

Pre-Analytical Requirements

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Summary

- Correct test selection: a test must have the potential to alter patient management and have the specificity and sensitivity appropriate to the pretest probability of disease.
- Correct dynamic test procedure: dynamic tests may assist diagnosis and protocols must be readily available.
- Correct patient preparation: fasting, or other patient preparation, may reduce variability. Clear communication, to both patients and staff, of any such requirements is essential.
- Correct sample collection: the tube type (for blood) or container (for urine) must be appropriate for the analyte; there must be sufficient volume, avoidance of venous stasis, contaminants and haemolysis; and adequate labelling.
- Correct sample handling: the time and temperature before and after separation, and the centrifugation and separation procedures, must be suitable for the analyte. Accept/reject criteria must be defined.
- Methods require thorough evaluation of patient-related pre-analytical factors, and quantification of the effects of time, temperature, haemolysis, anticoagulant type and minimum allowable volume on sample suitability.

Introduction

A large proportion, possibly a majority, of errors in laboratory medicine occur in the pre-analytical phase of the testing process.¹ Hence in the assessment and evaluation of biochemical assays, pre-analytical factors need as thorough a consideration and investigation as the more traditional direct analytical factors. A key goal must be ‘correct first time, every time’.

This brief review will consider only those pre-analytical factors that need to be considered when introducing a new analytical test. It will not discuss in detail those general pre-analytical requirements common to all analytes, including the correct identification of the patient, the correct labelling of the sample and the correct sample registration.

An extensive (though not fully comprehensive) compendium of the known effects of pre-analytical variables in clinical chemistry is *Young's Effects*.²

Correct Test Selection

A diagnostic test should only be requested when the result could alter the management of the patient. Since tests generally have specificities and sensitivities less than 100%, the greatest benefit from performing a test is likely to occur when the pretest probability of disease is intermediate.³ If the

pretest probability is too low, false positives will outnumber true positives, leading to unnecessary further testing; or if the pretest probability is very high, there is likely to be little to gain from another test and there is the risk of a potentially confusing false negative result. Screening tests, where the pretest probability may be very low, require a high specificity in order to avoid unnecessary impacts on the health system and on those screened.

Consequently, the laboratory should be proactive in the production and dissemination of guidelines for the appropriate selection of tests by clinicians.⁴ For example, in our laboratory it became apparent that plasma insulin analyses were being requested, mainly by general practitioners, in numbers far beyond any possible clinical need. The vast majority of requests were investigations of “insulin resistance” or “the metabolic syndrome”, particularly in the context of polycystic ovary syndrome. We then included a webpage link with every insulin report explaining that insulin measurement was not helpful in these contexts.⁵ Insulin request numbers have now dropped by more than 50% from their peak in 2003.

Similarly, we publish guidelines for screening for common endocrine disorders⁶ and for endocrine testing in general practice.⁷

For some analytes, guidelines or controls on the frequency of testing may also be advisable.⁸

Correct Dynamic Test Procedure

A number of biochemical tests are part of a dynamic test of a patient's physiological functioning. Common examples include plasma glucose measurement as part of the glucose tolerance test and plasma cortisol measurements as part of the Synacthen test.

The laboratory should develop dynamic tests when appropriate for the analyte, and ensure that the documentation is readily available since such tests can be complex. For example the 8 mg dexamethasone suppression test requires multiple blood sampling over two days and drug administration at midnight.⁹ For complex dynamic testing, specialist staff may be desirable.

Correct Patient Preparation

To reduce variability, biochemical tests may benefit from special instruction to, or preparation of, the patient before the samples are taken. This preparation may include:

- Dietary restriction. Examples are fasting (lipid profile, C-peptide), fluid restriction (urine cortisol¹⁰), and avoiding certain foods (5-HIAA).
- Drug restriction. A common error is to overlook withholding steroids for at least 8 hours prior to a Synacthen test.
- Attendance at a particular time of day (0800 for serum cortisol, 0800-1000 for aldosterone-renin ratio).
- Activity/posture/stress (ambulant for 30 minutes for plasma aldosterone, non-stimulated and recumbent for 30 minutes for plasma catecholamines).
- Other investigations (rectal examination for PSA).

Correct Sample Collection

Clear instructions must be readily available for sample collection. Comprehensive standard procedures for venepuncture are published by the Clinical and Laboratory Standards Institute.¹¹ There are many variables in sample collection that may influence test results; the following lists include the more commonly considered:

Blood (Venepuncture)

- Avoid use of drip arm and hence dilution by intravenously administered fluids ("drip arm" specimen).
- Venous stasis. This should be minimised, especially for calcium and protein.
- Tube type. Serum, being of greater clarity than plasma, may be less likely to clog automated instruments. EDTA however tends to improve the

stability of peptides¹² by chelating metal ions and thus inhibiting proteases; but EDTA can interfere in some enzymatic tests, or in those for metals. Heparin and fluoride were found to interfere in assays for ACTH and IGFBP-3 respectively.¹² Special low-contamination tubes may be required for trace elements, and preservatives may be required for labile analytes, for example aprotinin and leupeptin for parathyroid hormone-related peptide.

- "Order of draw". A typical tube order to minimise risk of interferences is: plain → citrate → Li heparin → EDTA → fluoride. A more comprehensive chart is given by the Gippsland Pathology Service.¹³
- Haemolysis should be avoided. This is common and often interferes with analyses;¹⁴ for example, raising serum potassium (by contamination with red cell K) and lowering serum insulin (by increasing proteolysis).¹⁵ Clinically significant changes in AST, chloride, LDH, potassium and sodium occur at a level of haemolysis (0.6 g Hb/L) barely detectable visually.¹⁴
- Collection volume. This should be sufficient per tube to give the correct concentration of additives and sufficient in total to allow for repeat analyses.
- Stress. If venepuncture is unusually stressful, or the patient faints, this should be recorded, especially for stress-responsive hormones. These include cortisol, ACTH, GH, prolactin, catecholamines and AVP (arginine vasopressin).
- Labelling. When multiple samples are collected during a dynamic test, samples should be labelled with both the clock time and the time elapsed during the test.

Urine

- Timed or spot. A 24-hour collection is often preferable but collection is more difficult and patient reliability can be an issue.
- Preservative. This may be needed for some analytes, for example hydrochloric acid for catecholamines. Addition of glycerol stabilises gonadotrophins in frozen urine.¹⁶
- Special container. For instance, acid-washed containers for copper determinations.

Correct Sample Handling

Many samples, particularly for less common analyses, may need to be transported some distance to the laboratory where the analysis will be done. This raises questions about how much delay is acceptable and what the transport and storage temperatures should be. These and other factors that may influence analytical results include:

- Time before separation from cells (for plasma). For example; (i) glucose is well known to decrease during storage of EDTA whole blood, and (ii) there is significant loss of immunoreactive ACTH by 18 hours in whole blood at both 4 °C and 24 °C and a significant gain of AVP immunoreactivity within three hours at 24 °C.¹⁷
- Centrifugation conditions. The time must be sufficient to ensure complete sedimentation, the acceleration low enough to avoid haemolysis, and it may be desirable to control the temperature. Commonly, 1100-1500×g for 10 minutes is recommended.
- Special separation requirements. For example, contamination by the buffy coat must be avoided in plasma for catecholamine determinations as platelets contain high levels.
- Division of the sample into a sufficient number of aliquots of a sufficient volume. If the aliquot volume is too small some analysers will report a low result, but without an error message (see Quantification section for an example), and if there are too few aliquots unnecessary delays and freeze/thaws may result.
- Temperature and time between separation and analysis. Cooler is usually better, and frozen usually better than liquid. For example, compared to the frozen state, ACTH could be considered stable in EDTA plasma for 18 hours at 4 °C but for only 8 hours at 30 °C.¹²
However there are exceptions; chilled red cells release potassium, and renin is more stable at room temperature than at 0 °C because the lower the temperature (short of freezing) the more likely prorenin is to be activated.¹⁸ FSH and LH are less stable in urine at -20 °C and -25 °C than at 4 °C,¹⁶ presumably because of denaturation by concentrated urea.
- Effect of freeze-thaw cycles (for frozen samples). This is often tested but rarely found to have a significant effect.¹⁹
- Complete thawing, adequate mixing and centrifugation of frozen samples before analysis. Concentration gradients develop during freezing and thawing, and fibrin lumps may form during storage of plasma samples.

Correct Accept/Reject Criteria

When specimens arrive in the laboratory, their acceptability for the requested analyses must be determined. Analyte-specific rejection criteria should be developed for each analyte and might, for example, include:

- Haemolysis exceeding a critical level. Some

analysers can measure haemolysis, or samples may be visually checked against a photograph of standard levels of haemolysis.

- Wrong anticoagulant. When EDTA would interfere, we use eriochrome black T to check that it is absent.²⁰
- Insufficient volume.
- Thawed when should be frozen.

Quantifying the effects of pre-analytical factors

Typical procedures for quantifying commonly studied pre-analytical factors for blood samples are outlined below. They are readily adapted where appropriate to other matrices such as urine.

Time and temperature stability

Collect ten tubes of 10 mL of blood from each of ten volunteers using either plain or anticoagulant-containing tubes as required and immediately allow to clot or centrifuge as appropriate. For each subject, pool the serum/plasma from the ten tubes to obtain a pool of about 40 mL. Immediately store a zero time aliquot of each pool below -20 °C, (but for long-term studies, or urine with its higher solute content, -70 °C may be required) and incubate other aliquots for the times and at the temperatures under study. For example, if zero, one and five days at 4 °C and 30 °C are to be studied,¹² take five aliquots from each pool. When the allotted incubation time for each aliquot is up, freeze it, and, at the completion of the experiment thaw all aliquots and analyse concurrently.

If stability in whole blood rather than in serum/plasma is being studied, perform the centrifugations immediately after the incubation of the aliquots instead of immediately after blood collection.¹⁷

Express measured values for an analyte at a particular temperature as a percentage of the zero time value for each subject, omitting values that are less than twice the analytical detection limit. Estimate the mean rate of change (k), by fitting the equation

$$y = 100e^{kt} \quad [1]$$

where y is the percentage of the zero time value and t is the elapsed time.

A negative (positive) value of k corresponds to a loss (gain) in concentration. Use of a statistical package such as SigmaStat (Jandel Scientific, Chicago) allows the estimation of k and its standard error (s), by least-squares. Assuming 10 subjects, the value of k is considered significantly different from zero if the ratio k/s exceeds 2.26, the value of Students t for nine degrees of freedom for a two-tailed test at the 0.05 probability level.

Where k is not significantly different from zero, the analyte can be considered to be stable for at least the longest time studied. If k does differ significantly from zero, the analyte can be considered to be “stable” for a time t_s , where

$$t_s = \frac{\ln(1 - d/100)}{k} \quad [2]$$

where d is the maximum acceptable mean percentage change in analyte concentration and has the same sign (positive or negative) as k . For hormones we have used a value of 10% for d . By way of illustration, Figure 1 shows equation 1 fitted to the ACTH values obtained at 24 °C in our study of the stability of hormones in whole blood.¹⁷ The value of $k \pm s$ was -0.0061 ± 0.0010 hours⁻¹ and hence, for a 10% maximum change, t_s , the stability time, is 17 hours.

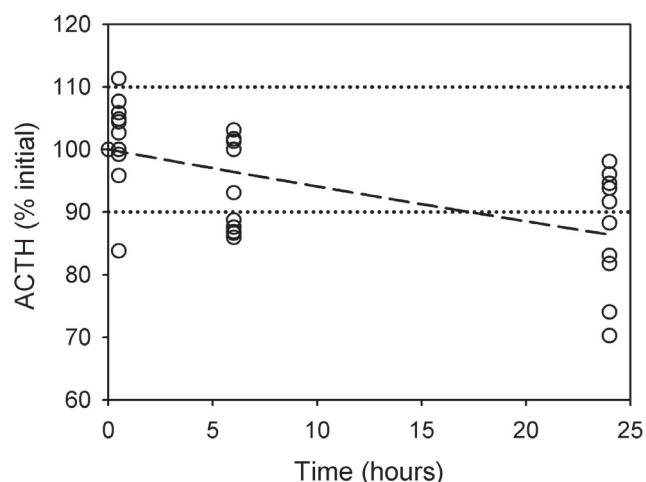


Figure 1. ACTH concentrations in EDTA whole blood from ten subjects after incubation for 0.5, 6 or 24 hours at 24 °C. The dashed line is the best fit of the function $ACTH = 100 * e^{k * Time}$, for which $k = 0.00610$ hours⁻¹. The dotted lines show the $\pm 10\%$ range within which the mean ACTH concentration is defined as “stable”. Consequently ACTH is considered to be stable at 24 °C in EDTA whole blood for 17 hours (the time at which the dashed line crosses a dotted line). ACTH was measured using the manual Nichols (Nichols Institute, Ca, USA) IRMA method.

Note that t_s is only a measure of average stability; samples from different individuals may differ in stability, as can be seen in Figure 1. A much larger study would be required to quantify the variation in stability between individuals.

Haemolysis

The effect of haemolysis may be immediate, for example, the raising of potassium concentrations, or may be time dependent, as for insulin degradation. Thus ideally the effects of haemolysis should be examined at several time points, as

for the effects of time and temperature above, but with the addition of varying amounts of haemolysed whole blood to the patient’s plasma/serum.

Whole blood can be efficiently haemolysed by freezing to between -10 °C to -40 °C for at least one minute.²¹ The degree of haemolysis, expressed as haemoglobin concentration, can be measured by the absorbance after centrifugation, using an absorbance coefficient of $0.53 \text{ l} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ at 556 nm.²²

Anticoagulant

The effect of differing anticoagulants (or none) is readily combined with the study of the effect of time and temperature on stability by drawing the volunteer blood samples into differing anti-coagulant tubes and comparing the measured zero-time concentrations.¹¹

Minimum volume

The determination of minimum volume is illustrated by our investigation of the effect of sample volume on parathyroid hormone (PTH) measurement. We presented varying volumes of three pools of EDTA plasma to a Roche Elecsys 2010 analyser in conical-bottomed 0.5 mL microfuge tubes, and observed (Figure 2) that when sample volumes were below about 70 μL , the measured PTH values began to decrease without an indication of an error condition. On this basis, and allowing for a margin for error, we selected 100 μL as the minimum acceptable volume for analysis.

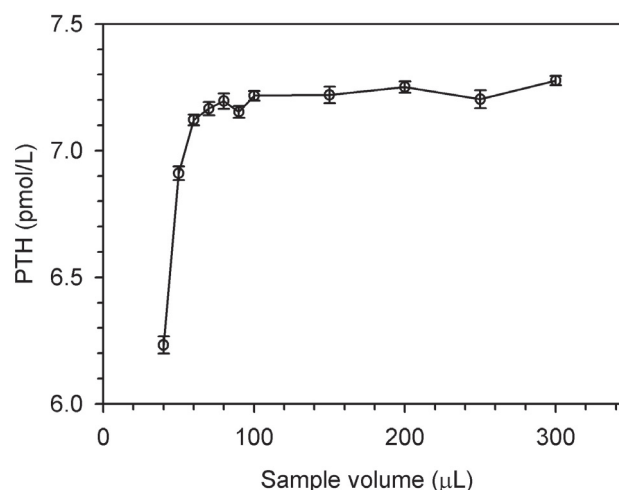


Figure 2. PTH concentrations (mean \pm sem of 10 replicates) as a function of the volume of pooled EDTA plasma presented to the Roche Elecsys 2010 analyser in 0.5 mL conical-bottomed microfuge tubes. Only for volumes below 40 μL was an error condition indicated.

Conclusion

Effective and efficient use of a biochemical test is only possible if the potential influences of pre-analytical factors on both the variability of sample collection, and the integrity of the sample itself, have been thoroughly investigated.

Acknowledgements

We would like to thank the Canterbury District Health Board for support, and Donna Willocks for technical assistance.

Competing Interests: None declared.

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