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# Statistical Variation of Selected Clinical Pathological and Biochemical Measurements in Rodents (42555)

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*Abstract.* The variability in clinical pathological and biochemical measurements among replicates is often greater than the effects under study. To minimize the effects of this variability, it is recommended that replicated concurrent analyses (randomized blocks) of groups of animals be used. That is, some samples from each of the groups of animals to be compared are analyzed at the same time. This process is replicated until a sufficient number of animals are sampled. If replicated concurrent analyses are not conducted and clinical measurements are made at different times for different groups of animals, the biases may be large.

Clinical data were examined from several experiments to illustrate that the problems of clinical measurements are not confined to a particular endpoint, species, or sex. In one case, four times as many animals would have been required using nonconcurrent analyses to achieve the same precision as for concurrent analyses. Permutation analyses of two of the data sets indicate that statistical conclusions concerning the comparison of average clinical levels in different groups of animals would have been incorrect, falsely indicating higher or lower levels in a group over one-half of the time with nonconcurrent analyses. © 1987 Society for Experimental Biology and Medicine.

Considerable variability is often noted in selected clinical pathological and biochemical measures in rats and mice. Frequently, the variability in these measurements from replicate to replicate (from day to day) is as great as that of the effects under study from the administration of a chemical to laboratory animals. In spite of improvements resulting from standardized animal care, phlebotomy techniques, use of carbon dioxide as an anesthetic, bleeding of animals during the same time period each day, and adherence to instrument calibration schedules and internal quality control programs such as the multi-rule Shewhart Chart (1) to monitor analytical accuracy and precision, consistent results often are not obtained from day to day. Enzyme levels in rodents are extremely sensitive to feeding schedules, housing, handling conditions, and assay environment. To minimize these problems, it is recommended that replicated concurrent analyses (randomized block design) of groups of animals be used. For example, if necropsies are restricted to the same 2- to 3-h period each day and enzyme levels for up to 12 animals can be measured concurrently on the day of sacrifice, then these 12 measurements represent one replicate (block). If an experiment contained four dose levels for which enzyme levels are to be compared, analyses of three animals per dose for the four dose groups can be conducted concurrently. The time of sacrifice can be almost the same for these 12 animals analyzed concurrently. Thus, environmental biases (variations) can be minimized for comparisons of dose effects among these 12 animals. Since three animals per dose generally will not provide adequate precision of clinical pathology measurements, the whole procedure can be replicated until adequate data are obtained.

Alternatively, if replicated concurrent analyses are not conducted and measurements are made at one time for animals from one dose group and another time for another dose group, differences in the results among dose groups may include sizable biases introduced by conducting the analyses at different times. As will be illustrated later, even for analyses conducted at the same time of day, variation from day to day in enzyme levels may obscure the true effects.

**Materials and Methods.** Clinical pathological and selected biochemical parameters for



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rats and mice for six sets of data from five different experiments are used to illustrate that the wide fluctuations obtained in these measurements are not confined to a particular endpoint, species, sex, or treatment condition. Analyses of variance are conducted to obtain estimates of components of variance (2) in order to estimate the relative efficiency of replicated concurrent analyses. In addition, all possible permutations of two of the data sets are formed to show the range of results that could have been obtained without replicated concurrent analyses.

Prior to sacrifice, animals were fasted for 12–18 h with ad lib access to water. During this period, animals were housed in a holding room about 10 yd from the necropsy room. Animals were bled from the retroorbital sinus after hypoxia induction by 70% carbon dioxide.

Following is a brief description of the analytical procedures used. Asparate aminotransferase and alanine aminotransferase were measured as kinetic rate reactions using a centrifugal analyzer (3). Albumin was quantitated by densitometry after cellulose acetate electrophoresis of serum and total serum protein was measured by the biuret method (4). For glutathione *S*-transferase, the assay (5–7) used 1-chloro-2,4-dinitrobenzene as the substrate and the reaction was run at pH 6.5, where protein was quantified by the method of (8). Hepatic reduced glutathione (GSH) (Sigma Chemical Co., St. Louis) was proportional to the reduction of 5,5′-dithiobis (2-nitrobenzoic) acid at 412 nm, 30°C (9, 10), where 5 mM GSH was used as a standard. Monocytes were determined by counting the number of monocytes per 100 leukocytes at 100× under a microscope and the peripheral blood smear was fixed in methanol and stained with Wright–Giemsa stain on an automatic stainer.

*Aspartate aminotransferase (AST) in male rats.* Data for AST in male rats were selected for illustration because of the relatively large variation from day to day in an experiment in which Fischer 344 rats were administered 0, 100, 600, and 1200 ppm of methapyrilene in neutral drinking water for 90 days. Twelve animals per dose were used. Clinical chemistry analyses were performed on 4 successive days using three animals per day (replicate) from each of the four dose groups. That is, four replicated concurrent analyses were conducted. Thus, comparisons of the results among dose groups could be made within a day (replicate) and then averaged over the 4 days.

*Alanine aminotransferase (ALT) in female rats.* From the same experiment described above, data for ALT in Fischer 344 female rats were selected for illustration because of the relatively large variation from day to day.

*Glutathione S-transferase (GST) in female agouti mice.* Data were selected, illustrating relatively large day-to-day variation, from an experiment in which female agouti $A/a$ (Balb/c × VY) $F_1$ hybrid mice were administered single doses of 0, 150, and 200 mg/kg of streptozotocin, a drug which decreases insulin production. Ten animals per dose were used. GST analyses were performed on 5 days using two animals per day (replicate) from each of the three dose groups. That is, five replicated concurrent analyses were conducted. Each animal sample was analyzed in triplicate and the mean was computed. Thus, comparisons of the results among dose groups could be made within a day (replicate) and then averaged over the 5 days.

*Hepatic reduced glutathione in agouti and yellow mice.* Data were selected, illustrating relatively large day-to-day variation, from an experiment with male agouti and yellow $A^{VY}/A$ (Balb/c × VY) $F_1$ hybrid mice. Sixteen animals per phenotype were used. Clinical chemistry analyses were performed on 4 days using four animals per day (replicate) from each of the phenotypes. That is, four replicated concurrent analyses were conducted. Thus, comparisons of agouti and yellow mice could be made for each day (replicate) and then averaged over the 4 days.

*Blood and serum chemistry analyses in female rats.* Data were selected, illustrating relatively large day-to-day variation, from an experiment for female Fischer 344 rats used as controls in a 2-year bioassay. Blood analyses were performed on 10 different days at the termination of the experiment. Data for percentage monocytes (MONO) and albumin concentration (ALB) were selected for illustration.

**Results.** *AST in male rats.* The two-way analysis of variance for AST in male Fischer 344 rats is shown in Table I. There were statistically significant differences ($P < 0.036$)

TABLE I. ANALYSIS OF VARIANCE FOR AST IN MALE RATS

| Source of variation | Degrees of freedom | Mean square | $F$ ratio | $P$ level |
|---|---|---|---|---|
| Replicates | 3 | 286.0080 | 3.22 | 0.036 |
| Doses | 3 | 7383.7874 | 81.98 | 0.001 |
| Reps × doses | 9 | 90.0665 | 1.02 | n.s.[a] |
| Animals within groups | 32 | 88.6981 | | |
| Total | 47 | | | |

[a] Not statistically significant.

among replicates for AST. The estimate of the variance of the mean level of AST for the 12 animals administered the same dose of methapyrilene is given by the mean square for replicates × dose divided by 12:

$$V(\text{dose mean}) = 90.0665/12 = 7.5055.$$

The standard error of a dose mean is $\sqrt{7.5055} = 2.74$ using replicated concurrent analyses.

If replicated concurrent analyses had not been conducted, an estimate of the variance among nonconcurrent replicates within dose groups is given by

$$\frac{(\text{Replicate degrees of freedom}) \times (\text{Replicate mean square}) + (\text{Replicate} \times \text{dose degrees of freedom}) \times (\text{Replicate} \times \text{dose mean square})}{(\text{Replicate degrees of freedom}) + (\text{Replicate} \times \text{dose degrees of freedom})}$$

$$\frac{(3 \times 286.0080) + (9 \times 90.0665)}{3+9} = 139.0519.$$

The variance of the mean level of AST for the 12 animals administered the same dose of methapyrilene is given by the mean square for nonconcurrent replicates within doses divided by 12:

$$V(\text{dose mean}) = 139.0519/12 = 11.5877.$$

The standard error of a dose mean is $\sqrt{11.5877} = 3.40$ if replicated concurrent analyses are not used. That is, 11.587/7.5055 = 1.5 times more animals (18 animals per dose group rather than 12) would have to be used if replicated concurrent analyses has not been used in order to achieve the same precision.

*ALT in female rats.* As was done above, a two-way analysis of variance for ALT in female Fischer 344 rats was performed. There were statistically significant differences ($P < 0.001$) among replicates for ALT. The estimate of the variance of the mean level of ALT for the 12 animals administered the same dose of methapyrilene was 1.2701.

If replicated concurrent analyses had not been conducted, an estimate of the variance of the mean level of ALT for the 12 rats administered the same dose of methapyrilene is 2.9191. Thus, 2.9191/1.2701 = 2.3 times more animals (28 animals per dose rather than 12) would have to be used if replicated concurrent analyses had not been used in order to achieve the same precision.

*GST in female mice.* For the two-way analysis of variance for GST in female agouti $A/a$ (Balb/c × VY) $F_1$ hybrid mice, there were statistically significant differences ($P < 0.001$) among replicates for GST. The estimate of the variance of the mean level of GST for 10 animals administered the same dose of streptozotocin was 0.0664.

If replicated concurrent analyses had not been conducted, an estimate of the variance of the mean level of GST for the 10 mice administered the same dose of streptozotocin is 0.2694. Thus, 0.02694/0.0664 = 4.1 more animals (41 animals per dose group rather than 10) would have to be used if replicated concurrent analyses had not been used in order to achieve the same precision.

*GSH in male mice.* The two-way analysis of variance for GSH in male agouti $A/a$ and yellow $A^{VY}/A$ (Balb/c × VY) $F_1$ hybrid mice did not indicate a statistically significant difference ($P < 0.10$) among replicates. The estimate of the variance of the mean level of GSH for the 16 animals of a given phenotype is 0.0374.

If replicated concurrent analyses had not been conducted, an estimate of the variance of the mean level of GSH for the 16 mice of a given phenotype is 0.1269. Thus, 0.1269/0.0374 = 3.4 times more animals (54 animals per phenotype rather than 16) would have to be used if replicated concurrent analyses had not been used in order to achieve the same precision. Note that there is still a large gain in using replicated concurrent analyses even though the differences among replicates were not statistically significant.

*Monocytes in female rats.* The one-way analysis of variance for monocytes (MONO) in control female rats is shown in Table II. The analysis is computed on the transformed measure, 2 arc sine $\sqrt{p}$, where $p$ is the proportion of monocytes, in order to stabilize variance. There is a statistically significant ($P < 0.001$) difference among days (replicates). That is, animals measured on different days give statistically different averages for MONO even though they were all control animals. If an experiment were conducted so that control animals were measured on one day and treated animals were measured on another day, differences in MONO between the groups may falsely arise or be obscured by differences of measurements obtained on different days.

The estimate of the variance of transformed MONO measurements among animals made on the same day is $\hat{\sigma}_a^2 = 0.0107$. From Table II, the estimate of the variance of transformed MONO measurements on different days is $\hat{\sigma}_d^2 = (0.1636 - 0.0107)/9.84 = 0.0155$. The estimate of the variance of the mean of 12 MONO measurements made on one day is

$$V(\text{MONO mean}) = \frac{\hat{\sigma}_a^2}{12} + \hat{\sigma}_d^2 = \frac{0.0107}{12} + 0.0155 = 0.0164.$$

If a study contained four dose levels so that concurrent analyses were conducted on three animals per dose in each batch of 12 analyses done per day, then the estimated variance of the mean of 12 animals obtained from four different days is

$$V(\text{MONO mean}) = \frac{\hat{\sigma}_a^2}{12} + \frac{\hat{\sigma}_d^2}{4} = \frac{0.0107}{12} + \frac{0.0155}{4} = 0.0048.$$

Thus, 0.0164/0.0048 = 3.4 times more animals (41 animals per group rather than 12) would have to be used to obtain the same precision if replicated concurrent analyses had not been used.

*Albumin in female rats.* For the one-way analysis of variance for albumin (ALB) in control female rats there is a statistically significant ($P < 0.001$) difference among days (replicates). That is, animals measured on different days give statistically different averages for ALB even though they were all control animals. If an experiment were conducted so that control animals were measured on 1 day and treated animals were measured on another day, differences in ALB between the groups may falsely arise or be obscured by differences of measurements obtained on different days.

The estimate of the variance of the mean of 12 ALB measurements made on 1 day is 0.6098. If a study consisted of four dose levels so that concurrent analyses were conducted on three animals per dose in each batch of 12 analyses made per day, the estimated variance of the mean of 12 animals measured on 4 different days is 0.2143. Thus, 0.6098/0.2143 = 2.85 times more animals (34 animals per group rather than 12) would have to be used to obtain the same precision if replicated concurrent analyses had not been used. That is,

TABLE II. ANALYSIS OF VARIANCE FOR MONO IN FEMALE MICE (2 ARC SINE $\sqrt{\text{proportion monocytes}}$)

| Source of variation | Degrees of freedom | Mean square | Expected mean square | $F$ Ratio | $P$ Level |
|---|---|---|---|---|---|
| Days (reps) | 9 | 0.1636 | $\sigma_a^2 + 9.84\ \sigma_d^2$ | 15.3 | 0.001 |
| Animals within days | 91 | 0.0107 | $\sigma_a^2$ | | |
| Total | 100 | | | | |

equal precision is expected for 12 animals (3 animals per day × 4 days) or 34 animals all measured on 1 day.

**Discussion.** The six sets of data were chosen as examples to illustrate the importance of using replicated concurrent analyses when comparing clinical measurements among groups of rats or mice. It was not the intent of this paper to demonstrate that one species or sex is more erratic than another nor that the variation is more severe for one endpoint than another. Other sets of data could have been selected for the same endpoint in the same strains which exhibit more or less variation. The parameters chosen for illustration were not necessarily the most variable parameters. In fact, in other experiments measurements on these particular parameters may be relatively consistent from day to day while extreme variation may be present in other parameters. It has been our experience that it is rather unpredictable which parameters will exhibit considerable variation from day to day in a particular species or sex. Hence, the routine use of replicated concurrent analyses will reduce the problem of variation due to test conditions and animals.

For these six sets of data, the precision from the use of replicated concurrent analyses was up to four times better than not using replicated concurrent analyses. That is, in one case four times as many animals would have been required using nonconcurrent analyses to achieve the same precision as for concurrent analyses. In each instance concurrent analyses significantly reduced the number of animals required.

The enzymes AST, ALT, and GST are quantitated in International Units per liter where IU = micromoles of substrate converted to product per minute. The range for male rats ALT is 88 ± 3 U/liter, for female rats ALT is 33 ± 3 U/liter, and for female agouti mice GST is approximately 2 U/mg liver cytosol protein. AST and ALT are measured in serum. GST is measured in a liver extract which has been homogenized, centrifuged, and diluted over several hours of sample preparation (preanalytical sources of variation). The number of animals required to reach equivalent precision between concurrent and nonconcurrent models reflects the quantitative level of the enzyme and the manipulations required to prepare the sample. A similar case exists for GSH, which is expressed as microgram per milligram cytosolic liver protein, and for monocytes, which in normal rats exist at a level of around 2 per 100 leukocytes. The point is that replicated concurrent analysis is most valuable where the absolute level of the analyte is low so that any variation becomes most significant.

Where differences exist between measurements made on different days, differences in average levels of measurements between control and treated groups may falsely arise or be obscured by measurements made on different days for the two groups.

The data from these six examples can also be examined by forming all possible permutations of the replicates to show the biases and chance of incorrect conclusions which could result from nonconcurrent analysis. For purposes of illustration two examples are subjected to this more intensive examination.

Consider the results for GST in female agouti mice. The average values of GST for two animals (only one animal per dose on Day 5) analyzed concurrently on each day for each dose level are given in Table III. There is not

TABLE III. AVERAGE GST LEVELS (U/mg) IN FEMALE AGOUTI MICE

| Replicate (day) | Streptozotocin 0 mg/kg | Streptozotocin 150 mg/kg | Streptozotocin 200 mg/kg | Average |
|---|---|---|---|---|
| 1 | 1.74 | 0.80 | 3.11 | 1.88 |
| 2 | 3.37 | 3.68 | 3.65 | 3.57 |
| 3 | 2.34 | 2.22 | 2.35 | 2.30 |
| 4 | 0.94 | 0.90 | 2.70 | 1.51 |
| 5 | 4.05 | 3.06 | 4.70 | 3.94 |
| Average ± SE | 2.49 ± 0.26 | 2.13 ± 0.26 | 3.30 ± 0.26 | 2.64 |

a statistically significant difference in the average levels of GST between the controls (2.49) and the 150 mg/kg dose group (2.13) with SE 0.26. The experiment utilizing replicated concurrent controls did show a statistically significant increase in GST at 200 mg/kg (3.30 ± 0.26). The question is whether or not the same conclusions would have resulted if replicated concurrent analyses had not been conducted.

Suppose concurrent analyses for each dose group were not used and all the animals in the zero dose group were measured on 1 day, all animals in the 150 mg/kg dose group were measured on another day, and clinical chemistry analyses on all animals in the 200 mg/kg dose group were done on still another day. For example, if the control had all been measured on Day 1, the best estimate of the average GST level is 1.74. If all the 150 mg/kg dose group were measured on Day 2 the best estimate of the average GST level is 3.68 and for the 200 mg/kg dose group on Day 3 is 2.35. If the experiment had been conducted in this manner the conclusion would have been that the 150 mg/kg group showed a significant increase in GST level but the 200 mg/kg dose group did not. This would have been difficult to explain biologically and is just the opposite of what is indicated by the replicated concurrent analyses in Table III.

It is useful to consider all possible $5 \times 4 \times 3 = 60$ permutations of results of running the three dose groups on 3 different days rather than concurrently. The 60 permutations are constructed in the following manner. Suppose the 0 mg/kg dose group was measured on the first day and gave an average GST level of 1.74. For nonconcurrent analyses, the 150 mg/kg dose group could be measured on any one of the 4 days other than the first day. Suppose the 150 mg/kg dose group was measured on the second day and gave an average GST level of 3.68. Then, the 200 mg/kg dose group could have been measured on any 1 of the 3 remaining days, giving average GST levels of 2.35, 2.70, or 4.70. The remainder of the permutations are constructed in a similar manner.

The average of the replicated concurrent analyses indicate that there was no statistically significant change from controls (2.49 ± 0.26) in the GST level for the 150 mg/kg dose group (2.13 ± 0.26) and an increase for the 200 mg/kg dose group (3.30 ± 0.26) (Table III). An examination of the 60 possible permutations shows that only six cases would lead to the same conclusions. That is, with nonconcurrent analyses only 6 out of 60 experiments would have given results similar to those obtained with concurrent analyses. Less than half of the nonconcurrent experiments would have indicated an increase in GST with increasing doses of streptozotocin. In fact, 12 out of the 60 nonconcurrent experiments would have indicated a tendency of decreasing GST levels with increased doses. Not only do nonconcurrent analyses result in less precision, but they can also lead to incorrect conclusions a high percentage of the time.

As a second example investigating all possible permutations of results which could result from nonconcurrent analyses, consider the data for GSH in male agouti and yellow mice. The average values of GSH for the four animals analyzed concurrently are given in Table IV. There are no statistically significant differences in the average levels of GSH for the agouti and yellow male mice by concurrent analyses. The question is whether or not the same conclusions would have resulted if replicated concurrent analyses had not been conducted.

Suppose concurrent analyses were not run and all 16 agouti mice were analyzed for GSH on 1 day and the 16 yellow mice were measured on a different day. For example, if the agouti mice had all been measured on Day 1 with an average GSH level of 2.98 and the yellow mice were all measured on Day 2 with an average GSH level of 3.33, no statistically significant difference would have been obtained. This is in agreement with the replicated concurrent analyses results. However, if the yellow mice had all been measured on Day 3

TABLE IV. AVERAGE GSH LEVELS (μg/mg) IN MALE AGOUTI AND YELLOW MICE

| Replicate (day) | Agouti | Yellow | Average |
|---|---|---|---|
| 1 | 2.98 | 3.63 | 3.30 |
| 2 | 2.04 | 3.33 | 2.68 |
| 3 | 2.20 | 2.24 | 2.22 |
| 4 | 1.64 | 1.90 | 1.77 |
| Average ± SE | 2.22 ± 0.19 | 2.78 ± 0.19 | 2.50 |

with an average GSH level of 2.24, it would have been concluded that GSH levels are statistically higher in the agouti mice. All possible 12 permutations of the results which could have been obtained by running analyses for the two phenotypes on different days are shown in Table V.

The average of the replicated concurrent analyses indicate that there is not a statistically significant difference in GSH levels for agouti and yellow male mice. For nonconcurrent analyses, cases 1, 5, 6, and 9 would have led to the same conclusion. Cases 2 and 3 would have led to the conclusion that the average GSH levels in the agouti mice were higher than those in the yellow mice. In the six remaining cases, the GSH levels for the agouti mice would have been considered significantly lower than for the yellow mice. Again, not only do nonconcurrent analyses result in less precision, but they frequently also lead to incorrect conclusions regarding average clinical levels.

**Conclusions.** These six sets of data illustrate the increase in precision that can be accomplished by using replicated concurrent analyses. In one case, four times as many animals would have been required with nonconcurrent analyses to achieve the same precision. Two sets of data were analyzed more extensively to construct all the possible expected experimental outcomes had nonconcurrent analyses been conducted. These studies indicate that incorrect conclusions concerning the comparison of average enzyme levels in different groups of animals would have been incorrect over one-half of the time. Undoubtedly, current literature must contain many cases of lack of correlation or spurious correlation of clinical pathology levels with disease entities or chemical exposures because analyses for groups of animals to be compared were conducted at different times. The method of replicated concurrent analyses (randomized blocks) can result in increased accuracy and precision of clinical pathology measurements in the study of the mechanisms of disease, increase the confidence in the analysis and interpretation of the toxicological data, and decrease the number of analyses and animals required for production of useful data.

TABLE V. PERMUTATIONS OF GSH RESULTS FOR NONCONCURRENT ANALYSES

| Case | Agouti ($\mu$g/mg) | Yellow ($\mu$g/mg) |
|---|---|---|
| 1 | 2.98 | 3.33 |
| 2 | 2.98 | 2.24 |
| 3 | 2.98 | 1.90 |
| 4 | 2.04 | 3.63 |
| 5 | 2.04 | 2.24 |
| 6 | 2.04 | 1.90 |
| 7 | 2.20 | 3.63 |
| 8 | 2.20 | 3.33 |
| 9 | 2.20 | 1.90 |
| 10 | 1.64 | 3.63 |
| 11 | 1.64 | 3.33 |
| 12 | 1.64 | 2.24 |

1. Westgard JO, Groth T. A multi-rule Shewhart control chart for quality control in clinical chemistry. Clin Chem **27**:493–501, 1981.
2. Anderson RL, Bancroft TA. Statistical Theory in Research. New York, McGraw-Hill, 1952.
3. Bergmeyer HU, Scheibe P, Wahlefeld AW. Optimization of methods for aspartate aminotransferase and alanine aminotransferase. Clin Chem **24**:58–73, 1978.
4. Silverman LM, Christenson RH, Grant GH. Amino acids and proteins. In: Tietz NW, ed. Textbook of Clinical Chemistry. Philadelphia, Saunders, 1986.
5. Habig WH, Pabst MJ, Jakoby WB. Glutathione *S*-transferases. The first step in mercapturic acid formation. J Biol Chem **249**:7130–7139, 1974.
6. Agius C, Gidari AS. Effects of streptozotocin on the glutathione *S*-transferases of mouse liver cytosol. Biochem Pharmacol **34**:811–819, 1985.
7. Jaeschke H, Wendel A. Manipulation of mouse organ glutathione contents I: Enhancement by oral administration of butylated hydroxyanisole and butylated hydroxytoluene. Toxicology **36**:77–85, 1985.
8. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **193**:265, 1951.
9. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys **82**:70–77, 1959.
10. Jollow DJ, Mitchell JR, Zampaglione N, Gillette JR. Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3,4-bromobenzene oxide as the hepatotoxic metabolite. Pharmacology **11**:151–169, 1974.

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