

Assay Interference: A Brief Review and Helpful Hints

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Laboratory Directors and laboratorians are responsible for the quality of data reported from their laboratories, but they rely on the assay manufacturer for a great deal of information. The quality of interference testing data provided by the manufacturer is seldom questioned, and, in-house interference testing is not normally performed. Only 8% of laboratories have performed in-house interference studies (2). Therefore, the burden is on the manufacturer to provide the highest quality interference data possible. While relying on the manufacturer's interference claims is considered sufficient to meet regulatory requirements, this information may not translate to performance in a specific clinical laboratory, and individual laboratories should be encouraged to verify manufacturer's data by performing their own in-house interference testing.

In addition, the laboratory must implement an effective detection system to identify specimens with clinically important interferences and a specific policy to prevent reporting of inaccurate results. One study reported that 9.7% of all specimens contained at least one visible interferent; 76% were lipemic, 16% were hemolyzed, and 6% were icteric (3), yet visual inspection of samples alone is not effective. The use of automated, spectrophotometric measurement of bilirubin, hemoglobin, and lipemia (serum indices), along with clearly defined decision rules, is effective (4), but must include an understanding of the relationship between index scale and the impact on each test. Interestingly, only 38% of laboratories were using automated serum indices for assessment of interference (2). Manufacturers should encourage their customers to use serum indices and provide training to help end users better understand how indices can better detect problem specimens.

It is also important for the laboratory to understand how the indices were determined by the manufacturer, and for the manufacturer to describe their interference testing protocols in detail. It is important to correlate icterus and hemolysis indices with bilirubin and hemoglobin concentrations. Lipemia index is typically based on the spectrophotometric response of Intralipid[®], a synthetic lipid emulsion, and cannot be correlated with the response of human triglyceride-rich lipoproteins. The manufacturer should assess lipemia using intact human lipoproteins, primarily chylomicrons, instead of Intralipid.

Information provided by the manufacturer may not be adequate for the laboratory to gauge the impact that interference will have on their results. Often, the data is reported without defining a "significant" change or what analyte concentration was tested along with the interferent, and interpretation is often based on an arbitrary 10% rule (5). The experimental design may be vague, such that the user does not have adequate information to make an informed decision about the adequacy of interference testing (5).

There are several reasons for encouraging a laboratory to perform in-house interference testing experiments, such as: verifying the commutability of interference data from the manufacturer to their specific instrument/reagent system; to provide additional information that may not be provided by the manufacturer, such as assessing interference with at least two levels of analyte, as recommended by CLSI due to potential interdependence of interferent and analyte concentrations; and determining the applicability of data generated from testing that used artificial materials, such as Intralipid, instead of human lipoproteins to test lipemia interference.

Common Interferents

Interference from **hemolysis** (hemoglobin and /or red blood cell contents), **lipemia** (triglyceride-rich lipoproteins or turbidity), **icterus** (unconjugated or conjugated bilirubin), and **proteins** (albumin, gamma-globulins, and paraproteins) are so common with routine chemistry assays, that validation by manufacturers always includes interference testing for these substances. Newer instruments and reagent formulations are better able to limit the effect of common interferents; sample blanking, bichromatic measurements, and additives to inhibit interferents have all been effective to a certain degree, but problems have not been eliminated. Unfortunately, we cannot develop universal rules regarding interference, as instruments and reagent systems can vary widely in the response to specific interferents (6, 7). Some investigators have developed a mathematic formula for the correction of interference, but this is generally not a good idea. The best approach is for laboratorians to understand the limitations of the assay in their laboratory, and not report results that are inaccurate to a degree that they will impact clinical interpretation.

Hemolysis

Hemolysis occurs in about 3% of all specimens received in the laboratory and accounts for 40% to 70% of all unsuitable specimens (8). Dimeski describes four mechanisms for interference from hemolysis: additive, spectral, chemical, and dilutional (9). Interference may be due to hemoglobin or to other red cell constituents that are released into plasma or serum. These substances can directly or indirectly interfere with a number of assays, including ALT, AST, creatinine, CK, iron, LDH, lipase, magnesium, phosphorus, potassium, urea, albumin, ALP, chloride, GGT and sodium (10-12).

Differentiating between *in vivo* and *in vitro* hemolysis is vitally important to patient management. It is sometimes possible to differentiate between the two biochemically. Free hemoglobin in the circulation binds to haptoglobin and the complex is rapidly cleared. Therefore, a decrease in serum haptoglobin is consistent with *in vivo* hemolysis. Increased bilirubin and reticulocyte counts are also characteristic of *in vivo* hemolysis. The bilirubin is derived from the breakdown of heme, while the presence of reticulocytes indicates the physiological response to anemia. Another clue differentiating *in vitro* and *in vivo* hemolysis is the concentration of intracellular contents: *in vitro* hemolysis is typically characterized by an increase in cellular components such as K, LDH, and AST, which would be quickly normalized during *in vivo* hemolysis. Given the clinical significance of hemolysis, the cause of hemolysis should be investigated and documented. Procedures for handling and reporting hemolyzed

specimens should be available, including appropriate rejection criteria. As a rule of thumb, when most or all specimens from a patient are hemolyzed, *in vivo* hemolysis should be suspected and reported to the physician. When intermittent specimens are hemolyzed, it is likely to be preanalytical, *in vitro* hemolysis. *In vivo* hemolysis is relatively rare, accounting for only about 3% of all hemolyzed specimens (10), which is all the more reason to be vigilant.

Hemoglobin, which absorbs light strongly at around 550 nm, can cause an apparent increased analyte concentration when monitored near this wavelength. Bichromatic readings and sample blanking will minimize interference; however, the high background absorbance may still be problematic (13). Hemoglobin may also have “pseudo” peroxidase activity which may interfere with bilirubin measurement by diazonium methods (3). Artifactual release of red cell constituents will have obvious effects on the accuracy of serum concentrations, especially K, Mg, P, LDH, AST and ALT (10); AST activity is approximately 40-fold higher in erythrocytes than in plasma, so that even slight hemolysis can alter results (10). Potassium is typically 25 times more concentrated in red blood cells than in plasma. Adenylate kinase released from red cells can increase creatine kinase (CK) activity (10), although the addition of AMP or other analogs can inhibit adenylate kinase (11). Although most phosphate in red blood cells is organic, it can stimulate the release of inorganic phosphate in serum (6). It is inappropriate to use purified hemoglobin to assess interference from hemolysis because only the direct effect of hemoglobin is evaluated. The effect of dilution and other red cell components should be considered, as well. CLSI therefore recommends interference testing by spiking hemolysate into a non-hemolyzed serum pool (14).

Various techniques have been used to reduce or correct for hemolysis interference. Deproteinization by ultrafiltration or precipitation can remove hemoglobin but cannot correct for the release of intracellular contents. Sample blanking and bichromatic measurement will decrease the absorbance effect of hemoglobin but, again, will not correct for the release of intracellular contents. It is critically important that the laboratory have a clear understanding of the effect of hemolysis on each laboratory test and clearly know when a test result cannot be accurately reported.

Lipemia

Lipemia is best defined as turbidity in a sample caused by elevated triglycerides, mostly as chylomicrons and very low-density lipoproteins (VLDL), and caused by recent dietary fat intake, abnormal lipoprotein metabolism, the infusion of lipids (parenteral or enteral feeding), and heparin therapy. Interference from lipemia is due to nonspecific light scattering or absorbance or, with severe lipemia, volume displacement (9). Turbidity is visually evident with triglycerides above about 300 mg/dL. Chylomicrons, because they are larger and more triglyceride-rich, are the more common source of turbidity, while VLDL contributes much less (15). Other causes of turbidity include elevated proteins (monoclonal gammopathies) and cold agglutinins.

Lipemia interferes with almost all spectrophotometric measurements by absorbing and scattering light. Sample blanking and bichromatic measurements help but do not eliminate this interference.

Triglyceride-rich lipoproteins in large concentrations also have a volume depletion effect, whereby the apparent concentration of analyte is decreased because lipoproteins replace the volume of available water. In other words, the volume taken up by lipoproteins is included in the analyte concentration. With immunoassays, the analyte may not be accessible to antibody, resulting in falsely low results.

For interference testing, lipemia is often simulated using Intralipid[®], an emulsion containing 20% soybean oil, 1.2% egg yolk phospholipids, 2.25% glycerin, and water. Unfortunately, the photometric response to this synthetic “fat” differs from physiological lipemia, making it inappropriate to use Intralipid[®] for interference studies (16). It is far more accurate to assess lipemia using intact human triglyceride-rich lipoproteins (15) as lipids are far more complex than Intralipid[®]. CLSI recommends use of a high-triglyceride serum sample to evaluate lipemia interference (14).

To prevent lipemia it is advisable to request fasting specimens (8-12 hour fast) and to halt parenteral feedings for 8 hours prior to specimen collection. If a turbid sample is received in the laboratory and the requested test(s) are affected, it is possible to remove lipoproteins by centrifugation. Other approaches such as extraction of lipids with organic solvents, precipitation of lipoproteins with polyanions, cyclodextrin, or polyethylene glycol, and delipidation with detergents, lipase, and deoxycholate, have all been suggested. It is important to document the process used to remove the turbidity from the sample and to remember to measure cholesterol, triglycerides, and apolipoproteins before centrifugation or other means of lipid removal. Laboratories will also want to ensure that their process to eliminate lipid interference does not introduce additional errors. Although equations can be used to correct for volume depletion, the equations do not consider other interfering effects, such as light scattering, that may also introduce error. Therefore, these equations are not recommended.

Icterus

Icterus is another name for jaundice, but it also specifically refers to serum or plasma with elevated levels of conjugated or unconjugated bilirubin. Bilirubin occurs in serum as relatively insoluble free bilirubin, as water soluble conjugate (mono- and di-glucuronides), and covalently bound to albumin. Conjugated bilirubin is often seen in urine when present in high concentrations in serum. Unlike hemolysis and lipemia, icterus is not easy to detect visually. Icterus has been reported to interfere with many assays, including creatinine (Jaffe reaction), phosphate (molybdate), albumin (dye-binding), and assays based on oxidase or peroxidase reactions (glucose, cholesterol, triglycerides, and uric acid). Hyperbilirubinemia is relatively common in hospitalized patients (ICU, gastroenterology, surgical, pediatrics), making assessment of bilirubin interference, and choice of methods, especially important.

Bilirubin causes spectrophotometric interference because of its strong absorbance between 340 and 500 nm (3). Spectrophotometric interference is due to the fact that the absorption of bilirubin and the chromophores typically used for reaction with hydrogen peroxide overlap (17). Because the response to conjugated and unconjugated bilirubin may differ, it is important to evaluate interference from both conjugated and unconjugated bilirubin.

Possible solutions to the problem of icterus interference have been proposed, including use of different chromogens, longer wavelengths, and additives such as ferrocyanide (17, 18) or bilirubin oxidase (18). Unfortunately, none of the methods fully resolve the problem. The best guidance is for laboratorians to evaluate or understand the effect of icterus on their test systems and ensure that inaccurate results are not reported.

Proteins

Most protein interference is caused by paraproteins (monoclonal immunoglobulins) that may affect many different assays, including bilirubin, phosphate, HDL cholesterol, GGT, CRP, and glucose (19). The interference may be due to physical or chemical alteration in the sample or it may be due to a volume displacement effect. A very high protein concentration will, like very high concentrations of chylomicrons and VLDL, reduce the available water (9). The practical effect of these “solids” is volume depletion; the aspirated sample is diluted with protein so that the analyte concentration is artifactually low. As with lipemia interference, the use of equations to calculate for volume depletion is discouraged. The equations do not consider other interfering effects, such as light scattering, that may also introduce error and the mean specific volumes are estimates that can vary widely between individuals.

Viscosity is a common problem with specimens containing monoclonal immunoglobulins, especially IgM, and this may also affect sample delivery.

Serum Indices

Visual inspection alone is not an effective method for screening for interferents that may be present in a sample. Serum indices are semi-quantitative estimates of hemoglobin, bilirubin, and lipemia (turbidity) in a sample using spectrophotometry. These indices are typically expressed in arbitrary units (+1, +2, etc) but can also be correlated to actual concentrations of hemoglobin, bilirubin, and triglycerides. Absorbances are recorded at several wavelengths to allow the calculation of the indices, and although they are similar, each manufacturer has a unique procedure. In general, we can assume that absorbance for turbidity is first monitored at wavelengths >600 nm. Then absorbance at ~450-575 nm is measured to assess bilirubin (icterus), and absorbance at 400-600 nm is used to assess hemoglobin interference. Algorithms are used to resolve overlapping areas and calculate indices. Although carotenoids and other pigments may interfere with this assessment, automated measurement of serum indices is necessary for an effective interference detection program (3).

Increase Understanding and Evaluation

The responsibilities of ensuring assay results are accurate falls on both the instrument/reagent manufacturer *and* the laboratory. Manufacturers should provide specific details of their experiments including the materials used, the protocols followed and the levels of interferent and analyte tested. Manufacturers must provide a full description of the experimental design and statistical methods used

to assess interference with each assay (5). When serum indices are listed, the procedure used to determine the indices should be clearly outlined.

Laboratories should verify how their instruments and assays perform in their environment, with their patient population, and compare that to the claims of their manufacturer. They must assess the quality and completeness of the data supplied by the manufacturer. There should be clear methods for identifying samples with interferents and procedures for dealing with affected samples. Labs must understand assay limitations and interference thresholds so that inaccurate results are not reported.

CLSI guidance document EP-7A outlines procedures for evaluating assays for interference and is intended for both manufacturers and laboratories. The recommendation includes an initial screening for interferents and then, if necessary, a dose response experiment to determine affect at different interferent concentrations.

Using the guidance outlined in the CLSI document, EP-7A (14), Sun Diagnostics has developed an interference test kit to aide manufacturers and laboratories in evaluating interference from the substances described in this paper. ASSURANCE™ Interference Test Kit contains human triglyceride-rich lipoproteins for evaluation of **lipemia** interference; human hemolysate for evaluation of **hemolysis** interference; a mixture of human serum albumin and gamma-globulins to evaluate **protein** interference; and conjugated and unconjugated bilirubin to evaluate interference from **icterus**. The samples are highly concentrated (up to 20x; the CLSI recommendation). Additionally, Sun Diagnostics has developed two very useful tools to accompany the interference test kit: a recommended testing protocol that clearly outlines the steps for both screening and dose response interference experiments and a data analysis spreadsheet that performs the calculations necessary to help determine if a substance is interfering with an assay.

With the availability of a kit, interference testing becomes more practical. Manufacturers and laboratories no longer need to find patient samples that fit their needs, store untested patient samples, or obtain artificial interferent substitutes, such as IntraLipid®. ASSURANCE™ contains stable, highly concentrated, purified and/or human-sourced materials that are representative of actual interferents encountered during testing. The human-sourced material is tested and found negative/non-reactive for infectious disease. The recommended testing protocols and data analysis tools can be worked into the routine procedure for interference testing. ASSURANCE™ Interference Test Kit from Sun Diagnostics will simplify interference testing.

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