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The analysis of dose-response curve from bioassays with quantal response: Deterministic or statistical approaches?

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HIGHLIGHTS

- The dose-response relations can be analyzed deterministically or statistically.
- The deterministic approach is based on the law of mass action.
- The statistical approach is based on the probabilities distribution of phenotype.
- Deterministic equations must be used to analyze dose-response in simple systems.
- Conversely, statistical models must be used in systems with quantal responses.

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ABSTRACT

Dose-response relations can be obtained from systems at any structural level of biological matter, from the molecular to the organismic level. There are two types of approaches for analyzing dose-response curves: a deterministic approach, based on the law of mass action, and a statistical approach, based on the assumed probabilities distribution of phenotypic characters. Models based on the law of mass action have been proposed to analyze dose-response relations across the entire range of biological systems. The purpose of this paper is to discuss the principles that determine the dose-response relations.

Dose-response curves of simple systems are the result of chemical interactions between reacting molecules, and therefore are supported by the law of mass action. In consequence, the shape of these curves is perfectly sustained by physicochemical features. However, dose-response curves of bioassays with quantal response are not explained by the simple collision of molecules but by phenotypic variations among individuals and can be interpreted as individual tolerances. The expression of tolerance is the result of many genetic and environmental factors and thus can be considered a random variable. In consequence, the shape of its associated dose-response curve has no physicochemical bearings; instead, they are originated from random biological variations. Due to the randomness of tolerance there is no reason to use deterministic equations for its analysis; on the contrary, statistical models are the appropriate tools for analyzing these dose-response relations.

1. Introduction

Dose-response relations and their associated parameters are crucial data in pharmacological and toxicological research. These relations are described by obtaining a dose-response curve from which parameters characterizing the molecule or the tissue-molecule system are estimated, i.e. lethal doses, potency, efficacy, affinity, etc (Kenakin, 2004). Dose-response curves can be obtained from systems at any structural level of biological matter, from the molecular (e.g. drug-receptors interactions) to the organismic level (e.g dose of toxic-proportion of affected individuals).

There are two types of approaches for analyzing the dose-response relations and obtaining the corresponding dose-response curves: the mechanistic or deterministic approach, based on the law of mass action (e.g. Michaelis-Menten equations), and the

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probabilistic or statistical approach based on the assumed probabilities distribution of certain phenotypic characters (e.g. probit regression) (Finney, 1978; Greco et al., 1995; Chou, 2006). Some authors propose models based on the law of mass action for analyzing the dose-response relations obtained from studies carried out along the entire range of biological systems (Chou, 2006). The law of mass action explains molecular interactions based on the probability of collision of the reacting molecules and therefore can be used to describe the dose-response curve at a molecular level. However, can models based on this law explain the dose-response curve at higher biological level? In this paper we show that the deterministic models can not explain the dose-response relations along the entire range of biological systems. To achieve that goal we discuss the different principles that determine the dose-response relation at two levels of biological organization: molecular level and organismic level. We used the drug-receptor system as an example of the molecular level, and bioassays with organisms responding quantally (e.g. bacterial or animal lethality bioassay) as an example of the organismic level. The aim of the manuscript is not to propose a novel method of analysis, but to discuss in which cases the use of statistical models is required for the analysis of the data. In summary, we show that dose-response curve obtained from bioassay with quantal response is explained by biological variation occurring at random (not by the law of mass action). In consequence, those dose-response relations should not be analyzed by deterministic approaches but via statistical approaches.

2. Biological action of chemical agents

The biological effect of chemical agents results from the interaction between the active compound and specific molecules in the biological structure, i.e. the site of action or receptor (Ariëns et al., 1979). However, the action of drugs involves a sequence of processes that can be grouped into pharmacokinetic and pharmacodynamic phases. The pharmacokinetic phase includes the processes of absorption, distribution, biotransformation, and excretion, and therefore determines the concentration of the active agent in the target tissue. The pharmacodynamic phase comprises the molecular interaction between active molecules and their specific site of action. This initiates the sequence of biochemical processes that finally ends in the biological effect measured (Ariëns et al., 1979). The main steps of the pharmacodynamic phase are: 1) drug-receptor interaction, inducing an initial stimulus, 2) transduction and amplification processes, transmitting the initial stimulus to the molecular effector system, and 3) generation of an effect by the activity of the molecular effector (Ariëns et al., 1979).

The effect of a drug on an organism clearly results from a series of biochemical and physiological process, from the initial absorption to the interaction with the site of action, and involves many molecular interactions.

3. Law of mass action and the dose-response curve

Molecular interactions such as drug-receptor can be considered chemical reactions; hence, the classical tools of chemical kinetics, particularly the law of mass action, can be used to analyze them. Thus, the efforts to describe the dose-response curves at the molecular level require some form of application of the law of mass action, and a variety of partially overlapping theoretical models has been developed (Ariëns et al., 1979; Clarke and Bond, 1998). A.J. Clark was the first to apply mathematical principles to the action of drugs and proposed that the fractional response caused by a drug is equal to the fractional occupancy of the receptors by the drug (Clarke and Bond, 1998; Kenakin, 2004; Maehle, 2005). In that simple model, if drug D combines with receptor R to form a complex D-R that produces a response, then:

$$D + R \underset{k_{-1}}{\overset{k_1}{=}} DR \quad (1)$$

where k_1 and k_{-1} = velocity constants. From the laws of mass action and mass conservation the following equations were derived:

$$E_D/E_m = [DR]/[R_T] = [D]/[D] + K_D \quad (2)$$

where E_D = effect, E_m = maximal response, [DR] = drug-receptor complex concentration, $[R_T]$ = total concentration of receptors, [D] = drug concentration, K_D = affinity-related parameter (Hathway, 1984).

If

$$E_D = E_m[D]/[D] + K_D \quad (3)$$

then Eq. (3) is an identical function to the Michaelis-Menten equation,

$$V = V_{max}[S]/K_M + [S] \quad (4)$$

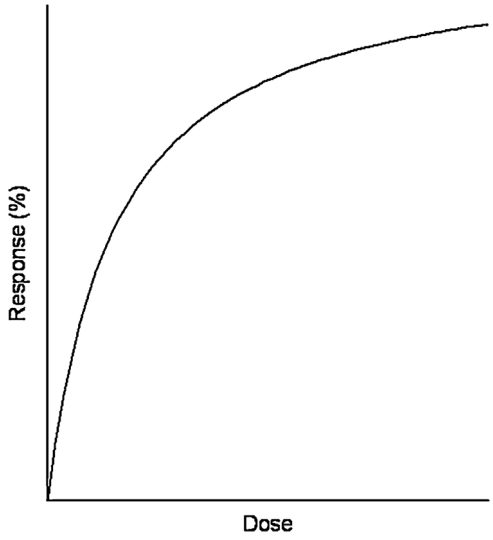

Fig. 1. Hyperbolic curve obtained when the response is plotted as a function of drug concentration.

where V = reaction velocity, V_{max} = maximum reaction velocity, [S] = substrate concentration, K_M = Michaelis-Menten constant (Marmasse, 1977).

When the effect is plotted as a function of drug concentration in equation (2), a hyperbolic curve is obtained (Fig. 1). If the effect is then plotted as a function of log [D] the result is a sigmoid curve (Fig. 2). This simple model was modified several times to explain a wider range of empirical observations. All those theoretical models allowed building the *classical receptor theory* (Ariëns et al., 1979; Clarke and Bond, 1998). The following sequence of reversible reactions is a condensed form covering essential aspects of these models:

$$D + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} DR \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} DR^* \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} D + R^* \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \underline{R} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} R \quad (5)$$

where D = drug, R = receptive receptor, R* = activated receptor, $\underline{R}$ = nonreceptive and nonactivated receptor. Receptor species DR* or R*, depending on the model considered, contribute to produce a receptor stimulus (Ariëns et al., 1979).

The development of these models included the proposal of the concept of *receptor stimulus* and the evolution of the concepts of *intrinsic activity*, *efficacy* and *intrinsic efficacy* (Stephenson, 1956; Ariëns et al., 1979; Clarke and Bond, 1998; Kenakin, 2004; Maehle, 2005). An example of this is Furchgott's model:

$$E = f(S) = f\,[\varepsilon_A R_T / 1 + K_D/[D]] \quad (6)$$

where E = effect, [D] = drug concentration, R_T = total receptors, K_D = affinity-related parameter, $_A$ = intrinsic efficacy, S = receptor stimulus, and f(S) = ability of the tissue to convert the receptor stimulus into a response (Clarke and Bond 1998).

In the classical receptor theory the drug has to activate the receptor via conformational changes in order to produce the receptor stimulus, however alternative models where the receptors exist in equilibrium between active and inactive conformations have been developed (Clarke and Bond 1998; Kenakin 2004).

The similarities among the equations of the most important biochemical and biophysical principles, such as Hill, Michaelis-Menten, Scatchard and Henderson-Hasselbach equations, lead to the development of a model that unified all these principles in a single equation. This model, known as the *General theory of dose and effect*, is mathematically expressed by the median effect equation (Chou, 2006):

$$f_a/f_u = (D/D_m)^m \quad (7)$$

where f_a = fraction affected by the drug, f_u = unaffected fraction (1-f_a), D = dose, D_m = median dose effect (e.g. ED_{50}, LD_{50}), and m = coefficient signifying the shape of the curve.

When f_a is plotted as a function of D, a dose-response curve is obtained. The shape of this dose-response curve can be hyperbolic, sigmoidal or flat-sigmoidal, depending on the m coefficient ($m = 1$, >1, <1 respectively) (Fig. 3a).

Applying logarithm to Eq. (7) results in:

$$\mathrm{Log}\,(f_a/f_u) = m\mathrm{Log}\,(D) - m\ \mathrm{Log}\,D_m \quad (8)$$

Plotting $\mathrm{Log}\,(f_a/f_u)$ vs $\mathrm{Log}\,(D)$, the curves obtained from (7) can be linealized with a resulting slope equal to m (Chou 2006) (Fig. 3b).

All the above are deterministic models, since the dose-generated effects are not random variables, and the shape of the curves obtained from their mathematical functions is sustained by physicochemical features, particularly by the law of mass action (Chou 2006). It is clear that dose-response curves resulting from molecular systems are supported by the law of mass action as measured responses are the result of chemical interactions between the molecules involved in the system. Moreover, the estimated parameters have the potential to be biologically meaningful (Greco et al., 1995). These models have been proposed for analyzing dose-effect data at all levels of biological material organization. In this way, typically hyperbolic curves are obtained in simple systems (e.g. enzyme-substrate or receptor-ligand systems) while sigmoidal curves usually occur in complex systems (e.g. cellular, multicellular, or animal lethality bioassays) (Chou 2006). However, complex systems should be occur with millions of interactions explained by mass action. Then, the biochemical origin of deterministic approaches will usually not facilitate mechanistic insight into complex systems (Greco et al., 1995), and therefore are used in an empirical manner due to the similarity of dose-response curves. Moreover, experimental and conceptual differences exist in the studies preformed on organisms showing quantal response, where the mass action do not explain the shape of dose-response curve.

4. Quantal response in individual lethality bioassays

The analysis of the experimental procedure allows us to understand how the dose-response curve emerges from studies performed on groups of organisms. The effect of a drug on an organism can be of two different types, *graded* or *quantal* (Hewlett and Plackett, 1956). In graded responses, a quantitative result is

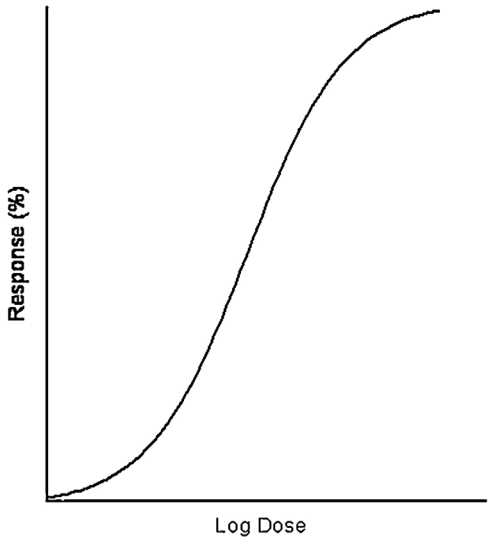

Fig. 2. Sigmoid curve obtained when the response is plotted as a function of dose logarithm.

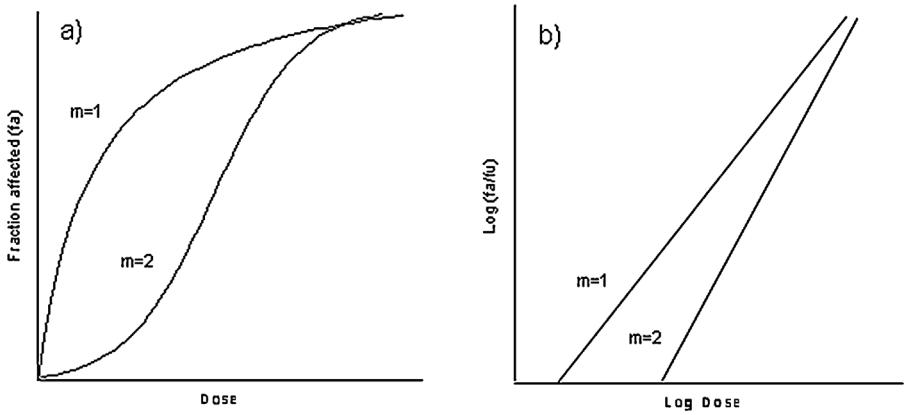

Fig. 3. Dose-response curves (a) and their corresponding linear forms (b) according to the median-effect equation for two different drugs. Coefficient *m* represents the shape of the dose-response curve (a) ($m = 1$ for hyperbolic curve and $m > 1$ sigmoid curve) and the slope of the linear form (b) (Chou, 2006).

observed for a single organism. Quantal responses occur when the result on a single organism is all-or-none. In that case, the organism is classified as having responded or not responded at a certain time after administration of the drug (e.g. dead or alive when the potency of an insecticide against an insect pest is determined). When a quantal response is measured, groups of organisms of the same species are treated with a range of doses of the experimental drug (Busvine, 1971). Each group receives a determined dose and the response is registered as the proportion of individuals that respond out of the total number of individuals treated. A proportion will be registered for every dose and a variation in the proportion of the response will occur as a function of the dose. Higher concentrations of the drug are expected to produce greater proportions of response. When the proportion of responses is plotted against the dose, an asymmetric sigmoid curve is usually obtained. On the other hand, a symmetric sigmoid curve is the result when the proportion of responses is plotted against log-dose (Hewlett and Plackett, 1978; Robertson et al., 2007) (Fig. 2).

Clearly, the curves obtained from dose-response relations have sigmoid shapes in both simple and complex systems. The similarity in the shape of the curves would allow using the same mathematical procedures to analyze both relations, e.g. procedures obtained from deterministic models. However, a deterministic model should be used if the model explains the process observed. Within this context, can the dose-response relation obtained from bioassays be interpreted in terms of deterministic models?

5. The dose-response relation in individual lethality bioassays

In the preceding paragraphs we have shown how the law of mass action determines dose-response relations in molecular

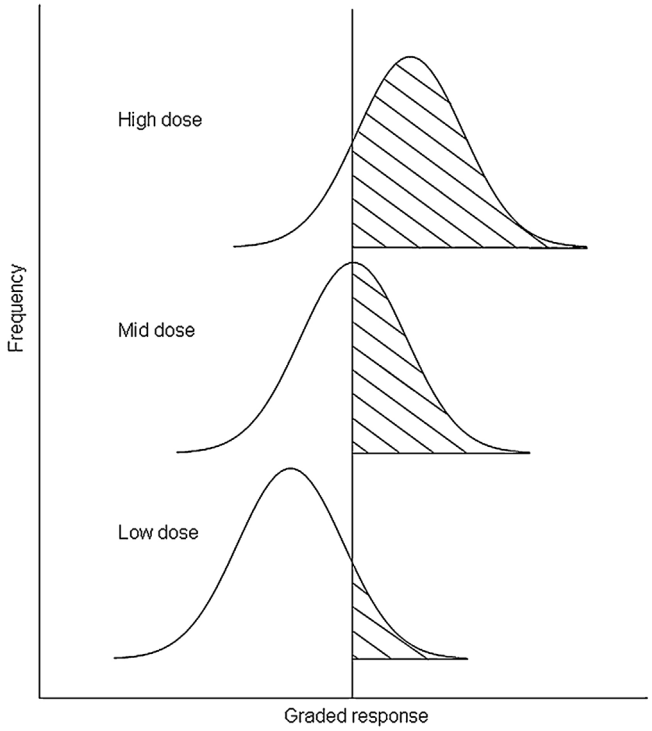

Fig. 4. The symmetric sigmoid curve that relates the percentage of response to log-dose is a cumulative normal distribution of log-tolerances (Hewlett and Plackett, 1978).

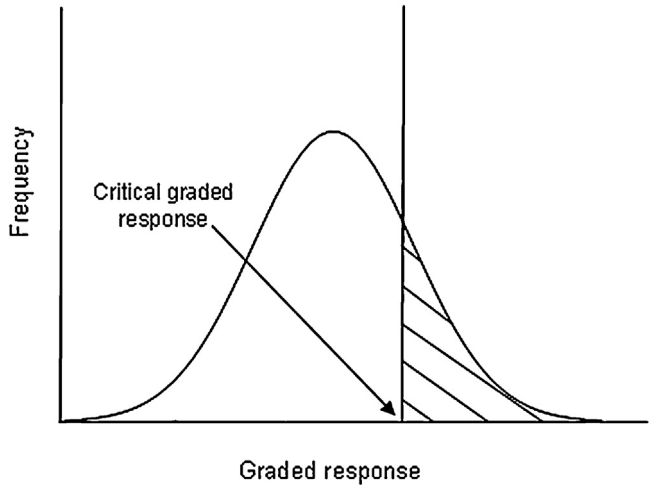

Fig. 5. Normal distribution of the graded response of individuals exposed to a determined dose of the drug. The proportion of individuals that reach the critical graded response (lined area) responds quantally. In this graph, the critical graded response is constant for all individuals.

systems. However, a different situation occurs when observing the response of groups of organisms to different doses of a drug. Although mathematically similar, the dose-response curve of a complex system does not emerge from the simple collision of molecules. The dose-response curve obtained from an individual lethality bioassay is the result of phenotypic variations among individuals and can be interpreted in terms of individual tolerances.

Hewlett and Plackett (1956) defined the *tolerance* of an individual organism (or other unit of biological material) as the dose of a drug that is just insufficient to show the quantal response concerned. The difference in the response observed (i.e. proportion of organisms responding quantally) to different doses results from the individual variation of biological characters that determine the individual tolerance. Therefore, the dose-response curve emerges from the variation of individual tolerances in the population, where the symmetric sigmoid curve relating the percentage of response with log-dose is a cumulative normal distribution of log-tolerances (Hewlett and Plackett, 1978) (Fig. 4).

But what is the connection between dose-response relations from quantal and graded responses?

An individual organism responds quantally when an underlying quantitative change, resulting from administrating the drug and that can be regarded as a graded response, reaches certain levels of intensity, namely the *critical graded response* (Hewlett and Plackett, 1956). If the dose of a drug is insufficient to bring a quantitative change to the critical level, the quantal response will not occur. Graded responses result from biochemical and physiological processes occurring during pharmacokinetic and pharmacodynamic phases (Hewlett and Plackett, 1978). Remarkably, the graded response varies randomly among the individuals of a population due to genetic and/or environmental factors just as any other phenotypic characters vary (Futuyma, 1998). Specifically in the graded response, it is possible speculate that genetic and environmental factors generate individual differences in pharmacological processes (e.g. rate of biotransformation), or in kinetic parameters (e.g. affinity or intrinsic efficacy) related to the effect of the drug.

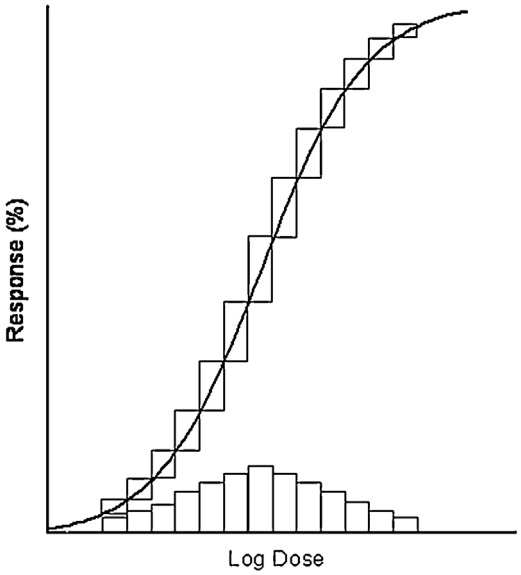

Fig. 6. Different proportions of individuals reach the critical graded response (lined area) at different doses. In this graph, the critical graded response is constant for all individuals.

As a result of individual variation, the intensity of the graded response show a frequency distribution, generally fitting a normal probability distribution (Sokal and Rohlf, 1980), among the individuals of the exposed group to a determined dose of the drug. At that dose only a proportion of exposed individuals reach the intensity needed to elicit a quantal response (Fig. 5). As discrete binary variable, that proportion show a binomial distribution when several groups are exposed at a single dose, with a mean equal to probability of measured outcome (e.g. dead) at that dose (Sokal and Rohlf, 1980). Due that the probability of outcome increases with the dose, higher proportions of quantal responses are obtained as the dose increases (Fig. 6). Consequently there is a relation between the proportion of individuals responding and the dose of the drug, and that fits to a symmetric sigmoid curve when the cumulative mean response is plotted against log-dose. This is the cumulative normal distribution of tolerance.

Hence, the variation of tolerance in log-dose (the shape of the dose-response curve) and the variation of proportion at a single dose clearly arise from the randomly variation of the intensity of the graded response at a single dose. In this way, the structural model and the variation model included in the concept called *generalized nonlinear modeling* (Greco et al., 1995) arise from the same biological variation. Due to the randomness of both tolerance and graded response, there is no reason to use deterministic equations for their analysis; on the contrary, statistical models (e.g. probit regression, non-linear regression, etc) are the correct tools for analyzing these variables (Finney, 1978; Sokal and Rohlf, 1980).

6. Conclusions

Like other phenotypic characters (e.g. height or body weight), the expression of tolerance in a population of organisms is due to many genetic and environmental factors. It can therefore be considered a random variable that fits a normal distribution. Clearly, the variation of tolerance in a population and the shape of its associated dose-response curve do not have physicochemical bearings, but they come from a random biological variation. On the contrary, dose-generated responses in molecular systems are not random variables because they are governed by the law of mass action. In consequence, although the same mathematical procedures can be applied for analyzing dose-response data from both molecular and organismic systems due to the similarity of their curves, organismic systems should to be analyzed using statistical tools.

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