Essential laboratory knowledge for the clinician

Laboratory testing forms an integral part of patient management.

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As clinicians place huge emphasis on the numerical values obtained from the clinical laboratory, it is imperative that they have a sound understanding of the factors that may impact on these results. This paper will address some of these issues.

Result comparability between laboratories

The result obtained when a sample is measured is not a perfect value. Usually this measured value is some distance from the true value (referred to as systematic error or bias) (Fig. 1).[1] This bias differs between analysers, implying that every analyte should ideally have analyser-specific reference intervals. Reasons for the poor agreement between analysers include the use of different methodologies, reagents and assay conditions (e.g. temperature, reagent concentrations, detection methods, wash steps and antibody epitopes).

To accommodate these differences, methods need to be calibrated to be traceable to reference methodologies (which accurately measure the true result) enabling comparability among laboratories.

Creatinine

Creatinine standardisation is a good example. Creatinine has been measured for decades using the Jaffe reaction, a method not specific for creatinine (may be interfered with by compounds such as ketones, protein and bilirubin). Numerous modifications by various manufacturers have led to improved specificity. However, in 2003 a College of American Pathologists (CAP) proficiency testing survey found that a large number of laboratories showed biases varying between -5.3 and 27 µmol/l at a creatinine concentration of 80 µmol/l, translating into a clinically significant error in the calculated glomerular filtration rate (GFR) (up to 27%).[2] This led to standardisation of creatinine assays to a reference method (Isotope Dilution Mass Spectrometry (IDMS)). It should be noted, however, that methods traceable to IDMS have lower creatinine levels than older unstandardised methods, an important fact when calculating GFR, as not all formulae have been adapted for use with standardised creatinine methods.[3]

HbA_{1c}

Standardisation is especially important in analytes such as HbA_{1c}, where clinical management is based on medical decision limits that are not method-specific. In the landmark Diabetes Control and Complications Trial (DCCT) study, HbA_{1c} was shown to correlate with the development and progression of chronic complications of diabetes[4] and specific diabetes treatment goals were developed based on this. The methodologies used in HbA_{1c} measurement portray varying degrees of accuracy. In fact, in 1993, CAP data showed that the result in a single sample could differ by as much as 7.1% depending on the method used, which made it impossible for clinicians to relate their results to DCCT guidelines.[5] A reference method was therefore established by the International Federation of Clinical Chemistry (IFCC), to which all HbA_{1c} methods must be traceable. However, HbA_{1c} was measured by a less specific method in the DCCT trial, providing levels higher than the IFFC method. Therefore, in order to utilise DCCT guidelines, HbA_{1c} methods, apart from being traceable to the IFCC reference method, also employ a conversion factor to harmonise results to the DCCT trial.

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Other analytes requiring standardisation

One area of laboratory medicine in which standardisation has been difficult to achieve is immunoassays.[6] Analytes such as hormones and auto-antibodies are inherently heterogeneous and therefore difficult to standardise. In peptide hormone assays such as parathyroid hormone (PTH), different methods employ antibodies directed at different epitopes, which leads to measurement of different forms of the hormone, some of which may or may not be biologically active,

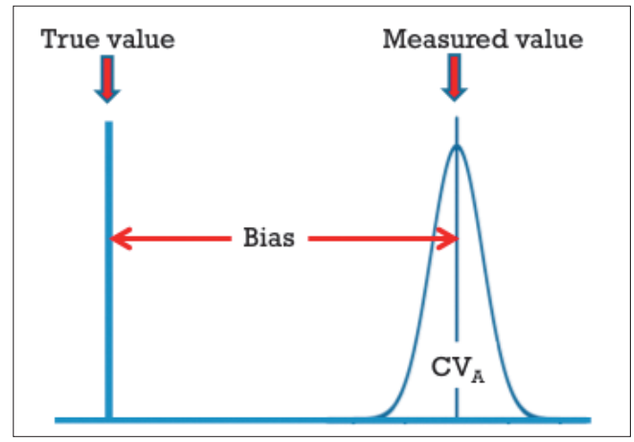

Fig. 1. Every result generated in the laboratory is associated with bias (systematic error) and imprecision (random analytical error – CV_A).

making medical decision limits across assays impossible.[7] Results are usually not comparable across laboratories, and reference intervals (and sometimes even units) differ among analysers.

Analytical variation

Each laboratory result is associated with random error termed analytical variation (Fig. 1), the magnitude of which depends on the methodology or analyser used. It can be calculated by repeatedly measuring one sample and calculating the analytical coefficient of variation (CV_A) from the mean and standard deviation.[1] The smaller this CV_A the smaller is the uncertainty around the result (Fig. 2).

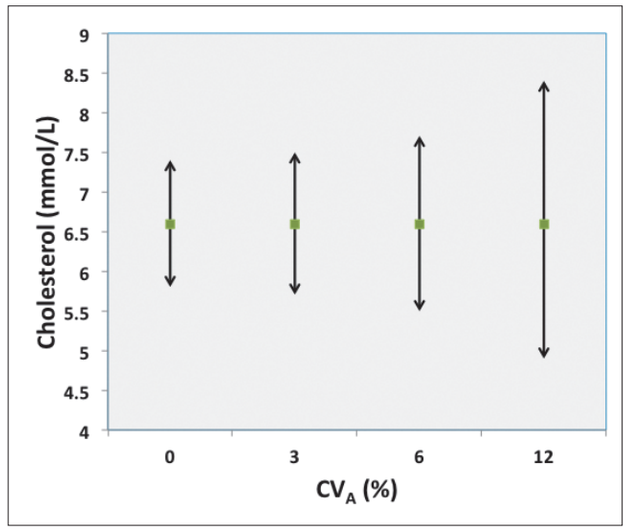

Fig. 2. With increasing analytical variation (represented by CV_A), the uncertainty around the result obtained increases. The smaller the CV_A, the more precise the laboratory method and the more certain we are of the result obtained.

Biological variation[8]

The concentrations of most analytes fluctuate around a homeostatic set-point within individuals (intra-individual biological variation or CV_I). This CV_I is considered to be random, and the homeostatic point differs between individuals (between individual biological variation or CV_G).

Much like the CV_A causes a degree of uncertainty around a laboratory result, the CV_I causes a degree of 'uncertainty' around the concentration of an analyte in an individual over time; e.g. the CV_I for creatinine is 6%, meaning that obtaining a blood result of 88 µmol/l in an individual implies that the creatinine concentration in this specific individual at any given time could be between 81 and 95 µmol/l, without a real change in renal function. Some analytes have a larger biological variation than others, e.g. calcium 1.9% v. aspartate aminotransferase (AST) 11.9%.[9]

When interpreting results, both the analytical and biological variation should be taken into account. The sum of these variations (CV_T) is expressed mathematically by the following equation

$$CV_T = \sqrt{CV_A^2 + CV_I^2}$$

As we are usually interested in a 95% significance level, the interval within which the analyte concentration falls is represented by:

$$result \pm 1.96 \times CV_T = result \pm 1.96 \times \sqrt{CV_A^2 + CV_I^2}$$

There are many applications for biological variation data. The most important are the index of individuality (II), reference change values (RCV) and setting of quality goals in the laboratory.

Standardisation is especially important in analytes such as HbA_{1c}, where clinical management is based on medical decision limits that are not method-specific.

Index of individuality (CV_I/ CV_G)

The II compares the biological variation of an analyte within an individual to that between all individuals. The index predicts the usefulness of a reference interval. Most analytes (including

Table 1. Index of individuality (II) for commonly measured analytes

Analytes	CV_I[9]	CV_G[9]	II
Na^+	0.7	1.0	0.7
K^+	4.8	5.6	0.9
Urea	12.3	18.3	0.7
Creatinine	6.0	14.7	0.4
HbA_{1c}	1.9	5.7	0.3
Glucose	6.1	6.1	1.0
Insulin	21.1	58.3	0.4
Alanine aminotransferase	24.3	41.6	0.6
Albumin	3.1	4.2	0.7
Ca^{2+} (total)	1.9	2.8	0.7
Ca^{2+} (ionised)	1.7	2.2	0.8
Cholesterol	5.4	15.2	0.4
Cortisol	20.9	45.6	0.5
Ferritin	14.2	15.0	0.9
Lactate	27.2	16.7	1.6
pH [H+]	3.5	2.0	1.75

CV_I = intra-individual biological variation; CV_G = between individual biological variation.

calcium, cholesterol and creatinine) have marked individuality (low II) meaning that the biological variation within an individual is much smaller than that between all individuals (Table 1). In such cases, reference intervals are not useful in deciding whether a result is normal or not. It would perhaps be more appropriate to compare the result with the patient's own reference interval – a very costly (and probably unfeasible) option!

Reference change values

The term RCV, also called the critical difference, refers to the minimum difference between two consecutive results, which needs to be exceeded in order for a significant change to have taken place. This difference should exceed the variations associated with each individual result. As there are two blood results to consider, there are two sets of variations which need to be combined to produce the RCV (Fig. 3). As the same analyser is usually being used, this combined variation is represented by the following equation:

$$CV_T = RCV = 2.77 \times \sqrt{CV_A^2 + CV_I^2}$$

This can be better illustrated with an example. Serum creatinine measurement repeated 3 days apart in a patient about to receive chemotherapy yielded the following results, respectively: 96 and 108 µmol/l (reference interval: 60 - 100). Are these two results significantly different? The difference between the two results = 12.5% and RCV = 17.8% (CV_I = 6%; CV_A = 2.3%), therefore the two results are not significantly different.

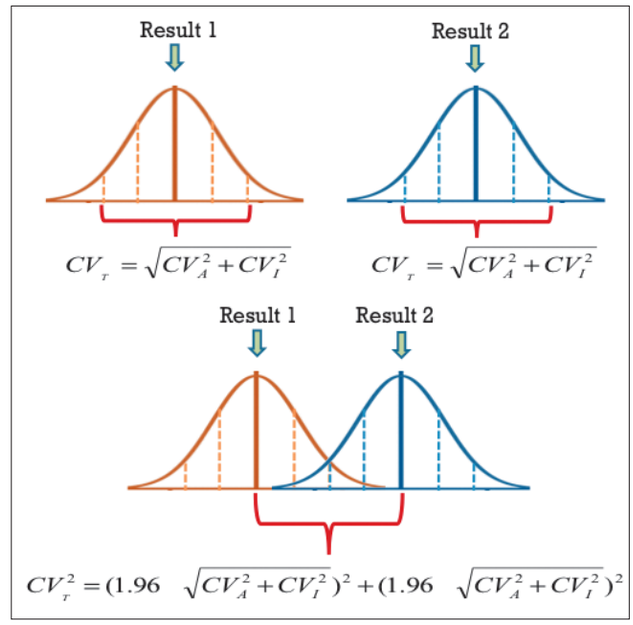

Fig. 3. Each result is associated with analytical (CV_A) and biological (CV_I) variation. The reference change value (RCV) encompasses the sum of these uncertainties associated with both results, as can be seen in the figure.

While some laboratories abroad provide the RCV along with laboratory results, this is not yet common practice in South Africa. See Table 2 for the RCV of commonly measured analytes. These will differ among laboratories.

Table 2. Reference change values (RCV) for various commonly measured analytes

Analyte	Arbitrary value	RCV (%)	RCV (absolute)
Na^+	140 mmol/l	3.2	4.4
K^+	4 mmol/l	13.5	0.5
Urea	5 mmol/l	34.6	1.7
Creatinine	100 µmol/l	17.9	18
HbA_{1c}	4%	5.8	0.2
Glucose	40 IU/l	20.5	8.2
Insulin	10 mU/l	58.7	5.9
Alanine aminotransferase	40 IU/l	67.5	27
Albumin	35 g/l	10.1	3.5
Ca^{2+} (total)	2.0 mmol/l	7.2	0.1
Ca^{2+} (ionised)	1.2 mmol/l	6.9	0.1
Cholesterol	4.0 mmol/l	16.0	0.6
Cortisol	400 nmol/l	58.3	233
Ferritin	200 ng/l	42.6	85
Lactate	2 mmol/l	75.4	1.5

Assay interference

Assay interference refers to the presence of a substance or factor in the sample that leads to a false decrease or increase in the analyte concentration measured (i.e. a bias). These factors can be endogenous or exogenous. The most common of these are listed in Table 3.

Table 3. Substances or factors that may lead to assay interference

Endogenous substances or factors	Exogenous substances

• Haemolysis
• Lipaemia
• Icterus (bilirubinaemia)
• Monoclonal antibodies and auto-antibodies
	• Test tube additives
	  • Ethylenediaminetetracetic acid (EDTA)
	  • Citrate
	  • Sodium fluoride (NaF)
	  • Oxalate
	• Drugs
	• Contrast media
	• Intravenous fluids

Endogenous substances

Haemolysis

In vitro haemolysis, the leading cause for sample rejections, may result from incorrect specimen collection, handling and transportation techniques. Haemolysis causes spurious results due to the release of intracellular substances, the concentrations of which may be much higher (e.g. K^+, phosphate, Mg^{2+}, ammonia, lactate dehydrogenase (LDH), adenylate kinase (AK) and AST or much lower (e.g. Na^+) than serum concentrations.[1]

It also causes altered results by interference with assays, leading to falsely increased creatine kinase (CK), CK-MB (AK interferes with measurement), total protein, alkaline phosphatase (ALP) and gamma-glutamyltransferase (GGT) (spectral interference of haemoglobin), and falsely decreased direct bilirubin levels (chemical interference of haemoglobin).

Haemoglobin-based oxygen carriers (e.g. Hemospan) lead to the presence of free haemoglobin in serum, which, above a certain serum concentration, interferes with a variety of assays in a preparation and analyser specific manner.[10] In patients receiving such therapy, serum haemoglobin should thus be measured before analysis of other analytes.

Bilirubin

Bilirubin interferes with a number of assays due to its spectral and chemical properties. These properties may lead to falsely increased levels in various colorimetric assays such as paracetamol and AST, and falsely decreased levels in H_2O_2-linked assays such as uric acid, glucose, cholesterol and triglyceride. Bilirubin also interferes negatively in creatinine assays (both Jaffe and H_2O_2-linked enzymatic methods). Most assays can accommodate a certain degree of bilirubinaemia.

Lipaemia

Lipaemia refers to the presence of large lipid particles, usually chylomicrons or very-low-density lipoprotein, in the serum sample. These particles lead to increased turbidity and electrolyte exclusion. The increased turbidity interferes with assays relying on spectral absorbance (leading to a decreased measuring range due to increased assay blanking). Dilution or ultracentrifugation of the sample may overcome the turbidity.

Severe lipaemia leads to electrolyte exclusion, resulting in falsely low sodium measurement (pseudohyponatraemia) when an indirect method, such as found in automated analysers, is used (dilution-calculation error). This phenomenon also occurs in samples with exceptionally high protein concentrations. Blood gas analysers avoid this error by analysing sodium directly (without diluting the sample).

Antibody interference

Antibody interference is the most well-known form of assay interference. Antibodies may cause *in vivo* interference by binding to other analytes, increasing their circulating half-life, and therefore concentrations, e.g. macro-prolactin, macro-CK, macro-amylase and macro-troponin. These peptides/proteins may be biologically inactive in their antibody-bound state with the elevated result therefore providing false clinical information. The presence of macroprolactin is common and it should be excluded in all samples where elevated prolactin levels are incongruous with the clinical picture.[11]

Heterophile antibodies, specific auto-antibodies or rheumatoid factor can interfere in numerous immunoassays, by binding to reagent antibodies or particles, causing falsely increased or decreased results (Fig. 4). Such interference has led to misdiagnosis and unnecessary treatment (including surgery) and should be

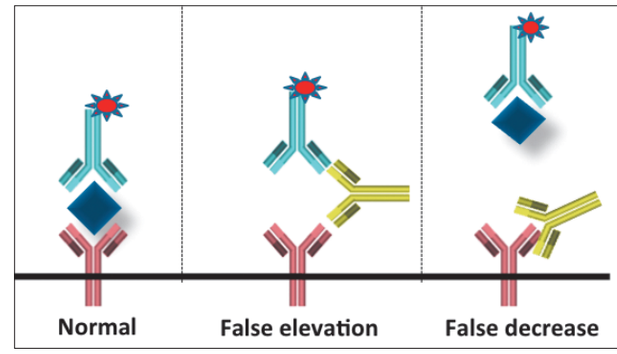

Fig. 4. Antibody interference in sandwich-based immunoassays. A sandwich formed between the capture antibody, antigen (e.g. hormone being measured) and labelled antibody allows for measurement of the hormone. The signal is directly proportional to the concentration. Antibodies may mimic this sandwich, increasing the signal and leading to falsely elevated hormone concentrations. They may also prevent this sandwich formation, thereby causing falsely decreased hormone concentrations.

suspected when the laboratory results correlate poorly with the clinical findings. Various methods can be employed to exclude antibody interference, including antibody precipitation or using alternative methods (different reagent antibodies and particles) for analysis.[12]

Each laboratory result is associated with random error termed analytical variation.

High-dose hook effect[12]

While this is not due to antibody interference, it is worth noting as it must be considered in analytes with very wide reference intervals, especially when these analytes are measured using a sandwich method, e.g. human chorionic gonadotropin (hCG) and alpha-fetoprotein (AFP). βhCG levels in the normal non-pregnant state are in the order of <25 IU/l, in pregnant women in the 100 000s and in patients with choriocarcinoma, in the millions. While the assay may perform well at lower levels, samples with concentrations in the millions may be affected by the low reagent concentration and therefore give falsely low results (Fig. 5).

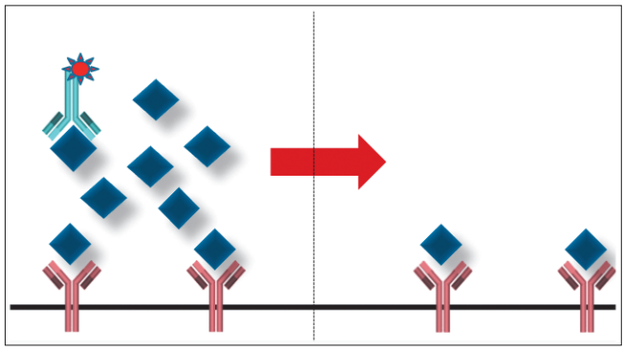

Fig. 5. The high-dose hook effect in sandwich-based immunoassays. Excess antigen (e.g. hormone measured) present leads to decreased sandwich formation and therefore decreased detection.

Detection of this effect relies heavily on a high clinical suspicion, and it may be overcome by performing serial dilutions. Many laboratories reflexively perform dilutions with every sample in the measurement of such analytes.

Exogenous substances

Exogenous compounds that most commonly lead to altered blood results are test tube additives. EDTA, the preservative found in purple top tubes for the prevention of sample clotting, chelates divalent cations such as Ca^{2+}, Mg^{2+} and Zn^{2+}, therefore lowering these analyte concentrations, as well as ALP levels (Zn is the co-factor required for ALP activity). Citrate (blue top tubes) and oxalate (grey top tubes) have a similar effect, while NaF (grey top tubes) which stabilises glucose levels by arresting glycolysis, may lead to spuriously elevated sodium and chloride levels (interference in the sodium and chloride ion selective electrodes). Heparin and EDTA may cause falsely low troponin levels by altering the epitopes required for antibody binding.[12] This is, however, assay dependent.

Certain drugs may cause assay interference, e.g. paracetamol in serotonin metabolite measurement,[13] and dopamine, ascorbic acid and paracetamol in glucose measurement using certain handheld devices.[14] Contrast media may interfere in protein electrophoresis methods (appearing as a false protein peak). Ideally, sample analysis should occur after cessation of the interfering drug or compound.

Conclusion

As is obvious from the above, there are numerous factors to bear in mind when interpreting laboratory results. Clinicians need to be aware of these factors so as to more readily consider the possibility of interference and to better interpret their results within the context of biological variation and current assay limitations.

Incongruous results should be discussed with a chemical pathologist, so that further testing may be considered.

The doors of a chemical pathologist are always open for consultation.

References and further reading available at www.cmej.org.za

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- The result obtained from the laboratory is not perfect. It is associated with bias and analytical variation.
- There is considerable variation in measurement of certain analytes across methodologies and therefore concentrations may not be comparable across laboratories.
- Standardisation addresses this variability. Creatinine and HbA_{1c} are good examples of method standardisation. Peptide hormones remain a challenge to standardise.
- All analytes have inherent biological variation which, along with the analytical variation, must be considered when interpreting results.
- The reference change value determines whether there has been a significant change between two serial results.
- Haemoglobin, bilirubinaemia and lipaemia interfere with analyte measurement, increasing some analyte values while decreasing others. This interference is method-specific.
- Substances released from red blood cells also lead to spuriously elevated results.
- Antibodies interfere either *in vivo* (binding substances in circulation) or *in vitro* in the assay and may lead to spuriously high or low results.
- The high-dose hook effect typically occurs in sandwich methods measuring substances such as βhCG.
- Collection tube additives are a common and avoidable cause of assay interference.