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Research Articles

Determination of Drug Absorption Rates Without Chemical Assay

By GERHARD LEVY and KAREN E. MILLER*

This study illustrates the possibility of determining absorption rates of certain drugs without using chemical assays. The method is based on the determination of the time of onset of a suitable pharmacologic response under conditions where a constant drug concentration gradient across the absorbing membranes is maintained. While particularly suitable to studies with fish and other aquatic animals, the method may also be applicable to mammals for determining the absorption rate of certain volatile substances or aerosols administered by the pulmonary route and of certain dissolved drugs administered by intestinal perfusion.

ONE OF THE most important considerations in the pharmacologic and toxicologic evaluation of chemotherapeutic agents, pesticides, and other chemicals is their ability to pass across biologic membranes. Absorption studies ordinarily require chemical analysis of blood, urine, intestinal content, tissues, or of the solution from which the drug is being absorbed. At times, this requirement can represent an almost insurmountable barrier because of the lack of a sufficiently sensitive or specific analytical method. Recently, we have developed and tested a mathematical model which describes the relationship between drug absorption rate, drug concentration in the aqueous medium, and time of occurrence of a suitable pharmacologic effect in fish (1). This model is the basis for a novel method for the determination of drug absorption rates without chemical analysis and is described in this report.

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Levy and Gucinski have shown (1) that the time of death (T_L) of fish due to passive absorption of a drug is related to the concentration (C) of that drug in the aqueous medium in the following manner

$$\frac{1}{T_L} = \frac{DA}{L} C \qquad \text{(Eq. 1)}$$

where L is the lethal dose of the drug, D is the absorption rate constant, and A is the area of the absorbing membrane. This relationship is based on the following requirements: (*a*) absorption occurs by passive diffusion and therefore is not a saturable process; (*b*) the drug concentration gradient across the absorbing membranes remains essentially constant during the experiment; (*c*) the permeability characteristics of the membrane do not change with time or drug concentration over the time and concentration range of the experiment; (*d*) drug elimination is negligible during the time of the experiment; and (*e*) the pharmacologic end point (death) occurs without significant delay after a given amount of drug (the lethal dose) has been absorbed.

In essence, the requirement that absorption occur by passive diffusion is fulfilled by most nonphysiologic substances; an essentially constant concentration gradient can be maintained by using sufficiently high drug concentrations and relatively large

volumes of solution; drug elimination need not be considered in fish since apparently they do not metabolize drugs but eliminate them solely by passive diffusion through the gills (2); this effect would be negligible in magnitude under the experimental contions employed, in view of the high concentration gradient in the opposite direction. In species capable of metabolizing drugs, the requirement for negligible elimination during the time of the experiment can be satisfied in most instances by using drug concentrations high enough to cause death in a relatively short time. Fortunately, the experimental data will in themselves indicate if the specified requirements have been fulfilled, since a plot of reciprocal time of death *versus* drug concentration (which should be linear and intersect the origin) will not yield a straight line if the necessary conditions are not present. Compounds for which the presently described method is not suitable include (*a*) substances which affect the permeability of the membrane by denaturing or precipitating proteins or mucus, or which have an erosive or otherwise directly toxic effect (such substances would present similar difficulties when other methods are used to study their absorption); (*b*) substances which have a delayed, inductive, or other indirect pharmacologic effect.

As apparent from Eq. 1, the slope of a plot of reciprocal time of death *versus* drug concentration represents the value DA/L. If the lethal dose, L, is known, DA can be calculated. It is not possible in most instances to determine the absorption area, A, with any degree of accuracy. However, using animals of equal size (weight) in each experiment, it is possible to combine D and A to obtain a relative absorption rate constant K

$$K = DA \qquad \text{(Eq. 2)}$$

The purpose of the present study was (*a*) to determine if the lethal dose, L, of a drug absorbed by fish from an aqueous medium is essentially equal to the lethal dose when the drug is administered parenterally and—if this was so—(*b*) to test the proposed method by determining the relative rates of absorption of a series of four barbiturates known to differ in their absorption rates and partition coefficients (3).

EXPERIMENTAL

Goldfish, *Carassius auratus*, common variety, weighing about 7 Gm. (for the absorption study) and about 25 Gm. (for the determination of lethal dose as a function of route of administration), were used. In each of the studies all fish were from the same lot.

Secobarbital Assay.—The assay consisted of the extraction of secobarbital from homogenized goldfish tissue (adjusted to pH 6) into chloroform, followed by extraction of the chloroform phase with aqueous buffer of pH 10.2. Secobarbital in the aqueous buffer phase was determined by differential spectrophotometry, based on the difference in ultraviolet absorption spectra of barbiturates in strong alkali and in solutions of pH 10.2, respectively, as reported by Goldbaum (4).[1]

Individual goldfish, which died after immersion in secorbarbital solution, were homogenized in the semimicro container of a Waring Blendor after addition of 25 ml. of phosphate buffer (0.05 *M*, pH 6.0) and 3 Gm. of sodium chloride per 25 Gm. of fish tissue. The homogenate was extracted five times with 15-ml. portions of chloroform. A 30-ml. aliquot of the chloroform phase was reduced in volume to 5 ml. by evaporation, then extracted three times with 10-ml. portions of phosphate buffer (0.05 *M*, pH 10.2). Five milliliters of 3.75 *N* NaOH was added to one 10-ml. aliquot of the combined pH 10.2 phosphate buffer phase (solution *A*), while 5 ml. of phosphate buffer (pH 10.2) was added to another 10-ml. aliquot (solution *B*). The absorbance of solution *A* at 262 mμ was determined with a Beckman DU spectrophotometer, using solution *B* as a blank. A small correction had to be made for the apparent absorbance of sodium hydroxide, since each of the various lots of reagent grade sodium hydroxide available produced a small but reproducible and concentration-dependent absorbance.[2] Average recovery of secobarbital (in the range around 0.05 mg. per Gm. fish tissue) was 78%, and the data were corrected accordingly.

Determination of Lethal Dose by Injection.—Each goldfish was weighed in a tared beaker of water, and the appropriate amount of aqueous drug solution was injected intraperitoneally with a 0.25-ml. capacity tuberculin syringe with a 27-gauge, 1/4-in. regular point hypodermic needle. The concentration of the injected drug solution was such that 0.01 ml. of solution was injected per gram of body weight. In preliminary experiments, injections were also made intramuscularly at a point slightly lateral to the anterior point of the dorsal fin. After injection, the fish were placed in 1-gal. jars of aerated water, with separate jars used for each group of fish (on the basis of the administered dose). The jars were checked periodically, and dead fish were removed. A final count was made after 24 hours, although deaths occurred usually within the first 2–3 hours. Each drug was administered in from 6 to 10 logarithmically equally spaced dose levels, sufficient that at least the lowest dose caused no deaths, and the highest dose resulted in deaths of all fish in that group. Ten fish were used for each dose level (*i.e.*, 60–100 fish per drug). LD_{50} values were calculated by the Spearman-Karber method, as described by Irwin and Cheeseman (5) and amplified by Epstein and Churchman (6).

Determination of Time of Death.—Barbiturate solutions of various concentrations were prepared by dissolving the respective drugs in tris(hydroxymethyl)aminomethane (tris) buffer (0.05 *M*, pH 5.9),[3] then readjusting the pH to 5.9 where necessary. One-hundred-milliliter portions of the solutions were placed in 250-ml. capacity beakers. The temperature of the solutions was maintained at 22 ±1°. Single goldfish were placed in each beaker; the time of death, evidenced by cessation of gill and mouth movement, was recorded.

To eliminate bias in the time of death determina-

[1] Goldbaum actually used a buffer solution of pH 10.5. In our study, the use of such solutions for extraction yielded a turbid aqueous phase which made accurate spectrophotometric analysis impossible. Lowering the pH to 10.2 prevented the occurrence of this turbidity.

[2] *Caution:* Strong NaOH solutions will damage quartz cells on prolonged contact. Such solutions should remain in the cells for a period as short as possible, and the cells should be rinsed promptly with dilute acid.

[3] Tris was used despite its relatively poor buffer capacity at pH 5.9 because of the known innocuousness of this material to fish (1) and because it maintained the desired pH without difficulty under the experimental conditions.

TABLE I.—EFFECT OF ROUTE OF ADMINISTRATION ON LETHAL DOSE OF SECOBARBITAL

i.p. Injection, LD_{50}	1.49×10^{-4} mM/Gm.
	(S.D. $+ 0.09 \times 10^{-4}$, -0.07×10^{-4} mM/Gm.)
Av. drug content at death due to immersion in 25 mg. % secobarbital soln.	1.46×10^{-4} mM/Gm.
	(S.D. 0.09×10^{-4} mM/Gm.)

TABLE II.—EFFECT OF ROUTE OF INJECTION OF SECOBARBITAL ON LETHALITY OF GOLDFISH

Date	Dose, mM/Gm.	Lethality[a] i.p.	Lethality[a] i.m.
10–18–1963	1.54×10^{-4}	2/5	1/5
10–21–1963	1.92×10^{-4}	7/8	4/8
10–26–1963	1.92×10^{-4}	8/8	4/8
10–30–1963	1.92×10^{-4}	8/8	4/8

[a] Number of deaths/number of fish injected.

tions, each beaker of drug solution was marked solely with a code number by an individual not involved in the study. This code was broken only after all determinations had been completed.

Partition Coefficients.—Partition coefficients were determined using 0.1 *N* HCl and olive oil as the aqueous and organic phases, respectively. The phases were shaken at room temperature (about 22°) until equilibrium was attained (24 to 48 hours). The aqueous layer was removed, centrifuged, diluted with 0.5 *N* NaOH, and assayed spectrophotometrically.

RESULTS AND DISCUSSION

To determine the effect of route of administration on the lethal dose of a barbiturate, goldfish weighing about 25 Gm. were injected intraperitoneally with graded doses of secobarbital sodium. A number of other fish of the same lot was placed in an aqueous solution of 25 mg.% secobarbital, and each fish was removed immediately after death. The fish were then rinsed with water and assayed individually.[4] Results are recorded in Table I. The drug content of the fish at death due to immersion in secobarbital solution varied only slightly between individual fish, and a similar extremely narrow distribution was apparent in the mortality data after intraperitoneal injection of the drug. Strictly speaking, comparison of lethal doses observed upon drug administration by different routes requires that the per cent mortality *versus* injected dose data be expressed in terms of a weighted average lethal dose. However, in view of the steep rise of mortality with a small increase of administered dose, it was possible to use the LD_{50} values as a measure of the average lethal dose. The respective LD_{50} values were in no case more than 5% below the apparent weighted average lethal doses. Use of the former values was preferred because they could be calculated by objective and well-known procedures, while the latter required somewhat subjective manipulations. (This simplified approach cannot be used if lethal dose distribution is wide and if it differs significantly from normal.)

As evident from the data in Table I, the lethal dose of secobarbital upon intraperitoneal administration was the same as the lethal dose (body drug content) upon immersion of fish in drug solution. This is not surprising since the drug enters directly into the circulation by either route. In contrast, differences in lethal dose as a function of route of administration are quite common in higher animals for a number of reasons [reviewed by Levy (7)]. For example, lethal doses, upon oral administration of a drug, may be higher than parenteral lethal doses because of partial degradation of the drug in the gastrointestinal tract, incomplete absorption, partial biotransformation in the intestinal wall, different initial distribution, and sufficiently slow absorption (though rapid elimination), resulting in maximum drug levels considerably lower than those after parenteral administration. None of these factors apply to fish since absorption takes place predominantly across the gills, biotransformation of drugs does not occur [at least in the case of those drugs, including barbiturates, studied to date (2)], and elimination (which is by passive diffusion across the gills and requires a favorable concentration gradient) is negligible under the conditions of the experiment. Actually, elimination is favored in the experiments where the drug is administered parenterally since the fish are placed in pure water after they have been injected. However, death occurs rapidly, and elimination of barbiturates is hindered not only by protein binding but also by partial ionization of these drugs in the tissue fluids, so that this factor does not appear to be important. These results make possible the use of lethal dose data obtained by intraperitoneal injection of graded doses, together with slope values (DA/L) determined from time of death *versus* drug concentration data, to calculate relative absorption rate constants (DA or K).

In initial experiments, parenteral injections were made by the intramuscular route. Lethal dose values obtained in this manner were considerably higher than lethal dose values from immersion of fish in drug solution. The data in Table II demonstrate that, when equal doses of drug were administered by the intraperitoneal route, the lethality was consistently greater than when administered by the intramuscular route. It was found, in agreement with observations by Bergmann *et al.* (8) and Chavin (9), that some leakage of drug solution occurred after intramuscular injection and was responsible for the apparently higher lethal doses when drug was administered by this route. That physical injury caused the observed differences was ruled out since no deaths occurred when animals were injected by both routes with comparable volumes of water.

The relationship between reciprocal time of death and drug concentration in the aqueous medium is shown in Fig. 1 for four barbiturates. Since the absorption rate of weak acids by fish is affected by the degree of ionization of these drugs (1), the pH of the solutions was adjusted to 5.9, so that all the barbiturates were in essentially nonionized form. Five concentration levels (10 fish per concentration) were used in the cases of secobarbital and pentobarbital. Only two concentration levels (but 15 fish per concentration) were used for phenobarbital and barbital, respectively, because of limitations imposed by solubility and the long death times at low concentrations. Concentrations yielding times of death

[4] The use of relatively large (25 Gm.) fish was necessary to permit assay of individual fish. In all other parts of the study it was more convenient to use smaller (7 Gm.) fish.

greater than 100 minutes were not used because death occurred gradually, end points proved too difficult to determine, and results were accordingly variable.

Figure 2 is a plot of per cent mortality *versus* intraperitoneal dose (logarithmic scale) for the four barbiturates. Although each drug was administered in between six to 10 logarithmically equal spaced doses, only the highest of the LD_0 and the lowest LD_{100} values were plotted in addition to the intermediate values. As mentioned previously, the distribution of the mortality as a function of dose was extremely narrow. The LD_{50} for secobarbital was the same for 7 Gm. and 25 Gm. goldfish, although this could have been fortuitous.

Relative absorption rate constants for the four barbiturates were calculated from slope values (from data in Fig. 1) and LD_{50} values (from data in Fig. 2) and are shown in Fig. 3. Also shown in the figure are the relative absorption rates of these compounds from the rat colon, as reported by Schanker (3). Such data are comparable because, under the experimental conditions of the rat study (virtual colonic membrane pH 6.5), the drugs were predominantly nonionized (phenobarbital is 89% nonionized, while the other three barbiturates are 96% or more nonionized at pH 6.5). A perfect rank-order correlation is shown between the results obtained from fish and rats.

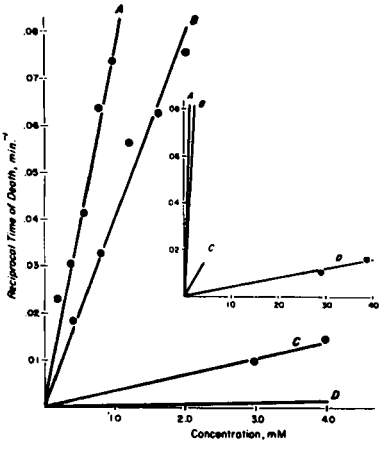

Fig. 1.—Reciprocal time of death of goldfish as a function of barbiturate concentration in pH 5.9 buffer at 22°. Key: *A*, secobarbital; *B*, pentobarbital; *C*, phenobarbital; *D*, barbital. Each data point represents an average of 10 fish (*A* and *B*) or 15 fish (*C* and *D*).

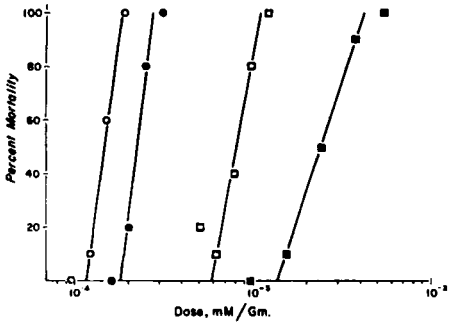

Fig. 2.—Mortality of goldfish as a function of dose of barbiturate injected intraperitoneally. Key: ○, secobarbital; ●, pentobarbital; □, phenobarbital; ■, barbital.

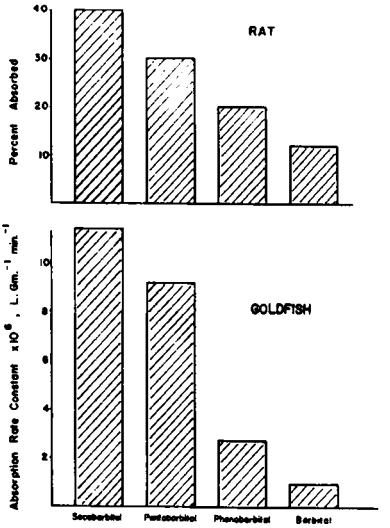

Fig. 3.—Relative absorption rates of four barbiturates in goldfish and from the rat colon. [Rat data from Schanker (3)]. The drugs were essentially nonionized under the conditions of both studies.

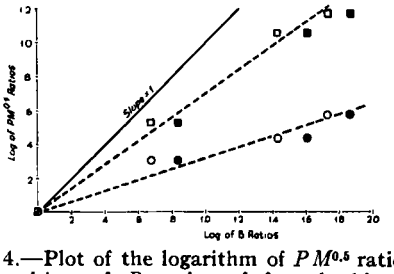

Fig. 4.—Plot of the logarithm of $PM^{0.5}$ ratios *vs.* the logarithm of *B* ratios of four barbiturates. (See *text* for explanation.) Squares represent goldfish data; circles represent rat data reported by Schanker (3). Solid symbols are based on chloroform: 0.1 *N* HCl partition coefficients; open symbols are based on olive oil: 0.1 *N* HCl partition coefficients.

From a theoretical study of the process of diffusion of substances across biologic membranes, Danielli (10) concluded that in the case of rapidly penetrating molecules

$$PM^{0.5} = B \cdot \text{Constant} \qquad \text{(Eq. 3)}$$

where *P* is the permeability, *M* is the molecular weight, and *B* is the lipoid-water partition coefficient of the penetrating substance. He pointed out that this relationship represents a first approximation, and that it is subject to appreciable deviation. Figure 4 is a plot of the logarithm of the $PM^{0.5}$ ratios (based on barbital $PM^{0.5} = 1$) *versus* the logarithm of the ratio of the respective partition coefficients (based on barbital $B = 1$) for the four barbiturates. Chloroform: 0.1 *N* HCl partition coefficients are those of Schanker (3), while olive oil:0.1 *N* HCl

TABLE III.—RATIO OF LD_{50} AND CT VALUES OF FOUR BARBITURATES IN GOLDFISH

Drug	LD_{50} mM/ Gm. $\times 10^{-4}$	Ratio of LD_{50} Values	CT, mM Min.	Ratio of CT Values
Secobarbital	1.46	1.0	12.8	1.0
Pentobarbital	2.26	1.5	24.7	1.9
Phenobarbital	7.85	5.4	290	22.6
Barbital	24.3	16.7	2614	204

partition coefficients were determined in the present study. Schanker's absorption data were corrected to account for the 11% ionization of phenobarbital and the lesser ionization of the other barbiturates under the conditions of his study. The goldfish absorption data are closer to a slope of unity (predicted by theory) than the rat absorption data obtained by Schanker. This may be due to the more severe and somewhat unphysiologic conditions of the rat perfusion method (involving surgery and prolonged perfusion) which may cause the intestinal membranes to become a somewhat less perfect lipoidal barrier. Alternatively, these results could reflect a true species difference or a number of other factors.

A frequently used index of relative toxicity for pesticides, compounds employed in chemical warfare, and other toxic agents is the CT value, which is the product of concentration of the poisonous agent and time of death due to exposure to that concentration of the poison. Levy and Gucinski (1) have shown, on the basis of theoretical considerations, that the CT value is a function not only of the inherent lethality but also of the rate of absorption of a compound. This fact is demonstrated rather convincingly by the results of the present study, as shown in Table III. For example, in a comparison of secobarbital to barbital, based on LD_{50} values, the former is 16.7 times more toxic than the latter. According to the CT values, secobarbital would be judged 204 times as toxic as barbital. The difference is due to the fact that, from Eq. 1

$$CT_L \approx \frac{L}{DA} \qquad \text{(Eq. 4)}$$

and, where animals of equal size are used and A cancels out,

$$\frac{C_1T_{L1}}{C_2T_{L2}} = \frac{L_1/D_1}{L_2/D_2} \qquad \text{(Eq. 5)}$$

where the subscripts refer to two different substances. Thus, the lower rate of absorption of barbital compared to secobarbital causes the ratio of CT values to be greater than the ratio of LD_{50} values. Obviously, the opposite would occur if the less toxic drug is absorbed more rapidly.

The results of this study demonstrate that the rate of absorption of certain drugs can be determined without chemical assay. The potential advantages of such a method are obvious: it may permit absorption rate studies with drugs which cannot be studied otherwise due to the lack of a sufficiently sensitive or specific analytical method. However, the theoretical considerations outlined in this and in previous report (1) should make it quite clear that the method is not suitable for all drugs. While it is particularly useful for fish and certain other aquatic animals, and although experimental verification has only been carried out with fish so far, the method is also applicable *in principle* to mammals, provided a constant drug concentration gradient across the absorbing membranes can be maintained. (This is often possible with aerosol application and with intestinal perfusion.) However, for reasons referred to previously, there may be practical limitations due to differences in lethal dose as a function of route of administration and/or due to rapid drug elimination. Further studies in this area are in progress.

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