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Model analysis of bidirectional interference in two-stage labeled-ligand immunoassays

Eleanor R. Lewin[a], Amitava Dasgupta[b], Roland Valdes Jr.[c], Douglas F. Stickle[a,*]

[a] Department of Pathology, Jefferson University Hospital, Philadelphia, PA, USA
[b] Department of Pathology and Laboratory Medicine, University of Texas-Houston Medical School, Houston, TX, USA
[c] Departments of Pathology and Laboratory Medicine and Biochemistry and Molecular Genetics, University of Louisville School of Medicine, Louisville, KY, USA

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ABSTRACT

Objectives: Immunoassays involving sample incubation followed by a wash step prior to introduction of labeled analyte are potentially subject to both positive and negative interference (bidirectional interference) by a competing ligand. We examine this phenomenon from a theoretical standpoint using a mathematical model for sequential-step immunoassays in the presence of interferent.

Design & methods: Competitive binding to antibody between analyte and interferent was modeled for sequential-step immunoassays. A primary assumption was that the ratio of affinity constants between the intended analyte and the interferent reflected the ratio of dissociation rate constants, with the higher dissociation rate constant for the lesser affinity ligand.

Results: Relationships of parameters (relative affinity constants, relative concentrations) for analyte and interferent were determined for conditions in which bidirectional interference can occur, for both steady-state and non-steady-state sample incubation conditions. Non-steady state sample incubation conditions can enhance the effects of an interferent. Homogeneous assay formats utilizing labeled ligand without a wash step can also demonstrate bidirectional interference, but positive interference is favored under such formats.

Conclusions: Model calculations demonstrate the theoretical basis for bidirectional interference in two-stage immunoassays. Results delineate constraints on conditions in which bidirectional interference can occur.

1. Introduction

Sequential step immunoassays, in which unoccupied receptor antibodies are labeled and measured after a first-stage incubation with sample, are in principle subject to bidirectional (negative and positive) interference by unintended, low affinity receptor ligands [1]. Bidirectional interference has been observed most notably in digoxin assays [2–7]. Digoxin assays are susceptible to interference in part because the therapeutic range of digoxin concentration is relatively low (< 2 ng/mL) [8]. As a result, an interferent with just 1% cross-reactivity with a digoxin assay may be a positive interferent for digoxin measurement if present at 100 ng/mL concentrations. In contrast, positive interference would be insignificant for the same degree of cross reactivity with a phenytoin assay, for which the therapeutic range is of the order of 10 μg/mL [9]. The basis for negative or bidirectional interference is considerably more complex, however. The explanation for bidirectional interference is understood in general terms as follows: after an initial stage of incubation with sample, the differential rates of dissociation of low-affinity vs. high-affinity ligands (viz., interferent vs. intended analyte, respectively) from the antibody receptor can occur during the interval of a wash step and/or labeled-ligand incubation step. Under certain circumstances of the relative concentrations and affinities of analyte and interferent, this phenomenon can lead either to positive or negative interference with measurement of the analyte [1].

Although the phenomenon of bidirectional interference is understood in outline, to our knowledge it has not been modeled formally in mathematical terms. Our objective in this study was to use a kinetic mass balance model for the states of the antibody receptor in different stages of such assays to demonstrate bidirectional interference, and to examine the range of conditions (relative concentrations of analyte and interferent, relative affinities of analyte and interferent for the receptor) in which positive and negative interference can occur. Additionally, the model is used to examine how interference is affected by whether the primary sample incubation phase is conducted as a steady-state vs. a non-steady-state. We also examine bidirectional interference for the case of a homogeneous sequential-step assay format that does not include a wash step.

* Corresponding author at: Department of Pathology, Jefferson University Hospital, 117 S 11th St, Pavilion 401, Philadelphia, PA 19107, USA.
E-mail address: douglas.stickle@jefferson.edu (D.F. Stickle).

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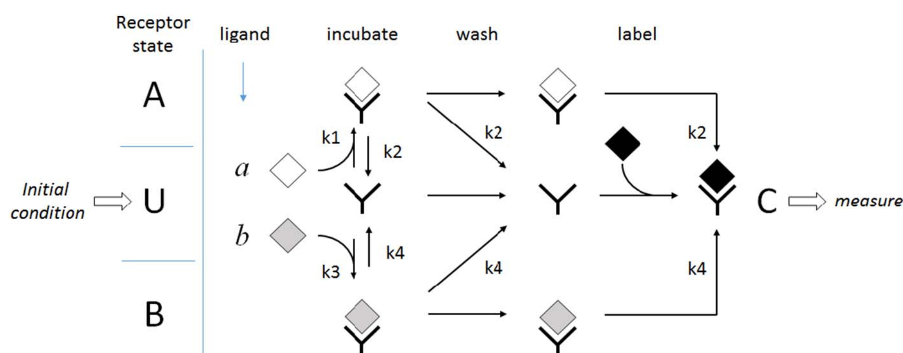

Fig. 1. Schematic diagram of a sequential step immunoassay involving a wash and label phase, with and without presence of an interfering ligand. Analyte (*a*) and interferent (*b*) can bind to unoccupied solid-state receptor (U) to produce receptor states A (bound to *a*) or B (bound to *b*) during the sample incubation phase. During the wash and label phases, both A and B are subject to loss back to U. Addition of excess label ensures that all U is converted to C, which is the measurable labeled receptor state.

2. Overview of a sequential step immunoassay

The type of sequential binding immunoassay under consideration is shown schematically in Fig. 1. In outline, the immunoassay works as follows. An initial incubation with sample allows for binding of the analyte (ligand *a*) to a solid-phase antibody receptor; after this incubation period, a wash step is performed followed by addition of an excess concentration of labeled ligand so as to bind immediately to all unoccupied receptors. The intent is to be able to measure receptors that were unoccupied by ligand *a* in the initial incubation. The final signal for occupancy by labeled ligand decreases as $[a]$ increases.

The range of the immunoassay signal is compressed somewhat from the range of potential occupancies after the incubation step, for the simple reason that the combined periods of wash and loading of label are finite, during which time there is some loss of receptor occupancy by ligand *a* due to dissociation to create some number of additional unoccupied receptors. That is to say, what becomes labeled is the sum of receptors unoccupied at the end of the sample incubation period, along with receptors that become unoccupied during the wash and label steps. Correspondingly, there will never be circumstances in which the lowest occupancy of receptors by label is zero, even when the sample concentration of *a* is sufficient to saturate the receptors during the sample incubation step. This detailed aspect of the immunoassay is not terribly important in the general case wherein the intended analyte is the only ligand for the receptor within the sample. In that circumstance there will be a simple standard curve relating signal to analyte concentration over some range of analyte concentrations.

The situation is more complicated, however, when an unintended ligand *b* that can bind to the receptor is present in the sample. As shown in the diagram, the final measure of unoccupied receptors is affected by the occupancy of receptor by ligand *b* after the initial incubation period and by its dissociation from the receptor during the wash and label intervals. Were there no wash and label interval, the presence of ligand *b* could only act as a positive interferent in measurement of $[a]$. As shown below, however, the wash and label interval creates conditions in which different rates of dissociation from the receptor between ligands *a* and *b* can cause negative interference, viz., a condition in which the apparent concentration of $[a]$ is less than its actual concentration.

The assay format in Fig. 1 follows the design of numerous past and present immunoassays, such as the Abbott MEIA digoxin assay. Below, we will delineate a formal kinetic mass balance model for the states of the receptor for the assay configuration depicted in Fig.1, in order to demonstrate interplay of variables by which the presence of ligand *b* may cause bidirectional interference in measurement of $[a]$.

3. Mathematical model for a sequential step immunoassay: kinetic mass balance model equations for changes in receptor states

A kinetic mass balance model of the system shown in Fig. 1 is developed as follows. For simplicity, it is assumed that free ligand concentrations, $[a]$ and $[b]$, are effectively unchanged by binding to the receptor. Simple reversible binding of ligands to receptor is assumed; the affinity of ligand $[a]$ for the receptor is assumed to be characterized by a dissociation constant, Kd_a ($\equiv$ concentration), which is the ratio of a dissociation rate constant, k_2 ($\equiv$ 1/time), and an association rate constant, k_1 ($\equiv$ 1/time/concentration): $Kd_a = k_2/k_1$. Similarly for $[b]$, $Kd_b = k_4/k_3$. Throughout the ensuing model analysis, we will assume that Kd_a and Kd_b are differentiated solely by their dissociation rate constants (k_2 and k_4), and that association rate constants (k_1 and k_3) are equal [1,10]. The lower affinity ligand (higher Kd) will have a proportionally higher dissociation rate constant; that is, the ratio Kd_b/Kd_a is taken to be equal to the ratio of the dissociation rate constants, k_4/k_2.

A kinetic mass balance model of the system shown in Fig. 1 is as follows. There are three possible receptor states in the sample incubation phase: A (bound to ligand *a*), B (bound to ligand *b*) and U (unbound); these are treated as state variables. These variables will have values ranging from 0 to 1 (viz., probabilities that a given receptor is in a given state), such that the sum $(A + B + U) = 1$, and $d(A + B + U) / dt = 0$.

Rate of change equations for the state variables A, B and U during the sample incubation phase interval are as follows:

$$dA/dt = k_1 [a] U - k_2 A$$

$$dB/dt = k_3 [b] U - k_4 B$$

$$dU/dt = -(dA/dt + dB/dt)$$

with initial conditions $A = B = 0$, and $U = 1$.

Rate of change equations for A, B and U in the combined interval of the wash and label phases are as follows:

$$dA/dt = -k_2 A$$

$$dB/dt = -k_4 B$$

$$dU/dt = -(dA/dt + dB/dt)$$

Below we will discuss equation solutions and assay properties for two sets of assay conditions: 1. conditions in which the incubation phase endpoint is assumed to be a steady-state; and 2. conditions in which the incubation phase duration is shorter than that needed to achieve a steady-state.

4. Assay properties when the sample incubation phase is assumed to be a steady-state

Below we will consider solutions to the mass balance equations for conditions in which the endpoint of the sample incubation phase of the assay is assumed to be in steady-state ($dA/dt = dU/dt = 0$).

4.1. Standard curve (assay measured response curve for [b] = 0)

A standard curve is produced for the immunoassay under conditions in which there is no competing ligand ($[b] = 0$). Assuming steady-state (ss) for the sample incubation phase ($dA/dt = dU/dt = 0$), then the

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