⟨111⟩ DESIGN AND ANALYSIS OF BIOLOGICAL ASSAYS

General

The potency of several Pharmacopeial drugs must be determined by bioassay. A controlling factor in assay design and analysis is the variability of the biological test system, which may vary in its mean response from one laboratory to another, and from time to time in the same laboratory. To control this type of variation, the response to a Pharmacopeial drug is compared with a USP Reference Standard or other suitable standard. For convenience, each such preparation will be called the "Standard" and each preparation under assay, or Sample, the "Unknown," and these will be designated respectively by the symbols S and U. (The Sample is sometimes referred to as the "test preparation.")

After elimination of extraneous variables from the comparison of the Standard and the Unknown, an error variance is computed from the remaining variation, which, while uncontrolled, can nevertheless be measured. The error variance is required in calculating the confidence interval of the assayed potency. The confidence interval, known also as the fiducial interval, is so computed that its upper and lower limits are expected to enclose the true potency of the Unknown in 19 out of 20 assays. Many assay procedures fix the acceptable width of the confidence interval, and two or more independent assays may be needed to meet the specified limit. The confidence limits of the individual component assays usually overlap.

The aim of this chapter is to present a concise account of biometrical procedures for the USP bioassays. Its various sections are interrelated. Although the procedures are planned primarily for the assay of a single Unknown, equations for the joint assay of several Unknowns are given in context throughout the chapter and are summarized in the last section. Proof that an assayed potency meets its required confidence limits may be based also upon other recognized biometric methods that have a precision equivalent to that of the methods outlined herein.

A glossary of the terms used in the equations is provided at the end of this chapter.

Steps Preceding the Calculation of Potency

Designs for Minimizing the Error Variance—Variation in response is reduced as much as is practicable by the limitations imposed on body weight, age, previous handling, environment, and similar factors. In a number of assays, the test animals or their equivalent are then assigned at random but in equal numbers to the different doses of the Standard and Unknown. This implies an objective random process, such as throwing dice, shuffling cards, or using a table of random numbers. Assigning the same number of individuals to each treatment simplifies the subsequent calculations materially, and usually leads to the shortest confidence interval for a given number of observations.

In some assays, the potential responses can be assembled into homogeneous sets in advance of treatment. The differences between sets are later segregated, so that they do not affect adversely either the computed potency or its confidence interval. One unit within each set, picked at random, receives each treatment. Examples of randomized sets are the cleared areas on a single plate in the plate assay of an antibiotic, and four successive paired readings in the same rat in the Vasopressin Injection assay. Sets of two occur where each test animal is used twice, as in the assays of Tubocurarine Chloride Injection and Insulin Injection. In these cases, neither the average differences between individuals nor the order of treatment can bias the potency or precision. In the microbial assays for vitamin B_{12} activity and for calcium pantothenate, replicate tubes are assigned to two or more separate, complete sets, preferably with the tubes arranged at random within each set. This restricts the variation due to position or order within a set to the differences within each complete replicate.

Rejection of Outlying or Aberrant Observations—A response that is questionable because of failure to comply with the procedure during the course of an assay is rejected. Other aberrant values may be discovered only after the responses have been tabulated, but can then be traced to assay irregularities, which justify their omission. The arbitrary rejection *or* retention of an apparently aberrant response can be a serious source of bias. In general, the rejection of observations solely on the basis of their relative magnitudes is a procedure to be used sparingly. When this is unavoidable, each suspected aberrant response or outlier may be tested against one of two criteria:

1. The first criterion is based upon the variation within a single group of supposedly equivalent responses. On the average, it will reject a valid observation once in 25 or once in 50 trials, provided that relatively few, if any, responses within the group are identical. Beginning with the supposedly erratic value or outlier, designate the responses in order of magnitude from y_1 to y_N, where N is the number of observations in the group. Compute the relative gap $G_1 = (y_2 - y_1)/(y_N - y_1)$ when N = 3 to 7, $G_2 = (y_3 - y_1)/(y_{N-1} - y_1)$ when N = 8 to 13, or $G_3 = (y_3 - y_1)/(y_{N-2} - y_1)$ when N = 14 to 24. If G_1, G_2, or G_3 exceeds the critical value in *Table 1* for the observed N, there is a statistical basis for omitting the outlier.

This criterion is applicable also in a microbial assay where each treatment is represented by a transmittance in each of two separate complete sets. Subtract each transmittance in the first set from its paired value in the second set, and record each difference with its sign, either plus or minus. Beginning with the most divergent difference, designate the N differences in order of magnitude from y_1 to y_N and compute the relative gap G_1, G_2, or G_3. If this exceeds its critical value in *Table 1*, one of the two transmittances giving the aberrant difference is suspect and may be identified on inspection or by comparison with its expectation (see next column). Repeat the process with the remaining differences if an outlier is suspected in a second pair.

2. The second criterion compares the ranges from a series of k = 2 or more groups. Different groups may receive different treatments, but all f responses within each group represent the same treatment. Compute the range from each group by subtracting the smallest response from the largest within each of the k groups. Divide the largest of the k ranges by the sum of all the ranges in the series. Refer this ratio R* to *Table 2*. If k is not larger than 10, use the tabular values in the upper part of *Table 2*; if k is larger than 10, multiply R* by (k + 2) and interpolate, if necessary, between the tabular values in the lower part of *Table 2*. If R* exceeds the tabular or interpolated value, the group with the largest range is suspect and inspection of its components will usually identify the observation, which is then assumed to be aberrant or an outlier. The process may be repeated with the remaining ranges if an outlier is suspected in a second group.

Official from May 1, 2012
Copyright (c) 2011 The United States Pharmacopeial Convention. All rights reserved.

Table 1

Test for outliers. In samples from a normal population, gaps equal to or larger than the following values of G_1, G_2, and G_3 occur with a probability P = 0.02 where outliers can occur only at one end, or with P = 0.04 where they may occur at either end.											
N	3	4	5	6	7						
G_1	.976	.846	.729	.644	.586						
N	8	9	10	11	12	13					
G_2	.780	.725	.678	.638	.605	.578					
N	14	15	16	17	18	19	20	21	22	23	24
G_3	.602	.579	.559	.542	.527	.514	.502	.491	.481	.472	.464

Table 2

Test for groups containing outliers. Compute the range from the f observations in each of k groups, where all groups in the series are equal in size. The observed ratio R* of the largest range to the sum of the k ranges will equal or exceed the following critical values at a probability of P = 0.05.									
No. of Ranges k	**Critical R* for Ranges Each from f Observations**								
	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
2	0.962	0.862	0.803	0.764	0.736	0.717	0.702	0.691	0.682
3	.813	.667	.601	.563	.539	.521	.507	.498	.489
4	.681	.538	.479	.446	.425	.410	.398	.389	.382
5	.581	.451	.398	.369	.351	.338	.328	.320	.314
6	0.508	0.389	0.342	0.316	0.300	0.288	0.280	0.273	0.267
7	.451	.342	.300	.278	.263	.253	.245	.239	.234
8	.407	.305	.267	.248	.234	.225	.218	.213	.208
9	.369	.276	.241	.224	.211	.203	.197	.192	.188
10	.339	.253	.220	.204	.193	.185	.179	.174	.172

No. of Ranges k	**Critical (k + 2)R* for Ranges Each from f Observations**								
	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
10	4.06	3.04	2.65	2.44	2.30	2.21	2.14	2.09	2.05
12	4.06	3.03	2.63	2.42	2.29	2.20	2.13	2.07	2.04
15	4.06	3.02	2.62	2.41	2.28	2.18	2.12	2.06	2.02
20	4.13	3.03	2.62	2.41	2.28	2.18	2.11	2.05	2.01
50	4.26	3.11	2.67	2.44	2.29	2.19	2.11	2.06	2.01

Replacement of Missing Values—As directed in the monographs and in this section, the calculation of potency and its confidence interval from the total response for each dose of each preparation requires the same number of observations in each total. When observations are lost or additional responses have been obtained with the Standard, the balance may be restored by one of the following procedures, so that the usual equations apply.

1. Reduce the number of observations in the larger groups until the number of responses is the same for each treatment. If animals have been assigned at random to each treatment group, either omit one or more responses, selected at random, from each larger group, or subtract the mean of each larger group from its initial total as often as may be necessary. The latter technique is preferred when extra animals have been assigned deliberately to the Standard. When the assay consists of randomized sets, retain only the complete sets.

2. Alternatively, an occasional smaller group may be brought up to size when the number of missing responses is not more than one in any one treatment or 10% in the entire assay. Estimate a replacement for each missing value by either *Method a* or *Method b*. One degree of freedom (n) is lost from the error variance s^2 for each replacement by either method, except in a microbial assay where each response is based on the sum of two or more transmittances and only one transmittance is replaced.

(a) If animals have been assigned to treatments at random, add the mean of the remaining responses in the incomplete group to their total. In a microbial assay, when one of two transmittances is missing for a given treatment, add the mean difference between sets, computed from all complete pairs, to the remaining transmittance to obtain the replacement.

(b) If the assay consists of randomized sets, replace the missing value by

$$y' = \frac{fT_r' + kT_t' - T'}{(f-1)(k-1)}, \qquad (1)$$

where f is the number of sets, k is the number of treatments or doses, and T_r', T_t', and T' are the incomplete totals for the randomized set, treatment, and assay from which an observation is missing.

If the assay consists of n′ Latin squares with k rows in common, replace a missing value by

$$y' = \frac{k(n'T_c' + T_r' + T_t') - 2T'}{(k-1)(n'k-2)} \qquad (1a)$$

where n′ is the number of Latin squares with k rows in common, k is the number of treatments or doses, and T_c', T_r', T_t', and T' are respectively the incomplete totals for the column, row, treatment, and assay from which an observation is missing.

If more than one value is missing, substitute the treatment mean temporarily in all but one of the empty places, and compute y′ for the other by *Equation 1*. Replace each of the initial substitutions in turn by *Equation 1*, and repeat the process in successive approximations until a stable y′ is obtained for each missing observation.

Official from May 1, 2012
Copyright (c) 2011 The United States Pharmacopeial Convention. All rights reserved.

Table 3

Probits (normal deviates + 5) corresponding to percentages in the margins.										
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
0	—	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

Calculation of Potency from a Single Assay

Directions for calculating potency from the data of a single assay are given in the individual monographs. In those assays that specify graphical interpolation from dosage-response curves but that meet the conditions for assay validity set forth herein, potency may be computed alternatively by the appropriate method in this section.

Planning the assay involves assigning to the Unknown an assumed potency, to permit administering it in dosages equivalent to those of the Standard. The closer the agreement between this original assumption and the result of the assay, the more precise is the calculated potency. The ratio of a given dose of the Standard, in µg or in USP Units, to the corresponding dose of the Unknown, measured as specified in the monograph, is designated uniformly by R. The log-relative potency in quantities assumed initially to equal those of the Standard is designated as M′.

Ideally, M′ should not differ significantly from zero. The log-potency is equation 2

$$M = M' + \log R \qquad (2)$$

or

$$\text{Potency} = P_* = \text{antilog } M = (\text{antilog } M')R$$

Assay from Direct Determinations of the Threshold Dose—Tubocurarine Chloride Injection and Metocurarine Iodide are assayed from the threshold dose that just produces a characteristic biological response. The ratio of the mean threshold dose for the Standard to that for the Unknown gives the potency directly. The threshold dose is determined twice in each animal, once with the Standard and once with the Unknown. Each dose is converted to its logarithm, the difference (x) between the two log-doses is determined for each animal, and potency is calculated from the average of these differences.

In the *Bacterial Endotoxins Test* ⟨85⟩, the geometric mean dilution endpoint for the Unknown corresponding to the geometric mean dilution endpoint for the Standard (multiplied by a dilution factor, where applicable) gives the concentration of endotoxin in the test material.

In these assays, the confidence interval depends upon the variability in the threshold dose.

Indirect Assays from the Relationship between the Log-Dose and the Response—Generally, the threshold dose cannot be measured directly; therefore, potency is determined indirectly by comparing the responses following known doses of the Standard with the responses following one or more similar doses of the Unknown. Within a restricted dosage range, a suitable measure of the response usually can be plotted as a straight line against the log-dose, a condition that simplifies the calculation of potency and its confidence interval. Both the slope and position of the log-dose response relationship are determined in each assay by the use of two or more levels of the Standard, or, preferably, of both the Standard and the Unknown.

In the assay of Heparin Sodium, the interval between the dose at which clotting occurs and that which produces no clotting is so small that the dosage-response curve is not determined explicitly. Moving averages are used instead to interpolate the log-dose corresponding to 50% clotting for both the Standard and the Unknown, leading to the log-potency (see *Calculation* under *Heparin Sodium*). The precision of the potency is estimated from the agreement between independent assays of the same Unknown.

For a drug that is assayed biologically, the response should plot as a straight line against the log-dose over an adequate range of doses. Where a preliminary test is required or the assay depends upon interpolation from a multi-dose Standard curve, plot on coordinate paper the mean response of the Standard at each dosage level on the ordinate against the log-dose x on the abscissa. If the trend is basically linear over the required dosage range, the initial response unit may be used directly as y; if, instead, the trend is clearly curvilinear, a suitable transformation of each initial reading may bring linearity.

One possible transformation is to logarithms; another, in microbial tube assays, where y = (100 – % transmittance) does not plot linearly against the log-dose x, is to probits. In this case, if absorbance cannot be read directly, the percent transmittance for each tube or test solution is first converted to absorbance, A = 2 – log(% transmittance). Each absorbance value, in turn, is converted to % reduction in bacterial growth as

$$\%\ \text{reduction} = 100(\overline{A}_C - A)/\overline{A}_C$$

where $\overline{A}_c$ is the mean density for the control tubes (without antibiotic or with excess of vitamin) in the same set or tube rack. Percent reduction is then transformed to a probit (see *Table 3*) to obtain a new y for all later calculation. The probit transformation offers the advantage of extending the working range of linearity even where a portion of the dosage-response relationship is nonlinear in the original units of percent transmittance, provided that the incubation period does not extend beyond the logarithmic phase of growth of the control tubes.

The LD_{50} in the *Safety* test for *Iron Dextran Injection* is calculated with log-doses and probits. The four doses of the Injection, in mg of iron per kg of body weight, are transformed to $x_1 = 2.574$, $x_2 = 2.699$, $x_3 = 2.875$, and $x_4 = 3.000$. The probits corresponding to the number of deaths observed in each group of 10 mice are designated y_1, y_2, y_3, and y_4, respectively, and are given in *Table 3* for mortalities from 10 to 90 percent. For observed deaths of 0 and 10 adjacent to doses giving an intermediate mortality, use the approximate probits 3.02 and 6.98, respectively; omit the

Official from May 1, 2012
Copyright (c) 2011 The United States Pharmacopeial Convention. All rights reserved.

Table 4

Coefficients x_* for computing the responses y_L and y_H predicted by least squares at the lowest and highest of k log-doses when these are spaced at equal intervals.								
No. of Doses	**Predicted End y**	**Coefficient x_* for Mean Response $\bar{y}_t$ at Log-Dose**						**Divisor**
		1	**2**	**3**	**4**	**5**	**6**	
3	y_L	5	2	–1				6
	y_H	–1	2	5				6
4	y_L	7	4	1	–2			10
	y_H	–2	1	4	7			10
5	y_L	3	2	1	0	–1		5
	y_H	–1	0	1	2	3		5
6	y_L	11	8	5	2	–1	–4	21
	y_H	–4	–1	2	5	8	11	21

Table 5

Coefficients x_1 for computing the slope b of a log-dose response curve when the doses are spaced on an arithmetic scale as shown.								
	Coefficients x_1 for Computing b from the Responses y at Doses, in mL, of							
No. of Doses	**1**	**1.5**	**2**	**3**	**4**	**5**	**Divisor $e_b'i$**	**Mean Log-Dose $\bar{x}$**
4	—	–29	–12	12	29	—	14.4663	0.38908
5	–34	—	–9	5	15	23	24.7827	0.41584
5	—	–20	–11	2	11	18	13.3249	0.45105
6	–15	–8	–3	4	9	13	14.1017	0.37588

end value (at x_1 or x_4) if not adjacent to an intermediate mortality. Since the information in a probit varies with its expectation, assign each probit an approximate relative weight w for computing the LD_{50} of the Injection, as shown in the accompanying table.

No. of Deaths	0 or 10	1 or 9	2 or 8	3 or 7	4 to 6
Weight, w	0.3	0.7	1.0	1.2	1.3

Calculate the weighted means

$$\bar{x} = \Sigma(wx)/\Sigma w$$

and (2a)

$$\bar{y} = \Sigma(wy)/\Sigma w$$

from the sum of the weights, Σw, of the four (or three) acceptable responses and the corresponding weighted sums of the log-doses, Σ(wx), and of the probits, Σ(wy). From the sums of the weighted products, Σ(wxy), and of the weighted squares, $\Sigma(wx^2)$, compute the slope b of the log-dose-probit line as

$$b = [\Sigma(wxy) - \bar{x}\Sigma(wy)]/[\Sigma(wx^2) - \bar{x}\Sigma(wx)] \qquad (2b)$$

The LD_{50} for this safety test, in mg of iron per kg of body weight, is calculated as

$$LD_{50} = \text{antilog}[\bar{x} + (5 - \bar{y})/b] \qquad (2c)$$

In quantal assays not included in this Pharmacopeia, such as the mouse assay for insulin, the calculation with probits involves other adjustments that are omitted here.

When the mean response $\bar{y}_t$ for each dose of Standard plots linearly against the log-dose, and the k doses are spaced at equal intervals on the logarithmic scale, the predicted responses (Y_L and Y_H) at the extreme ends of the line of best fit can be computed directly with the coefficients x_* in *Table 4*, which correspond to the k successive log-doses, as

$$Y_L = \Sigma(x_*\bar{y}_t)/\text{divisor}$$

and (3)

$$Y_H = \Sigma(x_*\bar{y}_t)/\text{divisor}$$

where Σ stands uniformly for "the sum of" the values that follow it. When Y_L and Y_H are plotted against the low and high log-doses, X_L and X_H, respectively, they may be connected by a straight line with the slope

$$b = (Y_H - Y_L)/(X_H - X_L) \qquad (4)$$

At any selected log-dose x of *Standard*, the predicted response is

$$Y = \bar{y} + b(x - \bar{x}) \qquad (5)$$

where $\bar{x} = \Sigma x/k$, and $\bar{y} = (Y_L + Y_H)/2$, or, for predictions within a set, $\bar{y}$ is the mean response for the Standard within the set.

When the log-dose response relationship is linear, but the k doses (expressed in mL) are spaced substantially in an arithmetic sequence as in *Table 5* (which refers to the microbial assays set forth under *Antibiotics—Microbial Assays* ⟨81⟩), the slope b of the straight line of best fit may be computed with the terms in *Table 5* and the mean response at each dose $\bar{y}_t$, or $T_t = f\bar{y}_t$ where the number of y's(f) is constant at each dose, as

$$b = \Sigma(x_1\bar{y}_t)/e_b'i = \Sigma(x_1T_t)/fe_b'i \qquad (6)$$

The coefficients x_1 are convenient multiples of the differences $(x - \bar{x})$ about the mean log-dose $\bar{x}$, and $e_b'i$ is the corresponding multiple of $\Sigma(x - \bar{x})^2$. The predicted response Y at a given log-dose x may be computed by substitution of the assay slope b in *Equation 5* and of the mean $\bar{y}$ either of all the responses on the Standard in the entire assay or of those for each set separately.

Official from May 1, 2012
Copyright (c) 2011 The United States Pharmacopeial Convention. All rights reserved.

Table 6

Factorial coefficients x_1 for analyzing a balanced bioassay, in which successive log-doses of Standard (S_i) and of Unknown (U_i) are spaced equally, each with the same number (f) of responses totaling T_i.											
		Factorial Coefficients x_1 for Each Dose									
Design	**Row**	S_1	S_2	S_3	S_4	U_1	U_2	U_3	U_4	e_t	T_i
2,2	a	–1	–1			1	1			4	T_a
	b	–1	1			–1	1			4	T_b
	ab	1	–1			–1	1			4	T_{ab}
3,3	a	–1	–1	–1		1	1	1		6	T_a
	b	–1	0	1		–1	0	1		4	T_b
	ab	1	0	–1		–1	0	1		4	T_{ab}
	q	1	–2	1		1	–2	1		12	T_q
	aq	–1	2	–1		1	–2	1		12	T_{aq}
4,4	a	–1	–1	–1	–1	1	1	1	1	8	T_a
	b	–3	–1	1	3	–3	–1	1	3	40	T_b
	ab	3	1	–1	–3	–3	–1	1	3	40	T_{ab}
	q	1	–1	–1	1	1	–1	–1	1	8	T_q
	aq	–1	1	1	–1	1	–1	–1	1	8	T_{aq}

			Value of Constant for Design		
For Computing	**Equation No.**	**Constant**	**2,2**	**3,3**	**4,4**
M′	8, 10	c	1	4/3	5
L	26, 29	c′	1	8/3	5

POTENCIES INTERPOLATED FROM A STANDARD CURVE—Where the log-dose response curve of the Standard in a given assay is curvilinear and is fitted graphically to the plotted points, the amount of Standard that would be expected to produce each observed response y of an *Unknown* is estimated by interpolation from the curve and then adjusted for the known concentration of its test solution.

When the response to the Standard can be plotted linearly against the log-dose, it is fitted numerically by a straight line, as described in the preceding section. For assays in randomized sets, a standard curve is computed with b for the assay and $\bar{y}$ for each set and the response y_U in each tube of a given *Unknown* in that set is converted to an estimated log-relative potency,

$$X = (y_U - Y_S)/b \qquad (7)$$

where Y_S is the response predicted by the standard curve at the assumed log-dose x of the *Unknown.* The average of the separate estimates from each of f sets, $M' = \Sigma X/f$, is the assayed log-relative potency of the *Unknown.*

Factorial Assays from the Response to Each Treatment—When some function of the response can be plotted linearly against the log-dose, the assayed potency is computed from the total response for each treatment, and its precision is measured in terms of confidence intervals. This requires that (1) in suitable units the response (y) depends linearly upon the log-dose within the dosage range of the assay, and (2) the number (f) of responses be the same at each dosage level of both Standard and Unknown. The y's are totaled at each dosage level of each preparation. In different combinations, these totals, T_t, lead directly to the log-relative potency and to tests of assay validity. The factorial coefficients in *Tables 6*, *7*, and *8* determine how they are combined. In a given row, each T_t is multiplied by the corresponding coefficient and the products summed to obtain T_i. The T_i's in the successive rows carry the same meaning in all assays.

T_a in the first row measures the difference in the average response to the Standard and to the Unknown. T_b in the second row leads directly to the combined slope of the dosage-response curves for both Standard and Unknown. The third to the fifth rows (ab, q, and aq) provide tests for the validity of an assay, as described in a later section. From the totals T_a and T_b, compute the log-relative potency of the Unknown, before adjustment for its assumed potency, as

$$M' = ciT_a/T_b \qquad (8)$$

where i is the interval in logarithms between successive log-doses of both the Standard and the Unknown, and the constant c is given separately at the bottom of each table. Each M′ is corrected to its log-potency M by *Equation 2*.

When doses are spaced unequally on a log scale, as in *Table 8*, use instead the constant ci at the bottom of the table.

In a fully balanced assay, such as the assay for corticotropin, compute *M*′ with the coefficients in *Table 6*. If one preparation has one less dose than the other but the successive log-doses of both Standard and Unknown differ by a constant interval i, use the factorial coefficients in *Table 7*, correcting for the actual difference between the observed mean log-doses, $\bar{x}_S$ and $\bar{x}_U$, by computing

$$M = \bar{x}_S - \bar{x}_U + M' \qquad (9)$$

In assays where the successive doses are not spaced at equal log-intervals, the log-relative potency of a single Unknown may be computed by *Equation 8* with the factorial coefficients and ci in *Table 8*.

In an assay of two or more Unknowns against a common Standard, all with dosage-response lines that are parallel within the experimental error, each log-relative potency may be computed with the same assay slope as follows. For each preparation, determine the slope factor $T_b' = \Sigma(x_1T_t)$ or $\Sigma(x_1y)$, where the values of x_1 are the factorial coefficients for the Standard in the appropriate row b of *Table 6* or *8*. The log-relative potency of each Unknown is

$$M' = cih'T_a/2\Sigma T_b' \qquad (10)$$

where h′ is the number of values of T_b' summed in the denominator.

Assays from Differences in Response—When doses of the Standard and Unknown are paired and the difference in response is computed for each pair, these differences are not affected by variations in the average sensitivity of the paired readings. The paired 2-dose insulin assay corresponds

Official from May 1, 2012
Copyright (c) 2011 The United States Pharmacopeial Convention. All rights reserved.

Table 7

Factorial coefficients x_1 for analyzing a partially balanced assay, in which successive log-doses of Standard (S_i) and of Unknown (U_i) are spaced equally, each with the same number (f) of responses totaling T_t. If the number of successive doses of the Unknown exceeds by one the number on the Standard, interchange S_i and U_i in the heading and reverse all signs in rows a, ab, and aq.										
		Factorial Coefficients x_1 for Each Dose								
Design	**Row**	S_1	S_2	S_3	S_4	U_1	U_2	U_3	e_i	T_i
2,1	a	–1	–1			2			6	T_a
	b	–1	1			0			2	T_b
3,2	a	–2	–2	–2		3	3		30	T_a
	b	–2	0	2		–1	1		10	T_b
	ab	1	0	–1		–2	2		10	T_{ab}
	q	1	–2	1		0	0		6	T_q
4,3	a	–3	–3	–3	–3	4	4	4	84	T_a
	b	–3	–1	1	3	–2	0	2	28	T_b
	ab	3	1	–1	–3	–5	0	5	70	T_{ab}
	q	3	–3	–3	3	2	–4	2	60	T_q
	aq	–1	1	1	–1	1	–2	1	10	T_{aq}

			Value of Constant for Design		
For Computing	**Equation No.**	**Constant**	**2,1**	**3,2**	**4,3**
M′	8, 10	c	1/2	5/6	7/6
L	26, 29	c′	3/4	25/12	49/12

Table 8

Factorial coefficients x_1 for analyzing assays with a 3- or 4-dose sequence of 1.5, 2.0, 3.0, and 4.0, each dose having the same number (f) of responses.											
		Dose of Standard				Dose of Unknown					
Design	**Row**	**1.5**	**2.0**	**3.0**	**4.0**	**1.5**	**2.0**	**3.0**	**4.0**	e_i	T_i
4,4	a	–1	–1	–1	–1	1	1	1	1	8	T_a
	b	–29	–12	12	29	–29	–12	12	29	3940	T_b
	ab	29	12	–12	–29	–29	–12	12	29	3940	T_{ab}
	q	1	–1	–1	1	1	–1	–1	1	8	T_q
	aq	–1	1	1	–1	1	–1	–1	1	8	T_{aq}
3,3	a	–1	–1	–1		1	1	1		6	T_a
	b	–25	–3	28		–25	–3	28		2836	T_b
	ab	25	3	–28		–25	–3	28		2836	T_{ab}
	q	31	–53	22		31	–53	22		8508	T_q
	aq	–31	53	–22		31	–53	22		8508	T_{aq}
3,3	a		–1	–1	–1		1	1	1	6	T_a
	b		–28	3	25		–28	3	25	2836	T_b
	ab		28	–3	–25		–28	3	25	2836	T_{ab}
	q		22	–53	31		22	–53	31	8508	T_q
	aq		–22	53	–31		22	–53	31	8508	T_{aq}

			Value of Constant for Design	
For Computing	**Equation No.**	**Constant**	**4,4**	**3,3**
M′	8, 10	ci	7.2332	5.3695
L	26, 29	c′i2	0.10623	0.06100

to the first design in *Table 6*, and requires four equal groups of rabbits each injected twice (see *Insulin Assays* ⟨121⟩). The difference (y) in the blood sugar response of each rabbit to the two treatments leads to the log-relative potency M′ (see the first two paragraphs of the section, *Calculation of Potency from a Single Assay*). The Vasopressin Injection assay follows a similar design, substituting two or more randomized sets of four successive pairs of injections into rats for the four treatment groups of rabbits in the insulin assay.

Oxytocin Injection is assayed from blood pressure changes in a single test animal following alternating injections of a single dose of Standard and of one of two doses of the Unknown. The calculation of potency from the differences in the response of the Unknown and to the average of the two adjacent responses to the Standard is equivalent to the first design in *Table 7* with S and U reversed, where i is the log-interval between the two dosage levels of the Unknown.

Experimental Error and Tests of Assay Validity

As the term is used here, "experimental error" refers to the residual variation in the response of biological indicators, not to a mistake in procedure or to an outlier that needs

Official from May 1, 2012
Copyright (c) 2011 The United States Pharmacopeial Convention. All rights reserved.

replacement. It is measured in terms of the error variance of a single response or other unit, which is designated uniformly as s^2, despite differences in the definition of the unit. It is required in tests of assay validity and in computing the *confidence interval.*

Error Variance of a Threshold Dose—The individual threshold dose is measured directly in some assays. In a Digitalis assay, designate each individual threshold dose by the symbol z, the number or frequency of z's by f, and the total of the z's for each preparation by T, with subscripts S and U for Standard and Unknown, respectively. Compute the error variance of z as

$$s^2 = [\Sigma z^2 - T_S^2/f_S - T_U^2/f_U]/n \qquad (11)$$

with $n = f_S + f_U - 2$ degrees of freedom. In the assay of Tubocurarine Chloride Injection, each log-threshold dose of the Unknown is subtracted from the corresponding log-dose of the Standard in the same rabbit to obtain an individual difference x. Since each x may be either positive or negative (+ or –), it is essential to carry the correct sign in all sums. Designate the total of the x's for the animals injected with the Standard on the first day as T_1, and for those injected with the Standard on the second day as T_2. Compute the error variance of x with $n = N - 2$ degrees of freedom as

$$s^2 = \{\Sigma x^2 - (T_1^2 + T_2^2)/f\}/n \qquad (12)$$

where N is the total number of rabbits that complete the assay, excluding any replacement for a missing value to equalize the size of the two groups.

Error Variance of an Individual Response—In the Pharmacopeial assays, differences in dose that modify the mean response are assumed not to affect the variability in the response. The calculation of the error variance depends upon the design of the assay and the form of the adjustment for any missing values. Each response is first converted to the unit y used in computing the potency. Determine a single error variance from the combined deviations of the y's around their respective means for each dosage level, summed over all levels. Doubtful values of y may be tested as described under *Rejection of Outlying or Aberrant Observations*, and proved outliers may be replaced as missing values (see *Replacement of Missing Values*).

In the simplest design, the units of response are assigned at random to each dosage level, as in the assay for corticotropin. If a missing value is replaced by adding the mean of the remaining y's at any given dosage level to their total, the degrees of freedom (n) in the error variance are reduced by one for each replacement but no other change is needed in the calculation. Assuming that f is then the same for all doses or groups, compute the error variance from the variation within doses of all the y's as

$$s^2 = \{\Sigma y^2 - \Sigma T_t^2/f\}/n \qquad (13)$$

where T_t is the total at each dose of the f values of y, there are k totals T_t and the degrees of freedom $n = \Sigma f - k$, with Σf diminished by 1 for each replacement.

If variations in f are adjusted by subtracting a group mean from its group total, compute the error variance from the observed y's and the *unadjusted* totals T_t as

$$s^2 = \{\Sigma y^2 - \Sigma(T_t^2/f)\}/n \qquad (14)$$

where $n = \Sigma f - k$.

In the calculation of the result of an assay using the coefficients of *Table 6* or *8*, s^2 may be computed from the response y for each of the h′ preparations, including the h Unknowns and the corresponding dosage levels of the Standard. For each preparation, compute $T' = \Sigma y$ and the slope factor $T_b' = \Sigma(x_1 y)$ where the values of x_1 are the factorial coefficients for the Standard in the appropriate row b of *Table 6* or *8*. The error variance for the assay is

$$s^2 = \{\Sigma y^2 - \Sigma T'^2/k - 2(\Sigma T_b')^2/h' e_b f\}/n \qquad (15)$$

where the degrees of freedom $n = h'(k - 1) - 1$, and e_b is the e_i from the same table and row as the coefficients x_1.

The Error Variance in Restricted Designs—In some assays, the individual responses occur in randomized sets of three or more. Examples of sets are litter mates in the assay of vitamin D, the cleared areas within each plate in an antibiotic assay, and the responses following four successive pairs of injections in the vasopressin assay. Arrange the individual y's from these assays in a 2-way table, in which each column represents a different treatment or dose and each row a randomized set. Losses may be replaced as described under *Replacement of Missing Values.* The k column totals are the T_t's required for the analysis of balanced designs. The f row totals (T_r) represent a source of variation that does not affect the estimated potency and hence is excluded from the assay error. Compute the approximate error variance from the squares of the individual y's and of the marginal totals as

$$s^2 = \{\Sigma y^2 - \Sigma T_r^2/k - \Sigma T_t^2/f + T^2/N\}/n \qquad (16)$$

where $T = \Sigma T_r = \Sigma T_t$, and the $n = (k - 1)(f - 1)$ degrees of freedom must be diminished by one for any gap in the original table that has been filled by computation.

When the order of treatment is an additional potential source of variation, its effect can be corrected by the dose regimen for a series of n′ Latin squares with k rows in common, such as that for the two Latin squares in the dose regimens 1 to 4 and 5 to 8 in the assay of Glucagon for Injection. List the observed responses y of each test animal in a separate column in the order of dosing. The responses to each of the k doses then occur equally often in each of the k rows and of the n′k columns, where n′ is the number of Latin squares. Total the responses y in each row (T_r) in each column (T_c), and, in a separate listing, for each dose or treatment (T_t). An occasional lost reading may be replaced by *Equation 1a* as described under *Replacement of Missing Values.* Compute the error variance from the squares of the individual y's and of the marginal and treatment totals as

$$s^2 = \{\Sigma y^2 - \Sigma T_r^2/n'k - \Sigma T_c^2/k - \Sigma T_t^2/n'k + 2T^2/N\}/n \qquad (16a)$$

where $T = \Sigma y = \Sigma T_r = \Sigma T_c = \Sigma T_t$, $N = n'k^2$, and the $n = (k - 1)(n'k - 2)$ degrees of freedom must be diminished by one for any gap in the original table that has been filled by computation.

In assays where the reactions occur in pairs, the differences between test animals or paired reactions are segregated automatically by calculating the assay with the difference within a pair as the response. With insulin, the response is the difference y in the blood sugar of a single rabbit following two injections (see *Insulin Assay* ⟨121⟩). After adjustment for rabbits lost during the assay, compute the error variance of y from the responses in all four groups and from the group totals $T_i = T_1$ to T_4 as

$$s^2 = \{\Sigma y^2 - \Sigma T_i^2/f\}/n \qquad (17)$$

where the number of rabbits f is the same in each group and the degrees of freedom, $n = 4(f - 1)$, are reduced by one for each replacement of a rabbit lost during the assay. In the Oxytocin Injection assay, each y represents the difference between the blood pressure response to a dose of the

Official from May 1, 2012
Copyright (c) 2011 The United States Pharmacopeial Convention. All rights reserved.

Table 9

Values of t, t^2, F_i and χ^2 for different degrees of freedom n that will be exceeded with a probability P = 0.05 (or 0.95 for confidence intervals).†											
n	**t**	**$t^2 = F_1$**	**F_2**	**F_3**	**χ^2**	**n**	**t**	**$t^2 = F_1$**	**F_2**	**F_3**	**χ^2**
1	12.706	161.45	—	—	3.84	19	2.093	4.381	3.52	3.13	30.1
2	4.303	18.51	19.00	19.16	5.99	20	2.086	4.351	3.49	3.10	31.4
3	3.182	10.128	9.55	9.28	7.82	21	2.080	4.325	3.47	3.07	32.7
4	2.776	7.709	6.94	6.59	9.49	22	2.074	4.301	3.44	3.05	33.9
5	2.571	6.608	5.79	5.41	11.07	23	2.069	4.279	3.42	3.03	35.2
6	2.447	5.987	5.14	4.76	12.59	24	2.064	4.260	3.40	3.01	36.4
7	2.365	5.591	4.74	4.35	14.07	25	2.060	4.242	3.38	2.99	37.7
8	2.306	5.318	4.46	4.07	15.51	26	2.056	4.225	3.37	2.98	38.9
9	2.262	5.117	4.26	3.86	16.92	27	2.052	4.210	3.35	2.96	40.1
10	2.228	4.965	4.10	3.71	18.31	28	2.048	4.196	3.34	2.95	41.3
11	2.201	4.844	3.98	3.59	19.68	29	2.045	4.183	3.33	2.93	42.6
12	2.179	4.747	3.89	3.49	21.03	30	2.042	4.171	3.32	2.92	43.8
13	2.160	4.667	3.81	3.41	22.36	40	2.021	4.085	3.23	2.84	55.8
14	2.145	4.600	3.74	3.34	23.68	60	2.000	4.001	3.15	2.76	79.1
15	2.131	4.543	3.68	3.29	25.00	120	1.980	3.920	3.07	2.68	146.6
16	2.120	4.494	3.63	3.24	26.30	∞	1.960	3.841	3.00	2.60	
17	2.110	4.451	3.59	3.20	27.59						
18	2.101	4.414	3.55	3.16	28.87						

†Adapted from portions of Tables III to V of "Statistical Tables for Biological, Agricultural and Medical Research," by R. A. Fisher and F. Yates, published by Oliver and Boyd, Ltd., Edinburgh.

Unknown and the average for the two adjacent doses of Standard. Compute the error variance of y as

$$s^2 = \{\Sigma y^2 - (T_1^2 + T_2^2)/f\}/n \qquad (18)$$

with $n = 2(f - 1)$ degrees of freedom, where T_1 is the total of the y's for the low dose of the Unknown and T_2 that for the high dose.

In a microbial assay calculated by interpolation from a standard curve, convert each difference between two paired responses to units of log-dose, X, by the use of *Equation 7*. With each difference X as the unit, a composite s^2 is computed from the variation in the f values of X for each *Unknown*, totaled over the h *Unknowns* in the assay, as

$$s^2 = \{\Sigma X^2 - \Sigma(T_x^2/f)\}/n \qquad (19)$$

where $T_x = \Sigma X$ for a single *Unknown* and the degrees of freedom $n = \Sigma f - h$.

Tests of Assay Validity— In addition to the specific requirements in each monograph and a combined log-dose response curve with a significant slope (see the statistic C in the next section), two conditions determine the validity of an individual factorial assay: (1) the log-dose response curve for the Unknown must parallel that for the Standard within the experimental error, and (2) neither curve may depart significantly from a straight line. When the assay has been completely randomized or consists of randomized sets, the necessary tests are computed with the factorial coefficients for ab, q, and aq from *Tables 6* to *8* and the treatment totals T_t. Sum the products of the coefficients in each row by the corresponding T_t's to obtain the product total T_i, where the subscript i stands in turn for ab, q, and aq, respectively. Each of the three ratios, $T_i^2/e_i f$, is computed with the corresponding value of e_i from the table and with f equal to the number of y's in each T_t. That in row ab tests whether the dosage-response lines are parallel, and is the only test available in a 2-dose assay. With three or more doses of both preparations, that in row q is a test of combined curvature in the same direction, and in row aq of separate curvatures in opposite directions. If any ratio in a 3- or 4-dose assay exceeds s^2 as much as three-fold, compute

$$F_3 = \Sigma(T_i^2/e_i f)/3s^2 \qquad (20)$$

For a 2-dose assay, compute instead

$$F_1 = T_{ab}^2/e_{ab} f s^2 \qquad (21)$$

and for a 3,2 assay *(Table 7)* determine

$$F_2 = \Sigma(T_i^2/e_i f)/2s^2 \qquad (22)$$

For a valid assay, F_1, F_2, or F_3 does not exceed the value given in *Table 9* (at odds of 1 in 20) for the degrees of freedom n in s^2.

An assay may fail the test for validity and still provide a contributory estimate of potency that can be combined profitably with the result of a second assay of the same Unknown, as described in a later section. An end dosage level for either the Standard or the Unknown, or both, may fall outside the linear zone. With three or more dosage levels and relatively large values of T_a, T_{ab}, and T_{aq}, the total response T_t at an end dose of one preparation may approach an upper or lower limit and be responsible for the large values of T_{ab} and T_{aq}. This T_t may be omitted and the assay recomputed with the appropriate design in *Table 7*. If the assay then meets the test in *Equation 20*, or *22*, the resulting potency, M, may be combined with that of a second assay in computing the log-potency of the Unknown (see under *Combination of Independent Assays*). If T_a is not significant but T_q shows significant combined curvature, the largest (or smallest) dose of both preparations may be too large (or too small). Their omission may lead to a valid assay with the factorial coefficients for the next smaller design in *Table 6* or *8*. A statistically significant T_q or $\Sigma T_q'$ may be neglected and all dosage levels retained without biasing the

Official from May 1, 2012
Copyright (c) 2011 The United States Pharmacopeial Convention. All rights reserved.

computed log-potency M′ and its confidence interval by more than 5% when the following inequality is true:

$$T_b^2/e_b > 100T_q^2/e_q$$

or

$$(\Sigma T_b')^2/e_b > 100(\Sigma T_q')^2/e_q \qquad (23)$$

where each T_b' and T_q' is computed with the T_t's (or y's) for a single preparation multiplied by the coefficients for the Standard in rows b and q, respectively. If both T_a and T_{ab} are significant in a 2-dose assay, one T_t may be outside the linear zone. Sometimes a preliminary or contributory estimate of potency can be computed from the remaining three values of T_t and the first design in *Table 7*. In assays of insulin and of other drugs in which the responses are paired, the test for parallelism is so insensitive that it is omitted. If the tubes in each set are arranged systematically instead of at random in a microbial assay, the tests for validity may be subject to bias from positional effects.

The Confidence Interval and Limits of Potency

A bioassay provides an estimate of the true potency of an Unknown. This estimate falls within a confidence interval, which is computed so that the odds are not more than 1 in 20 (P = 0.05) that the true potency either exceeds the upper limit of the confidence interval or is less than its lower limit. Since this interval is determined by a number of factors that may influence the estimate of potency, the required precision for most bioassays is given in the monograph in terms of the confidence interval, related either to the potency directly or to its logarithm.

General Calculation—Despite their many forms, bioassays fall into two general categories: (1) those where the log-potency is computed directly from a mean or a mean difference, and (2) those where it is computed from the ratio of two statistics.

(1) When the log-potency of an assay is computed as the mean of several estimated log-potencies that are approximately equal in precision, the log-confidence interval is

$$L = 2st / \sqrt{k} \qquad (24)$$

where s is the standard deviation of a single estimated log-potency, t is read from *Table 9* with the n degrees of freedom in s, and k is the number of estimates that have been averaged. The same equation holds where the log-potency is computed as the mean $\bar{x}$ of k differences x, with s the standard deviation of a single x. In either case, the estimated log-potency M is in the center of its confidence interval, so that its confidence limits are

$$X_M = M + {}^1/_2L \text{ and } M - {}^1/_2L, \text{ or } X_M = M \pm {}^1/_2L \qquad (25)$$

The upper and lower limits are converted to their antilogarithms to obtain the limits as explicit potencies.

(2) More often, the log-potency or potency is computed from a ratio, and in these cases the length of the confidence interval is typified by the log-interval in the equation

$$L = 2\sqrt{(C-1)\left(CM'^2 + c'i^2\right)} \qquad (26)$$

where M′ is the log-relative potency as defined (see *Calculation of Potency from a Single Assay*), i is the log-interval between successive doses, and c′ is a constant characteristic of the assay procedure. The remaining term C depends upon the precision with which the slope of the dosage-response curve has been determined. (This is sometimes expressed in terms of g = (C − 1)/C.) In factorial assays, it is computed as

$$C = T_b^2/(T_b^2 - e_b f s^2 t^2) \qquad (27)$$

where s^2 is the error variance of a single observation, t^2 is read from *Table 9* with the degrees of freedom in s^2, f is the number of responses in each T_t used in calculating T_b, and T_b and e_b are computed with the factorial coefficients for row b in *Tables 6* to *8*. The s^2 in *Equation 26* depends upon the design of the assay, as indicated for each drug in the next section. In a valid assay, C is a positive number.

In an assay of two or more Unknowns against a common Standard, all with dosage-response curves that are parallel within the experimental error, C may be computed with the error variance s^2 for the assay and with the assay slope as

$$C = (\Sigma T_b')^2/\{(\Sigma T_b')^2 - e_b f h' s^2 t^2/2\} \qquad (28)$$

The slope factor $T_b' = \Sigma(x_1 T_t)$ or $\Sigma(x_1 y)$ for each of the h′ preparations, including the Standard, is computed with the factorial coefficients x_1 for the Standard in the appropriate row b of *Table 6* or *8*. If a treatment total T_t includes one or more replacements for a missing response, replace $e_b f$ in *Equation 27*, or $e_b fh'/2$ in *Equation 28*, by $f^2\Sigma(x_1^2/f')$, where each x_1 is a factorial coefficient in row b of *Tables 6* to *8*, in this chapter, and f′ is the number of responses in the corresponding T_t *before* adding the replacement. With this C, compute the confidence interval as

$$L = 2\sqrt{(C-1)\left(CM'^2 + c'i^2h'/2\right)} \qquad (29)$$

In assays computed from a ratio, the most probable log-potency M is not in the exact center of the confidence interval. The upper and lower confidence limits in logarithms are

$$X_M = \log R + CM' + {}^1/_2L \text{ and } \log R + CM' - {}^1/_2L \qquad (30)$$

C is often very little larger than unity, and the more precise the assay, the more nearly C approaches 1 exactly. $R = z_S/z_U$ is the ratio of corresponding doses of the Standard and of the Unknown or the assumed potency of the Unknown. The upper and lower confidence limits in log-potencies are converted separately to their antilogarithms to obtain the corresponding potencies.

Confidence Intervals for Individual Assays—Since the confidence interval may vary in detail from the above general patterns, compute it for each assay by the special directions given under the name of the substance in the paragraphs following.

Antibiotic Assays—The confidence interval may be computed by *Equations 24* and *25*.

Calcium Pantothenate—For log-potencies obtained by interpolation from the Standard curve, the confidence interval may be computed with *Equations 19* and *24*. For log-potencies calculated with *Equation 8* or *10*, s^2 may be computed with *Equation 15*, C with *Equation 27* or *28*, and the confidence interval L with *Equation 26* or *29*.

Corticotropin Injection—Compute the log confidence interval by *Equations 26* and *27*, with the coefficients and constants in *Table 6* for a 3-dose assay, and s^2 as determined by *Equation 13* or *14*.

Digitalis—Compute the confidence interval as

$$L = 2\sqrt{(C-1)\left\{C\left(\bar{z}_S/\bar{z}_U\right)^2 + f_U/f_s\right\}} \qquad (31)$$

Official from May 1, 2012
Copyright (c) 2011 The United States Pharmacopeial Convention. All rights reserved.

where f_U and f_S are the number of observations on the Unknown and on the Standard, and

$$C = \bar{z}_U^2/(\bar{z}_U^2 - s^2t^2/f_U) \qquad (32)$$

is determined with s^2 from *Equation 11*. The confidence limits for the potency in USP Units are then

$$X_{P*} = R\{C(\bar{z}_S/\bar{z}_U) \pm {}^1/_2L\} \qquad (33)$$

in which R is as defined in the *Glossary of Symbols.*

Glucagon for Injection—Compute the error variance s^2 by *Equation 15a*, C by *Equation 27* with $e_bf = 16n'$, and the log confidence interval L by *Equation 26* with $c'i^2 = 0.09062$.

Chorionic Gonadotropin—Proceed as directed under *Corticotropin Injection.*

Heparin Sodium—If two independent determinations of the log-potency M differ by more than 0.05, carry out additional assays and compute the error variance among the N values of M as

$$s^2 = \{\Sigma M^2 - (\Sigma M)^2/N\}/n \qquad (34)$$

with n = N – 1 degrees of freedom. Given this value, determine the confidence interval in logarithms (L) by *Equation 24*.

Insulin Injection—Compute the error variance (s^2) of y by *Equation 16* and C as

$$C = T_b^2/(T_b^2 - s^2t^2N) \qquad (35)$$

where t^2 from *Table 9* depends upon n = 4(f – 1) degrees of freedom in s^2 and N = 4f is the total number of differences in the four groups. By *Equation 26*, compute the confidence interval L in logarithms, where $c'i^2 = 0.09062$. The upper and lower confidence limits in USP Units of insulin are given by the antilogarithms of X_M from *Equation 30*.

Oxytocin Injection—Compute the approximate log confidence interval by *Equation 26*, in which

$$C = (T_2 - T_1)^2/\{(T_2 - T_1)^2 - 4(f + 1)s^2t^2/3\} \qquad (36)$$

where s^2 is defined by *Equation 18,* and

$$c' = (4f - 1)/8(f + 1) \qquad (37)$$

Tubocurarine Chloride Injection—Compute the error variance by *Equation 12*, and the confidence interval by *Equation 24*.

Vasopressin Injection—Compute the error variance s^2 by *Equation 16*, C by *Equation 35*, and the log confidence interval by *Equation 26*, where c′ = 1 and i is the log-interval separating the two dosage levels.

Vitamin B_{12} Activity—Proceed as directed under *Calcium Pantothenate.*

Combination of Independent Assays

When the method permits, additional animals can be added to an insufficiently precise assay until the combined results reduce the confidence interval within the limits specified in the monograph. Where two or more independent assays are required, each leading to a log-potency M, the M's are combined in determining the *weighted mean potency* of the Unknown. Except in the Heparin Sodium assay, where the log-potencies are weighted equally, the relative precisions of the two or more independent M's determine the weight assigned to each value in computing their mean and its confidence interval.

Before combining two or more separate estimates of M, test their mutual consistency. If the M's are consistent, their respective confidence intervals will overlap. Where the intervals do not overlap or where the overlap is small, compute an approximate χ_M^2. Assign each of the h individual assays a weight w, defined as

$$w = 4t^2/L^2 \qquad (38)$$

where the length of the confidence interval L is computed with the appropriate equation from the preceding section, and t^2 is read from *Table 9* for the degrees of freedom n in the error variance of the assay. Sum the individual weights to obtain Σw. Then an approximate χ^2 with h – 1 degrees of freedom is determined as

$$\text{Approx. } \chi_M^2 = \Sigma(wM^2) - \{\Sigma(wM)\}^2/\Sigma w \qquad (39)$$

For two assays with log-potencies M_1 and M_2 and weights w_1 and w_2, *Equation 35* reduces to

$$\text{Approx. } \chi_M^2 = w_1w_2(M_1 - M_2)^2/(w_1 + w_2) \qquad (40)$$

with one degree of freedom. If the approximate χ_M^2 is well under the critical value for χ^2 in *Table 9*, use the weights w in computing the mean log-potency $\overline{M}$ and its confidence interval, L. If χ_M^2 approaches or exceeds this critical value, use instead the semi-weights w′ *(Equation 47)* when computing $\overline{M}$.

Compute the mean log-potency $\overline{M}$ of two or more mutually consistent assays as

$$\overline{M} = \Sigma(wM)/\Sigma w \qquad (41)$$

This is the most probable single value within a combined confidence interval of length L_c, defined as the square root of

$$L_c^2 = 4t_L^2/\Sigma w\ \{1 + (4/\Sigma^2 w)\Sigma(w(\Sigma w - w)/n'\} \qquad (42)$$

where each n′ = n – 4(h – 2)/(h – 1) and t_L^2 is interpolated from *Table 9* with the degrees of freedom

$$n_L = \Sigma^2 w/\Sigma(w^2/n)$$

For two assays (h = 2) with log-potencies M_1 and M_2 and weights w_1 and w_2, respectively, the above equation may be rewritten as

$$L_c^2 = 4t_L^2/\Sigma w\ \{1 + [1/n_1 + 1/n_2]4w_1w_2/\Sigma^2 w\} \qquad (43)$$

where $\Sigma w = w_1 + w_2$. Where L_c, the confidence interval for a combined estimate, does not exceed the requirement in a monograph, upper and lower confidence limits are taken ${}^1/_2L_c$ above and below M, to obtain approximately a 95% confidence interval.

Where the variation in the assayed potency between the h independent determinations, as tested by χ_M^2, approaches or exceeds P = 0.05, the several estimates are assigned semi-weights w′. From the weight w, compute the variance of each M as

$$V = 1/w = L^2/4t^2 \qquad (44)$$

Calculate the variance of the heterogeneity between assays as

$$v = \Sigma M^2 - (\Sigma M^2/h)/(h - 1) - \Sigma v/h \qquad (45)$$

or if h = 2,

$$v = (M_1 - M_2)^2/2 - (V_1 + V_2)/2 \qquad (46)$$

Where V varies so markedly that v calculated as above is a negative number, compute instead an approximate v by

Official from May 1, 2012
Copyright (c) 2011 The United States Pharmacopeial Convention. All rights reserved.

omitting the term following the minus sign in *Equations 45* and *46*. A semi-weight is defined as

$$w' = 1/(V + v) \qquad (47)$$

Substitute w′ and Σw′ for w and Σw in *Equation 41* to obtain the semi-weighted mean $\overline{M}$. This falls near the middle of a confidence interval of approximate length L_c', where

$$L_c'^2 = 4t^2/\Sigma w' \qquad (48)$$

and t^2 from *Table 9* has ΣN degrees of freedom.

Where $\chi_M 2$ in *Equation 39*, from h = 4 or more estimates of M, exceeds the critical level in *Table 9* by more than 50%, and the weights w differ by less than 30%, the h estimates of M may be checked for a suspected outlier with *Table 1*. Where significant, the outlying M may be omitted in computing $\overline{M}$ with w′.

Where the potency of a drug is determined repeatedly in a given laboratory by the same bioassay method, successive determinations of both the slope b and the error variance s^2 may scatter randomly within the sampling error about a common value for each parameter. Plotting estimates from successive assays on a quality control chart for each statistic and computing the midvalue and control limits defining the allowable random variation make it possible to check continuously the consistency of an assay technique. Where estimates of b and s^2 from a single assay fall within the control limits, they may be replaced by their laboratory means. Reject any assay in which these statistics fall outside the control limits, or accept it only after close scrutiny with respect to its validity.

Joint Assay of Several Preparations

Each monograph describes the assay of a single Unknown against the Standard. Although not provided explicitly, several different Unknowns are often included in the same assay and each is compared separately with the same responses to the Standard. This fact may warrant increasing the number of observations with the Standard. Given f observations at each dosage level of each of h different Unknowns, the number of observations at each dosage level of the Standard may be increased advantageously, if h is large, to

$$f\sqrt{h}$$

This rule can be applied only approximately where litter differences or their equivalent must be segregated, and in any case is merely suggestive.

If all of several assays conducted concurrently meet the requirements for validity, and have linear log-dose response curves with the same slope b and the same error variance s^2 about these lines, these two statistics may be considered as characteristic of the assay. Combining all of the evidence from the same assay into a single value of the assay slope results in a more stable and reliable estimate of b than if each Unknown were analyzed independently. The degrees of freedom and reliability of the error variance s^2 can be increased similarly. Confidence intervals computed with these composite values for b and s^2 are smaller on the average than if based upon only part of the relevant data. For the calculation or application of such assay estimates, see *Equations 10, 15, 16, 19, 28*, and *29*. The potency estimated with a slope computed from a single Unknown and the Standard agrees within a fraction of the confidence interval with that computed from the combined slope for the entire assay. Since it is based upon more evidence, the latter is considered the better estimate.

GLOSSARY

Glossary of Symbols

A	absorbance for computing % reduction in bacterial growth from turbidimetric readings.
b	slope of the straight line relating response (y) to log-dose (x) [*Equations 2b, 4, 5, 6*].
c	constant for computing M′ with *Equations 8* and *10*.
c′	constant for computing L with *Equations 26* and *29*.
ci	constant for computing M′ when doses are spaced as in *Table 8*.
$c'i^2$	constant for computing L when doses are spaced as in *Table 8*.
C	term measuring precision of the slope in a confidence interval [*Equations 27, 28, 35, 36*].
χ^2	statistical constant for testing significance of a discrepancy [*Table 9*].
χ_{M2}	χ^2 testing the disagreement between different estimates of log-potency [*Equations 39, 40*].
e_b	e_i from row b in *Tables 6* to *8*.
$e_b'i$	multiple of $\Sigma(x - \bar{x})^2$ [*Table 5*; *Equation 6*].
e_i	sum of squares of the factorial coefficients in each row of *Tables 6* to *8*.
e_q	e_i from row q in *Tables 6* to *8*.
f	number of responses at each dosage level of a preparation; number of replicates or sets.
f_S	number of observations on the Standard.
f_U	number of observations on the Unknown.
F_1 to F_3	observed variance ratio with 1 to 3 degrees of freedom in numerator [*Table 9*].
G_1, G_2, and G_3	relative gap in test for outlier [*Table 1*].
h	number of Unknowns in a multiple assay.
h′	number of preparations in a multiple assay, including the Standard and h Unknowns; i.e., h′ = h + 1.
i	interval in logarithms between successive log-doses, the same for both *Standard* and *Unknown.*
k	number of estimated log-potencies in an average [*Equation 24*]; number of treatments or doses [*Table 4*; *Equations 1, 13, 15, 16*]; number of ranges or groups in a series [*Table 2*]; number of rows, columns, and doses in a single Latin square [*Equations 1a, 16a*].
L	length of the confidence interval in logarithms [*Equations 24, 26, 29, 38*], or in terms of a proportion of the relative potency of the dilutions compared [*Equations 31, 33*].
L_c	length of a combined confidence interval [*Equations 42, 43*].
L_c'	length of confidence interval for a semi-weighted mean $\overline{M}$ [*Equation 48*].
LD_{50}	lethal dose killing an expected 50% of the animals under test [*Equation 2c*].
M	log-potency [*Equation 2*].
M′	log-potency of an Unknown, relative to its assumed potency.
$\overline{M}$	mean log-potency.
n	degrees of freedom in an estimated variance s^2 or in the statistic t or χ^2.
n′	number of Latin squares with rows in common [*Equations 1a, 16a*].
N	number; e.g., of observations in a gap test [*Table 1*], or of responses y in an assay [Equation 16].

Official from May 1, 2012
Copyright (c) 2011 The United States Pharmacopeial Convention. All rights reserved.

Glossary of Symbols *(Continued)*

P	probability of observing a given result, or of the tabular value of a statistic, usually P = 0.05 or 0.95 for confidence intervals [*Tables 1, 2, 9*].
P_*	potency, P_* = antilog M or computed directly.
R	ratio of a given dose of the Standard to the corresponding dose of the Unknown, or assumed potency of the Unknown [*Equations 2, 30, 33*].
R_*	ratio of largest of k ranges in a series to their sum [*Table 2*].
$s = \sqrt{s^2}$	standard deviation of a response unit, also of a single estimated log-potency in a direct assay [*Equation 24*].
s^2	error variance of a response unit.
S_i	a log-dose of Standard [*Tables 6, 7*].
Σ	"the sum of."
t	Student's t for n degrees of freedom and probability P = 0.05 [*Table 9*].
T	total of the responses y in an assay [*Equation 16*].
T′	incomplete total for an assay in randomized sets with one missing observation [*Equation 1*].
T_1	Σ(y) for the animals injected with the Standard on the first day [*Equations 18, 36*].
T_2	Σ(y) for the animals injected with the Standard on the second day [*Equations 18, 36*].
T_a	T_i for the difference in the responses to the Standard and to the Unknown [*Tables 6* to *8*].
T_{ab}	T_i for testing the difference in slope between Standard and Unknown [*Tables 6* to *8*].
T_{aq}	T_i for testing opposed curvature in the curves for Standard and Unknown [*Tables 6* to *8*].
T_b	T_i for the combined slope of the dosage-response curves for Standard and Unknown [*Tables 6* to *8*].
T_b'	$\Sigma(x_1T_t)$ or $\Sigma(x_1y)$ for computing the slope of the log-dose response curve [*Equations 10, 23, 28*].
T_i	sum of products of T_t multiplied by the corresponding factorial coefficients in each row of *Tables 6* to *8*.
T_q	T_i for testing similar curvature in the curves for Standard and Unknown [*Tables 6* to *8*].
T_r	row or set total in an assay in randomized sets [Equation 16].
T_r'	incomplete total for the randomized set with a missing observation in Equation 1.
T_t	total of f responses y for a given dose of a preparation [*Tables 6* to *8*; *Equations 6, 13, 14, 16*].
T_t'	incomplete total for the treatment with a missing observation in Equation 1.
U_i	a log-dose of Unknown [*Tables 6* to *8*].
v	variance for heterogeneity between assays [Equation 45].
V = 1/w	variance of an individual M [*Equations 44* to *47*].
w	weight assigned to the M for an individual assay [*Equation 38*], or to a probit for computing an LD_{50} [*Equations 2a, 2b*].
w′	semi-weight of each M in a series of assays [*Equations 47, 48*].
x	a log-dose of drug in a bioassay [*Equation 5*]; also the difference between two log-threshold doses in the same animal [Equation 12].
x*	coefficients for computing the lowest and highest expected responses y_L and y_H in a log-dose response curve [*Table 4*; Equation 3].
x_1	a factorial coefficient that is a multiple of $(x - \bar{x})$ for computing the slope of a straight line [*Table 5*; Equation 6].
$\bar{x}$	mean log-dose [*Equation 5*].

Glossary of Symbols *(Continued)*

$\bar{x}_S$	mean log-dose for Standard [*Equation 9*].
$\bar{x}_U$	mean log-dose for Unknown [*Equation 9*].
X	log-potency from a unit response, as interpolated from a standard curve [*Equations 7a, 7b, 19*].
X_M	confidence limits for an estimated log-potency M [*Equations 25, 30*].
X_P*	confidence limits for a directly estimated potency P_* (see *Digitalis* assay) [Equation 33].
y	an observed individual response to a dose of drug in the units used in computing potency and the error variance [*Equations 13* to *16*]; a unit difference between paired responses in 2-dose assays [*Equations 17, 18*].
$y_1 \ldots y_N$	observed responses listed in order of magnitude, for computing G_1, G_2, or G_3 in *Table 1*.
y′	replacement for a missing value [*Equation 1*].
$\bar{y}$	mean response in a set or assay [*Equation 5*].
$\bar{y}_t$	mean response to a given treatment [*Equations 3, 6*].
Y	a response predicted from a dosage-response relationship,often with qualifying subscripts [*Equations 3* to *5*].
z	threshold dose determined directly by titration (see *Digitalis* assay) [*Equation 11*].
$\bar{z}$	mean threshold dose in a set (see *Digitalis* assay) [*Equations 31, 32, 33*].

⟨115⟩ DEXPANTHENOL ASSAY

The following procedure is provided for the determination of dexpanthenol as an ingredient of multiple-vitamin preparations. It is applicable also to the determination of the dextrorotatory component of racemic panthenol and of other mixtures containing dextrorotatory panthenol.

Media may be prepared as described hereinafter, or dehydrated mixtures yielding similar formulations may be used provided that, when reconstituted as directed by the manufacturer or distributor, they have growth-promoting properties equal to or superior to those obtained from the formulas given herein.

USP Reference Standards ⟨11⟩—*USP Dexpanthenol RS*.

Standard Stock Solution of Dexpanthenol—Dissolve an accurately weighed quantity of USP Dexpanthenol RS in water, dilute with water to obtain a solution having a known concentration of about 800 µg per mL, and mix. Store in a refrigerator, protected from light, and use within 30 days.

Standard Preparation—On the day of the assay, prepare a water dilution of the *Standard Stock Solution of Dexpanthenol* to contain 1.2 µg of dexpanthenol per mL.

Assay Preparation—Proceed as directed in the individual monograph for preparing a solution expected to contain approximately the equivalent of the dexpanthenol concentration in the *Standard Preparation.*

Modified Pantothenate Medium—

Acid-Hydrolyzed Casein Solution	25 mL
Cystine–Tryptophane Solution	25 mL
Polysorbate 80 Solution	0.25 mL
Dextrose, Anhydrous	10 g
Sodium Acetate, Anhydrous	5 g

Official from May 1, 2012
Copyright (c) 2011 The United States Pharmacopeial Convention. All rights reserved.