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The Effect of Time of Death on Extravascular Tissue/Blood Secobarbital Concentration Ratios in the Rat

The concentration of a drug in the liver is often significantly higher than its concentration in the blood [*1*]. Curry and Sunshine [*2*] obtained data from 52 cases of barbiturate poisoning involving quick- or intermediate-acting barbiturates in which some information was available on the time between drug ingestion and death. It was observed that when the liver/blood ratio was greater than 4, death had usually occurred within 5 h of ingestion, and when the liver/blood ratio was less than 4, no relationship between time of drug ingestion and death was indicated.

The presence of blood in tissue may bias drug measurements. The blood content of human liver tissue has been estimated to represent 15% of its weight [*3*] and that of brain tissue to be 6 to 8% [*4*]. The blood content of rat tissues measured by various methods has been reported [*5-9*]. Muelheims et al [*9*], using ^{51}Cr-labeled red blood cells, determined the blood content of rat brain to be less than 1% of its weight and that of rat liver to be less than 5%. The present study was undertaken to investigate the relationship between extravascular liver/blood and extravascular brain/blood secobarbital concentration ratios and the interval of time between drug administration and the death of male Wistar rats. The effect of enzyme induction by pretreatment with phenobarbital was also studied.

Methods and Materials

Blood Volume Determination

All experiments used male Wistar rats weighing 180 to 200 g. For the measurement of tissue blood volume, a technique similar to the method of Muelheims et al [*9*] was employed. Three millilitres of blood was obtained from a donor rat by heart puncture on the morning of the experiment, and to this was added 0.4 ml of an acid-citrate-dextrose solution (dextrose, 12 mg/ml; sodium citrate, 25 mg/ml; and citric acid, 0.75 mg/ml) and 50 μCi ^{51}Cr.[3] The blood was allowed to incubate for 1 h at 37°C and was then washed twice with 0.9% saline and reconstituted to its original volume. A volume of 0.5 ml of this ^{51}Cr-labeled blood was injected through the tail vein of each rat at least 15 min prior to the start of the experiment to allow the ^{51}Cr-labeled red blood cells to be equally

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distributed throughout the rat's circulatory system. With this technique, blood volume was estimated in tissues according to the following equation:

$$\frac{\text{ml blood}}{\text{g tissue}} = \frac{\text{counts}}{\text{g tissue}} \div \frac{\text{counts}}{\text{ml blood}}$$

Correction was not made for small differences in hematocrit between blood obtained by heart puncture and from individual tissues. Each animal was treated as described before the drug treatment.

Secobarbital Assay

The method of Kananen et al [*10*] was modified for blood, brain, and liver secobarbital analyses. This procedure does not use direct solvent extracts of biological materials since artifacts extracted along with the barbiturate produce interfering chromatographic peaks. Analysis was carried out on 2 ml whole blood and 1 to 1.5 g brain or liver. A 3.5 mg/dl solution of sodium pentobarbital was used as the internal standard for all specimens. Brain and liver specimens were weighed, homogenized in a solution containing 2 ml 7% perchloric acid and 2 ml internal standard with a Polytron homogenizer, and refrigerated overnight. After centrifugation, the barbiturate assay was carried out on 2 ml of the supernatant. A Varian-Aerograph Series 2700 gas chromatograph equipped with a flame ionization detector and a 1.8-m (6-ft) by 2-mm inside diameter glass column packed with 3% SE-30 stationary phase on Gas-Chrom Q, 60-80 mesh, was used for the analyses. The nitrogen carrier gas had a flow of 30 ml/min, and the oven temperature was maintained at 175°C, the injection port at 250°C, and the detector at 270°C. Pentobarbital eluted from the column at 1.6 min and secobarbital at 2 min. Peaks were well separated and no tailing was observed. No interfering peaks were present in blank specimens of blood, brain, and liver. Standard curves in each of these tissues were prepared with each set of assays. Calibration curves in all three tissues were linear in the range of concentrations used in this study.

Calculation of Extravascular Tissue Secobarbital Concentration

From peak height ratios of unknown/internal standard, liver, brain, and blood concentrations were calculated from the standard curve. The brain/blood and liver/blood secobarbital concentration ratios were expressed as mg/g tissue per mg/ml blood. For the calculation of extravascular tissue concentration *ETC*, the amount of drug present in the vascular portion of brain and liver was subtracted from the total tissue drug concentration *TTC* as follows:

$$ETC = \frac{TTC - [\text{blood concentration} \times (\text{ml blood/g tissue})]}{1 \text{ g whole tissue} - 1.035 \text{ (ml blood/g tissue)}}$$

where the value 1.035 is the average density of rat blood at room temperature.

Statistical Methods

Statistical methods employed included regression analysis, analysis of variance, and a multiple range test. A probability level of less than 0.05 was considered statistically significant.

Results

Comparison of Ratios

An initial experiment was conducted to compare extravascular tissue/blood secobarbital concentration ratios to total tissue/blood ratios following an intraperitoneal dose of secobarbital. Animals were then given intraperitoneal doses of secobarbital bracketing and including the median lethal dose LD_{50} (72, 86, and 104 mg/kg body weight) and were killed 10 min later when most of the high-dose animals were near death. A specimen of blood was obtained by heart puncture. Brain and liver were removed, immediately wrapped in foil, frozen in liquid nitrogen, and stored in a freezer. On the same or next day, liver and brain specimens were weighed and counted for 2 min on a Packard automatic gamma counter. An aliquot of 0.5 ml blood from each animal was also counted. Secobarbital assays were performed on these same tissues.

The results of this experiment are shown in Table 1. Extravascular liver/blood ratios were greater than total liver/blood ratios by an average of 6%. Extravascular brain/blood ratios were greater than total brain/blood ratios by an average of 1%.

Total and extravascular liver/blood and brain/blood ratios were significantly higher ($P < 0.05$) in rats given secobarbital in a dose of 104 mg/kg body weight than in rats given 72 or 86 mg/kg body weight. There was no significant difference between extravascular or total tissue/blood ratios in rats given 72 or 86 mg/kg body weight in either brain or liver.

There was no significant effect of dose on the percentage of the difference between extravascular and total tissue/blood secobarbital concentration ratios.

Effects of Time

A total of 54 animals were randomly assigned into three groups. Following a 12-h fast, each group was given secobarbital by mouth in a dose of either 41, 50, or 60 mg/kg body weight. This range of doses was chosen because a preliminary study indicated that 60 mg/kg body weight was the maximum dose which allowed survival of all animals for the duration of the experiment. At intervals of ¼, 1, 2, 3, 4, or 6 h after administration of the drug, animals from each dose level were killed. Blood volume was measured in brain and liver, and these tissues along with blood were assayed for secobarbital.

As before, extravascular liver/blood ratios were an average of 6% higher than total liver/blood ratios and extravascular brain/blood ratios were an average of 1% higher

TABLE 1—*Comparison of extravascular tissue/blood and total tissue/blood secobarbital concentration ratios.*

Dose, mg/kg	n	Extravascular Tissue/Blood, Mean (± SE)[a]		Total Tissue/Blood, Mean (± SE)		Difference,[d] %	
		Liver[b]	Brain[c]	Liver[b]	Brain[c]	Liver	Brain
72	9	3.45 (0.06)	1.59 (0.05)	3.29 (0.05)	1.57 (0.05)	4.8	1.3
86	9	3.82 (0.32)	1.53 (0.03)	3.56 (0.30)	1.52 (0.03)	7.3	0.6
104	10	4.65[e] (0.35)	1.77[e] (0.06)	4.40[e] (0.32)	1.75[e] (0.06)	5.7	1.1

[a]SE = standard error.
[b]mg secobarbital/g liver ÷ mg secobarbital/ml blood.
[c]mg secobarbital/g brain ÷ mg secobarbital/ml blood.
[d][(Extravascular − total) ÷ total] × 100.
[e]Significantly higher at 104 mg/kg body weight than at low or middle dose ($P < 0.05$).

than total brain/blood ratios. The results of this experiment are shown in Fig. 1. For each time period, data are pooled over all doses since preliminary analysis of variance indicated there was no significant effect of dose on tissue/blood ratios at any given time period.

There was a significant time effect ($P < 0.05$) with the liver/blood ratios shown by regression analysis; however, there was no significant difference between any time periods by analysis of variance. It appears that for animals killed within 1 h of administration of the drug, the liver/blood ratio is greater than for animals killed after longer intervals. There was no significant time effect with brain/blood ratios, suggesting that these ratios are not indicative of the interval of time between drug administration and death.

Effect of Phenobarbital Pretreatment

Thirty-six rats were randomly assigned to three groups and pretreated with phenobarbital, 80 mg/kg body weight, daily for four days. On the fifth day, following a 12-h fast, each group was dosed with secobarbital orally with either 150, 216, or 311 mg/kg body weight. This range of doses was chosen since a preliminary study indicated that 311 mg/kg body weight was the maximum dose that allowed survival of animals pretreated with phenobarbital. These animals were able to tolerate higher doses than the animals in the previous study that had not been pretreated because of the effect of enzyme induction on tissue drug levels. Animals from each dose level were killed at intervals of ¼, 1, or 4 h after the drug administration. As in previous experiments, blood volume was measured in brain and liver, and brain, blood, and liver were assayed for secobarbital.

The results of this study are shown in Fig. 2. As before, data were pooled over all

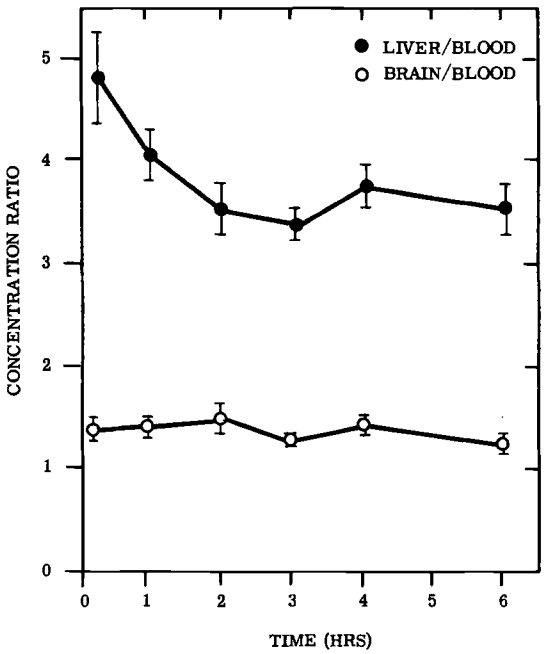

FIG. 1—*Extravascular liver/blood (●) and extravascular brain/blood (○) secobarbital concentration ratios versus time in hours between secobarbital administration and death. Vertical bars represent ±1 standard error of the mean for nine animals.*

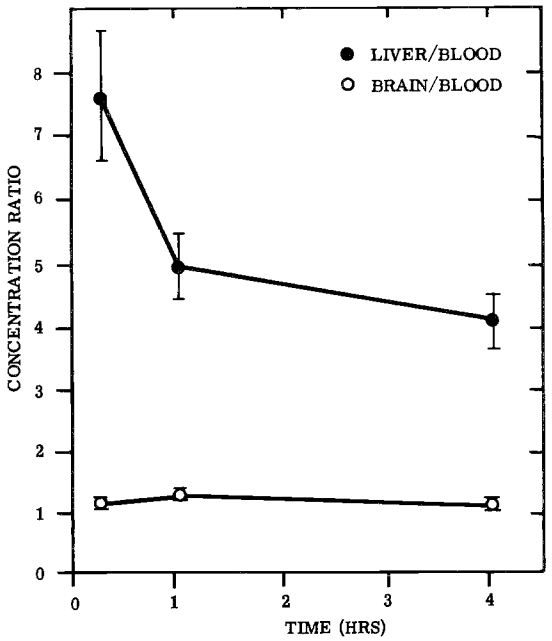

FIG. 2—*Extravascular liver/blood (●) and extravascular brain/blood (○) secobarbital concentration ratios versus time in hours between secobarbital administration and death in rats pretreated with phenobarbital, 80 mg/kg body weight daily for four days. Vertical bars represent ±1 standard error of the mean for twelve animals.*

doses since a preliminary analysis of variance indicated that there was no significant effect of dose on tissue/blood ratios at any given time period.

There was a significant time effect ($P < 0.01$) (by regression analysis) with the liver/blood ratios. In addition, the liver/blood ratio at ¼ h was significantly higher ($P < 0.05$) than at 1 or 4 h.

There was no significant regression, and hence no time effect, with the brain/blood ratios.

The tissue/blood ratios in pretreated and non-pretreated animals are compared in Fig. 3. Liver/blood ratios appear higher in pretreated animals than in non-pretreated animals. However, the P value was 0.1. Brain/blood ratios are similar at all time periods.

Liver, brain, and blood secobarbital concentrations in pretreated and non-pretreated animals are shown in Table 2. Although pretreated animals received doses up to four times greater than non-pretreated animals, tissue levels of secobarbital were lower or nearly the same in pretreated compared to non-pretreated animals at ¼ and 1 h, demonstrating the effect of enzyme induction on decreasing secobarbital levels at these time periods. At 4 h, however, tissue levels in pretreated animals were significantly higher ($P < 0.01$) than in those of non-pretreated animals.

Summary

Extravascular liver/blood and brain/blood ratios were found to be an average of 6% and 1% higher, respectively, in all experiments than total liver/blood and brain/blood ratios. This difference may be informative in establishing true tissue levels. There was a significant time effect ($P < 0.05$) with the extravascular liver/blood ratios but not with the extravascular brain/blood ratios. Extravascular liver/blood ratios were slightly higher

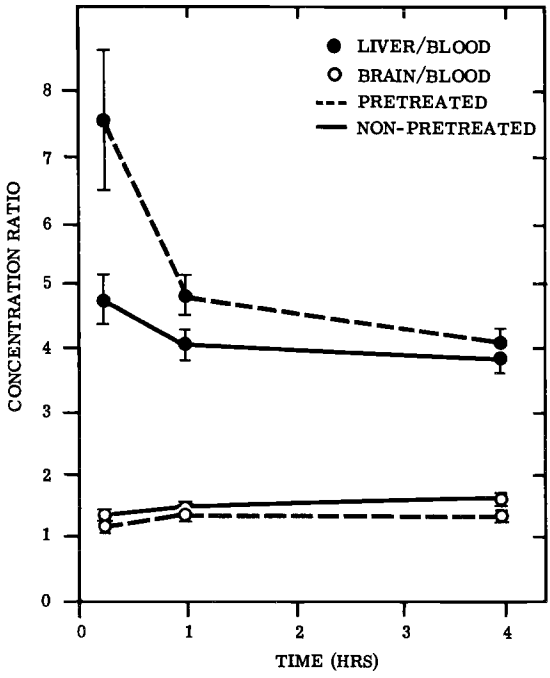

FIG. 3—*Comparison of extravascular liver/blood (●) and extravascular brain/blood (○) secobarbital concentration ratios versus interval of time between secobarbital adminstration and death in phenobarbital-pretreated (- - -) and non-pretreated (——) rats.*

in phenobarbital-pretreated animals than in non-pretreated animals. Tissue secobarbital levels in pretreated and non-pretreated animals are not different at ¼ or 1 h, even though pretreated animals received higher doses than non-pretreated animals. Tissue levels are significantly higher ($P < 0.01$) in pretreated animals than in non-pretreated animals at 4 h. It is possible that, at this time period, the barbiturate-metabolizing enzymes have become saturated or exhausted.

TABLE 2—*Liver, brain, and blood concentrations in phenobarbital-pretreated and non-pretreated animals at ¼, 1, and 4 h after administration of secobarbital.*

	¼ h		1 h		4 h	
Specimen	Non-Pretreated, Mean (±SE)[a]	Pretreated, Mean (±SE)	Non-Pretreated, Mean (±SE)	Pretreated, Mean (±SE)	Non-Pretreated, Mean (±SE)	Pretreated, Mean (±SE)
Liver[b]	71.9 (10.5)	61.4 (7.8)	54.1 (6.0)	69.7 (9.8)	27.0 (7.4)	62.2 (8.5)[c]
Brain[d]	22.1 (3.2)	13.8 (3.0)	27.2 (6.2)	21.3 (2.9)	10.9 (3.2)	20.2 (3.3)[c]
Blood[e]	16.2 (2.1)	10.8 (2.0)	14.8 (1.9)	15.3 (1.8)	7.4 (2.0)	16.3 (2.5)[c]

[a] SE = standard error.
[b] mg secobarbital/g total liver.
[c] Significantly higher than non-pretreated at 4 h ($P < 0.01$).
[d] mg secobarbital/g total brain.
[e] mg secobarbital/ml blood.

Conclusion

In agreement with Curry and Sunshine's findings in humans [2], we observed that the liver/blood secobarbital concentration ratio is correlated with the time between administration of the drug and death in the rat. However, the variability in these measurements precludes an accurate prediction of this interval. It also does not appear to be necessary to correct for vascular secobarbital concentration in this type of study.

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References

[1] Wright, J. T., "The Value of Barbiturate Estimations in the Diagnosis and Treatment of Barbiturate Intoxication," *Quarterly Journal of Medicine,* Vol. 24, No. 93, Jan. 1955, pp. 95-108.

[2] Curry, A. S. and Sunshine, I., "The Liver/Blood Ratio in Cases of Barbiturate Poisoning," *Toxicology and Applied Pharmacology,* Vol. 2, No. 5, Sept. 1960, pp. 602-606.

[3] Braver, R. W., "Liver Circulation and Function," *Physiological Reviews,* Vol. 43, No. 1, Jan. 1963, pp. 115-147.

[4] Brante, G., "Studies on Lipids in the Nervous System," *Acta Physiologica Scandinavica,* Vol. 18, Supp. 63, 1949, pp. 1-189.

[5] Caster, W. O., Simon, A. B., and Armstrong, W. D., "Evans Blue Space in Tissues of the Rat," *American Journal of Physiology,* Vol. 183, No. 2, Nov. 1955, pp. 317-321.

[6] Lewis, A. E., Goodman, R. D., and Shuck, E. A., "Organ Blood Volume Measurements in Normal Rats," *Journal of Laboratory and Clinical Medicine,* Vol. 39, No. 5, May 1952, pp. 704-710.

[7] Dellenbeck, R. J. and Muelheims, G. H., "Red Blood Cell Volume and Distribution Before and After Bleedout in the Rat," *American Journal of Physiology,* Vol. 198, No. 6, June 1960, pp. 1177-1180.

[8] Everett, N. B., Simmons, B., and Lasher, E. P., "Distribution of Blood (Fe^{59}) and Plasma (I^{131}) Volumes of Rats Determined by Liquid Nitrogen Freezing," *Circulation Research,* Vol. 4, No. 4, July 1956, pp. 419-424.

[9] Muelheims, G. H., Dellenback, R., and Rawson, R., "Red Cell Volume and Distribution After Bleed-out in the Rat as Determined by Cr^{51}-Labeled Red Cells," *American Journal of Physiology,* Vol. 196, No. 1, Jan. 1959, pp. 169-172.

[10] Kananen, G., Osiewicz, R., and Sunshine, I., "Barbiturate Analysis—A Current Assessment," *Journal of Chromatographic Science,* Vol. 10, No. 5, May 1972, pp. 283-287.

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