Pharm Res (2012) 29:637–642
DOI 10.1007/s11095-011-0612-5

PERSPECTIVE

'Null Method' Determination of Drug Biophase Concentration

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Received: 22 June 2011 / Accepted: 17 October 2011 / Published online: 29 October 2011

ABSTRACT PK/PD modeling is enhanced by improvements in the accuracy of its metrics. For PK/PD modeling of drugs and biologics that interact with enzymes or receptors, the equilibrium constant of the interaction can provide critical insight. Methodologies such as radioliogand binding and isolated tissue preparations can provide estimates of the equilibrium constants (as the dissociation constant, *K* value) for drugs and endogenous ligands that interact with specific enzymes and receptors. However, an impediment to further precision for PK/PD modeling is that it remains a problem to convert the concentration of drug in bulk solution (*A*) into an estimate of receptor occupation, since *A* is not necessarily the concentration (*C*) of drug in the biophase that yields fractional binding from the law of mass action, *viz.*, *C*/(C + K). In most experimental studies *A* is much larger than *K*, so the use of administered instead of biophase concentration gives fractional occupancies very close to unity. We here provide a simple way to obtain an estimate of the factor that converts the total drug concentration into the biophase concentration in isolated tissue preparation. Our approach is an extension of the now classic 'null method' introduced and applied by Furchgott to determination of drug-receptor dissociation constants.

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KEY WORDS biophase concentration · receptor · dissociation constant · null method

ABBREVIATIONS

A	drug concentration in bulk solution in absence of receptor blockade
A'	drug concentration in bulk solution in presence of receptor blockade
C	drug concentration in biophase
K	dissociation constant of ligand-receptor interaction
k_1	forward rate constant of ligand-receptor interaction
k_2	reverse rate constant of ligand-receptor interaction
PK	pharmacokinetics
PD	pharmacodynamics
R_t	total number of receptors
r	receptor concentration
x	bound drug concentration
ξ	unblocked fraction of receptors
Φ	constant relating bulk and biophase concentrations

INTRODUCTION

The equilibrium established between a receptor and complementary ligand, *e.g.*, hormone, neurotransmitter, or drug (small molecule or biologic), is a reversible interaction based on the net attractive chemical forces that establish the affinity between ligand and receptor (for a review of the historical development of the concept of affinity, see Raffa and Tallarida (1)). Such an interaction results in either stimulation of transduction of a biological signal *via* 2nd-messenger pathways (*i.e.*, the drug is an agonist) or can inhibit endogenous receptor tone (*i.e.*, the drug is an antagonist or partial agonist) (2,3). The drug-receptor interaction can be viewed as a bimolecular reversible reaction between drug A and receptor R (the

analysis is the same if the reaction mechanism is more complex):

$$A + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} AR \quad (1)$$

It is a convention in the receptor theory literature to use the reciprocal of the familiar equilibrium constant, known as the dissociation constant (K_A) (4,5). Thus for the simplest case,

$$K_A = \frac{[A][R]}{AR}, \quad (2)$$

namely, the ratio of the product of the drug and receptor concentrations and concentration of the associated molecules (the drug-receptor complex), and $K_A = k_2/k_1$. The greater the affinity of drug for receptor, the smaller the magnitude of K_A. The dissociation constant is now a standard metric used to measure affinity and for the identification and classification of receptors. It is determined by methods that quantify effect against concentration or by radioligand binding methods that use tissue homogenates.

When the drug-receptor dissociation constant is determined from data obtained employing radioligand binding methods, the concentration of bound and unbound drug are assumed to be known accurately. When the dissociation constant is determined from data obtained employing pharmacological methods, the concentrations used in the calculations are acknowledged to be approximate and termed 'apparent' (6). (For example, in isolated tissue bath experiments the bath concentration of drug is often used.). However, in both cases, this is only an approximation, because the concentration of drug at the receptor is the concentration of drug in the 'biophase', not in the bulk solution. The 'biophase' is the physical region (environment) in which the receptor is located. At the macro level, the bulk region might be the plasma and the biophase might be brain tissue for a centrally acting drug. At the micro level, the biophase for all drugs is the layer (domain) of cell-attached and adsorbed proteins, glycosaminoglycans, and other macromolecules that often extend hundreds of nm from the cell surface into the bulk region (Fig. 1) (7). Thus the biophase is a region with physiochemical properties different from those of the bulk region, and every drug differentially distributes from bulk into biophase according to its own physiochemical properties and attains a concentration different from that of the bulk concentration. Since a drug distributes differently in the biophase domain (adjacent to the receptor) than in the bulk domain, it is biophase concentration that yields a more accurate parameter estimation for receptor occupation in PK/PD modeling.

Unfortunately, direct measurement of the biophase concentration is frequently not possible. In this paper we demonstrate the use of a methodology that uses pharmacological methods to yield estimates of the biophase concentration. The method is an extension of an elegant approach first described by Furchgott (8), known as the null method, and used for other purposes. The null method takes advantage of a fundamental tenet of pharmacology: that equal pharmacologic effects corresponds to equal receptor occupancy (9).

MATERIALS AND METHODS

Animals, Isolated Tissue Preparation, Drugs

All protocols received prior approval from the Temple University Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and AAALAC-International standards. Male Sprague Dawley rats, 250–350 g, were purchased from Ace Animals, Inc. (Boyertown, PA). Following a period of acclimation to the animal facility conditions for a minimum of 1 week, the rats were euthanized by CO_2 asphyxiation, and their thoracic aorta was excised and placed in ice-cold Krebs-Henseleit solution (a bicarbonate-buffered physiological salt solution that maintains a pH of 7.6 when bubbled with 5% CO_2/95% O_2), consisting of 120 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM $NaHCO_3$. A section of the thoracic aorta from just below the aortic arch to the diaphragm was then dissected for use. Minimal manipulation of connective tissue was done in order to avoid damage to the adventitia and smooth muscle cells. The aorta segments were then sectioned into rings approximately 3 mm in length, and suspended between two stainless steel hooks in a water-jacketed 25 ml glass chamber maintained at 37°C. One of the hooks was anchored to a fixed position and the other hook was attached to a force-displacement transducer (Grass Instruments, West Warwick, RI). The transducers were connected in series to signal amplifiers, an analog-to-digital converter, and a computer for data acquisition. The data were recorded using Chart software (AD Instruments; Colorado Springs, CO).

NULL METHOD AND BIOPHASE DETERMINATION

Determination of Fractional Receptor Occupancy

This method uses concentration-effect data of an agonist under normal conditions of full receptor concentration and

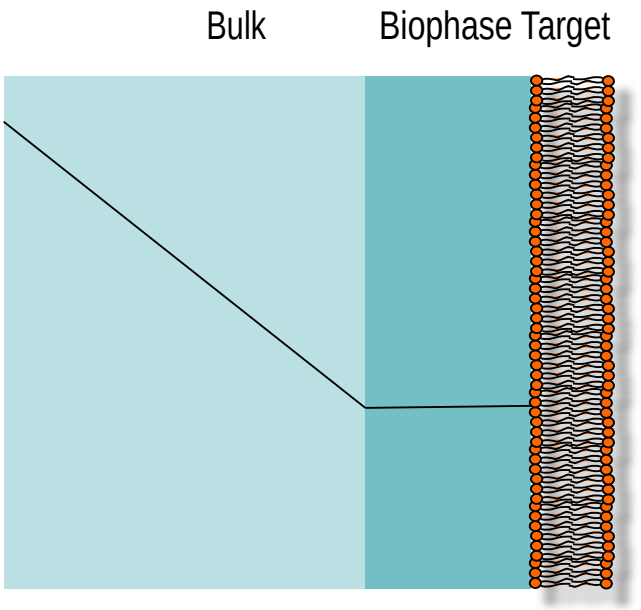

Fig. 1 Differential distribution of drug in the bulk domain and biophase (linear shown for illustrative purposes only). For a typical cell, the biophase is a domain consisting of glycoproteins and other macromolecules that extend beyond the cell surface membrane up to several hundred nm. The differential distribution is established due to the different physicochemical properties of the bulk and biophase regions.

also under the condition of partial occlusion of the receptor by an irreversible blocking compound that reduces receptor number to some fraction (ξ) of its original value. The methodology uses two concentration-effect curves for the agonist, one in the absence and one in the presence of the irreversible blocking compound (8,9). Equi-effective concentrations of agonist in the presence (A') and the absence (A) of block are related by the equation

$$\frac{1}{A} = \frac{1}{\xi}\left(\frac{1}{A'}\right) + \frac{\left(\frac{1}{\xi} - 1\right)}{K_A}. \tag{3}$$

Hence, for multiple equieffective pairs of A and A', a double-reciprocal plot of $1/A$ against $1/A'$ is a straight line of slope = $1/\xi$ and intercept = (slope – 1)/K_A. The basis for Eq. 3 is that equal drug-induced effects result from equal receptor occupancy by the drug. The latter requires knowledge of the drug concentration at the receptor site (the biophase concentration). It should be noted for our subsequent derivations, though, that the slope of the curve still yields the value of $1/\xi$ as shown below.

Biophase Concentration in Relation to the Null Method

Receptor occupation based on external tissue concentrations A and A' (as in an isolated tissue muscle bath) are commonly used in applications of the null method according to

$$\frac{R_t A}{A + K_A} = \frac{\xi R_t A'}{A' + K_A}, \tag{4}$$

where K_A is the apparent dissociation constant and whose value is derived from the double reciprocal line as *(slope −1)/intercept*. Since the biophase concentration (which is the same factor, Φ times A, in the presence and absence of antagonist) is required for occupancies to be equated, we equate occupancies as

$$\frac{\Phi R_t A}{\Phi A + K_A} = \frac{\xi \Phi R_t A'}{\Phi A' + K_A}, \tag{5}$$

so that K_A is now the true dissociation constant. When the above is transformed to double reciprocal form we get

$$\frac{1}{A} = \frac{1}{\xi}\left(\frac{1}{A'}\right) + \frac{\Phi}{K_A}\left(\frac{1}{\xi} - 1\right), \tag{6}$$

from which we see that $1/\xi$ (the slope of the double-reciprocal plot) is the same quantity whether the bath concentration or the biophase concentration is used. However, the true value of K_A is related to the apparent K_A as $K_{\text{true}} = \Phi\left(K_{\text{apparent}}\right)$.

Use of Effect-Time Curves

The time course of effect due to agonist receptor occupation may be different from the time course of drug-receptor occupation due to a threshold occupation. If the time required to attain this threshold is approximately the same in the (a) normal and (b) partially blocked preparation, then the time course of the effect closely approximates the time course of receptor occupancy and therefore the same effect magnitude in cases (a) and (b) implies equal receptor occupancy. The law of mass action governs the rate of receptor occupation so that

the attainment of this same occupation takes a longer time in case (b).

Determination of Biophase Concentration

From the *dose-response* curves constructed in the absence and presence of an irreversible antagonist, a single concentration of agonist A is selected that produces an effect both in the presence and absence of partial irreversible blockade. Under the same experimental conditions as above, two *effect-time* curves for agonist A at the concentration selected are obtained: one curve in the absence of the irreversible antagonist and one curve in the presence of the irreversible antagonist. A horizontal line is then constructed that intersects the curves at points corresponding to times t_1 and t_2, respectively as illustrated in Fig. 2.

To illustrate the simplest (bimolecular) reaction, the relation between bound concentration of drug (x) to receptor concentration (r) in the normal (unblocked) case is given by the differential equation $dx/dt = k_1A(r - x) - k_2x$, a standard model whose solution is

$$x = \frac{k_1Ar}{k_1A + k_2}\left[1 - e^{-(k_1A+k_2)t}\right] \tag{7}$$

at time t (see, for example, Tallarida and Jacob, 1979, p. 52 (10)). The relation for the partially blocked case with lesser concentration (ξr) is given by

$$x = \frac{k_1A\xi r}{k_1A + k_2}\left[1 - e^{-(k_1A+k_2)t}\right]. \tag{8}$$

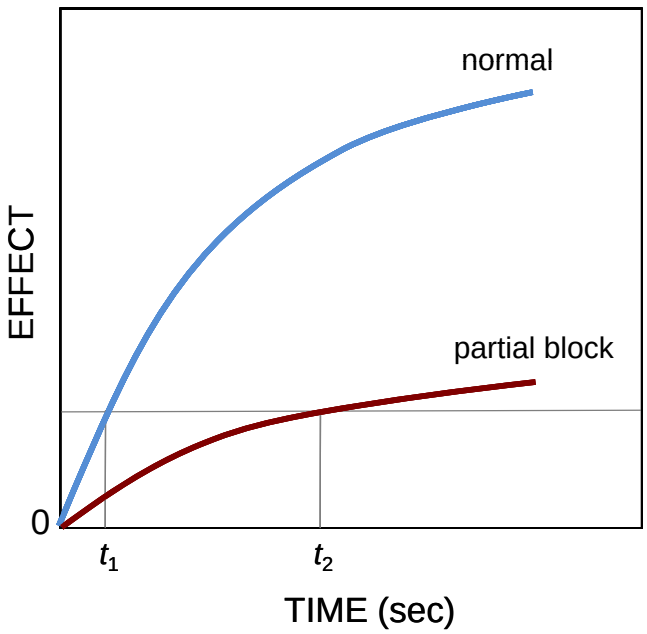

Fig. 2 Effect-time curve illustrating the times of equal effect in the normal and partially receptor blocked conditions.

Since by definition the same effect level under the two conditions occurs at times t_1 in the normal and t_2 in the partially blocked preparation, and because equal effects mean equal receptor occupation, we equate Eqs. 7 and 8 at these two times thereby obtaining the following:

$$1 - e^{-(k_1A+k_2)t} = \xi\left[1 - e^{-(k_1A+k_2)t_2}\right]. \tag{9}$$

In deriving the above expression it is assumed that the concentration A remains the same during the short time interval (t_1 to t_2) and also that the time course of the effect is a good approximation to the time course of receptor occupancy. Both assumptions seem reasonable for a rapidly acting molecule such as norepinephrine that will be subsequently illustrated.

Values of k_1 and k_2 will generally be obtainable from radioligand binding experiments, and ξ can be determined using the standard Furchgott null method. Therefore, the experimental determination of times t_1 and t_2 allows a calculation of the biophase concentration (A), by substitution in Eq. 9. We illustrate this calculation in the following simple example of an isolated tissue preparation whose data were obtained in an experiment in our laboratory that examined norepinephrine constrictor action in an *in vitro* preparation of rat aorta. These data from a limited number of experiments serve the purpose of illustrating the calculations.

Example

For convenience we denote $(k_1A + k_2)$ by β. Thus, Eq. 9, expressed as

$$1 - e^{-\beta t_1} = \xi\left(1 - e^{-\beta t_2}\right), \tag{10}$$

and for which experiment showed $t_1 = 10$ s and $t_2 = 60$ s (Fig. 3), the null method gave $\xi = 0.83$ (Fig. 4). Thus, $1 - e^{-10\beta} = 0.83\left(1 - e^{-60\beta}\right)$, whose solution, found using the Newton Raphson iterative procedure, is $\beta = 0.18\ \mathrm{s}^{-1}$. In order to calculate the biophase concentration A, we insert the values of k_1 and k_2 in the equation $(k_1A + k_2) = 0.18\mathrm{s}^{-1}$ using $k_1 = 10^6\,\mathrm{M}^{-1}\mathrm{s}^{-1}$ (11). For k_2 we first use a value obtained from $K_A = k_2/k_1 = 10^{-7}\,\mathrm{M}$ reported by Strecker *et al.* (12), which leads to $k_2 = 0.1\ \mathrm{s}^{-1}$; this calculation gives $A = 1.8 \times 10^{-8}\mathrm{M}$. We also used the K_A derived from the experiment described here (a different preparation from Strecker's) which gave $K_A = (4.4 \pm 1.6) \times 10^{-8}\mathrm{M}$. Use of this K_A value leads to $k_2 = (0.44 \pm 0.16) \times 10^{-8}\mathrm{s}^{-1}$, and this yields $A = (11.4 \pm 0.2) \times 10^{-8}\mathrm{M}$. Because the bath concentration in our experiment was 10^{-6} M, it is seen that the biophase concentration values calculated are approximately between 2 and 11% of the bath concentration. This finding, which is the principle aim of this communication, is addressed further in the Discussion.

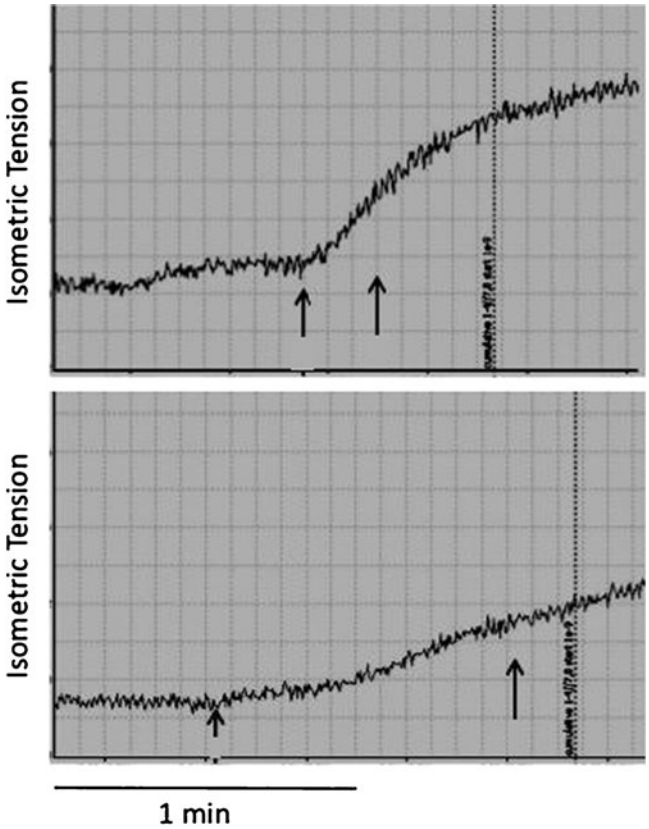

Fig. 3 Tension-time curves for 1×10^{-6} M norepinephrine in rat aorta with endothelium removed illustrating full receptor density (upper) and partial inactivation (lower). Arrows indicate intervals $0 - t_1$ and $0 - t_2$.

absence of an antagonist of this kind one cannot apply this method to other ligands that act on blood vessel cells. The method also assumes that the time course of observed effect is a good approximation of the time course of receptor occupancy. This assumption seems valid in our norepinephrine experiment in light of the rapidity of the onset of effect following dosing. Although we report only on two examples, it is the method and its demonstration that is the message here. Each example, and the values derived from these, indicate that there is a large difference between bath concentration and biophase concentration. This large difference in biophase concentration should stimulate additional efforts to obtain such values where possible.

In vivo experiments present an additional challenge in getting the biophase concentration. In a recent experiment with the analgesic drug tapentadol in the rat (13) we determined the whole brain concentration of this drug and estimated from that and other information that the biophase concentration is approximately four percent of the whole brain concentration for that drug. Having this concentration and the K value gives the fractional receptor occupation and thereby allows the conversion of concentration-effect curves into occupancy effect curves, thereby providing a more intimate view of drug action. It should also be mentioned that extensive data are available on ß-adrenoceptor antagonists in PK-PD experiments for which *in vivo* estimates of the K value have been made, and compared to determinations made *in vitro* (14,15).

DISCUSSION

Receptor occupation requires the value of K (the drug-receptor dissociation constant) as well as the biophase concentration of the ligand. Precision in the determination of forward and reverse rate constants for drug-receptor interactions has greatly increased due to the application of radioligand binding methods. Certain databases, *e.g.*, PDSP (Psychoactive Drug Screening Program, National Institute of Mental Health) list the values of dissociation constants for a number of drug-receptor complexes in various species. Less available, are estimates for the biophase concentration. The approach described here provides a method that is based on the null method of Furchgott, to obtain an estimate of this concentration. It is notable that even in the controlled conditions of the example tissue bath experiment that the calculated biophase concentration is significantly different from the drug concentration in the bath.

The method described here requires the use of an irreversible competitive antagonist and thus it is limited to situations for which such an antagonist exists. In the

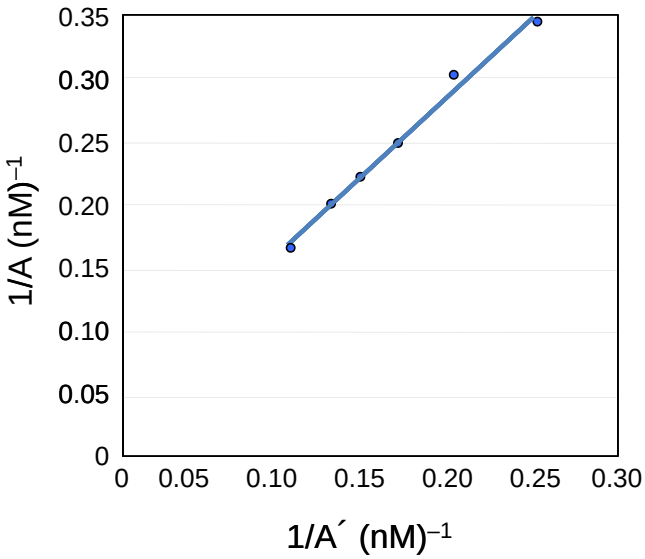

Fig. 4 The null method applied to norepinephrine constriction in the simple example of an isolated preparation of rat aorta that has endothelium removed. The equation has slope 1.22 ± 0.06 and intercept $= 0.0484 \pm 0.01$ ($r = 0.99$).

CONCLUSION

For estimation of receptor occupancy in pharmacologic and PK/PD modeling applications, biophase concentration is optimal. The approach presented here provides a methodology that can be used in experiments that produce quantifiable records of effect against time after dosing. Further, these results demonstrate that the biophase concentration can be much different than the bulk concentration in isolated tissue preparations – in the example experiment, the biophase concentration was one to two orders of magnitude less than the bulk concentration – and suggest also that they are likely to be different than blood (or brain) drug concentrations determined *in vivo*.

ACKNOWLEDGMENTS & DISCLOSURES

R.J.T. was supported by NIH/NIDA grant DA013429 and N.L. was supported by NIH/NIDA training-grant DA07237-17.

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