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Do we need to examine the quantitative data obtained from toxicity studies for both normality and homogeneity of variance?

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Abstract: *Most of the statistical techniques used to evaluate the data obtained from toxicity studies are based on the assumption that the data show a normal distribution and homogeneity of variance. Literature review on toxicity studies on laboratory animals reveals that in most of the cases homogeneity of variance alone is examined for the data obtained from these studies. But the data that show homogeneity of variance need not always show a normal distribution. In fact, most of the data derived from toxicity studies, including hematological and biochemical parameters show a non-normal distribution. On examining normality of data obtained from various toxicity studies using different normality tests, we observed that Shapiro-Wilk test is more appropriate than Kolmogorov-Smirnov test, Lilliefors test, the normal probability paper analysis and Chi square test. But there are situations, especially in the long-term toxicity studies, where normality is not shown by one or more than one of the dosage groups. In this situation, we propose that the data may be analyzed using Dunnett multiple comparison test after excluding the data of the groups that do not show normality. However, the biological relevance of the excluded data has to be carefully scrutinized. We also observed that the tendency of the data to show a normal distribution seems to be related to the age of the animals. Present paper describes various tests commonly used to test normality and their power, and also emphasizes the need of subjecting the data obtained from toxicity studies to both normality and homogeneity tests. A flow chart suggesting the statistical techniques that may be used for both the types of data showing a normal or non-normal distribution is also proposed.*

Key words*: Shapiro-Wilk test, Normality, Normal distribution, Toxicity study, Homogeneity of variance*
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Introduction

It has been stated that a lot of fixed quantity data derived from healthy human and other living things generally show a normal distribution. Several statistical techniques, for example the *t*-test and variance analysis, are based on the assumption that the data show a normal distribution and homogeneity of variance. It is a common practice followed by most of the toxicologists to examine the data for homogeneity of variance before subsequently treating the data with any specific statistical tool. But, on examining various reports on toxicity studies, one can find that there is no uniform approach to it. Toxicity study reports with data subjected to homogeneity test (Hanley *et al*., 2000; EPA, 2006) and not subjected to homogeneity test (Bondy *et al*., 2000; Arts *et al*., 2004; Kumar *et al*., 2005; Srivastava *et al*., 2006) are available in the literature. The data that show homogeneity of variance need not always show a normal distribution. To analyze the difference between the control group and dosage groups, the decision tree, tree-type algorithm has long been used in most of the countries. For example, in Japan, the decision tree has been used since 1982 and is still being used with several modifications to the initial one (Yamazaki *et al*., 1981; Hamada *et al*., 1998; Kobayashi *et al*., 2000; Sakaki *et al*., 2000; Kobayashi, 2001). The decision tree commonly used in Japan is given in Table 1. The decision tree used in most of the countries follow a common pattern, *i.e*., based on the result of homogeneity of variance test, the tests are divided into the parametric or nonparametric; then, the difference between the dosage groups and the control group are compared.

In a report on the toxicology and carcinogenesis study of the National Toxicology Program, USA (NTP, 2006), two approaches were employed to assess the significance of pair-wise comparisons between the dosage and control groups in the analysis of continuous variable. Organ weight and body weight data, which have approximately normal distributions, were analyzed using the parametric multiple comparisons procedures of Dunnett and Williams. Hematology, clinical chemistry, spermatids and epididymal spermatozoa counts, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison method.

Considering the above it is felt to be appropriate to carry out a study on the distribution (normality and homogeneity) of data obtained from toxicity studies. An attempt was also made to compare among various statistical techniques used for testing normality and homogeneity of the data and propose a decision tree to analyze the data.

Materials and Methods

Tests for normality: Kolmogorov-Smirnov, Lilliefors and Shapiro-Wilk tests were used for testing normality and for goodness of fit, Chi distribution test was used (Muto, 2000). The comparison among the powers of the Kolmogorov-Smirnov, Lilliefors, Shapiro-Wilk tests for normality was done using the normal probability paper and Chi distribution. The area under the curve was calculated by Chi distribution using software and STATISTICA (Muto, 2000).

Influence of number of samples on normality examined by Shapiro-Wilk test: In toxicity studies, body weight of the animals is one of the quantitative parameters usually shows a normal distribution. Hence this parameter was chosen to study the influence of number of samples on normality. For this, body weight data of male control groups of 10 long-term toxicity studies were examined for normal distribution using Shapiro-Wilk test. For studying the influence of number of samples on non-normal distribution, platelet count data of F344 control male rats at week 104 obtained from 7 toxicity studies were examined using Shapiro-Wilk test.

Measured items in long-term studies that do not show normal distribution: The measured items considered for this study were body weight, food consumption, hematocrit, hemoglobin, red blood cell, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets, white blood cell (WBC), protein, albumin, glucose, triglyceride, total cholesterol, fasting cholesterol, nonesterified fatty acids (NEFA), phospholipids, blood urea nitrogen (BUN), creatinine, total bilirubin, aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), alkaline phosphatase (ALP), gamma-guanosine triphosphate (gamma-GTP), creatine phopsphokinase (CPK), calcium, inorganic phosphorus, sodium (Na), potassium (K), chlorides (Cl), urine volume, urine specific gravity, absolute and relative weights of brain, kidney, testes, heart, spleen, liver and adrenals of F344 male rats of control group of 2-year combined oncogenicity and chronic toxicity with pesticides. Distribution of above measured items was examined for normality using Shapiro-Wilk test.

Tests for homogeneity of variance and their power: In order to test homogeneity of variance, Bartlett's test (Bartlett and Kendall, 1946) was used for the data with a non-normal distribution and for the data with a normal distribution, O'Brien (O'Brien, 1979), Brown-Foresythe (Brown and Forsythe, 1974) and Levene (Levene, 1960) tests were used. The power of these tests was compared.

Results and Discussion

Tests for normality: Body weight of F344 male rats at week 104 obtained from a carcinogenicity study was examined for normal distribution (Fig. 1; Table 2). Visual examination of the figure indicates that the data show a normal distribution. These data were further examined using various tests (Table 3). From the table it may be stated that Lilliefors test and the Chi square test with width of class 5 showed a non-normal distribution. Kolmogorov-Smirnov test, Shapiro-Wilk test and Chi square test with width of class 15 and 10 showed normal distribution. In Fig. 2 and Table 4, hemoglobin concentration (g/dl) of F344 male rats at week 104 obtained from the carcinogenicity study is given. Visual examination indicates that, a non-normal distribution may be attributed for this data. When the data were further examined, it was found that Lilliefors test, Shapiro-Wilk test and Chi square test with width of class 7 showed a non-normal distribution (Table 5), where as, Kolmogorov-Smirnov test and the Chi square test with width of class 5 and 13 showed a normal distribution.

For the data derived from toxicity studies that follow a normal distribution as revealed by visual examination of the normality curve, Kolmogorov-Smirnov test and Shapiro-Wilk test seem to be more apt to test the normality. Lilliefors test and Shapiro-Wilk test seem to be more suitable to examine the data that do not follow a normal distribution. Though Chi square test also could be used to examine the data for the above purpose, setting of the width of the class can influence the judgment.

Influence of number of samples on normality examined by Shapiro-Wilk test: It is believed that increase in number of samples tend to show a normal distribution. On the other hand, as evidenced in the body weight data, that increase in number of samples in the groups changed the normal distribution to the non-normal distribution in some cases (Table 6). Similarly, in the data of platelet count, increase in number of samples in the groups, did not change a non-normal distribution to a normal distribution pattern (Table 7).

Table - 1: Decision tree commonly used in Japan

Developer	Analytical method
Yamazaki *et al.* (1981)	Bartlett, ANOVA, Dunnett, Scheffé, Kruskal-Wallis, Dunnett-type rank and Scheffé-type rank tests
Sano and Okayama (1990)*	Bartlett, ANOVA, Dunnett, Kruskal-Wallis and Dunnett-type rank tests
Hamada *et al.* (1998)	Scatter plots or box-plot, Bartlett, Log-transformation, Check outliner, Dose-dependency linearity and Dunnett tests
Kobayashi *et al.* (1999, 2000)	Bartlett, Dunnett and Steel's tests
Sakaki *et al.* (2000)	Williams and Steel's tests
Gad and Weil (1986)	Bartlett, Scatter gram, ANOVA, Dunnett, Duncan, Kruskal-Wallis and Distribution free multiple comparison

* Improved version of Yamazaki *et al.* (1981)

Note: Bartlett: Bartlett's test; ANOVA: Analysis of variance; Dunnett: Dunnett's multiple comparison test; Scheffe : Scheffe's multiple comparison test; Kruskal-Wallis : Kruskal-Wallis's H test

Table - 2: Moments of body weight data given in Fig. 1

Mean (g)	390.6
Standard deviation (g)	22.4
Standard error of mean (g)	3.5
Skewness	0.4328
Kurtosis	-0.665
N	40

Table - 3: Power of various tests on the body weight data with normal distribution of F344 male rats at week 104

Tests	Calculated value	*p* value
Kolmogorov-Smirnov test	*D*= 0.143164	> 0.20
Lilliefors test	*D*= 0.143164	< 0.05
Shapiro-Wilk test	*W*=0.958435	0.148039
Normal probability paper	-	Normal distribution byvisual examination
Chi-distribution calculated	Width of class= 15, Chi value= 9.21795	0.51155
at different width classes	Width of class= 10, Chi value= 9.25489	0.15974
	Width of class= 5, Chi value= 10.32634	0.01599

Table - 4: Moments of hemoglobin data given in Fig. 2

Mean (g/dl)	15.2
Standard deviation (g/dl)	1.4
Standard error of mean (g/dl)	0.2
Skewness	-1.618
Kurtosis	4.794
N	38

Table - 5: Power of various tests on the hemoglobin data with non-normal distribution of F344 male rats at week 104

Tests	Calculated value	*p* value
Kolmogorov-Smirnov test	*D*= 0.161718	> 0.20
Lilliefors test	*D*= 0.161718	< 0.05
Shapiro-Wilk test	*W*=0.880189	0.000736
Normal probability paper	-	Non-normal distribution by visual examination
Chi-distribution calculated	Width of class= 13, Chi value= 9.82503	0.19871
at different width classes	Width of class= 7, Chi value= 5.34626	0.06904
	Width of class= 5, Chi value= 1.11368	0.29128

Measured items in long-term studies that do not show normal distribution: The items to skew to the right distribution with sharp kurtosis by non-normal distribution were MCV, MCH, platelet, ALT, AST, ALP, Gamma-GTP, CPK, protein, free cholesterol, potassium and absolute spleen weight and absolute weight/body weight ratio of heart, spleen and adrenal gland. The items to skew to the left distribution with sharp kurtosis by a non-normal distribution were the food consumption, hematocrit, hemoglobin, red blood cell and MCHC (Table 8). Items like body weight, WBC, albumin, glucose, triglyceride,

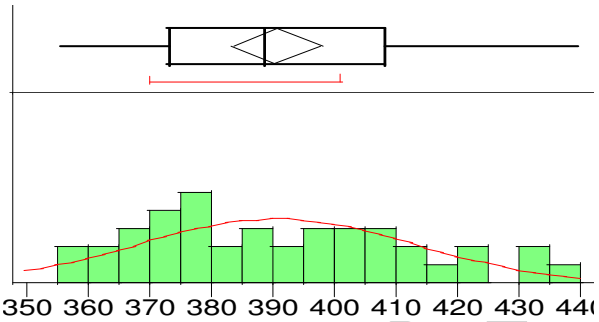

Fig. 1: Body weight data (g) of F344 male rats at week 104
Note: Histograms are the number of animals in different body weight ranges. Curve on the histograms indicate normality

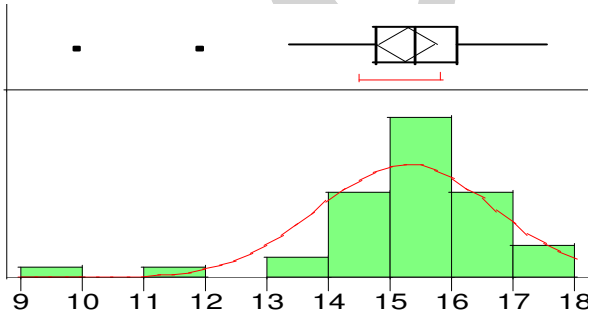

Fig. 2: Hemoglobin concentration data (g/dl) of F344 male rats at week 104
Note: Histograms are the number of animals in different hemoglobin weight ranges. Curve on the histograms indicate non-normality

Fig. 3a: Normal distribution in two groups

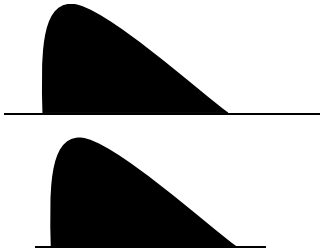

Fig. 3b: Non-normal distribution and homogeneous of variance

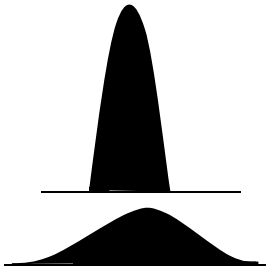

Fig. 3c: Non-normal distribution and heterogeneous of variance

Table - 6: Influence of number of samples on normal distribution of body weight (g) of F344 control male rats at week 52 obtained from 10 toxicity studies

Item	Study numbers									
	1	2	3	4	5	6	7	8	9	10
Mean±S.D. (g)	355±20	396±26	344±24	351±21	361±22	384±20	355±18	358±18	371±29	358±16
N	59	70	50	60	49	69	50	50	50	49
CV (%)	5.6	6.6	7.0	6.0	6.1	5.2	5.1	5.0	7.8	4.5
Skewness	-0.70911	0.33990	0.02128	-0.14887	0.40923	0.26216	0.23044	0.39612	-0.28367	-0.19555
Kurtosis	5.775852	-0.38485	-0.40960	-0.23998	0.14793	0.01995	-0.12768	0.72421	-0.11968	0.50738
W value	0.958792	0.972164	0.980042	0.792880	0.974313	0.978060	0.976035	0.976787	0.977877	0.978780
P (Prob<*W*)	0.0912	0.3140	0.7299	0.3964	0.5262	0.5499	0.5809	0.6089	0.6496	0.6895
P (Prob<*W*) for cumulative effect	0.1069									
	0.1218									
	0.0144									
	0.0070									
	0.1449									
	0.0460									
	0.0141									
	0.0365									
	0.0153									

Table - 7: Influence of number of samples on non-normal distribution of platelet counts (10^3/μl) of F344 control male rats at week 104 obtained from 7 toxicity studies

Item	Study numbers						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Mean±S.D.	611±136	648±137	647±104	797±194	679±125	724±115	733±150
N	41	38	40	37	40	38	41
CV (%)	22	21	16	24	18	16	20
Skewness	1.972014	1.708086	1.290418	0.240257	-1.178269	-1.864571	1.457151
Kurtosis	7.148736	7.770058	4.467973	5.235356	6.786090	8.887765	4.885960
W value	0.787694	0.848761	0.917248	0.869061	0.873100	0.836600	0.850105
P (Prob<*W*)	<0.0001	<0.0001	0.0069	0.0003	0.0002	<0.0001	<0.0001
P (Prob<*W*) for cumulative effect	0.0000						
	<0.0001						
	0.0000						
	<0.0001						
	<0.0001						
	0.0000						

Table - 8: Measured items of F344 male rats of control group of chronic and carcinogenicity studies showing non-normal distribution

Distribution	Skewness	
	To right distribution (+)	**To left distribution (-)**
Kurtosis: Sharply (+)	MCV, MCH, platelet, AST, ALT, ALP,Gamma-GTP, CPK, protein, free cholesterol, potassium, absolute spleen weight and relative weights of heart, spleen and adrenals	Food consumption, hematocrit, hemoglobin, red blood cell and MCHC
Kurtosis: Uniformly (-)	None	None

Table- 9: Number of measured items showing non-normal distribution at different weeks of dosing (Shapiro-Wilk test)

Measured Items	Number of non-normal items at different weeks of dosing			
	Week 26	Week 52	Week 78	Week 104
Hematology	2	7	6	8
Biochemistry	4	8	3	12
Urinalysis	1	1	0	0
Organ weight	0	1	0	4
Relative organ weight	0	2	1	5

Table - 10: Power of different tests for homogeneity of variance of water consumption (g/week) in male B6C3F1mice at week 13

Group	N	Mean ± S.D. (g/week)	Calculated *p* value			
			O'Brien	Brown-foresythe	Levene	Bartlett
1	10	43.8±9.0	0.0459	0.0340	0.0014	<0.0001
2	10	35.4±3.4				
3	10	31.9±1.5				
4	10	30.7±2.1				

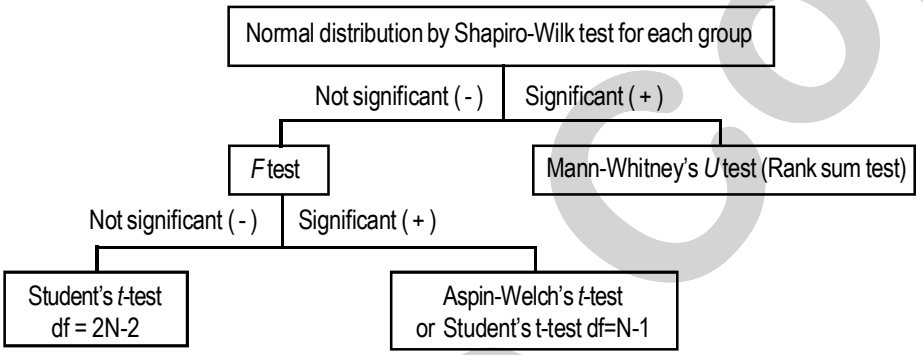

Fig. 4a: Flow chart for selecting the statistical tool when the data show a normal or non-normal distribution (Number of groups > 2)

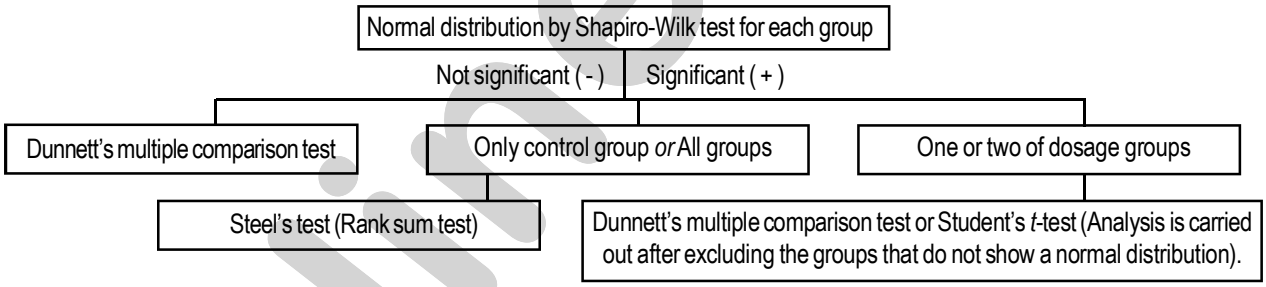

Fig. 4b: Flow chart for selecting the statistical tool when the data show a normal or non-normal distribution (Number of groups > 2)

NEFA, phospholipids, BUN, creatinine, total bilirubin, calcium, inorganic phosphorus, sodium, chlorides, urine volume, urine specific gravity, absolute and relative weights of brain, kidney, testes and liver and absolute weight alone of adrenals and heart showed a normal distribution. It is interesting to note that several measured items that showed a normal distribution in the early stage of the studies showed a non-normal distribution in the later stages. From the data given in the Table 9, it could be stated that aging affects a normal distribution.

Combinations of normal distribution and homogeneity of variance: Different combinations of a normal distribution and homogeneity of variance of data obtained from the toxicity tests are given in Fig. 3a to 3c (Ichihara, 1994). An ideal combination is the one given in Fig. 3a. But in practice, in toxicity studies the experimental groups exhibit effect of the treatment, whereas the control group does not show any undue effect. Usually, the number of samples and variances are different in the treatment groups compared to the control group. As a result, in toxicity studies, most of the cases, data show distributions as given in Fig. 3b and 3c.

It has been stated that most of the data derived from living things follow normal distribution pattern (Katabami, *et al.*, 1997). Therefore, in the analysis of data obtained from toxicity studies, the

homogeneity of variance is confirmed initially, then the data are analyzed for a normal distribution. If data show a normal distribution, then the parametric tests are used for the analysis. If the data do not show a normal distribution, Kruskal-Wallis H test is used if the number of groups is three or more than three and Welch's *t*-test or Mann-Whitney *U* (Ichihara, 1994)*for* two groups. But the specific statistical tool to be used to examine a normal distribution is not clearly stated in most of the books on biostatistics. Also, the statistical tool to be applied for the comparison among the groups after the Kruskal-Wallis H test is not clearly described.

Tests for homogeneity of variance and their power: Homogeneity of the variance of water consumption of mice in the toxicity study at week 13 was examined using four different tests (SAS JMP, 1996) and given in Table 10. All the four tests showed a significant difference at 5% level, but Bartlett's test had the highest power followed by Levene, Brown-Foresythe and O'Brien.

Most of the toxicologists adopt a conservative approach for analyzing the data. The data are examined for homogeneity of variance and if the variance is homogeneous, parametric tests are used and for heterogeneous variance nonparametric tests are used. Usually, the data are not examined for a normal distribution, though it is a fact that for most of the statistical tools, one of the important prerequisite requirements is that data must show a normal distribution. If at all the data are examined for a normal distribution, it is not vividly explained in most of the books on biostatistics, what nonparametric statistical tools should be chosen for the data that show a non-normal distribution. Shapiro-Wilk test seems to be more appropriate for testing normal distribution, as this test can be used for both the data types that show normal or non-normal distribution by visual examination of the graph. On the basis of above, we propose a flow chart describing the statistical tool that may be used for the analysis of the data showing a normal or non-normal distribution (Fig. 4a and 4b).

It is important to examine the data for both homogeneity of variance and normal distribution. Though the Bartlett's test is used to examine for homogeneity of variance, it is more sensitive to heterogeneous data (Finney, 1995). We propose that when normality of each group is guaranteed by Shapiro-Wilk test, Dunnett multiple comparison test may be used for further analysis. When the control group or all groups do not show normality, Steel test of separate type in Dunnett nonparametric test may be used. When normality is not shown by one or two of the dosage groups, the data may be analyzed using Dunnett multiple comparison test after excluding the group/s that do not show normality. However, the biological relevance of the excluded item has to be carefully scrutinized by the toxicologist.

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