Unexpected Suppression of Immunoassay Results by Cross-Reactivity: Now a Demonstrated Cause for Concern

More than other analytical procedures, immunoassays have afforded a wealth of knowledge about biochemical physiology over the last 40 years. These assays have posed major analytical challenges, which most likely stem from the delicate balance between chemical equilibrium and our ever-increasing quest for speed of analysis, simplicity of procedure, and improved reliability. In attempts to enhance these criteria, we often face limitations imposed by the fundamental principles of thermodynamics: balancing chemical equilibrium, the underlying kinetics, and that ever-present variable, time.

Cross-reactivity in competitive immunoassays can be measured by various methods, including 50% displacement, equal displacement, or the gradient approach (1). Typically, a cross-reactant is conceptually regarded as an interferent causing positive bias in the assay results. When an immunoassay is “rushed” (i.e., the signal is measured well before reaching equilibrium), the effect of the cross-reactant is enhanced, in essence increasing the positive bias (2). That is the expected phenomenon. The unexpected is the observation that cross-reactivity can also lead to suppression in recovery (3), i.e., a negative bias. In other words, the result obtained for a constant amount of the analyte in a sample is lower when the cross-reactant is present than when it is absent. This indicates that rigorous characterization of cross-reactivity requires studies in the presence of the analyte (1).

Suppression of assay results induced by cross-reactivity was first described in Clinical Chemistry in 1996 for digoxin immunoassays (4). In retrospect, however, previous observations hinted at this problem. Kanan et al. (5) reported that in plasma obtained from patients suspected of having increased concentrations of digoxin-like immunoreactive factor, recovery of digoxin was lower in one assay than in others. Additional immunoassays have also been shown to be subject to suppression of results caused by cross-reactivity (6, 7). In addition to digoxin-like immunoreactive factor, other cross-reactants, such as progesterone (7), digitoxin (8), or oleandrin (3), have been reported to suppress recovery of digoxin in various immunoassays.

A proposed mechanism for the observed suppression of results lies in the physical design (i.e., architecture) of the assay and is fundamentally based on the fact that for binding of small ligands to antibodies, the rates of association are comparable for primary ligand and cross-reactant. It is the rate of dissociation that is different and accounts for the lower binding affinity of the cross-reactant molecule (2). The microparticle enzyme immunoassay (MEIA) for digoxin (Abbott Diagnostics) demonstrates a thermodynamic justification for this phenomenon. In this assay, digoxin in the sample (shown as gray boxes in Fig. 1A) binds to anti-digoxin polyclonal antibodies complexed to microparticles (MP in Fig. 1A). An aliquot of this reaction mixture is transferred to the matrix cell where the microparticle binds to glass fiber. The matrix cell is then washed to remove the unbound material. The enzyme (alkaline phosphatase) complexed to digoxin serves as the tracer binding to unoccupied antibody sites. The instrument measures the conversion of substrate (4-methylumbelliferyl phosphate) to a fluorescent product by the enzyme. The amount of product formed is inversely proportional to the concentration of digoxin present in the sample. A cross-reactant (shown as hatched boxes in Fig. 1B) such as progesterone competes with digoxin for binding to the antibody sites with similar association rate constants. However, during the wash step, the dissociation rate for the cross-reactant is greater.

Fig. 1. Mechanism of suppression of results by cross-reactivity.
(A), format when only the primary ligand (gray boxes) is present. MP denotes a microparticle with anti-digoxin antibodies attached to it. In this assay, the amount of product generated as a result of enzymatic activity is inversely proportional to the concentration of the analyte in the sample. (B), when a cross-reactant (hatched boxes) is also present along with the primary ligand, it will compete for binding to the antibodies. For a detailed description of the mechanism of suppression in this assay, see text. Alk-Phos, alkaline phosphatase.
than that for the primary ligand and allows more unoccupied sites to become available to bind tracer. The outcome of such a time-dependent competition is reduced recovery attributable to increased tracer binding and product formation. Therefore, the components necessary to create the environment for this to occur are the presence of a cross-reacting substance in the sample and a wash (or separation) step in the assay protocol. Short assay incubation times can exaggerate the extent of interference. What is very relevant is that the phenomenon of suppression caused by cross-reactivity is not limited to any particular cross-reactant or to any particular immunoassay as long as the above-mentioned criteria are met. To our knowledge, other explanations or plausible mechanisms have not been reported.

The clinical importance of this analytical problem was not appreciated until recently. Steimer et al. reported in Lancet (9) that misleading, subtarget concentrations of digoxin measured by the MEIA assay (AxSYM falsely guided therapy and led to intoxication of a 71-year-old patient. In this individual’s serum sample, potassium canrenoate (given for ascites) and the metabolite of this drug (i.e., canrenone) cross-reacted with the anti-digoxin antibody, leading to reduced recovery of digoxin. The extent of this suppression became evident when a sample originally measuring 0.9 µg/L by the MEIA was reanalyzed by two other methods, both of which measured digoxin as >5.5 µg/L. They further confirmed similar interference in serum collected from additional patients on potassium canrenoate therapy.

In this issue of Clinical Chemistry, Steimer et al. (10) have further studied the clinical consequences of the suppression of digoxin results caused by cross-reactivity of several steroid-like compounds, including spironolactone, canrenone, and their metabolites. Although potassium canrenoate is not currently prescribed in the US, spironolactone therapy has been increasing and is now listed as one of the top 200 drugs prescribed in the year 2000 (11). Their investigation shows that the steroids tested for cross-reactivity could increase, decrease, or have no effect on the nine digoxin immunoassays tested. Two of the three assays with decreased results use the same reagents and assay design (i.e., MEIA) and have been noted to have decreased results in the presence of cross-reactants. Falsely low values were seen in ~4% of patients and >8% of intensive care unit patients. Alarmingly, three of four individuals with digoxin values in the therapeutic range by the MEIA assay actually had toxic concentrations of the drug in their serum. The study by Steimer et al. (10) shows that negative bias introduced by a cross-reactant is a serious clinical problem and that several modern digoxin assays are prone to this problem.

It may be wise to reevaluate those immunoassays currently on the market that by virtue of their design may be susceptible to this kind of a problem. All of these recent findings now point to potential concerns over clinically important matters such as published reference intervals for hormones (e.g., those with structurally similar endogenous compounds whose concentrations fluctuate with time), dosing regimens based on therapeutic drug monitoring results, and variations in measured elimination half-life of drugs.

In summary, the unexpected finding of a negative interference from a cross-reactant was reported in 1996, its mechanism was proposed in 1997 (3, 4) and subsequently confirmed in 1998 (8), and the interference was shown to cause a serious health risk for a patient in 1999 (9). Now, in this issue, Steimer et al. (10) have taken this further and demonstrate the broader impact of this type of interference. An important lesson is that unexpected phenomena, when finally recognized as real, lead to new thinking, new processes, and new approaches—all of which translate to improved patient care.

References

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