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EVALUATION OF TOXICITY DOSE LEVELS BY CLUSTER ANALYSIS

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ABSTRACT — Determination of 'no observed adverse effect level' (NOAEL) is one of the prime objectives of a repeated subchronic experiment. The data collected in such experiments are huge, and it is a difficult and time-consuming task to determine NOAEL. In such situations, cluster analysis could be used as a valuable tool. In the present paper the determination of NOAEL is demonstrated using the data of a 28-day repeated oral toxicity study carried out in rats. Groups (10/sex/group) of Crj: CD rats were administered the test substance at low, middle, high and top dose levels by gastric intubation daily for 28 days. A concurrent control group was also maintained. In-life measurements included general behavior, body weight, food and water consumption. At termination various hematological and biochemical parameters were determined in the blood of individual animals in all groups. Urinalysis was also carried out at termination. Animals were sacrificed for microscopic and macroscopic findings. Analysis of data showed that 40 measurements (data 1) in the treatment groups were different from the control and 48 (data 2) were not treatment-related. Cluster analysis was carried out separately on data 1 and combined data 1 and data 2. This study revealed that for better judgment of NOAEL performing a cluster analysis on the data which alone showed a significant difference compared to control is more advisable than performing the cluster analysis on all data collected in the study. The advantage of cluster analysis is that quantitative data and qualitative data can be analyzed in annexation. In addition, cluster analysis assists in the result of the univariate analysis and displays NOAEL at once quite obviously.

KEY WORDS: Cluster analysis, Statistics, NOAEL, Decision tree

INTRODUCTION

Data collected in repeated toxicity studies are enormous and are either qualitative or quantitative in nature. No observed adverse effect level (NOAEL) of the test substance is judged as based on these data. Sometimes the toxicity effects manifested are not dose-dependent, which makes judging NOAEL as a difficult task. I think cluster analysis is extremely useful for a decision of NOAEL by using it together with univariate analysis. Now the question is whether to consider only those data which show a significant difference compared to control for the cluster analysis or all data collected in the study, irrespective of whether their difference from the control is significant or not.

DATA AND GENERAL ANALYTICAL METHODS

Groups (10/sex/dose) of seven-week-old Crj: CD rats were administered the test substance at low, middle, high and top doses by gastric intubation daily for 28 days. A concurrent control group was also maintained. Rats were examined daily for general behavior. During the dosing period, body weight, food consumption and water consumption of the animals were measured. Animals were sacrificed on Day 29 after overnight starvation for assessment of hematology, blood biochemistry, serum protein electrophoresis, urinalysis, myelogram and ophthalmologic and pathological (organ weight measurement as well as gross and histopathology) examinations.

Salivation in both sexes in the high dose group, staggering gait in the top dose group, slight suppression of the body weight gain in males in the top dose group, slight anemic trend in both sexes in the top dose group, higher values in alkaline phosphatase in both sexes in the high dose and top dose groups, lower values in albumin in males in the top dose group and in females

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in the high dose and top dose groups, bone fractures, mobilization of the sinusoidal cell and extramedullary hematopoiesis in the liver in both sexes in the top dose group and squamous hyperplasia and erosion of the fore-stomach in both sexes in the high and top dose groups were observed as the main changes attributable to the repeated oral administration of the test substance. Based on the above observations and determinations, the NOAEL was considered to be the middle dose for both males and females.

The data obtained in the study were analyzed statistically. Continuous data were subjected to automatic analysis (Fig. 1) by Bartlett's test for homogeneity of variance following the decision tree (Kobayashi *et al*., 2000) using the two-sided analysis. Data for macroscopic and microscopic findings were analyzed by the Fischer's exact test (Gad and Weil, 1986). The levels of significance were set at $p<0.05$.

CLUSTER ANALYSIS

Cluster analysis is a method which examines a collection of variables to see if individuals can be formed into any natural system of groups (Kirkwood, 1989). It serves to sort a heterogeneous collection of objects into a series of sets, that is, to identify sets and allocate objects to these sets simultaneously. The cluster analysis is applied in the field of toxicogenomics (Hamadeh *et al*., 2002). The technique using the personal computer software was described in detail by Shinmura (2000). The cluster analysis includes the Ward method, the Nearest neighbor, the Furthest neighbor, the Centroid method and the Median method, analyzed according to the hierarchical method. I chose the Ward method (Milligan, 1980) which could be divided into a minimum section. This technique groups the variable and the case as well as the principal component analysis. After the grouping, the feature of each cluster was considered. The cluster analysis used the software JMP (version 5) of the SAS (SAS Institute, Japan).

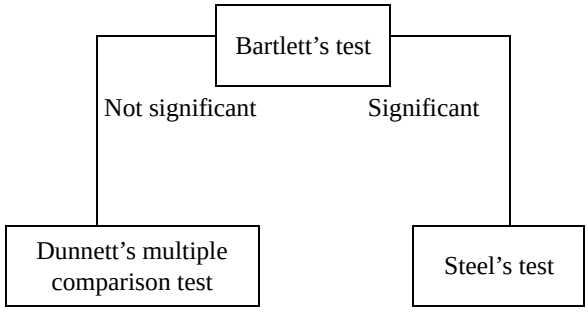

Fig. 1. Analytical method by general decision tree.

Cluster analysis -1

Items where significant difference ($p<0.05$) was detected in the dosed groups from control group were 40 in total.

Body weight gain, food efficiency, hematocrit, hemoglobin, red blood cell count, platelet count, neutrophil (%), lymphocytes (%), lymphocyte count, blood urea nitrogen, total protein, alanine aminotranferase, alkaline phosphatase, glucose, prothrombin time, albumin, alpha-2, beta, albumin/globulin ratio, inorganic phosphorus in urine, lung weight, relative weight of lung, liver, kidneys and testes, gross findings "A, B, C, D, E, F, G and H" and microscopic findings "A, B, C, D, E, F and G" were analyzed.

Cluster analysis -2

Here the 40 items which were subjected to cluster analysis -1 and the items which did not show a significant difference compared to control were put together. Altogether, the cluster analysis was carried out in 88 items, out of which the following 48 items did not show a significant difference compared to control:

Food and water consumption for 28 days, leucocyte count, lymphocyte count, reticulocyte count, activated partial thromboplastin time, total cholesterol, free cholesterol, triglyceride, phospholipid, non-esterified fatty acid, creatinine, total bilirubin, sodium, potassium, chloride, calcium, inorganic phosphorus, alnine aminotransferase, lactate dehydrogenase, alpha-1 (%), gamma (%), urine volume, urine specific gravity and sodium, potassium, chloride, calcium and inorganic phosphorus in urine, sodium, potassium, chloride, and calcium (mmol/day) and weights of brain, heart, liver, kidneys, spleen, adrenals, testes, thyroid and thymus and relative weight of brain, heart, spleen, adrenals, thyroid and thymus were analyzed.

RESULTS

Cluster analysis -1

Table 1 and Fig. 2 depict the result of analyzing 40 items that showed a significant difference compared with the control group. The group was classified into 2 when greatly classifying it. When Group 1 was subdivided, it was divided into 2 groups. Therefore, it was possible to classify it into 3 by containing the subdivision group. As for the animals of the high dose group,

Table 1. Results of cluster analysis obtained in 40 items which showed a significant difference ($p<0.05$) compared to control.

Dose level	Number of animals		
	Group 1		Group 2
	Subgroup-1	Subgroup-2	
Control	10	0	0
Low	10	0	0
Middle	10	0	0
High	2	8	0
Top	0	4	6

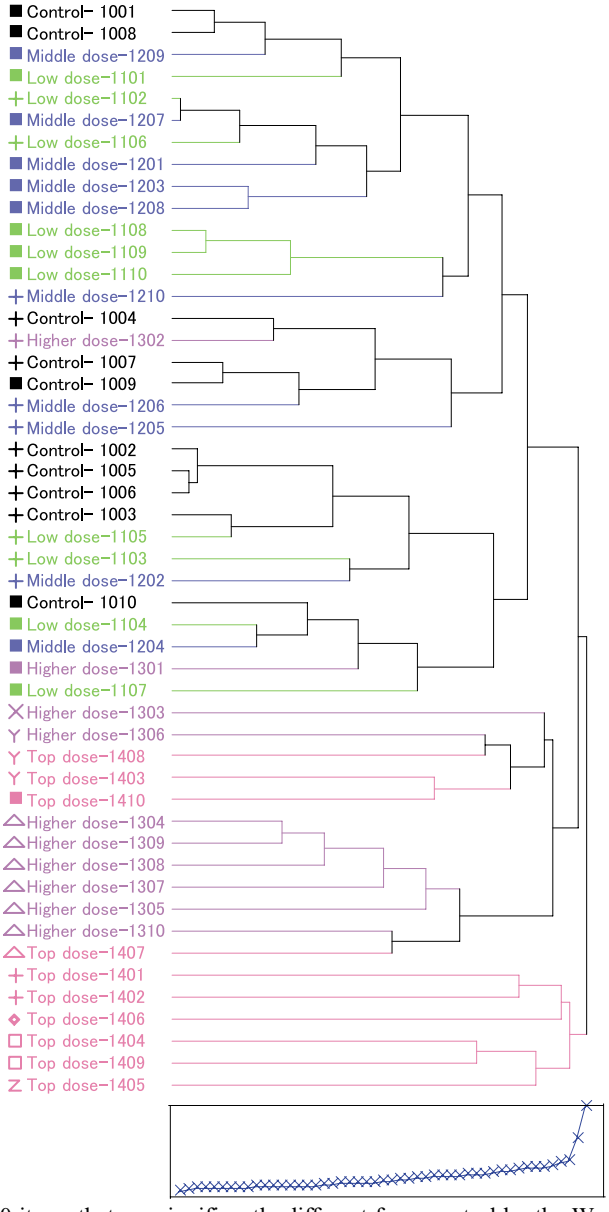

Fig. 2. Dendrogram obtained in 40 items that are significantly different from control by the Ward method. The smaller the distance (combination of the left sides), the stronger the relation. Each group contained 10 rats on Day 29. Animal identification mark, dose and animal number are given.

Table 2. Results of cluster analysis obtained in 88 items of all data.

Dose group	Number of animals			
	Group 1		Group 2	
	Subgroup-1	Subgroup-2	Subgroup-1	Subgroup-2
Control	8	2	0	0
Low	6	4	0	0
Middle	7	3	0	0
High	5	0	5	0
Top	0	0	5	5

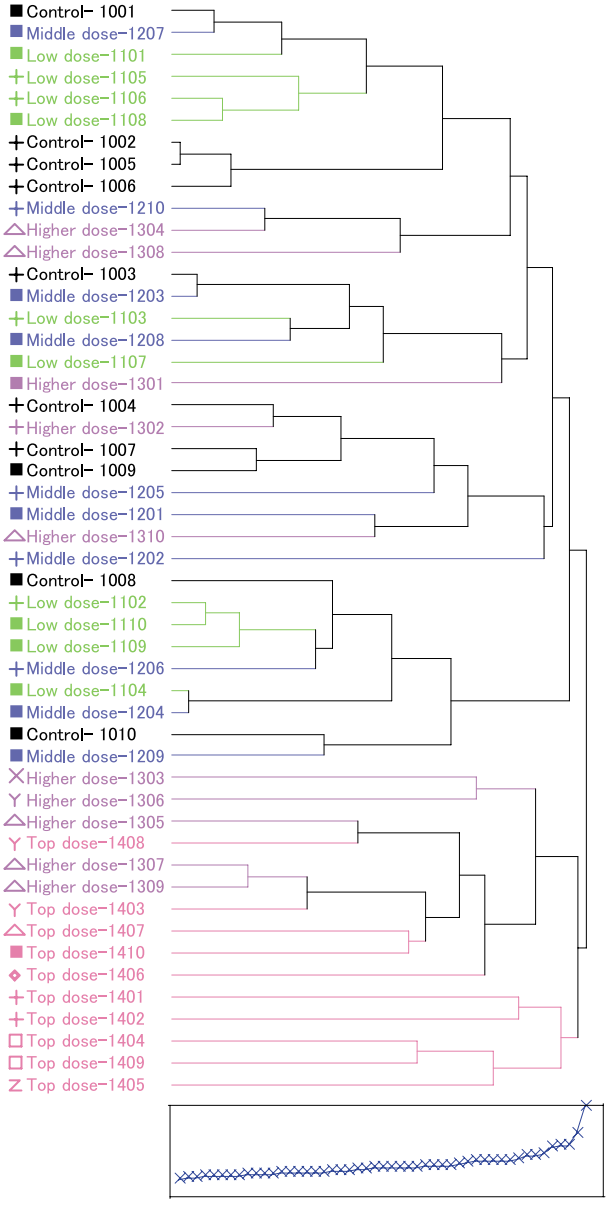

Fig. 3. Dendrogram obtained in 88 items. The smaller the distance (combination of the left sides), the stronger the relation. Each group contained 10 rats on Day 29. Animal identification mark, dose and animal number are given.

2 were observed in subgroup-1 of Group 1. The NOAEL was judged as the middle group and the toxic dose level was judged as greater than the high dose.

Cluster analysis -2

The group was classified into two when greatly classifying it. Groups 1 and 2 were divided into two subgroups. Therefore, it was possible to classify it into 4 by containing the subdivision groups. As for the animals of the high dose group, 5 and 0 were observed in subgroups-1 and -2 of Group 1, respectively. The middle dose was judged as NOAEL and the high dose was judged as the toxic level from this analysis (Table 2; Fig. 3).

DISCUSSION

Cluster analysis is a useful technique to determine NOAEL in long-term studies. In the present study, cluster analysis was carried out in two sets of data: 1. Only those items in dosed groups, which showed a significant difference compared to control, and 2. All the items, irrespective of their difference from the control as significant or not. The NOAEL determined using cluster analysis for data 1 and data 2 is the middle and high dose, respectively. The judgment made based on all findings of the study, without applying cluster analysis, was similar to the results of cluster analysis performed for data. Therefore, it may be presumed that for a better judgment of NOAEL, performing cluster analysis on the data, which alone showed a significant difference compared to control, is more advisable than performing cluster analysis on all data collected in the study. The advantage of the cluster analysis is that the quantitative data and the qualitative data can be analyzed in annexation. In addition, the cluster analysis assists in the result of the univariate analysis, and displays NOAEL at once quite obviously. It contributes to the NOAEL decision by executing the univariate analysis if a clear classification cannot be done by cluster analysis. I think that cluster analysis is extremely useful for the NOAEL decision by using it together with the univariate analysis.

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