T}, x_{2T}, \ldots, x_{n_T T}\}$ are i.i.d. $N(\mu_T, \sigma^2)$, and

$\{x_{1S}, x_{2S}, \ldots, x_{n_S S}\}$ are i.i.d. $N(\mu_S, \sigma^2)$

We have assume that the standard and test responses have equal variances; and this can be tested using $F = s_S^2/s_T^2$ which distributed as $F(n_S-1, n_T-1)$ under the null hypothesis

ANALYTIC DILUTION ASSAYS

- Analytic dilution assays are those for which the test and standard preparations behaved as though they are identical, except for the concentration; that is $X_S = \rho X_T$.
- It can be seen that the homoscedascity assumption is no longer valid because $Var(X_S) \neq Var(X_T)$ if $\rho \neq 1$, the cases that we are interested in; previous method for C.I., even poor, **does not apply**.

AN EXAMPLE

A standard preparation and an unknown or test preparations of a lethal drug are infused into cats. The (measured) response is the amount of this drug (in cc) per kilogram of body weight of the cats needed to produce cardiac arrest.

	Standard	Test
	2.42	1.55
	1.85	1.58
	2	1.71
	2.27	1.44
	1.7	1.24
	1.47	1.89
	2.2	2.34
Total	13.91	11.75
Mean	1.987	1.679
Variance	0.1136	0.1265

Perhaps the case of a **comparative assay**

POSSIBLE SOLUTION FOR
ANALYTIC DILUTION ASSAYS

- From $X_S = \rho X_T$, taking the log we get: $\log X_S = \log \rho + \log X_T$; then we can preserve the homogeneity variances for log doses.
- But that is like assuming the dosages are distributed as log-normal with equal variability.
- The advantages of doing analysis on log dosages are (i) variance estimates can be pooled to have more precise estimation and (ii) relative potency is obtained as the antilog of the difference of means rather than the ratio, an easier procedure.

RESULTS FOR DILUTION ASSAYS

Let $\overline{z}$'s be the means and s_p the (pooled) standard deviation on the "natural log scale", the point estimate and the 95% confidence interval for the relative potency ρ are, where $t_{.975}$ is the 97.5th percentile of the t distribution with (n_s+n_T-2) degrees of freedom:

$$R = \exp(\overline{z_S} - \overline{z_T})$$

$$95\%\,\mathrm{CI} = \exp[(\overline{z_S} - \overline{z_T}) \pm t_{.975} s_p \sqrt{\frac{1}{n_S} + \frac{1}{n_T}}]$$

EXAMPLE, Part A

If we approximate the sampling distribution of r by normal, we can form a 95% CI the usual way; but result is rather poor:

	Standard	Test
	2.42	1.55
	1.85	1.58
	2.00	1.71
	2.27	1.44
	1.70	1.24
	1.47	1.89
	2.20	2.34
Total	13.91	11.75
Mean	1.987	1.679
Variance	0.1136	0.1265

$$SE(r) \cong \frac{s_p}{\bar{\bar{x}}_T}\{\frac{1}{n_S}+\frac{r^2}{n_T}\}^{1/2}$$

$$95\%\ \text{C.I.}: r \pm 1.96 SE(r)$$

$$1.18 \pm (1.96)\frac{.3464}{1.679}\sqrt{\frac{1}{7}+\frac{(1.18)^2}{7}}\}$$

$$= (0.94, 1.42)$$

EXAMPLE, Part B

Same example, but on natural log scale:

	Standard	Test
	0.884	0.438
	0.615	0.457
	0.693	0.536
	0.821	0.365
	0.531	0.215
	0.385	0.637
	0.788	0.849
Mean	0.674	0.451
Variance	0.031	0.041

$$R = \exp(\overline{z}_S - \overline{z}_T)$$

$$= \exp(.674 - .451) = 1.25$$

$$95\%\ \text{C.I. is } \exp[(\overline{z}_S - \overline{z}_T) \pm t_{.975} s_p \sqrt{\frac{1}{n_S} + \frac{1}{n_T}}]$$

$$= \exp[.223 \pm (2.179)(.190)(2/7)^{1/2}$$

$$= (1.02, 1.59)\ \textit{versus exact result: } (.94, 1.42)$$

But this is a case where log transformation is not needed

WHAT DO WE DO WITH RATIOS?

(1) We take the log of the point estimate

(2) Form Confidence interval on log scale

(3) Then exponentiating the endpoints

$$R = \exp(\overline{z_S} - \overline{z_T})$$

$$95\%\ \mathrm{CI} = \exp[(\overline{z_S} - \overline{z_T}) \pm t_{.975} s_p \sqrt{\frac{1}{n_S} + \frac{1}{n_T}}]$$

ABOUT STEP #1

In general, by first taking log of the point estimate - log of ratio of sample means in the case of "Direct Assays"– then we treat the "log of numerator" and "log of denominator" as normally distributed. In other words, we treat the sample mean as log normal in the next step, contradicting the Central Limit Theorem. This may be more serious.

THE COMBINED RESULT

- Together, the two-step procedure produce confidence **intervals which are often too long**.

- Focusing on Risk Ratio (ratio of 2 proportions, Lui (Contemporary Clinical Trials, 2006) found that the log transformation method could lead to intervals which are many times longer than those by competing methods - as much as 40 times in some configurations – an obvious loss of "efficiency".

LET START OVER

$$\rho = \frac{Dose_S}{Dose_T} = \frac{\mu_S}{\mu_T}$$

$$\ln\rho = \ln\mu_S - \ln\mu_T$$

Here the question is not an "if a log transformation is needed"; the question is "how should we do it right"? if not handled well, even the point estimate may be "off".

"USUAL" ESTIMATION OF LOG NORMAL MEANS

Data :

$$\{z_i\}_{i=1}^{n} \Rightarrow \{x_i = \ln x_i\}_{i=1}^{n}$$

Is it confidence Interval for Mean?

$$\exp\{\bar{z} \pm z_{1-\alpha/2} \frac{s_z}{\sqrt{n}}\}$$

By this "usual method", if X is distributed as Lognormal with mean θ, We estimate $\ln\theta$ by $\bar{z}$, then form "Geometric Mean", $\exp(\bar{z})$, to estimate θ (Is this all the reason we create "geometric mean"?). However,

$\text{X is Lognormal} \rightarrow \text{Z is N}(\mu, \sigma^2)$

$\text{Mean of } \theta \text{ of X satisfies: } \ln\theta = \mu + \frac{\sigma^2}{2}$

Therefore:

(1) Instead of estimate (μ) then exponentiating

$$\exp\{\bar{z} \pm z_{1-\alpha/2} \frac{s_z}{\sqrt{n}}\}$$

(1) We should estimate $(\mu + \frac{\sigma^2}{2})$ then exponentiating

$$\exp\{\bar{z} + \frac{s_z^2}{2} \pm z_{1-\alpha/2} \sqrt{\frac{s_z^2}{n} + \frac{s_z^4}{2(n-1)}}\}$$

According to Land (Technometrics, 1972), result #2 (constructing confidence intervals for $\ln(\theta)$ then exponentiating endpoints) was proposed (in a personal communication to Land) by Cox and Land called it "Cox's method").

Zhou and Gao (Stat Med, 1997) showed that result #1 (usual method) is inappropriate (very wrong coverage) and recommended Cox method (result #2) for moderate to large sample.

Think of cases with large σ^2!

The lesson learned is, if X is distributed as Lognormal with mean θ, We estimate $\ln\theta$ by $\exp(\bar{z} + s_z^2/2)$ - can call this "corrected geometric mean" - where $Z = \ln X$.

The ratio of these two "Corrected Geometric means" will serve as **point estimate** of "Relative Potency" using data from Direct Bioassays.

CORRECTED RESULTS

$$R = \exp[(\overline{z_S} + \frac{s_S^2}{2}) - (\overline{z_T} + \frac{s_T^2}{2})]$$

$$95\%\ \text{CI} = \exp\{[(\overline{z_S} + \frac{s_S^2}{2}) - (\overline{z_T} + \frac{s_T^2}{2})] \pm z_{1-\alpha/2}\sqrt{[\frac{s_S^2}{n} + \frac{s_S^4}{2(n-1)}] + [\frac{s_T^2}{n} + \frac{s_T^4}{2(n-1)}]}$$

If the two samples have equal variances on the log scale, then the original estimate – the ratio of sample means – turns out **accidentally correct**!

Conclusion:

There are more than one way to estimate the relative potency, which is a ratio. It could be more interesting if the Standard and Test samples could be assayed in the same individuals!

The following is an interesting related problem where we have to deal with the estimation of a ratio:

A tobacco product $[D_{10}]$PheT can be administered in 2 different ways: oral or smoking; these are given in random order to 16 healthy individuals. The substance is then monitored repeatedly from sample of blood and urine; with a long washout period between administrations. The next slides give these data; each number is a conventional pharmacokinetic parameter: Area under the Curve (AUC).

For each type of samples, blood or urine, the parameter of interest if the smoking to oral ratio

MODEL & STATISTICAL PROBLEM

Oral consumption would lead to measurement X, from plasma or urine, with negligible error because the whole amount was consumed where as smoking would lead to a measurement Y or more considerable error because different people smokes differently. Therefore the data would be suitable to frame as a "Regression through the Origin" (no intercept). And the parameter of interest is the slope; the question is how would we obtain an optimal estimate:

$$Y = \beta X$$

GENERAL SOLUTION

For each mode of administration, we have a "ratio" for each individual, $r_i = y_i/x_i$ with "variance" $Var(r_i)$; an optimal estimate of the ration across individuals is the "**weighted average**" of individual ratios – each is weighted by the inverse of the variance:

$$Var(r_i) = \frac{Var(y_i)}{x_i^2}$$

$$r = \frac{\sum \left[\frac{x_i^2}{Var(y_i)}\right]\left[\frac{y_i}{x_i}\right]}{\sum \frac{x_i^2}{Var(y_i)}}$$

$$r = \frac{\sum \left[\frac{x_i^2}{Var(y_i)}\right]\left[\frac{y_i}{x_i}\right]}{\sum \frac{x_i^2}{Var(y_i)}}$$

$$= \frac{\sum \frac{x_i y_i}{Var(y_i)}}{\sum \frac{x_i^2}{Var(y_i)}}$$

We consider 3 cases: (1) $Var(y_i)$ is a constant, (2) $Var(y_i)$ is proportional to x_i, and (3) Var(yi) is proportional to x_i^2

Case #1: $Var(y_i)$ is a constant

$$Var(y_i) = k$$

$$r = \frac{\sum \frac{x_i y_i}{Var(y_i)}}{\sum \frac{x_i^2}{Var(y_i)}}$$

$$= \frac{\sum x_i y_i}{\sum x_i^2}$$

This is the "Least Squares" estimate of the slope in the regression model without intercept

Case #2: Var(y_i) is proportional to x_i

$$\mathrm{Var}(y_i) = kx_i$$

$$r = \frac{\sum \frac{x_i y_i}{\mathrm{Var}(y_i)}}{\sum \frac{x_i^2}{\mathrm{Var}(y_i)}}$$

$$= \frac{\sum y_i}{\sum x_i}$$

This is the "ratio of the (sample) means"

Case #3: Var(y_i) is proportional to x_{i2}

$$\mathrm{Var}(y_i) = k x_i^2$$

$$r = \frac{\sum \frac{x_i y_i}{\mathrm{Var}(y_i)}}{\sum \frac{x_i^2}{\mathrm{Var}(y_i)}}$$

$$= \frac{\sum \frac{y_i}{x_i}}{n}$$

This is the "(arithmetic) mean of the ratios"

EXERCISES

6.1 For each type of assays, comparative and dilution, how do we test for null hypothesis H_0: $\rho=1$? And what does the answer imply?

6.2 The following table on the left gives the doses (cc per 100g of body weight) obtained from two groups of mice for two preparations of insulin, labeled as A and B. Estimate the relative potency (treating A as standard) and interpret the result, including testing for homodasticity.

Standard(A)	Test(B)
2.4	5.2
1.9	8
2	4.8
2.3	6.5
1.7	7
	8.1
	6

C	A	B
21	18	35.5
26	13	39
19	13.5	38.5
16	11.5	37
22	15	34

6.3 For the data in exercise 2.2, find the ratio of standard deviation estimator. How do we find the 95% confidence interval for relative potency using this estimator?

6.4 The table on the right provide the data from three preparations; preparation C is the standard and A and B were compared with C, (a) Estimate the relative potency of A to C and B to C, including testing for homodasticity, (b) Is there any difference between A and B relative to C? Should we compare the estimates?