IMPACT OF ULTRA-SENSITIVE TECHNOLOGY AND CONTEMPORARY THERAPY ON LABORATORY RESULTS

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ABSTRACT

The introduction of ultra-sensitive labels for immunoassays has exposed some of the limitations inherent in them. Thus, for instance a major problem with acridinium esters used, as chemiluminescence label is the formation of the so called "pseudobases" at an alkaline pH, which if not suppressed can affect the rate of the chemiluminescence reaction. Chemiluminescence labels such as luminol can also be problematic when attached to antibodies and small molecules to the extent that the sensitivity of the assay can be reduced by the decrease in the intensity of chemiluminescence. The increased use of molecular methods such as polymerase chain reaction (PCR) and post PCR methods to study mutations have various pitfalls, which if unrecognized and uncontrolled can lead to incorrect results and misinterpretation. Patients who are exposed to mouse immunoglobulins through imaging or therapeutic techniques can develop antibodies to mouse immunoglobulins (human anti-mouse antibodies or HAMA) which can be a major problem for non optimized immunoassays using murine monoclonal antibodies. The types of therapy such as anticoagulant used in anticoagulant therapy, blood substitute and drug therapy can impact on the measurements of some of the biochemical and other analytes. One must separate the transient physiological effects introduced by therapy from long-term biochemical alterations due to disease.

INTRODUCTION

Pitfalls in technology can affect the quality of laboratory results. Likewise the effects of therapy can influence the laboratory results. In this selective review I will attempt to illustrate with examples the influence of technology and therapy on clinical laboratory results.

EFFECT OF TECHNOLOGY

1. Chemiluminescent assays

In recent years the detection limits of immunoassay technology have been considerably improved with the introduction of ultrasensitive chemiluminescent labels.

Acridinium esters, luminol, adamantyl dioxetane phenyl phosphate (AMPPD), and ruthenium (II) tris (2, 2' bipyridyl) are some of the widely used

chemiluminescent labels used in the clinical laboratory. The last named label (ruthenium bipyridyl complex) is an electrochemiluminescent label.

There are problems associated with conventional acridinium esters. The drawback is that when acridinium esters are attached to small molecules in a competitive assay, poorly chemiluminescent products with very short half-lives are formed. A major problem is the formation at alkaline pH of the so-called "pseudobases" which are non-chemiluminescent and, as such, can affect the sensitivity of the reaction. However, since a strong alkaline pH (12.0 - 13.0) is required to initiate the chemiluminescent reaction in presence of hydrogen peroxide it is imperative that the acridinium esters be first preincubated in a pH range of 5-7 with hydrogen peroxide. This step will reconvert any pseudobase back to the acridinium ester. Subsequently pH is raised rapidly with strong alkali (sodium hydroxide) to initiate the

chemiluminescence reaction (1,2).

As a chemiluminescent label luminol when directly attached to antibodies and small molecules can reduce the amount of chemiluminescence generated and thus decrease the sensitivity of the assay. The reagent blank itself can contribute to a nonspecific high light emission. Scrupulous care need to be taken to ensure that the water used in the preparation of buffer is free from contaminants as otherwise the background signal itself can be high. In addition, one must ensure that the type of buffer used does not contribute to a high background signal or noise.

Some of the problems associated with luminol can be partially circumvented by labeling the antibody with an enzyme such as horseradish peroxidase. Luminol is just used as a chemiluminescent substrate when it is oxidized by hydrogen peroxide and alkali in presence of peroxidase label to generate chemiluminescence.

With adamantyl dioxetane phenyl phosphate (AMPPD) as substrate alkaline phosphatase is used to cleave the phosphate group in AMPPD to generate the adamantyl dioxetane phenyl anion (AMPD) which is weakly stable and fragments to yield adamantanone and the excited species methyl-meta-oxybenzoate anion which yields chemiluminescence. The efficiency of the chemiluminescence of the meta-oxy-benzoate anion can further be enhanced by energy transfer to fluorescein surfactants, such as micelles formed from cetyltrimethylammonium bromide and 5- (N-tetradecanoyl) amino fluorescein (3). The implication of surfactants promoting energy transfer is that contamination with surfactants in a non-optimized assay system without enhancers will have the effect of apparently overestimating a constituent due to the increased intensity of chemiluminescence that is generated.

Similarly contaminants at electrode surface can affect the electro chemiluminescent signal generated during the interaction of ruthenium (II) - tris (bipyridyl) complex and a reductant such as tripropylamine at

the electrode surface.

In general, specimen matrix effects can contribute to either enhancement or quenching of the chemiluminescent reaction. The efficiency of washing step in the immunoassay is critical to the elimination of specimen matrix effects. Larger the sample volume greater is the likelihood of interference. The type of solid matrix such as antibody coated magnetic beads versus antibody coated wells is also a consideration in the assessment of interference in ultrasensitive chemiluminescent immunoassays.

Another drawback with conventional chemiluminescent labels is their insolubility in aqueous buffers which makes the labeling of antibodies or proteins complicated. These labels due to their instability have a limited shelf life and contribute to inconsistent background signal and noise.

Attempts have been made to increase the stability and water solubility of acridinium derivatives. One example is the use of acridinium (N-sulfonyl) carboxamides, which provides increased quantum yield of chemiluminescence thus enhancing the sensitivity of the assay. The sulfopropyl substituent has the advantage of reducing background interference, in addition to increasing water solubility and providing longer shelf life (4).

2. Molecular techniques

Increasing the sensitivity of amplification techniques such as the polymerase chain reaction (PCR) can lead to false positive results especially if messenger RNA (mRNA) for prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSM) are found in cell lines of non-prostate origin as well as normal blood cells. Thus in reverse transcriptase PCR (RT-PCR) for PSA and PSM, increasing the number of amplification cycles to greater than 2 x 19 cycles causes an increase in false positive signal. However, if the number of amplification cycles is limited to 2 x 19 cycles, while no positive signal is seen in normal donors the limit of detection is greatly compromised (5).

Post-PCR methods such as single strand conformational polymorphism (SSCP) that are used for studying mutations have their pitfalls which needs to be overcome. The fact that the DNA-amplifying enzyme taq polymerase in PCR can add a non-base paired adenine residue at some, but not all, of the 3' ends of double-stranded DNA can result in extraneous bands on SSCP electrophoretic gel complicating their interpretation especially if fluorescent labels are introduced during PCR. This problem can be overcome by post-PCR labeling of amplified products (6,7).

The sensitivity of molecular biology techniques for the detection of mutations and other molecular events may be inadequate in the face of mosaicism, a phenomena where not all cells carry a mutation. In such cases while some cells sampled from a particular body site or a fraction of leukocytes do not carry a mutation thus testing negative, the mutation responsible for a disease or malignancy may still be present at the tissue level (8).

The fact that numerous chromosomal translocations characteristic of leukemia and lymphoma are found in normal tissues and cells from healthy persons raises the possibility of obtaining false positive results especially when the sensitivity of molecular biology techniques is pushed to its limits (9).

3. Exposure to therapeutic techniques using monoclonal antibodies

Patients who are either receiving radioimmunotherapy with mouse monoclonal antibodies or those who are exposed to imaging techniques such as murine monoclonal antibodies or those who handle experimental animals such as mice are apt to develop human anti mouse antibodies (HAMA). These antibodies can be problematic in two-site immunoassays using murine monoclonal antibodies. Binding of HAMA to both the capture and the labeled detection antibody can mimic the presence of analyte, thus giving either a false-positive result in the absence of an analyte or overestimation in the presence of normal amounts of analyte (2). A negative interference can also occur due to the size of the

immune complex that is formed when HAMA is in excess or when binding of the indicator antibody to the analyte bound by the capture antibody is reduced by HAMA.

Incorporation of mouse immunoglobulins in the assay mixture to absorb HAMA may not always be adequate especially when the added immunoglobulins are not sufficient to neutralize all of the HAMA present or when the appropriate immunoglobulin class, subclass or fragment type may not have been added to neutralize HAMA present in serum (2). Other strategies to overcome the effect of HAMA range from protein precipitation with polyethyleneglycol (130 g/L) to the use of human / mouse chimeric antibody as tracer in the two-site immunometric assay (2, 10, 11). The latter strategy is based on the premise that most of the circulating HAMA is directed toward the Fc portion of mouse IgG. As such the use of tracers with chimeric antibodies where the Fc portion is of human origin can overcome the interfering effect of HAMA.

4. Anticoagulant therapy

The fact that low molecular weight heparin (LMWH) is less reactive to platelets unlike unfractionated heparin, has made the use of the former more attractive especially when it can be administered subcutaneously once or twice a day. However, like unfractionated heparin, LMWH activates the enzyme triglyceride lipase, which, in turn, converts triglycerides to glycerol and free fatty acids (FFA). The fact that FFA competes with free drugs and free hormones for binding sites on albumin can lead to their displacement thus resulting in an increase in free drug and free hormone levels. Indeed in one study where blood was collected three hours after the injection of a LMWH preparation (enoxaparin) from nine healthy subjects and serum was analyzed within nine hours of collection, an increase in FFA was demonstrated (12).

However, the increase in FFA was not sufficient to displace thyroxine (T_4) bound to albumin and spuriously elevate free T_4 (FT_4) levels. But if the assay is delayed for up to 48 hours with samples stored at room temperature, levels of FT_4 were spuriously

increased from 2 to 63% ($\times 2.6 \pm 0.8$ pmol/L) when compared to samples analyzed within 9 hours of collection. The increase in FT_4 was directly correlated with the triglyceride and FFA level. In the same study similar data were obtained on 10 cardiac hospitalized patients with increase in FT_4 ranging from 10 to 161% in blood specimens collected 2 hours after LMWH injection and analyzed after storage at room temperature for 48 hours (12). However if blood is collected at least 10 hours after the last LMWH dose and specimen is analyzed within 24 hours, this in vitro phenomena of release of FFA from triglycerides by the enzyme lipoprotein lipase can be avoided. Storage of serum or plasma at 4°C retards the activity of lipoprotein lipase and thus minimizes further the in vitro release of FFA, thereby preventing spurious increase in the concentration of the free fraction of drugs and hormones displaced from albumin.

5. Therapy with blood substitutes

Applications for blood substitutes range from its use as oxygen carriers to the oxygenation of tumor during treatment with radiation or chemotherapy. Although several blood substitutes are undergoing clinical trials, some have been discontinued due to their side effects. Even so, the use of oxygen carriers is particularly attractive since it can be used along with transfused blood or blood products or even temporarily while one is waiting for matched blood to arrive.

Oxygen carriers used as blood substitutes can be classified into two types. One belongs to the category called polymerized hemoglobin solutions, while the other are 2nd generation perfluorocarbon (PFC) emulsions.

Polymerized hemoglobin solutions serving as hemoglobin based oxygen carriers (HBOC) have a serious drawback as far as the laboratory is concerned since it imparts a red color to the plasma or serum, while the plasma resulting from the use of PFC emulsions is milky white or lipemic.

Hence plasma derived from the use of a HBOC will simulate a grossly hemolyzed specimen. Plasma

hemoglobin concentration varies depending upon the amount of HBOC infused and the time elapsed after infusion. Thus when compared to pre-infusion plasma hemoglobin concentration of less than 5 mg/dL, at the end of 250 mL of infusion of HBOC the plasma hemoglobin concentration increased to 600 mg/dL, while at the end of 750 mL of infusion the plasma hemoglobin concentration rose to as high as 1450 mg/dL (13). Two hours after the 750 mL of HBOC infusion, the plasma hemoglobin concentration had dropped to 650 mg/dL, while at 24 and 48 hours after infusion the plasma hemoglobin concentration had dropped to 300 and 80 mg/dL respectively. The pre-infusion plasma hemoglobin concentration of less than 5 mg/dL was only reached 7 days after the completion of the HBOC infusion.

The practical implications of increased plasma hemoglobin after HBOC infusion is the interference that would be expected in the assay of some biochemical analytes as well as tests performed in the blood bank. It is important that the laboratory be informed of the time of infusion of HBOC to ensure that the blood bank does not misinterpret the interference as a hemolytic reaction. Agglutination reactions, however, are not obscured by the red color. While many of the biochemical analytes are unaffected by HBOC, both positive and negative interferences have been reported for some analytes, which may be related to both the analyzer methodology and the type of HBOC infused (13).

In as much as the HBOC contributes to the total hemoglobin concentration, hemoglobin concentration is expected to be overestimated. Red cell indices MCH and MCHC which are calculated using the hemoglobin value would be spuriously increased.

Both positive and negative interferences have been reported for PFC emulsions with few biochemical analytes and in co-oximetry measurements using hemolyzing co-oximeters (13, 14). However, non-hemolyzing co-oximeters that measure both total and various forms of hemoglobin (oxy, carboxy and methemoglobin) are not generally affected by the turbidity of PFC emulsions (13).

6. Effect of thyroid replacement therapy

Thyroid stimulating hormone (TSH) measurements are not particularly helpful for following the progress of thyroid replacement therapy especially if blood is collected within 6 weeks after the initiation of therapy. Since the pituitary gland takes 6 to 8 weeks to respond to thyroid replacement therapy (pituitary lag) blood specimens collected prior to 6 weeks after the initiation of therapy can yield spuriously elevated TSH levels in the hypothyroid range while the Free T4 (FT4) concentration would have risen to the normal range. In fact FT4 levels come back to normal as early as 3 weeks after the commencement of thyroid replacement therapy (15).

7. Therapy with selected drugs

Many drugs that are used in therapy have toxicity for liver and kidney, making it imperative for the patient receiving such drugs to monitor both liver and renal function. One has to merely read the drug manufacturer's instructions to note such adverse effects.

Thus anti-hyperlipidemic drugs such as hydroxymethyl glutaryl CoA reductase (HMGCoA) inhibitors (statins) which interfere in the synthesis of cholesterol by inhibiting an early step in the cholesterol synthesis pathway (the conversion of HMGCoA to mevalonic acid) can affect liver function. 3 to 12 months after therapy with these inhibitors AST and ALT levels can reach three times the ULN (upper limit of normal) (ULN 40 u/L). When HMGCoA reductase inhibitors are administered together with triglyceride reducing drugs such as gemfibrosil or with greater than 1g/day nicotinic acid, creatinine concentration can increase due to renal failure from rhabdomyolysis. In a very few patients (1 in 1000) who are administered HMGCoA reductase inhibitor such as lovastatin, myopathy can develop raising creatine kinase levels to greater than 10 times the ULN (ULN 150 U/L). Some drugs have proven to be so toxic that they have been removed from the market. An example of such a drug is rezulin (troglitazone) which turned out to be very hepatotoxic when it was used to treat patients with type II diabetes mellitus.

As a rule the laboratory should ascertain the nature of the drugs that a patient is receiving especially when significant alteration in laboratory results reflective of liver and kidney function are noted three to six months from the initial base line testing.

The laboratory should also be alert to significant reduction in the concentration of an analyte, bringing down the analyte value from the high abnormal to within the normal range. An example of such a drug is finasteride, which has been used to treat male pattern baldness. It is also used to decrease the size of the prostate gland in patients who have an enlarged prostate (benign prostatic hyperplasia or BPH). In men with BPH, the use of finasteride can cause a 50% drop in the concentration of prostate-specific antigen (PSA) (16).

8. Preanalytical concerns in monitoring therapy

Apart from the absorptive effects of some drugs to the specimen container, the anticoagulant chosen for specimen collection, and failure to promptly separate the cells from plasma can affect laboratory results (17).

For instance the assessment of the efficacy of in vivo platelet activation requires minimization of invitro platelet activation. The extent of in vitro platelet activation depends on the anticoagulant used for specimen collection. Thus while the release of platelet factor 4 (PF4) as a measure of platelet activation in platelet-poor plasma was 1,180 ng/mL in specimens collected with unfractionated heparin, it was 469 ng/mL with hirudin, 440 ng/mL with citrate and 217 ng/mL with EDTA-plasma (17, 19). EDTA appears to suppress platelet degranulation. Incidentally PF4 concentration obtained with a low-molecular-weight heparin preparation called Fragmin was comparable to that obtained with hirudin (18).

Finally, for monitoring of AIDS therapy it is imperative that plasma containing viral human immunodeficiency virus (HIV) RNA, be promptly separated from cells at least within 6 hours of collection and be frozen till analysis (7, 20). Apparently,

neutrophils have an inhibitory effect on the AIDS virus. It has been postulated that the stimulated neutrophil undergoes degranulation and releases the enzyme myeloperoxidase, which in turn reacts with hydrogen peroxide generated by the respiratory burst. As a result chloride is oxidized to form hypochlorous acid which is toxic to the AIDS virus (21).

Conclusion

As new technology is introduced, pitfalls associated with such technology needs to be thoroughly investigated and understood in order to

assess the bias introduced on laboratory results.

As the scope of therapy expands with new drugs, the effects of such therapy on laboratory results as well as preanalytical effects introduced by the choice of anticoagulants for specimen collection, specimen processing and specimen container effects need to be evaluated to understand the effect of such variables. Ultimately the quality of laboratory results is dependent on minimizing errors introduced by technology and therapy related variables.

REFERENCES

1. Littig, J.S. and Nieman, T.A. (1992) Quantitation of acridinium esters using electrogenerated chemiluminescence and flow injection. *Anal. Chem.* 64, 1140-1144.
2. Narayanan, S. (1988) Quality control in tumor marker analysis: preanalytical, analytical and post-analytical issues. *J. Clin. Ligand Assay.* 21, 11-17.
3. Narayanan, S. (1996) Concepts, principles and applications of selected molecular biology techniques in clinical biochemistry. *Adv. Clin. Chem.* 32, 1-38.
4. Pringle, M.J. (1999) Acridinium ester labels: Esters, sulfonamides, and their applications. *J. Clin. Ligand Assay.* 22, 105-122.
5. Gala, J., Heusterspreute, M., Loric, S., Hanon, F., Tombal, B., Van Cangh, P., De Nayer, P. and Phillippe, M., (1998) Expression of prostate-specific antigen and prostate-specific membrane antigen transcripts in blood cells: implications for the detection of hematogenous prostate cells and standardization. *Clin. Chem.* 44, 472-481.
6. Inazuka, M., Tahira, T. and Hayashi, K. (1996) One-tube post-PCR fluorescent labeling of DNA fragments. *Genome Res.* 6, 551-557.
7. Narayanan, S., (1997) Preanalytical and analytical pitfalls in molecular biology techniques. *J. Clin. Ligand Assay.* 20, 200-205.
8. Kwiatkowska, J., Widgowska-Sowinska, J., Napierala, D., Slomski, R. and Kwiatkowski, D.J. (1999) Mosaicism in tuberous sclerosis as a potential cause of the failure of molecular diagnosis. *New Engl. J. Med.* 340, 703-707.
9. Bagg, A. and Kallakury, BVS. (1999) Molecular pathology of leukemia and lymphoma. *Amer. J. Clin. Pathol.* 112 (suppl. 1), 576-592.
10. Hansen, H.J., La Fontaine, G., Newman, E.S., Schwartz, M.K., Malkin, A., Mojzisek, K., Martin, E.W. and Goldenberg, D.M. (1989) Solving the problem of antibody interference in commercial "sandwich-type" immunoassays of carcinoembryonic antigen. *Clin. Chem.* 35, 146-151.

11. Kuroki, M., Matsumoto, Y., Arakawa, F., Haruno, M., Murakami, M., Kuwahara, M., Ozaki, H., Senba, T. and Matsuoka, Y. (1995) Reducing interference from heterophilic antibodies in a two-site immunoassay for carcinoembryonic antigen (CEA) by using a human/mouse chimeric antibody to CEA as a tracer. *J. Immunol. Methods*, 180, 81-91.
12. Stevenson, H.P., Archbold, G.P.R., Johnson, P., Young, I.S. and Sheridan, B. (1998) Misleading serum free thyroxine results during low molecular weight heparin treatment. *Clin. Chem.* 44, 1002-1007.
13. Ma, Z., Monk, T.G., Goodnough, L.T., McClellan, A., Gawryl, M., Clark, T., Moreira, P., Keipert, P.E. and Scott, M.G. (1997) Effect of hemoglobin and perfluoron-based oxygen carriers on common clinical laboratory tests. *Clin. Chem.* 43, 1732-1737.
14. Shepherd, A.P. and Steinke, J.M. (1998) Co-oximetry interference by perfluoron emulsion: comparison of hemolyzing and non-hemolyzing instruments. *Clin. Chem.* 44, 2183-2190.
15. Narayanan, S. (1997) Current concepts of thyroid function and laboratory evaluation. *Indian J. Clin. Biochem.* 12, 25-34.
16. Oesterling, J.E., Roy, J., Agha, A., Shown, T., Krarup, T., Johansen, T., Lagerkvist, M., Gormley, G., Bach, M., Waldstreicher, J. and Finasteride. (1998) PSA Study Group. Biologic variability of prostate-specific antigen and its usefulness as a marker for prostate cancer: effects of finasteride. Finasteride study group. *Urology*. 51 (suppl 4-A), 58-63.
17. Narayanan, S. (2000) The preanalytic phase. An important component of laboratory medicine. *Amer. J. Clin. Pathol.* 113, 429-452.
18. Engstad, C.S., Guttentag, T.J. and Osterud, B. (1997) Modulation of blood cell activation by four commonly used anticoagulants. *Thromb. Haemost.* 77, 690-696.
19. Narayanan, S. and Hamasaki, N. (1999) Current concepts of coagulation and fibrinolysis. *Adv. Clin. Chem.* 33, 133-168.
20. Saag, M.S., Holodniy, M., Kuritzkes, D.R., O'Brien, W.A., Coombs, R., Poscher, M.E., Jacobsen, D.M., Shaw, G.M., Richmann, D.D. and Volberding, P.A. (1996) HIV viral load markers in clinical practice. *Nature. Med.* 2, 625-629.
21. Klebanoff, S.J. and Coombs, R.W., (1992) Viricidal effect of polymorphonuclear leukocytes on human immunodeficiency virus-1. Role of myeloperoxidase system. *J. Clin. Invest.* 89, 2014-2017.