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Interference from endogenous antibodies in automated immunoassays: what laboratorians need to know

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ABSTRACT

Full automation of laboratory procedures confers numerous advantages over semi-automated/manual tests because equipment, reagents and the computation of results are offered as an integrated package. Automation has allowed millions of immunoassay tests to be performed with good sensitivity and excellent precision but inaccuracy caused by interference from endogenous immunoglobulins/antibodies remained a problem (irrespective of the immunoassay's format). Interference leading to a falsely high or low result affects a specific sample and may not be obvious despite the strictest laboratory control schemes. Reporting and interpreting such potentially erroneous data remained however the responsibility of the clinical laboratory despite the limited information supplied by their providers. The focus of this review is on highlighting the potential downside of current disjointed and blurred arrangement between the developers/providers of immunoassays, and the laboratorians responsible for providing these data to their clinical colleagues. These limitations can be addressed by drawing attention to the importance of the key fundamentals underpinning these immunologically based analyses which, if carefully considered, could help to formulate pragmatic strategies to reduce errors in immunoassays. In this review, the inherent fallibility of the binding reaction between an antigen and antibody will be reiterated. The difficulties in defining reaction rate kinetics in non-equilibrium automated assays, the potential clinical error rate and the need for minimising analytical error rate of these automated technologies will be highlighted.

A primary role of the clinical laboratory is to provide data which must not lead to misdiagnosis of disease and/or mismanagement of illness. Generally, this goal has been approached for conventional/routine biochemical analyses but not for immunoassays with their inherent fallibility.^{1–15}

Immunoassay providers have focused on meeting the ever increasing workload and tests repertoire by developing highly sophisticated and fully automated “black-boxes” which have changed the analyst's role to essentially a highly skilled “machine operator”. This has allowed samples and reagent kits to be loaded at one end and results to be produced at the other. The product liability act has made it legally risky for the analysts to deviate from immunoassay protocols stipulated by the provider. Laboratorians who remained responsible for the integrity of these analyses have shifted their skills from being primarily “analyst/scientific” to “interpretive/clinical” using clinical validation as a pragmatic means to

identify inconsistencies between clinical correlates and immunoassay data. Such endeavour has favourably increased their clinical input and succeeded in recognising erroneous results in some cases. This approach however has a downside because validating and interpreting plausible, albeit analytically erroneous results in the first place by laboratorians can potentially undermine their role. It may be also unsustainable in view of the progressive rise in daily workload and the scant clinical information usually provided by the requesters. The clinical validation process could however be markedly enhanced if analytical interference from endogenous immunoglobulins/antibodies is reduced.

This review has four sections. The first two deal with the basic science underpinning antigen–antibody reaction which is fundamental to all immunoassays; and explain the inherent weakness and limitation of immunoassays as an analytical tool. The third and fourth sections highlight the current methods used in detecting interference and suggest manoeuvres to reduce or even eliminate their adverse effects in some cases; and finally examine the clinical sequelae of analytical interference without being prescriptive.

THE INHERENT FALLIBILITY OF MOLECULAR BINDING BETWEEN ANTIGEN AND ANTIBODY WHICH LEADS TO THE OPPOSING PHENOMENA OF HIGH SPECIFICITY AND CROSS-REACTIVITY

Generally, the molecular binding mechanism infers good specificity in the reaction between an antigen and antibodies which have undergone class switching and antigen-driven somatic mutation (ie, from the polyspecific IgM class of antibodies to IgG, IgA or IgE during a second or subsequent exposure). However, monospecificity between an antigen and antibody is untenable^{16–23} and specificity in immunoassays is not and could not be absolute. This is because molecular binding actually constitute a three-dimensional spatial/shape process which involves some of the variable subunits (or domains) on the light chain (V_L) and heavy chains (V_H) on each arm of the Y-shaped immunoglobulins.^{9–19} The spatial folding of these separate but close domains (three from the V_L and three from the V_H) is different, thus creating a cluster in space which forms the antigen-combining site (ACS) with its six hypervariable loops forming the complementarity-determining regions (CDRs). The CDRs determine both the contact surface on the antigen that binds to this site (ie, the epitope) and its antigenic determinants, namely its nature, shape and specific chemistry. The three-dimensional

shape of both the epitope and its contact surface on the antibody (its complementarity paratope) determines the number and strength of non-covalent bonds formed. Two mechanisms have been postulated for antigen and antibody interactions: the “lock and key” model in which there is no change of the structure of the antibody when it binds to the antigen; and the mutual or “induced fit” model in which both the antigen and antibody change their conformation on binding.²⁴ There is evidence that significant structural changes do occur at the binding site when binding of the antigen–antibody occurs; for example, small side chain rearrangements, segmental and relatively large movements of CDRs, or alterations in the relative disposition and rotation of the variable light and heavy chains. The concept of “conformational selection” has focused on the utility of the induced fit mechanism to accommodate conformational changes that occur to the antigen. Conformational selection mechanisms also help to explain antigen–antibody interaction when antigens without apparent structural complementarity acquire binding potential when they do not optimally fit themselves to begin with.²⁵ All in all, the induced-fit may be perceived as a pathway which results in a kinetically more competent antigen–antibody binding mechanism.

It is now generally accepted that both structural mimicry and the “induced fit” model play important roles in the antigen–antibody binding mechanism. The magnitude of this inducibility ultimately affects both the specificity and the affinity and would be dependent on the number and strength of non-covalent bonds formed. It is paramount to emphasise that undesirable cross-reactions to highly specific antibodies were a frequent finding affecting specificity since the earliest immunochemical studies. Cross-reaction is currently regarded by molecular immunologists as an intrinsic property by which antibodies participate in degenerate binding to differing antigens to amplify their functional diversity.¹⁶ Furthermore, in “heteroclitic” binding reactions, antibodies bind some of these unintended antigens with even higher affinity. It is of interest to point out that these unintended antigens involved in heteroclitic binding are not necessarily related to the original challenge of the immune response, that is, they are unrelated to the original antigen which triggered the antibody.¹⁶ Studies and conceptual models/mechanisms to explain the paradox of high specificity and broader cross-reactivity with different antigens remain speculative. It is therefore important to emphasise that despite the relentless innovations in immunoassays,^{26–29} cross-reaction(s) will continue to be manifested. Their adverse effects are however unpredictable, compounded by factors such as vastly different immunoassay formats.

THE KNOWN AND UNKNOWN IN ANTIGEN–ANTIBODY REACTION-RATE KINETICS IN AUTOMATED IMMUNOASSAYS

A considerable amount of seminal knowledge was developed in the 1960–70s regarding kinetic rate reactions, optimisation, precision and sensitivity^{30–33} of immunoassays. Such information was commonly shared by laboratorians because most immunoassays at that time were developed for in-house use and had a common denominator, namely “competitive, equilibrium radioimmunoassays”. Currently however, immunoassays are a heterogeneous mix of non-equilibrium, immunometric excess-reagent assays with vastly different formats. Because of this, the individual immunoassay became “almost unique”. Furthermore, information on each assay known to the providers of these assays (with its pros and cons) may not be fully shared with the users, being regarded with some justification as

commercially sensitive. The following two sections highlight the sort of information which is generally known to the providers and that which remains usually unknown to both the providers and the users.

Information generally known to the providers

- (1) Cross-reactivities from closely related moieties which produce a measurable effect in the dose–response curve are usually tested and established by the immunoassay developer.
- (2) The diffusion rate, temperature and reaction time would influence the chance of the antigen and antibody to essentially bump into each other. Immobilisation of capture antibody reagent on a solid phase (a common feature in almost all automated immunoassays) as well as conformational complexities of the antigen and/or the antibody determine the outcome of this key initial step. Assay volume, viscosity and addition of reactants (simultaneously or sequentially) may affect differently the diffusion rates of the immunoassay’s components. The developers of immunoassays usually optimise these factors.
- (3) Once the antigens bump into the capture reagent antibody, typically they spontaneously bind; however, larger molecules may require a particular orientation before binding. This forward/association binding rate reaction (K_1) is followed by a reverse unbinding/dissociation rate reaction (K_{-1}) because antigen–antibody binding follows a reversible bi-molecular thermodynamic principle. In immunoassays K_1 is quite dominant and fast. The reverse dissociation rate reaction (K_{-1}) determines the length of time the antigen remains bound to the antibody and is influenced by both the number and types of bonds that are formed between the antigen and antibody. Like the forward reaction, the reverse rate reaction can range over several orders of magnitude. The binding of antigen to antibody involves therefore molecular recognition followed by self-assembly stochastically favoured by non-covalent forces. Multiple non-covalent bonds determine the strength of the binding reaction and utilise intermolecular forces of interaction, such as: *hydrogen-bonding*, which involves reversible hydrogen bridges between various hydrophilic structures such as hydroxyl, amino and carboxyl groups; *electrostatic interactions*, which involve oppositely charge ionic groups which attract each other, eg carboxyl and amino groups; *hydrophobic interactions*, which involve the association of non-polar groups in aqueous solution; and *van der Waals forces*, which refer to interaction between external electron cloud that leads to induced dipole interactions. The electron cloud shapes of both the epitope and paratope influence the spatial complementarities and are paramount in the binding reaction. Distance affects the force of attraction between the antigen and the antibody; for example, van der Waals and electrostatic interactions are inversely proportional to the 7th power of the distance for the former and the square of the distance in the latter.
- (4) The overall strength of interaction between a single epitope on the antigen and a single paratope on the antibody is referred to as *affinity*, a mathematical notation expressed as 10^* , ie 10 to the power of the digit. It reflects how many of the above non-covalent bonds have been formed, and how strong are each of them. The *affinity constant* (K_a) is the ratio of the forward to the reverse rate constants (K_1/K_{-1}); in early equilibrium immunoassays this ratio was referred to as the equilibrium constant (K_{eq}). Because automated high throughput immunoassays are invariably non-equilibrium assays, the term K_a may be regarded as a *functional affinity*/

avidity which encompasses “non-constant” variables such as temperature, surface area, ionic strength and viscosity. The term K_a may also reflect the *functional avidity* between multiple epitopes on the antigen with more than one paratope on the antibodies. The affinity/avidity of most reagent antibodies ranges from $\sim 10^7$ to 10^{11} M^{-1} , ie a difference of 10 000-fold between the weakest and strongest.

Information usually unknown to both the providers and users

- (1) In an immunometric assay, interference from antibodies could also produce a measurable effect in the dose–response curve (positive or negative). However, unlike cross-reactivity from known moieties, the effect of endogenous immunoglobulins/antibodies is neither predictable nor easy to measure. The effect is method dependent and can produce a false positive result with one method but a lower result with another.^{8 11 14 15 28 34–50} Although immunoassay providers can never exclude or predict interference from such sources, they do incorporate buffers in their reagents to reduce adverse effects from endogenous interfering antibodies.
- (2) In immunoassays, two components, namely reagent antibody and antigen concentrations, are both critical in determining reaction rate kinetics. In the old competitive equilibrium immunoassays, the binding reaction obeyed a second-order rate and the dissociation followed a first-order rate (first-order reaction depends on the concentration of only one reactant; other reactants follow a zero-order rate reaction). A second-order reaction depends on the concentrations of two simultaneous first-order rate reactions. The reagent antibody in automated immunoassays is however present in large excess and because of this, only a relatively small amount of reagent antibodies is consumed as the binding reaction progresses. For this reason, its concentration can be considered to stay relatively constant and in such a case, the antigen and antibody binding follows a pseudo first-order rate reaction.⁹ This assumption may be justified for many straightforward immunoassays in which the antigen has a well defined homogenous epitope. However, when the antigen is complex with an array of multiple different epitopes (with different affinities), the reaction rate becomes complex and difficult to predict or to be correctly modelled or thermodynamically defined (because mixed-order or higher rate reactions have a fractional order of their rate).
- (3) Another example of poorly defined reaction rate is measurements of antibodies themselves in serum, such as rheumatoid factor (RF), thyroid peroxidase antibodies (TPO), and insulin antibodies which are heterogeneous.¹⁵ Measurements may utilise either the corresponding antigen or an antibody which binds to the endogenous circulating antibody. In immunoassays using the antigen format, the degree of previous saturation, ie the amount of antigen already bound to these endogenous antibodies prior to assay, could impact on immunoassay results. For example, excessive antigen production during an acute/fulminant phase of the disease could lead to a falsely low immunoassay result. The use of antibody for quantitation is also susceptible to considerable error because of the heterogeneity of circulating serum antibodies in term of class/subclass. Attempts to standardise the outcome of these immunoassays have failed, even when purified materials or recombinant antigens were used.⁵¹ In fact, some of these analyses could be justifiably regarded as empirically based immunoassays.

METHODS OF DETECTING INTERFERENCE FROM ENDOGENOUS ANTIBODIES AND SUGGESTED MANOEUVRES TO REDUCE OR EVEN ELIMINATE INTERFERENCE

It may be important to re-emphasise from the outset that interference is easily suspected when the immunoassay result is grossly wrong and without clinical correlates. However, when the immunoassay result is plausible or subtle, it can be difficult to suspect and has led to numerous instances of misdiagnosis of disease and mismanagement of illness.^{6 7 8 10 15 52 53} Needless to say, current method(s) cannot identify/detect or eliminate this insidious and pernicious source of interference in all samples.

When interference is suspected, it is important to take into account the form of analyte(s) under consideration and the nature of potentially interfering antibodies. Endogenous interfering antibodies may fall under two categories: (1) antibodies known to affect specific analytes (eg, anti-thyroglobulin antibodies in thyroglobulin immunoassays,⁵² and anti-insulin antibodies in the measurement of insulin, proinsulin or even C-peptide immunoassays⁵³); and (2) heterophilic antibodies which are unpredictable and could bind randomly to more than one antigen. Laboratorians could screen for the formers but not the latter. Screening samples for heterophilic endogenous antibodies such as human anti-mouse antibodies (HAMA) prior to analysis^{54 55} was found to be neither specific nor practical because antibodies with low affinity, though measurable, may exert little or no interference in immunoassay analyses.^{60 63}

Generally, heterophilic antibodies may cause interference by binding to the antigen itself or binding to the capture and/or sensor reagent antibodies and/or by binding to the antigen–antibody complex, ie metatope. In such cases, interference may lead to erroneous results (falsely high or low),^{1–15 52 53} the magnitude of which will be dependent on the affinity/avidity of interfering antibodies as well as their concentrations (ie, titres), both of which may vary with time. Interference may be transient over weeks (the half-life of immunoglobulins is ~ 1 month), arising from antibodies produced as a result of bacterial/viral infection, recent immunisation, treatment with monoclonal antibodies or a blood transfusion; or chronic (over years) in patients exposed to a wide range of animal proteins (through pets, including rodents, or via occupational exposure in veterinarians) or those with autoimmune disorders.^{4 5 8 15 53}

The incidence of interference in immunoassay varies from 0.4% to 4%,¹⁵ although a figure of 0.05% has been suggested, based largely on strong circumstantial evidence. These differences may be attributed to the specific nature of interference, its means of identification and the study design. However, in the largest prospective study involving more than five thousand patients in whom thyroid function tests were requested, the incidence of interference in thyroid stimulating hormone (TSH) immunoassays with potentially adverse clinical effect was found to be 0.4% of these patients.⁸ The overall incidence of interference was, however, slightly higher at 0.5%.

Suspecting and detecting interference from endogenous antibodies in immunoassays

Results on samples suspected of interference can be checked by three independent tests⁵⁶; for example, repeat analysis using a different immunoassay platform. Knowledge of methods bias is mandatory in such cases because interference would be highly likely when a reverse or exaggerated bias is found (see Ismail⁵⁶ for more details). Reanalysis using alternative technology such as liquid chromatography or tandem mass spectrometry^{57 58 68} should be considered if available.

Review

Another test is serial dilutions,⁵⁶ which could identify ~60% of samples with interference in which linearity and parallelism are lacking. However, linearity and parallelism can still occur despite the presence of interfering antibodies (detailed discussion elsewhere⁵⁹). The third useful test is the commercially available blocking antibodies. Statistically discrepant results before and after incubation with blocking antibodies would be indicative of interference.⁵⁶ However, ~20–30% of samples with interfering antibodies may yield similar results in this test too. Using these three tests could identify interference in ~90% of suspected samples.

Although clinical validation is undoubtedly a useful tool, an analytically based approach to ameliorate or even eliminate interference would be highly desirable. Several manoeuvres have been described to reduce analytical interference from antibodies; for example, humanisation of reagent antibodies, removal of the Fc fragment, and the use of F(ab)₂ or even smaller chain fragments as reagent antibodies.⁶⁰ Although such manoeuvres could reduce interference, they do not eliminate it. Analytical innovations to ameliorate or even eradicate interference can only be introduced by the immunoassay providers because it is outside the clinical laboratory domain.

All of the suggested procedures/manoeuvres are off-line. Furthermore, none could be universally applied to all analytes because of the very wide diversity of immunoassay measurements. Suggested manoeuvres are:

- ▶ Adding animal serum as blockers to immunoassay reagents. This is normally carried out by the immunoassay providers themselves, for example mouse sera to block human anti-mouse antibodies (HAMA) in immunoassays using monoclonal antibodies. The simple addition of mouse sera by laboratorians may not necessarily result in further neutralisation of HAMA if the mouse serum is from another strain of mice.⁶¹ Details of all buffering additives are considered to be commercially sensitive information, but since interference still occurs, it appears that such treatment does not neutralise all forms of heterophilic antibodies of human origin, animals or pathogens. Commercially available “blockers antibodies” formulated to neutralise interference from a wider range of endogenous antibodies was in my experience valuable,⁸ albeit producing false negative results in 20–30% of samples with known endogenous interfering antibodies.
- ▶ If endogenous interfering antibodies could be eliminated, there is no need to either detect or neutralise them.⁶⁰ Polyethylene glycol (PEG) is a reagent that could produce immunoglobulin-free samples prior to analysis.^{62–63} PEG was found to be effective in immunoassays such as prolactin, α -fetoprotein and follicle stimulating hormone. Its wider use in other immunoassays such as thyroxine, triiodothyronine, testosterone, sex hormone binding globulin, oestradiol, cortisol and growth hormone was encouraging, but 35–40% of leutinising hormone and thyroid stimulating hormone (TSH) was lost.^{62–63} Treatment of serum at room temperature with PEG at a final concentration of 125 g/l precipitated all IgM and IgG and 70% of IgA over a wide range of serum concentrations. This approach could eradicate almost entirely this potential and insidious source of interference. PEG, however, cannot be a universal agent, for example, when the analyte itself is an immunoglobulin/antibody, eg RF, TPO and/or those which co-precipitate.
- ▶ Chromatography prior to immunoassay, though effective, would be unsuitable for high throughput automated analyses.⁶⁰ Heat treatment could inactivate endogenous immunoglobulins prior to immunoassay; however, such

Take-home messages

- ▶ Interference in immunoassays from endogenous antibodies is a major unresolved and underestimated analytical problem, which has clinical consequences.
- ▶ Eliminating or reducing this insidious and pernicious source of error should be a primary goal of providers of immunoassays.
- ▶ In the meantime, laboratorians must remain alert to the analytical inaccuracy of immunoassays.

harsh treatment would be applicable to a few robust analytes.⁶⁰

- ▶ Intra-analytical: as mentioned previously, linearity and parallelism is a useful test which could detect interference in ~60% of samples with known endogenous interfering antibodies.⁵⁹ Parameters used in plotting the dose–response curve as well as serially diluted samples could also be utilised to construct a Scatchard plot.⁶⁴ The dose–response in immunoassay is curvilinear or sigmoid when the data are plotted on a linear or semi-logarithmic scale, respectively. Scatchard plotting is a popular method to linearise the binding data, and because current immunoassays use monoclonal rather than polyclonal reagent antibodies, the transformation produces a straight line. Key information could be deduced from a Scatchard plot, eg total amount of antibodies (Ab_t), its valency (n) and affinity/avidity constant (K_a). Significant discrepancy in one or more of these parameters between those obtained from the dose–response curve compared with the serially diluted sample would raise the possibility of interference even if linearity and parallelism remained intact⁵⁹ (ie, in ~40% of samples). This approach has limitations, however, because some information on the immunoassay parameters is not available to the users and the procedure necessitates dilutions. Ideally, analysers should have in-built software which could implement appropriate steps in sample handling and process such information on-line.
- ▶ A “nonsense” assay for analytes with a single epitope in which the original capture antibody is used as both capture and tracer antibody has been described by Boscatto and Stuart.⁶⁵ With no interference, readings would be in the blank range. Interference produced by human antibodies against animal antibodies used as reagent would be detectable and flagged. This approach, however, would be limited to few immunoassays and necessitates reengineering of analysers to run duplicate assays on each sample.

CLINICAL SEQUELAE AND CONCLUSION

Erroneous results in immunoassays causing adverse clinical sequelae have been reported in detail on numerous analytes.^{6–9 15 27 28 34–50 52 53} Although interference from endogenous antibodies is statistically low (about 0.4%), and the majority of immunoassay results are analytically accurate and clinically valid, laboratorians and clinicians alike must distinguish between analytical error rate and clinical error rate. This is because a relatively modest analytical error may be magnified when the disease prevalence in the population is taken into account (eg, predictive positives = true positives divided by the sum of true+false positives or vice versa for predictive negatives).⁶⁶ This well established statistical concept⁶⁶ was illustrated in a recent note on the potential of misdiagnosing the condition of subclinical hypothyroidism,⁶⁷ in which an analytical immunoassay false positive error rate of ~0.4% in

TSH measurement could potentially cause ~17% wrong diagnosis and lifelong treatment for a phantom disease when both the analytical error and the prevalence of subclinical hypothyroidism in the population (~2% true positives) are taken into account.⁶⁷

Until now, analyser design and excellent methodological formats have not overcome the longstanding pernicious and insidious problems of analytical interference leading to clinically misleading results in immunoassays (details elsewhere).^{15 66 69–72} Indeed, false results can in some cases be more dangerous than no results. Potential improvement is however possible, achievable and overdue. Meanwhile, laboratorians should always be aware of the inherent fallibility of immunoassays; and be ready to perform additional tests on any suspicious data to eliminate/reduce its adverse sequelae.^{56 59}

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Review

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