Clinical Laboratory Safety Data in Drug Studies

A. Craig

Introduction

Clinical laboratory measurements are an integral component of most drug studies for two major reasons, firstly to act as an efficacy endpoint in monitoring the success or otherwise of therapy and secondly to ensure patient safety on any new drug or reformulation. The pharmaceutical industry rarely employs physicians and scientists with expertise in clinical laboratory medicine. As such, there is a tendency for test selection, data-management and data interpretation to be based on historical knowledge. The industry rarely involves the clinical laboratory in study design, resulting in out-of-date parameters, with little appreciation of the limitation and advantages of specific test programmes.

In major clinical studies, the numerical element in a submission report may consist of 50 per cent laboratory data with many thousands of laboratory test results, which may increase to 80 per cent in phase 1, and early phase 2 studies. Thus, we are concerned with a vast volume of data, particularly related to the assessment of safety.

Why do we do laboratory tests?

- To provide information to support a clinical diagnosis.
- To extend a clinical diagnosis by offering information about causation.
- To indicate the presence of complications, including those due to treatment.
- To monitor prognostic information.
- To monitor the progress of the condition.
- To detect sub-clinical disease (screening).

Changes in serial results may be due to:

- the patient getting better
• the patient getting worse
• pre-analytical variation
• analytical variation
• biological variation

all of which demand considerable attention in any clinical study.

It is unfortunate that clinical laboratory systems evolved around automated analytical systems, which were designed for diagnostic and healthcare screening purposes. As such, these have tended to be used as basic screening procedures in drug-study safety monitoring. While it may have economical advantages to undertake a battery of health-care screening tests, is this really appropriate in drug studies? Should not greater attention be given to the chemical characteristics of the compound and the data available from pre-clinical toxicology studies to define the test requirements in later phases? Also, is it appropriate to employ identical tests at phase 1 similar to the later phases? In this respect, the most sensitive analytical procedures for the detection of toxicity should be employed as early as possible in development in order to eliminate compounds with a problem quickly rather than have this delayed to later phases with the resulting cost implications.

Efficacy testing differs significantly from safety monitoring, in that tests are being employed to demonstrate a change, which hopefully will occur in all subjects on active therapy. However, a full appreciation of the test limitations of the endpoint measurement is essential prior to statistical analyses, which must involve information from the clinical laboratory on analytical validation. Also, although analytical validation is a prerequisite, consideration must also be given to possible analytical method changes due to the drug or its metabolites. In safety monitoring, the data volume is large, and even in phase 3 studies only a few subjects may show toxicity effects, thus demanding critical examination of organ-specific tests.

Essentially, there are three different types of clinical laboratory providing services to the pharmaceutical industry. Local independent or hospital-based, central laboratory services and specialist core-research units, and all have a place in clinical studies. Over the last decade there has been favour in the use of centralized facilities, as these laboratories offer an all-encompassing service from study set-up and supplies, through analytical measurements to electronic data transfer to the sponsors facilities, and are compliant with the demands of good laboratory practices (GLPs). There is also demand from the pharmaceutical industry for central services to be provided on a global basis for consistency of data; but, as we shall see later, although global facilities are requested, Homo sapiens can hardly be considered to exhibit much degree of consistency. However, there will always be a requirement for local facilities, particularly when the study is concerned with acutely ill patients, e.g. myocardial infarction, stroke, etc., and test assessments are required immediately for patient care. Also, specialist analytical techniques, which may not always be available either locally or centrally, will demand the use of specialist core-clinical or bio-analytical laboratories.

The advantages of the central laboratory are:

• all-encompassing service
• one set of standards, units and reference ranges
• simplified data-management
• comprehensive range of tests
• uniform reporting and interpretation
• demonstrable quality and GLP compliance
• data and project management facilities
• monitoring facility for the sponsor.

The disadvantages are:

• no affiliation to local investigator
• lack of investigator confidence
• transport costs
• stability limitations and sample deterioration
• language issues.

The industry requirement for laboratory services from whatever sources include:

• comprehensive service – logistics, supplies, etc.
• analytical excellence and scientific excellence
• accreditation and documentation
• quality communication and response
• data management and electronic data transfer systems
• user friendly for investigators and monitors.

From this introduction, it should be apparent that the involvement of the clinical laboratory at an early stage would markedly enhance the completion of successful studies from the laboratory viewpoint and reduce potential problems related to logistics, test selection, data handling and interpretation.

Factors that influence interpretation of clinical laboratory data

Factors that may influence the interpretation of clinical laboratory results will be considered in the following sections:

• Pre-analytical factors
• Analytical standards
• Reference ranges and their value
• Intra-individual biological variation.
Pre-analytical factors

Table 5.1 details a short list of ‘environmental’ issues that can affect the interpretation of laboratory results, some of which are discussed below.

<table>
<thead>
<tr>
<th>Table 5.1</th>
<th>A list of the important factors that may bear influence on data interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age/gender</td>
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<tr>
<td>Alcohol intake</td>
<td></td>
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<td>Posture at sample collection</td>
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<td>Drugs</td>
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<td>Ethnic origin</td>
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<td>Effect of exercise</td>
<td></td>
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<tr>
<td>Menstruation</td>
<td></td>
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<td>Smoking</td>
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<tr>
<td>Stress</td>
<td></td>
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</tbody>
</table>

**Age/gender**

Many laboratory tests will show highly significant differences between male and female with or without changes over the growth life cycle.

Haemoglobin and most routine haematological measurements show little change with age, but there are significant differences between adult males and females. The haemoglobin reference range for males over 18 years of age is 13.5–18.0 g/dl, whereas females show a lower range at 12.5–16.0 g/dl and similar changes are noted with haematocrit and mean cell haemoglobin (MCH) levels.

Liver function tests do show minor changes with increasing age, with albumin lowering slightly from a mean value at the age of 30 years of 48 g/l to 44 g/l at 65 year of age. The enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyltranspeptidase (GGT) all show slightly higher values for males. However, our own data in examining phase 1 normal volunteers has demonstrated a marked difference in females pre and post the age of 50 years, with the mean GGT enzyme activity changing from 14.5 to 21.6 IU/l. One of the major changes in liver enzymes is that of alkaline phosphatase (AP), which after a reduction from pre-pubertal level remains extremely stable for males. However, the female shows a highly significant difference pre- and post-menopause, with a mean change of 49.8 to 59.7 IU/l. We are now aware from measurement of specific bone AP that this increase is due predominantly to the bone isoenzyme.

Creatinine demonstrates a highly significant difference between males and females and a small increase with increasing age. The mean value for a male subject at the age of 50 is approximately 100 μmol/l and at the corresponding age in the female the value would be approximately 80 μmol/l.

In terms of total cholesterol, there is a noticeable increase in males about the age of 30 with a mean increase from 4.7 to 5.5 mmol/l; thereafter there may be a slight increase or decline into the 65–70 age range. The female, on the other hand, shows a very significant
difference pre- and post-menopause. The mean female total cholesterol in phase 1 volunteers <50 years of age was 4.71 mmol/l and it was 6.05 mmol/l in subjects >50 years.

**Ethanol intake**

The intermediate and long-term effect of ethanol ingestion was studied by Shaper et al. (1985), who compared occasional drinkers against subjects consuming more than six units daily in 7735 middle-aged men and demonstrated a 70 per cent increase in the mean gamma glutamyltranspeptidase and an 18 per cent increase in HDL-cholesterol, and smaller changes in uric acid and AST. Also, there was an increase in the red cell index of the mean cell volume (MCV) from 88 to 91 fl. Other changes in creatine kinase and electrolytes have also been noted.

Physicians are likely to identify only 20–50 per cent of patients with alcoholism attending medical centres, and this thus demands a high degree of clinical suspicion. Alcohol is, of course, metabolized rapidly, and its measurement in identifying the alcoholic is of little value. Although a number of new markers have been suggested and reviewed by Sharpe (2001), in essence GGT remains the most sensitive marker in conjunction with ALT and MCV. From a clinical trial viewpoint it is important to identify chronic alcoholics, as ethanol is a risk factor in certain diseases and there may be associations with various drug therapies that could result in adverse drug reactions.

**Posture**

Samples are usually obtained from a subject in either the supine or upright sitting position. In moving from the supine position to standing there is an efflux of water and filterable substances from the intravascular space to the interstitial fluid. One of the most significant changes is that of plasma active renin, which will show a mean of 22.5 µU/ml in the upright position changing to a mean value of 14.8 µU/ml when recumbent, with a similar change in aldosterone levels.

The non-filterable substances, such as proteins, cellular elements and compounds bound to protein, will increase in concentration by 8–12 per cent. We recently reviewed differences in subjects attending a phase 1 clinic for pre-study screening with the values obtained once the volunteer was selected. The volunteers attended for pre-study screen in the evening and the results were compared with the samples collected in the phase 1 unit when the volunteers were at rest, fasting and early in the morning. These results are shown in Table 5.2. Highly significant differences were noted in the measurements of total protein, albumin, calcium and neutrophils. These changes are undoubtedly due to the effect of posture, but other factors may also be contributing.

Prolonged use of a tourniquet in venesection will result in elevated concentrations of albumin, cholesterol and calcium, and excessive use will show changes in erythrocyte enzymes, which are at much higher levels of activity than plasma and, on a similar basis, elevation of potassium. Ideally, minimum use of a tourniquet is recommended for reliable results.
Circadian and seasonal variation

Many biochemical compounds show significant variation over a 24 h period, of which the most pronounced is that of the adrenal steroid cortisol. Figure 5.1 shows mean data from a group of 20 males from which samples were taken every 20 min over a 24 h period. These measurements were carried out using an old method; although there may be changes in the magnitude of units with newer methodology, the pattern over the 24 h period will be identical. With current radioimmunoassay the reference range for samples collected between 07:00 and 08:30 is 200–700 nmol/l, whereas samples collected in the afternoon or evening will have a reference range of 140–400 nmol/l, there being only minimal crossover.

Table 5.2  Mean changes in clinical laboratory measurements from pre-study screen through baseline in 30 normal volunteers.

<table>
<thead>
<tr>
<th>Test</th>
<th>Pre-screen</th>
<th>Baseline</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein (g/l)</td>
<td>72.17</td>
<td>67.29</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>44.26</td>
<td>40.81</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Calcium (mmol/l)</td>
<td>2.502</td>
<td>2.473</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>White cell count (10^9/l)</td>
<td>7.61</td>
<td>5.76</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Neutrophils (10^9/l)</td>
<td>4.54</td>
<td>2.98</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lymphocytes (10^9/l)</td>
<td>2.2</td>
<td>1.96</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.53</td>
<td>1.11</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>56.4</td>
<td>50.58</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Figure 5.1  Diurnal variation of serum cortisol. Mean data from 20 individuals sampled at 20 min intervals over a 24 h period. The boxes show the calculated reference ranges at 8am and 4pm.

The pituitary hormones are released in a pulsatile fashion under the control of the hypothalamus, but some also demonstrate a diurnal variation. Thyroid stimulating hormone shows a distinct sleep–wake pattern with the highest levels being found overnight. One of the most pronounced sleep–wake patterns is found in the secretion of growth hormone, and Figure 5.2 shows the typical changes in this hormone.

In a study by Pocock et al. (1989), noticeable changes in some safety profile biochemical markers were recorded over the period from 09:00 until 18:00. Triglycerides increased by
some 30 per cent over the time period irrespective of food intake. Similarly, inorganic phosphate increased by 20 per cent but this increase is abolished by fasting. Potassium showed an increase of 0.3 mmol/l and creatinine showed a slight reduction. There was a noticeable fall in bilirubin over the time period. These data are shown in Figure 5.3.

In addition to changes within the 24 h clock, there are also significant seasonal differences in some measurements. Whilst we would expect to find changes in vitamin D between summer and winter, due to sunlight levels, in terms of tests in safety profiles, cholesterol has been shown to differ significantly. Rastam et al. (1992) reported that cholesterol values in males are 2.6–6.3 per cent higher in winter than summer, with the corresponding figures for females being 1.0–4.6%. Using the National Cholesterol Educational Programme upper limit guideline of 6.2 mmol/l, 25.4 per cent of men were above this level in winter, whereas only 13.5 per cent met the cutpoint in summer.
Therefore, attention to sample collection times is an important issue in data interpretation.

**Diet**

Prolonged fasting of 48 h duration will increase glucagon and decrease insulin levels. Owing to the increase in fat metabolism, ketone bodies will be found in blood and urine, and plasma glucose levels drop. Bilirubin, triglycerides and non-esterified fatty acids will all be elevated.

A meal high in fat content will increase the value of plasma alkaline phosphatase, presumably due to the intestinal isoenzyme. A diet high in protein will show an increase in urea and uric acid, with little change in creatinine.

**Drugs**

The possible effect of drugs on clinical laboratory measurements is beyond the scope of this presentation. For a review of this issue, readers may find the publication of Siest and Gaiteau (1988) of help in this direction.

**Ethnic origin**

The difference in race related to disease and biochemical measurements requires a lengthy review. Thus, this will be limited to a few general comments on chemical markers. Cholesterol values in the Japanese population tend to be lower than the equivalent levels in the Western population. The black african native show higher levels of immunoglobulins. There are highly significant differences in the enzyme creatine kinase between Afro-Caribbean, Asian and Caucasian populations (Sherwood et al. 1996). Alpha-fetoprotein, used diagnostically in the detection of spina bifida and Down’s syndrome, shows major difference between Caucasian and Asian populations. Also, there are genetic differences regarding the African population, as they show a significant neutropenia in comparison with the Western population (Shaper and Lewis, 1971).

With our increasing knowledge of pharmacogenetics and associated cytochrome P450 enzyme systems, there is a much greater awareness of the effects of race on metabolic systems, and particularly in relation to the metabolism of drugs. The cytochrome P450 enzyme group located in the liver is involved in the metabolism of many therapeutic agents. There are about 200 CYP enzymes, which can result in individuals being classified as slow, intermediate and ultra-fast metabolizers with significant differences in therapeutic effects and toxicity when standard drug regimes are employed. Although some 80 per cent of the population on this planet are slow metabolizers, there are highly significant differences between races. CYP2C19 deficiency is found in 2–6 per cent of Caucasians, but in 19–25 per cent of Asians, which accounts for reduced rates of metabolism in these populations. Some 7 per cent of the Caucasian population are ultra-fast metabolizers of CYP2D6 with a prevalence of up to 29 per cent ultra-fast metabolizers in Africans. Consequently, individuals who differ in genetic make-up may not respond to a standard regime of drugs. Thirty per cent of the population do not respond to beta-blockers, with a similar percentage in respect of statins, and it has been reported that over 50 per cent of the population show a poor response to tricyclic antidepressants.
This brings into question a number of issues. How can we justify a global database in clinical studies with such a diverse worldwide population? It is now considered that by 2005, genotyping will be a routine procedure prior to prescribing many drugs; how will the clinical laboratories meet this inevitable challenge?

**Exercise**

The effect of exercise, whether on trained athletes or untrained individuals on biochemical and haematological tests is well defined. Intensive or sustained exercise induces ‘sports anaemia’ (Radomski et al., 1980); however, even a short period of exercise has been shown to increase haemoglobin and leucocyte counts, which may take several days to return to baseline levels. Strenuous exercise may lead to dramatic increases in growth hormone, cortisol and the catecholamines, but these are usually of short duration and the values rapidly return to normal. There is also a highly significant increase in plasma lactate, which may influence acid–base balance, and rapid changes of short duration in antidiuretic hormone. More important is the release of muscle enzymes, in particular creatine kinase, which may take some 6–7 days post-exercise to return to basal levels; this is a point well worth noting in healthy volunteers taking part in phase 1 studies.

**Menstruation**

There are dramatic changes in hormones and steroids during the different phases of the normal menstrual cycle, and Figure 5.4 shows an example of the changes in pituitary follicle stimulating hormone (FSH) and ovarian 17β-oestradiol. These, together with other hormone changes, can elicit effects on body temperature, electrolytes and water balance. FSH offers one of the most discriminating tests of the perimenopausal period, with a marked increased secretion rising to extremely high levels, as shown in Figure 5.5.

![Figure 5.4](image)

**Figure 5.4** The changes in FSH and 17β-oestradiol during the normal menstrual cycle

**Smoking**

Tobacco smokers have a raised concentration of carboxyhaemoglobin, of the order of 8 per cent in comparison with 1 per cent in non-smokers. It has also been suggested that smokers
have an increased level of plasma cortisol and catecholamines, leading to increases in non-esterified fatty acids.

Over the last few years there have been several studies that show increased concentrations of the acute phase reactants fibrinogen and C-reactive protein in smokers, and this may well account for the added cardiovascular risk in this population.

**Stress**

Psychological stress appears to cause an increase in plasma catecholamines resulting in an elevation of non-esterified fatty acids (NEFAs). Plasma cortisol, prolactin and growth hormone are increased in stressful situations, including phlebotomy, and the peripheral white cell count can also be increased.

**So what is a normal volunteer?**

The points above illustrate a number of biological pre-analytical factors that may have an influence on the interpretation of clinical laboratory data. How, then, might we define a normal volunteer for participation in a phase 1 study? As:

“A 20–40 year old, who does not drink alcohol or smoke, is unstressed, does not participate in heavy sport, resides at sea level in a moderate climate, has limited stress or sexual activity, lies flat on his or her back, eats a normal diet and is preferably of Western European nationality”

**Sample collection procedure**

Many of the points relating to sample collection procedure, e.g. posture, tourniquet use, and time of phlebotomy, have been covered in the above section. Moving now to the actual
sample collection, most central laboratories will provide all necessary material for venesection, together with tube labels and comprehensive instructions on the procedure.

In the UK, two popular systems for blood collection are employed. Becton Dickinson Vacutainers or Sarstedt Monovette. Both companies provide an extensive range of blood collection containers, including tubes suitable for trace metals, containing special preservatives such as Aprotinin, and also for paediatric samples.

In diagnostic pathology testing there is a need for complex collection systems depending on the analyses being undertaken; therefore, comments on this section will be limited to the tests normally included in standard safety testing profiles.

**Stability**

There are considerable data available on the stability of safety profile tests. In terms of clinical chemistry measurements, our own laboratory has shown little change in the safety profile tests up to 96 h after collection. Some minor loss of ALT and AST activity is noted, but this is less than 10 per cent up to 96 h post-collection providing that the sample is treated as recommended. There is no apparent change in other enzymes, such as AP, GGT or creatine kinase. Whole blood samples for clinical chemistry will be unsatisfactory within a very short time due to leakage of the high concentration of potassium and phosphate and the activity of lactate dehydrogenase, ALT and AST for erythrocytes.

Up to 30 per cent of blood samples from general practice have raised potassium levels, and about 50 per cent of these are due to the sample being left overnight without separation of the red blood cells (Johnston and Hawthorne, 1997). Cooling these samples in a refrigerator accelerates the rate at which potassium leaks from the red cells.

Table 5.3 shows the results of samples from a single individual taken in duplicate in which analysis was carried out immediately on one sample and the second sample stored at 4°C, then separated from the red cells the following day and analysed. The changes in AST, ALT, potassium and phosphate are significant, with the second sample results being above the upper limit of the reference ranges. Glucose measurement was carried out on the serum sample, and virtually all the glucose had been utilized by the continuing metabolism of

<table>
<thead>
<tr>
<th>Table 5.3 Comparison of samples for biochemistry and haematology analysed within 2 h of collection against samples left untreated, separated the following day and analysed. Values in italics show highly significant differences</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Haematology</strong></td>
</tr>
<tr>
<td>WBC (10⁹/l)</td>
</tr>
<tr>
<td>RBC (10¹²/l)</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
</tr>
<tr>
<td>PCV (fl)</td>
</tr>
<tr>
<td>MCV (pg)</td>
</tr>
<tr>
<td>MCH (g/dl)</td>
</tr>
<tr>
<td>MCHC(10⁹/l)</td>
</tr>
<tr>
<td>Platelets(10⁹/l)</td>
</tr>
</tbody>
</table>

(continued)
the red cells. The MCV increases cannot be controlled, as within 6 h of sample collection the red blood cells will show swelling in size. Although there is adequate evidence for the stability of the clinical chemistry safety tests, much of this is derived from normal levels of test concentrations. The stability of test concentrations in grossly abnormal situations, e.g. obstructive jaundice with highly raised levels of bilirubin, AP, and GGT, needs further assessment.

Table 5.3 (continued)

<table>
<thead>
<tr>
<th>Clinical chemistry</th>
<th>Immediate</th>
<th>After 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (IU/l)</td>
<td>15</td>
<td>57</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>21</td>
<td>27</td>
</tr>
<tr>
<td>AP (IU/l)</td>
<td>197</td>
<td>201</td>
</tr>
<tr>
<td>GGT (IU/l)</td>
<td>27</td>
<td>30</td>
</tr>
<tr>
<td>Bilirubin (µmol/l)</td>
<td>6.7</td>
<td>5.8</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.6</td>
<td>0.3*</td>
</tr>
<tr>
<td>Urea (mmol/l)</td>
<td>7.0</td>
<td>7.5</td>
</tr>
<tr>
<td>Sodium (mmol/l)</td>
<td>142</td>
<td>145</td>
</tr>
<tr>
<td>Potassium (mmol/l)</td>
<td>4.4</td>
<td>6.6*</td>
</tr>
<tr>
<td>Chloride (mmol/l)</td>
<td>109</td>
<td>101</td>
</tr>
<tr>
<td>Phosphate (mmol/l)</td>
<td>1.1</td>
<td>2.9*</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>114</td>
<td>150</td>
</tr>
<tr>
<td>Uric acid (µmol/l)</td>
<td>311</td>
<td>317</td>
</tr>
<tr>
<td>Calcium (mmol/l)</td>
<td>2.41</td>
<td>2.46</td>
</tr>
<tr>
<td>Total protein (g/l)</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>47</td>
<td>48</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>7.9</td>
<td>8.0</td>
</tr>
</tbody>
</table>

*Highly significant changes.

Table 5.4  Factors that can result in false positive and negative results

<table>
<thead>
<tr>
<th>Albumin</th>
<th>+</th>
<th>Prolonged application of tourniquet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin</td>
<td>−</td>
<td>Prolonged exposure to light</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>−</td>
<td>Prolonged exposure to air</td>
</tr>
<tr>
<td>Calcium</td>
<td>+</td>
<td>Prolonged application of tourniquet</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>Use of wrong container – EDTA</td>
</tr>
<tr>
<td>Creatinine</td>
<td>+</td>
<td>Raised plasma acetoacetic acids</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>Not fasting</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>No fluoride preservative</td>
</tr>
<tr>
<td>Phosphate</td>
<td>+</td>
<td>Prolonged contact with RBC</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Haemolysis</td>
</tr>
<tr>
<td>Potassium</td>
<td>+</td>
<td>Use of wrong container – EDTA</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Haemolysis</td>
</tr>
<tr>
<td>Sodium</td>
<td>+</td>
<td>Contamination from IV fluids</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>High glucose pulls sodium into cells</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>+</td>
<td>Not fasting</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>Hyperlipidaemia</td>
</tr>
</tbody>
</table>
Many specialist tests will demand specific collection methods, and it cannot automatically be assumed that all tests will be stable if stored at temperatures of $-20\, ^\circ C$ or below. The slow migrating lactate dehydrogenase, predominantly of liver origin, is unstable at $+4\, ^\circ C$ and below, and some enzymes, such as alanine aminopeptidase in urine samples, will demand the use of ethylene glycol to stop the samples freezing, which destroys this enzyme. Table 5.4 details a number of situations that result in falsely measured safety profile test results.

**Analytical variation**

The variability of an analytical measurement surrounding a value is extremely small in respect of the tests performed in safety monitoring with modern instrumentation. Generally, laboratories undertake two types of quality control programme to monitor performance: internal and external assessments.

Internal quality control (IQC) is usually carried out with lyophilized human serum available from laboratory suppliers. Generally, two levels of control are provided: within and outside the reference interval. Initially these samples are analysed within and between different analytical batches to establish the mean value for each measurement and the two standard deviation (2SD) distribution. These data are then incorporated into the laboratory data-management system. The quality control samples are treated identically to patient samples on every analytical run and the data-management system will accept or reject the batch analyses based on Westgard rules; see Westgard *et al.* (1981) and [www.westgard.com/mltirule.htm](http://www.westgard.com/mltirule.htm).

The recommended Westgard rules generally applied are as follows:

- If both result within 2SD range, then **issue report**.

Reject if:

- One result exceeds 3SD.
- Both results exceed the same 2SD limit.
- Each result exceeds a different 2SD limit.
- The same material exceeds the same 2SD in a previous batch.
- The last 12 consecutive results are on the same side of the mean, rather than showing an equal distribution on both sides of the mean when the distribution is plotted graphically.

Thus, having established the rules and data within the laboratory data-management system, the computer will determine the acceptability of the performance. Table 5.5 shows a typical set of statistics from within the reference range quality-control sample over a period of 6 months.

There is no doubt that with each passing year the analytical precision of IQC improves, and this is closely monitored by laboratories to ensure reliable data on patient results. However, it must be remembered that as the acceptable data are extrapolated on 2SD ranges then at least 5 per cent of batches will be rejected on a statistical basis. Also, it is important
to remember that it is the IQC that is the deciding factor on the release of the clinical laboratory report.

Concurrently, clinical laboratories participate in national and international external quality control (EQC) programmes in which samples are forwarded from a central source to many laboratories. A description of these complex and highly professional systems is outside the scope of this chapter. In the UK-NEQAS (www.ukneqas.org.uk) programme the result of the test is compared with the consensus mean for the method group, the variance and bias determined on the sample, and over a period of the last 10 quality control samples. Also, the programme incorporates tests of reproducibility and recovery of test material.

As EQC is based on consensus mean data; anything that would change in the technology or reagents employed by a large number of participating laboratories might result in a distortion of the statistics. Thus, the performance of the laboratory in both IQC and EQC must be examined independently.

### Reference ranges

The subject of reference ranges is worthy of a separate review, and books are available on this topic (Grasbeck and Alstrom, 1981). There are a number of important issues that require comment at this stage. In the pharmaceutical industry the terminology of ‘normal’

<table>
<thead>
<tr>
<th>Test</th>
<th>Value</th>
<th>CV (%)</th>
<th>−2SD</th>
<th>+2SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST</td>
<td>30</td>
<td>2.70</td>
<td>28</td>
<td>32</td>
</tr>
<tr>
<td>ALT</td>
<td>27</td>
<td>3.40</td>
<td>25</td>
<td>29</td>
</tr>
<tr>
<td>AP</td>
<td>158</td>
<td>2.20</td>
<td>151</td>
<td>165</td>
</tr>
<tr>
<td>GGT</td>
<td>48</td>
<td>2.60</td>
<td>46</td>
<td>50</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>14</td>
<td>5.30</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.1</td>
<td>1.20</td>
<td>3.0</td>
<td>3.2</td>
</tr>
<tr>
<td>Urea</td>
<td>5.2</td>
<td>1.50</td>
<td>5.0</td>
<td>5.4</td>
</tr>
<tr>
<td>Sodium</td>
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<td>0.60</td>
<td>136.3</td>
<td>139.7</td>
</tr>
<tr>
<td>Potassium</td>
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<td>0.60</td>
<td>4.4</td>
<td>4.6</td>
</tr>
<tr>
<td>Chloride</td>
<td>104</td>
<td>0.70</td>
<td>102.5</td>
<td>105.5</td>
</tr>
<tr>
<td>Creatinine</td>
<td>96</td>
<td>2.70</td>
<td>90.8</td>
<td>101.2</td>
</tr>
<tr>
<td>Uric acid</td>
<td>330</td>
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<td>320</td>
<td>340</td>
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<tr>
<td>Calcium</td>
<td>2.52</td>
<td>1.60</td>
<td>2.44</td>
<td>2.60</td>
</tr>
<tr>
<td>Total protein</td>
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<td>1.70</td>
<td>64.5</td>
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</tr>
<tr>
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<td>1.40</td>
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<td>38.4</td>
</tr>
<tr>
<td>Cholesterol</td>
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<td>1.80</td>
<td>5.00</td>
<td>5.38</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>105</td>
<td>3.20</td>
<td>98.3</td>
<td>111.7</td>
</tr>
<tr>
<td>WBC</td>
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<td>2.30</td>
<td>6.4</td>
<td>7.0</td>
</tr>
<tr>
<td>RBC</td>
<td>4.22</td>
<td>1.10</td>
<td>4.1</td>
<td>4.3</td>
</tr>
<tr>
<td>HB</td>
<td>133</td>
<td>0.90</td>
<td>130.6</td>
<td>135.4</td>
</tr>
<tr>
<td>MCV</td>
<td>81.5</td>
<td>0.70</td>
<td>80.4</td>
<td>82.6</td>
</tr>
<tr>
<td>Platelets</td>
<td>202</td>
<td>5.50</td>
<td>179.8</td>
<td>224.2</td>
</tr>
</tbody>
</table>
ranges is still widely applied, and even when substituted with the word ‘reference’ in many cases the two are treated identically. The implication is that there is a gap between two populations, one healthy and the other diseased. This is not the case, and there is considerable overlap in the two populations.

Schneider (1960) concluded, ‘the routine assessment of individual laboratory tests by reference to conventional population-based normal ranges, should no longer remain an accepted automatic practice’. The International Federation of Clinical Chemistry (IFCC) comments that a reference range merely serves as the basis for a more or less intuitive assessment of the biological information given by an observed value. The IFCC has laid down extensive guidelines on the collection of data to obtain the reference interval, which includes the segmentation and selection of the population, which should be greater than 120 estimates. The reference interval is then calculated by taking 95 per cent of the estimates and applying 90 per cent confidence limits at each end of the interval. At this stage, perhaps an example will illustrate the calculation. Table 5.6 shows the results of bone-specific AP measurements on 253 peri- or post-menopausal women with a median age of 50 years (95th percentile ranges 43–54 years).

Table 5.6 The statistics of bone-specific AP on 253 peri-menopausal women

<table>
<thead>
<tr>
<th>Number</th>
<th>253</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum value</td>
<td>6</td>
</tr>
<tr>
<td>Maximum value</td>
<td>32.7</td>
</tr>
<tr>
<td>Mean</td>
<td>13.8</td>
</tr>
<tr>
<td>Standard deviation (SD)</td>
<td>4.36</td>
</tr>
<tr>
<td>−2SD</td>
<td>5.06</td>
</tr>
<tr>
<td>+2SD</td>
<td>22.5</td>
</tr>
<tr>
<td>Median</td>
<td>13.1</td>
</tr>
<tr>
<td>2.5th percentile</td>
<td>7.42</td>
</tr>
<tr>
<td>97.5th percentile</td>
<td>23.1</td>
</tr>
</tbody>
</table>

Examination of these data show the lowest estimate as 6 U/l, yet the 2SD lower range is 4.36 U/l, clearly implying that the distribution is not Gaussian (equal distribution of data frequency on both sides of the average value). Thus, prior to determining any calculation of the reference range, it is well worth plotting a frequency histogram in order to examine the distribution visually or preferably assess the asymmetry of these data by use of coefficients of skewness and kurtosis. Generally, biological compounds show a distinct shift to the right, as the distribution is not a random error. The results of the above data are shown in Figure 5.6 and confirm the skew to the right. Thus determination of the reference interval is not an easy exercise demanding careful use of statistics.

The resulting calculation of the reference interval on the bone-specific measurements on this study gave a lower limit of 7.42 with a 90 per cent confidence interval of 6.1–7.9 and at the upper limit a value of 23.1 with a 90 per cent confidence interval of 21.4–27.8

The basic problem with clinical studies is that, in terms of safety testing, we are looking at high volumes of data in which we are endeavouring to determine whether there is any adverse clinical laboratory event occurring. Thus, sensitivity is of course an important issue. Here, we are faced with the fact that there is no gap between healthy patients and those
suffering from disease. Indeed, in all situations there is a considerable crossover between these two populations. Considering that moderately sized trials may consist of 500 patients with sampling on five occasions and with safety profiles consisting of 40 test measurements, the number of estimates approaches 100,000 test results. Assuming that there is total independence in the measurements, then on the basis of pure statistics one would expect \( (1 - 0.95^{40}) \) that there is an 87 per cent chance that one result would be outside the reference interval. However, we are aware that there is an association between different test measurements, e.g. haemoglobin has a relationship to PCV, MCV, MCHC, and in terms of chemistry GGT relates to AST, ALT, AP, etc. Thus, these measurements cannot be considered as independent. It has been estimated that, in a profile of 40–50 tests, statistically there is a chance that five results will be outside the reference interval. Now, ideally what the industry requires from laboratory testing is that all results should be normal; but, based on statistics, this will be impossible.

Figure 5.7 shows the likely crossover of a laboratory test with a population of healthy and diseased individuals. From this type of presentation we can determine the sensitivity and specificity of a particular test in assessing a diseased state. For example, consider the presentation in respect of glucose measurements from normal and diabetic populations.
Increasing the effective reference range from 95 per cent of the population (black line) to 100 per cent (grey line) will enhance the sensitivity of the test, but with a resultant decrease in specificity and a greater number of false negatives. It is important, therefore, that the 95 per cent reference range be employed to maintain specificity.

Finally, many of the comments above relate to small changes outside of the reference ranges, which is naturally important in a drug study, where early warning of possible toxicity is important. However, the greater the distance from the upper or lower reference interval the greater is the likelihood of pathophysiology.

It is in the area of reference ranges that we encounter the major difference in the requirement between diagnostic pathology and the needs of the pharmaceutical industry in safety testing. Diagnostic pathologists are content to employ reference ranges based on 95 per cent of data from normal populations. However, as defined above, this results in a considerable number of single test results demanding clinical review in safety testing. In order to alleviate the number of so-called abnormals, a variety of approaches have been used. These include the increase of the 95th per cent percentile range to 99.7 per cent and also the application of multiplication factors to the upper and lower limit of the reference interval. As can be seen from Figure 5.7, whilst this may result in a lower number of ‘abnormals’ in safety profiles, it correspondingly alters the specificity of the analyte for the detection of disease with an increase in the false negative component.

For an excellent review of reference intervals, the definitive publication by Whitehead et al. (1994) in examining haematology and clinical chemistry data from some 80 000 subjects attending the BUPA screening programme should be consulted.

Intra-individual biological variation

In addition to the pre-analytical factors discussed above, there is an additional variation within any individual, as all biological compounds oscillate to a smaller or greater extent around an intrinsic homeostatic set-point. Williams et al. (1970) demonstrated that for many biochemical constituents the standard deviation of individual mean values was considerably greater than the average standard deviation of interpersonal fluctuations.

One of the early references to day-to-day variation was by Winkel et al. (1974), but a current listing of the intra- and inter-individual biological variation as a coefficient of variation for over 250 blood components can be found at http://www.westgard.com/biodatabase1.htm (Rius et al., 1999) Within the individual the mean variation surrounding a result is termed the intra-individual biological variation, and the difference in the set-points between different individuals is termed inter-individual biological variation.

In clinical studies we are looking at safety tests to monitor potential toxicities, and it is apparent that the use of the broad-spectrum population-based reference range does not offer the most sensitive assessment. What, then, is the alternative? For many years in diagnostic clinical chemistry we have employed the smallest significant difference (SSD) or critical difference (CD) for assessing changes in results from the same individual on different occasions. The clinician, looking at his patient reports, examines the changes in values against baseline reports to determine if there has been a significant difference in the analytical results due to time, therapeutic response, etc.

An original article in the British Medical Journal in 1989, discussing the interpretation of laboratory results, drew attention to the use of intra-subject variation and its relevance in
clinical decision making (Fraser and Fogarty, 1989), and the value of this measurement in clinical trial safety profiles was reported by Craig (1994). The use of this parameter thus treats the individual as his or her own reference point and offers greater sensitivity than broad-spectrum reference ranges.

The calculation of the SSD is based on the following formula:

$$SSD = \sqrt{CV_{av}^2 + CV_{libv}^2} \times 2.77$$

where $CV_{av}$ (%) is the coefficient of variation of the analytical variation and $CV_{libv}$ (%) is the coefficient of variation of the intra-individual biological variation; the equation is multiplied by 2.77 standard deviations to provide the 95 per cent confidence limits.

The SSD is similar in health and in stable chronic disease, and Fraser (1993) showed that the average within-subject variation in healthy elderly people and younger adults is similar and that there is no apparent variation with different ethnic groups. Thus, it is possible to build a significant database on biological variation.

Table 5.7 details the SSD of the tests normally employed in standard safety profiles. These data were obtained from the Westgard database on the intra-individual biological variation and recalculated using information of analytical variation and 95 per cent confidence limits. In a comparison with our own unpublished SSD values. There is a very close agreement.

A good example of the benefit of using the SSD is shown in Figure 5.8. This shows the reference range for serum creatinine and the SSD at 14%. Assuming that a subject enters at clinical trial with a baseline value of 80 \(\mu\)mol/l and the creatinine level increases by twice the SSD, then the result would still be within the reference range, yet it would have shown a highly significant difference from the subject’s baseline.

Although there will always be a need for population-based reference ranges, these should be restricted to preselection of patients and incorporated into investigator reports. In terms of data analyses and information on trends during clinical studies, the SSD offers a more meaningful and sensitive measurement.
Safety testing in drug development

The issue of clinical laboratory testing programmes in clinical trials is complex. It is important to appreciate that the most sensitive procedures should be employed as early as possible in clinical development. To reach phase 3 in the development programme and identify toxicity is expensive. Thus, at phase 1 and 2, careful selection of the correct tests is mandatory. The selection of the most appropriate tests should be based on information on the compound and details of the pre-clinical toxicology data.

There is a tendency in the industry to look at tests as individual entities related to specific biological functions, e.g. the relationship of calcium in bone metabolism. However, as calcium is in part bound to protein, any alteration in the protein concentration will significantly alter the serum calcium value. Thus, we can now consider calcium changes related to bone and protein metabolism, but also add that, as the kidney is involved in retaining calcium, pathophysiology of the renal mechanism will have an influence on the serum calcium result, and as the parathyroid glands produce the appropriate hormone to maintain calcium balance, a much broader picture is presented. Therefore, in the examination of abnormal results it is inappropriate to place too much credence on a single test measurement.

Rather than use an alphabetical listing of the safety tests, it is considered more convenient to group these as organ-related tests. First, we discuss aspects of Renal tests, e.g. urea, creatinine, creatinine clearance, uric acid, serum Cystatin C, β2-microglobulin, urinary N-acetyl-β-D-glucosaminidase (NAG), urinary microalbumin, electrolytes and glucose. The section on bone test then follows, and covers calcium, inorganic phosphate and new bone markers. Next, the section on liver tests includes discussion of enzymes, bilirubin, total protein and albumin, and prothrombin (PT) time. The lipids and lipoproteins section discusses the various forms of cholesterol and triglycerides. The various thyroid hormone screening tests are then mentioned. Finally, in the haematology section the discussion centres around the tests for haemoglobin, haematocrit, mean cell volume, mean cell haemoglobin, and various red and white cell count measures.

Renal

The kidneys can be considered as the controller of homeostatic mechanisms with the following major functions:
• Excretion of nitrogenous compounds, such as urea and creatinine.
• The retention of amino acids, glucose and other important compounds.
• The retention of bicarbonate for buffering purposes.
• The maintenance of electrolyte and water balance.
• Endocrine activities related to calcium, erythropoietin and renin–aldosterone axis, and the antidiuretic hormone (vasopressin).

**Urea**

• Urea is the major end product of nitrogen metabolism derived from protein and amino acids and is excreted in urine at high concentration.

• Its measurement may not always be due directly to renal impairment, as blood pressure and volume results in a low cardiac output with retention of urea.

• There is no significant difference in the values from males and females, but there is an increase with increasing age. Young healthy adults will show a value of about 4.0 mmol/l, which will increase to 7.0 mmol/l over the age of 65 years, presumably due to diminishing renal function in elderly subjects.

• In the USA the same measurement is undertaken but it is termed blood urea nitrogen (BUN). This is identical to blood urea but with the result expressed in terms of the nitrogen content (molecular weight 28) as opposed to the whole urea molecule (molecular weight 60). As different units are used to convert BUN to urea, the following formula applies:

\[
\text{BUN} \div 2.8 \text{ (mg/dl)} = \text{Blood urea (mmol/l)}
\]

The reference range is 2.5–6.8 mmol/l.

**Low values** are rare, but they may be seen in starvation and in conditions characterized by anabolic demand, such as pregnancy, malabsorption and severe liver damage.

**High values** occur in dehydration, acute and chronic renal disease, recent high protein intake, and decrease in effective circulating volume.

**Creatinine**

• Creatinine is a waste product of muscle metabolism that is excreted at a relatively constant rate in each individual. For this reason, urine measurement, e.g. of drugs, is usually expressed in terms of drug concentration per gram of creatinine.

• The measurement of creatinine is superior to urea, but nevertheless has limitations as a test of renal function. The reason for this is that there will be a reduction in the
glomerular filtration rate (GFR) to less that 60 ml/min (normal 120 ml/min) prior to any increase in serum creatinine being apparent.

- Essentially, creatinine concentration is directly related to body weight.
- Males tend to have higher values in comparable age ranges than females, due to the relative difference in muscle mass.
- Dietary protein does not influence creatinine to the same extent as urea.
- Some compounds interfere in its measurement, including acetoacetic acids, ascorbic acid, bilirubin and glucose in high concentration.

*Reference range*: 70–120 µmol/l. Both males and females show an increase with increasing age, and the female values tend to be lower than the male by about 12–15 per cent. 

*Low levels* may be found in severe muscle wasting and occasionally during pregnancy. 

*High levels* can be found in renal functional impairment, obstruction of the urinary tract, and may be encountered in acromegaly and hyperthyroidism.

There is a very large list of drugs, particularly antibiotics and chemical compounds, that are known to be nephrotoxic.

**Creatinine clearance**

- As mentioned above, the serum creatinine does not show significant changes until the creatinine clearance as a measure of glomerular filtration is about 40 per cent of normal. Therefore, the measurement of the GFR provides a much greater sensitivity of renal impairment.

- Although there are sophisticated measurements of GFR, in the clinical environment creatinine clearance is generally adequate.

- The full creatinine clearance demands the collection of a timed 24 h urine specimen with a sample for serum creatinine, which is inconvenient for subjects in clinical trials.

- For a prompt determination of the predicted creatinine clearance suitable for monitoring potential drug toxicities, the formula reported by Cockcroft and Gault (1976) using only the serum creatinine is of considerable value. The formula has been modified by a number of workers to include body surface area (BSA) as an additional parameter, but the basic formula is:

\[
\text{Predicted clearance (ml/min)} = \frac{[140 - \text{age}] \times \text{weight (kg)} \times k}{72 \times \text{Serum creatinine (µmol/l \times 0.0113)}}
\]

where \(k = 1.0\) for men and 0.85 for women.

*Reference range*: creatinine clearance values decline with increasing age and the
clearance levels are lower in females. Using the above formula and correcting for BSA, the following creatinine clearance values (ml/min) apply:

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults to 30 years</td>
<td>60–130</td>
<td>55–120</td>
</tr>
<tr>
<td>Adults to 50 years</td>
<td>55–110</td>
<td>50–90</td>
</tr>
<tr>
<td>Adults to 80 years</td>
<td>40–79</td>
<td>38–62</td>
</tr>
</tbody>
</table>

**Uric acid (urate)**

- The major clinical laboratory interest in urate is in the identification of subjects with gout, when monosodium urate crystals penetrate the joints.

- Approximately 75 per cent is excreted in the urine and the remainder destroyed by gut bacteria.

- In the kidney, urate is filtered and fully reabsorbed in the proximal tubule, and thus the urate in urine is derived from excretion from the distal tubule.

*Reference range*: 220–450 µmol/l.

*Low values* are of no clinical significance, although it has been reported in Wilson’s disease and some neoplasms.

*Raised values* are seen in renal failure, as well as in toxaemia of pregnancy, liver disease and sarcoidosis. Conditions that result in an increase in turnover of cells, such as pernicious anaemia, radiotherapy, polycythaemia, may all show elevation in uric acid. Also, increased levels can be found after exercise, and in hypothyroidism and hypoadrenalism.

A number of drugs are known to increase the plasma urate concentration, including diuretics, catecholamines, salicylates and ethambutol.

**Serum cystatin C**

- Simonsen *et al.* (1985) reported on the values of cystatin C as a measure of GFR, and since that time there has been increasing popularity in the measurement of this serum protein.

- It is produced in all nucleated cells, freely filtered in the renal glomeruli and almost completely reabsorbed in the proximal tubule.

- Serum cystatin C is an effective measure of GFR and avoids the need for the collection of 24 h urine samples.

- The values are not influenced by non-renal factors, such as muscle mass and protein intake.
The value of the measurement and a comprehensive review of the reference ranges was recently published by Finney et al. (2000). 

*reference range:* the reference range which applies to both sexes, is 0.5–1.1 mg/l.

**β2-Microglobulin**

- This is a 99 amino acid polypeptide present on the surface of nucleated cells and bound to class 1 HLA antigens. Over half the serum concentration originates from the lymphocytes.

- In normal subjects it is synthesized constantly at a concentration of 150 μg/24 h and eliminated exclusively through the kidneys and reabsorbed in the proximal tubules. In kidney disease, a comparison of the blood and urine results will help identify the source of damage. In glomerular disease the levels increase in blood and decrease in urine, whereas in tubular disease the urine concentration increases and the blood level falls.

- Increased serum levels may result from induction of autoimmune systems, high cell turnover or reduced GFR.

- Increased urine levels are encountered in renal impairment, usually prior to creatinine changes.

*Reference range:* normal serum concentrations are 0.7–3.0 mg/l; normal serum concentrations are 0.7–3.0 mg/l; normal urine concentrations are <20 mg/l.

**Urinary N-acetyl-β-d-glucosaminidase (WAG)**

- NAG has its origin in the epithelial cells of the proximal tubule which contain a large number of lysosomes, and due to its sizeable molecular weight (130 000) NAG cannot appear in the glomerular filtrate if the glomerular membrane is intact.

- NAG in urine is widely used for the assessment of renal disease and the detection of nephrotoxicity, particularly as related to hypertension, diabetes, arthritis, urinary tract infections, and to nephrotoxic drugs and environmental pollutants, many of which are attacked by lysosomes or by destruction of the cells in the proximal tubule.

- The enzyme is stable up to 50 °C for reasonable time periods, but owing to the nature of urine it is recommended that analysis takes place as soon after collection as possible. For short delays the sample can be collected with 0.1–0.3 per cent boric acid as a preservative.

- The second void sample of the day is preferred, and the enzyme can be measured in serum as well as urine.

*reference range:* urine NAG is usually expressed as μmol/h/mmol of creatinine and the reference range in that format is 7–28 μmol/h/mmol creatinine.
Urinary microalbumin

- The measurement of urinary microalbumin is of considerable interest as a sensitive marker of renal dysfunction, particularly in relation to diabetic nephropathy.

- Owing to the fact that type 2 diabetes is increasing in prevalence, and also that diabetic patients’ lifespans are increasing, this disease now represents the most common single cause of end-stage renal dysfunction.

- About 30 per cent of patients of type 1 and 2 diabetes develop nephropathy.

- There are major racial differences in the prevalence of diabetes.

- The earliest clinical evidence of nephropathy is the appearance of abnormal concentrations of urinary microalbumin.

- Microalbuminuria in diabetic subjects reflects the presence of glomerular involvement in early renal damage, but recent studies have shown that there is also a tubular component to the renal complications. Indeed, it may well be that tubular involvement precedes glomerular changes.

- The measurement can be carried out on a random, overnight or 24 h collection of urine samples, but it is generally performed on a random sample with the result expressed in terms of creatinine.

  Reference range: normal &lt;30 μg/mg of creatinine  
  microalbuminuria 30–300 μg/mg creatinine  
  albuminuria &gt;300 μg/mg creatinine

Electrolytes

Although the measurement of electrolytes is concerned primarily with mineral and water balance, owing to the close association with the renal endocrine systems, in particular the renin–aldosterone axis, the electrolytes may also be considered as tests of kidney function.

Sodium  This is the principal biological cation and is strongly related to the extra-cellular osmolality and volume. There is a close association between sodium and body water.

  Reference range: 135–145 mmol/l.

  Low levels are found after severe ingestion of water, such as in fluid replacement following sweating, diarrhoea, vomiting and diuretic abuse. Dilutional hyponatraemia may occur in cardiac failure, liver failure, nephrotic syndrome and due to changes in the posterior pituitary antidiuretic hormone.

  Raised levels are found in conditions related to water loss in excess of salt loss, as found dehydration and hyperminalocorticoidism.
**Potassium**  
This is the major intracellular cation and has a pronounced effect on nerve and muscle membrane activity.  
*Reference range*: 3.5–5.4 mmol/l.  
*Low levels*: are found in conditions of gastrointestinal loss, such as diarrhoea, certain renal tubular defects and overactivity of the adrenal cortex. Hypokalaemia can cause muscle weakness. Drugs such as diuretics frequently result in low potassium levels and the adrenergic beta agonists may cause hypokalaemia to such an extent as to result in cardiovascular effects.  
*Raised levels* may be found in conditions of excessive cell destruction, acute and chronic renal dysfunction and with the administration of potassium supplement therapy. In markedly increased hyperkalaemia there is a significant risk of cardiac arrest. Haemolysis and marked thrombocytosis may cause falsely elevated results. Spironolactone, tetracyclines and some antineoplastic agents may results in elevated potassium values.

**Glucose**

Although glucose deserves a devoted section, because the kidney is strongly involved in maintaining a normal blood glucose level it is included here for convenience.

- Blood glucose level is a controlled balance between the amount entering circulation and that being removed by metabolism. It can be considered as a metabolic fuel and is dependent on diet, absorption, insulin from the pancreatic beta cells, glucagon from the pancreatic alpha cells, adrenaline, adreno-corticotropic steroids and liver and muscle metabolism.

- Under normal circumstances glucose should not appear in urine, as all glucose in the glomerular filtrate is reabsorbed in the proximal tubule by active transportation. However, the active transportation is rate limiting and when the blood glucose attains a level of greater than 180 mg/dl (10 mmol/l) it exceeds the rate-limiting capacity and glycosuria results.

*Reference range*: there is a small but significant difference between the glucose concentration in whole-blood and serum due to the difference in the water content of the two fluids. To obtain whole-blood glucose samples it is essential that the collection tube includes a glycolytic inhibitor and fluoride/oxalate tubes are normally employed. Serum can satisfactorily be used providing that the sample is centrifuged and the serum separated within 1 h of collection. The fasting ranges are

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>whole blood</td>
<td>3.3–5.5 mmol/l</td>
</tr>
<tr>
<td>serum</td>
<td>3.8–6.0 mmol/l</td>
</tr>
</tbody>
</table>

As it is not always possible to obtain fasting samples from subjects in clinical studies, the *non-fasting reference range* employed is 3.7–7.8 mmol/l.  
*Low levels* may be found in insulin overdose, insulin-secreting tumours (insulinoma) starvation, severe exercise, hypothyroidism and Addison’s disease (adrenocortical hypoplasia).
Raised levels are usually found in diabetes mellitus, hyperthyroidism, Cushing’s disease (adrenocortical hyperplasia) acromegaly and post-vascular accidents.

Type 2 diabetes is on the increase in all countries, as sadly are the complications (both macro-vascular and micro-vascular) of the disease. Statistics from the USA in 1998 showed that 10.3 million people were diagnosed as diabetic and additionally there are probably 5 million undiagnosed. It has been estimated that by the year 2025 some 5 per cent of the worldwide population will have type 2 diabetes, offering a challenge to the industry for new products and to the diagnostic business for much improved testing programmes.

Although self-testing of glucose levels can now be performed accurately by patients, this merely represents glycaemic control at a single point in time. This must be supplemented by more long-term glucose control with the use of haemoglobin A1c, and the assessment of macro- and micro-vascular testing to monitor and prevent diabetic complications.

Bone

Bone is living connective tissue comprising of an organic protein and the inorganic mineral hydroxyapatite. Some 90 per cent of the organic protein is type 1 collagen derived from the triple-helical procollagen molecule. The collagen molecule is cross-linked at both the C and N terminals to other type 1 collagen strands, forming the basic fabric and tensile strength of bone tissue. The cross-linking is accomplished by pyridinoline and deoxypyridinoline (DPD) as a result of condensation of lysine and hydroxylysine residues on adjacent collagen molecules.

Bone is constantly undergoing a process of remodelling which includes a degradation stage of bone resorption by the action of osteoclasts and a building stage of formation mediated by the action of osteoblasts.

During the resorption of collagen, both free and peptide-bound DPD is released together with both C- and N-terminal telopeptides (CTx and CNx). These peptides are specific fragments of type 1 collagen and for bone resorption.

Osteocalcin comprises 10–25 per cent of the non-collagen protein in bone and is specific to this tissue source. Osteocalcin is involved in bone formation by binding calcium to γ-carboxyglutamic acid residues. The enzyme AP is involved in bone formation and is thought to indicate the activity of the osteoblast. Although this enzyme is present in serum from both bone and liver, the bone-specific isoenzyme can be measured as a separate entity.

Calcium

- Calcium is widely distributed in both intra- and extra-cellular fluids and is concerned with bone formation and coagulation; it is a necessary activator for many enzymes.

- Plasma calcium levels are controlled by the level of parathyroid hormone (PTH) secreted from the parathyroid glands by its action on the kidney, bone and the gut via 1,25 dihydroxycholecalciferol (vitamin D3). Calcitonin from the C cells of the thyroid gland decreases osteoclastic activity and has the opposite effect from PTH.

- Approximately half of the plasma calcium is bound to protein, predominately albumin, and only the unbound (free ionized) calcium is physiologically active.
• PTH acts to stimulate the action of osteoclastic bone resorption, releasing calcium into the extra-cellular fluid, and decreases the renal tubular reabsorption of phosphate and increases the reabsorption of calcium. The control of the PTH secretion is dependent on a feedback mechanism of the free ionized plasma calcium level.

• As 50 per cent of calcium is bound to albumin any change in the concentration of this protein will significantly alter the calcium level, and most laboratories will include a correction for the protein difference. The formula for correction is:

\[
\text{Corrected calcium} = \text{Measured } Ca^{2+} + \{0.02 \times [40 - \text{albumin concentration (g/l)}]\}
\]

*Reference range:* 2.25–2.65 mmol/l (uncorrected). There is a slight difference between the sexes with males showing higher values, due presumably to the slight difference in albumin between males and females.

*Low levels* may be found in vitamin D deficiency, renal insufficiency, acute pancreatitis, low protein levels, hypoparathyroidism and prolonged anticonvulsant therapy.

*High levels* may be encountered in primary hyperparathyroidism, bone metastases, malignancy, hyperthyroidism, Paget’s disease and acromegaly.

**Inorganic phosphate**

• Phosphorus is present as inorganic salts of phosphoric acid, and organic esters of glycerophosphate, nucleotide phosphate and lipid phosphorus.

• The erythrocytes are rich in phosphorus, the whole-blood concentration being about 10 mmol/l in comparison with plasma of about 4 mmol/l. Thus, any haemolysis of the samples renders it unsuitable for this measurement.

• Approximately 80 per cent is located in bone. It has a vital role in the transfer of energy.

• Phosphate is excreted by the kidney following glomerular filtration and tubular reabsorption. The latter process is inhibited by PTH, increasing phosphate excretion.

• As mentioned previously, inorganic phosphate levels increase significantly during the day and immediately after a meal there is a decline in phosphate due to the increase in insulin.

*Reference range:* 0.8–1.3 mmol/l.

*Low levels* are found in hyperparathyroidism, acute alcohol toxicity, hypokalaemia, malabsorption and hyperinsulinaemia. Antacid overuse and exercise may also demonstrate hypophosphataemia.

*Raised levels* are encountered in hypoparathyroidism, renal failure, thyrotoxicosis myeloma and excess vitamin D intake.
New bone markers

In terms of bone resorption, it is impossible to choose the single best collagen degradation marker. The publications from each diagnostic company inevitably favour their own product. Nevertheless, in terms of monitoring changes, in post-menopausal subjects on oestrogen or alendronate therapy all show significant changes, and these occur much more rapidly than bone mineral density measurements. Kyd et al. (1999) reviewed the clinical usefulness of DPD, CTx and NTx in patients on alendronate and showed that CTx and NTx were more sensitive than DPD, but that only baseline DPD showed an association with the bone mineral density measurement after 1 year. In clinical studies, sponsoring companies should communicate with the analytical laboratory to determine the most appropriate marker. Table 5.8 lists a number of the markers that are currently available.

<table>
<thead>
<tr>
<th>Name</th>
<th>Diagnostic supply company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone resorption</td>
<td></td>
</tr>
<tr>
<td>Deoxypyridinoline (free lysyl-pyridinoline) DPD</td>
<td>Metra (Quidel)</td>
</tr>
<tr>
<td>N-terminal telopeptide (NTx)</td>
<td>Ostex (Osteomark)</td>
</tr>
<tr>
<td>C-terminal telopeptide (CTx)</td>
<td>Osteometer (Crosslaps)</td>
</tr>
<tr>
<td>Bone formation</td>
<td></td>
</tr>
<tr>
<td>Bone-specific AP</td>
<td>Hybritech, Quidel</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>Many diagnostic companies</td>
</tr>
<tr>
<td>Carboxyterminal propeptide of collagen (PICP)</td>
<td>Orion, Quidel</td>
</tr>
</tbody>
</table>

Similarly, in terms of bone formation marker, it is now generally considered that both bone-specific AP and osteocalcin should be employed.

Osteoporosis is a common condition that can lead to fractures with a decreased quality of life. A woman’s lifetime risk of fractures is 40 per cent whereas a man’s risk is 13 per cent (Delmas and Fraser, 1999). Caucasians and Asians are the two ethnic groups with the highest risk of fractures (Lau and Cooper, 1996). The condition affects more than 25 million of the US population and results in 1.5 million fractures annually, and this is likely to increase with the increase in population age. The worldwide cost of osteoporotic fracture treatment is enormous and represents a challenge to the industry for efficacious therapeutic products and for the diagnostic clinical laboratory towards greater efficiency in testing programmes.

Liver

- The liver is located in the right hypochondrium and is the largest organ in the body, accounting for 5 per cent of body weight. It has little connective tissue but is highly vascular and metabolically extremely active. In the normal situation, 10–15 per cent of the total blood volume is in the liver and acts as a vascular reservoir.

- Roughly 80 per cent of blood entering the liver is venous blood from the portal vein, thus providing the liver with first choice of all material in the intestine. The remaining 20 per cent is arterial blood from the hepatic artery.
• The functions of the liver are principally bile production, protein, carbohydrate and lipid metabolism, ketone body formation, vitamin storage and inactivation of steroids, drugs and pharmacological agents.

• The functional unit in the liver is the hepatic lobule, a hexagonal arrangement of plates and layers of hepatocytes radiating outwards from a central vein in the centre of the lobule. Within the lobule are the sinusoids, consisting of endothelial cells and hepatocytes, similar to the structure in Figure 5.9.

‘Liver’ enzymes
• Although there are many enzyme measurements that can be undertaken to determine hepatitic dysfunction, these tend to be limited to AST, ALT, GGT and AP. It is important to remember that changes in liver enzymes often, but not always, indicate liver involvement. For example, elevation of AP may be due to bone disease or hyperthyroidism, and changes in the measured activity of AST and ALT to myocardial damage and the influence of muscle damage or exercise.

• GGT and AP are membrane-bound enzymes, ALT is located in the cell cytosol and AST in both the cytosol and the mitochondria, which may explain the variable pattern of enzyme changes found in different liver pathologies, dependent of the severity of the liver damage. Also, the enzyme activity within the cell is extremely high relative to the level encountered in plasma. The ratio of AST in the erythrocyte relative to plasma is 15:1, but in liver and heart cells it is approximately 8000:1.

• The classification of liver disease is complex, and the enzyme activities will change significantly over the course of a disease; thus, serial enzyme measurement may be required to attain a diagnosis.

• There are a large number of drugs, toxins and foodstuffs that can induce hepatic lesions, resulting in varied symptoms and enzyme profiles. In terms of drugs, it has been recorded that 5 per cent of all reported adverse events are associated with hepatic
dysfunction or injury, and that 2–3 per cent of all hospital admissions are due to adverse
drug effects linked with a hepatic reaction. Therefore, the detection of liver disease at
an early stage is of overriding importance, both in diagnostic pathology and in the
assessment of drug-induced reactions.

**Gamma glutamyltranspeptidase**  GGT is an enzyme present in kidney, liver and pancreas
but with little activity in other organs. Its measurement is particularly sensitive to liver cell
damage and cholestatic situations.

*Reference range:* 8–55 IU/l, but the distribution shows a marked skew to the right.

*Low levels* are of no significance.

*High levels* The greatest increase occurs in liver cell obstruction and malignancy
(hepatoma and cancer of the pancreas). It is a useful marker for metastasis from breast and
colon.

It is also raised in cirrhosis, hepatitis and infectious mononucleosis. Fatty liver (as in
obesity and badly controlled diabetes) may give elevated values, and enzyme induction with
a number of the anticonvulsant drugs (e.g. phenytoin and phenobarbitone) also shows raised
enzyme activity.

GGT is particularly sensitive to alcohol toxicity and may show significant increases in
persons who have more than three units of alcohol per day. In a recent review of old and
newer biochemical markers for the detection and monitoring of alcohol abuse (Sharpe,
2001) concluded that GGT continues to remain the test combining the greatest convenience
and sensitivity for monitoring alcohol, particularly when used in conjunction with AST,
ALT and the MCV.

**Aspartate aminotransferase and alanine aminotransferase**

- Both enzymes are located in many tissues, but predominantly in the hepatocytes,
  myocardial cells, erythrocytes and skeletal muscle cells.

- AST has a bias toward myocardial cells and ALT towards liver cells, and thus the
  relative ratio of the enzyme activities are important in establishing the source of tissue
damage.

- Moderate increases in both enzymes (less than 1.5 times the upper limit of the reference
  range) may be normal for the individual, due to ethnic origin or body mass index.

- In testing liver enzymes we are endeavouring to determine hepatocellular injury or
  cholestasis, and serial measurements may be required to confirm a diagnosis. Drugs that
  are hepatotoxic in certain individuals may give rise to significantly different pictures
  including cholestasis, viral-type hepatitis and often a mixture of both.

*Reference range:* ALT: 5–50 IU/l.

*Low values* are rare and of little significance.

*High values* are found in hepatic cirrhosis, but the levels may only be moderately
increased. Viral hepatitis can give markedly increased levels up to several thousand units. In
chronic hepatitis, varying enzyme activity may be encountered, dependent on the stage of
the disease. Cholestatic jaundice gives moderately raised levels, as can muscular trauma,
including severe exercise and *in vivo* and *in vitro* haemolysis. In myocardial infarction there will be increased plasma levels, usually less in magnitude than AST. Drugs such as heparin, salicylates, opiates, tetracycline and isoniazide have been known to give asymptomatic rises in aminotransaminases.

*Reference range*: AST: 4–40 I U/l.  
*Low values* are rare and of little significance.  
*High values* of the order of 100–700 I U/l are found in myocardial infarction, and generally lesser levels in congestive cardiac failure. Similar increases are seen in cirrhosis and chronic alcohol toxicity, but may be variable. In viral hepatitis, markedly increased plasma levels are noted. Trauma, including surgery and muscular disorder, can give elevated levels, and phenothiazines, erythromycin, methyldopa, halothane and anabolic steroids are known to increase the AST and ALT levels.

**Alkaline phosphatase**

- AP is present in most tissue sources, particularly in the osteoblasts of bone, the biliary canaliculi in the liver, intestine, renal proximal tubules, and the placenta.

- In the normal adult non-pregnant serum the major contribution of the enzyme is from liver and bone.

- Convenient and reliable methods are now available for the measurement of bone-specific AP.

- The interpretation of abnormal AP activity must be examined in conjunction with other liver function tests.

*Reference range*: 70–250 IU/l (extremely dependent on method employed).  
*Low values* are rare but may be encountered in children with growth hormone deficiency, severe anaemia and in congenital defects in phosphate metabolism.  
*High values* may be found in a variety of clinical conditions:

- Bone disease, including rickets, osteomalacia, Paget’s disease and secondary malignancy of bone.

- Hyperparathyroidism with bone involvement.

- Marked increase may be found in osteogenic sarcoma, a malignant tumour of the osteoblast.

- In liver disease the levels are increased moderately in acute hepatitis (usually less than threefold from the upper limit of the reference range). Infective mononucleosis with liver involvement can increase the AP by about five times, and in biliary cirrhosis and cholestasis marked increases may be found (10–15 times).

- Hyperthyroidism may show a slight elevation, as will samples from subjects in late pregnancy.
A large number of drugs have been implicated in cholestasis and hepatocellular toxicity. From a cholestatic aspect these include androgens, oestrogens, sulphonamides, tricyclic antidepressants, anticoagulants and diuretics; from a hepatocellular toxicity aspect these include aspirin, paracetamol, phenytoin and many antibiotics, in particular isoniazide. Therefore, considerable care and emphasis is necessary in all clinical studies in the interpretation of the liver enzymes.

**Bilirubin**

- The life span of erythrocytes is approximately 120 days; thereafter, they are broken down by the reticuloendothelial system, resulting in the release of haemoglobin. After the removal of the iron component for re-use, bilirubin is formed.

- The bilirubin formed is loosely bound to albumin and is transported to the liver, where it is conjugated. In normal circumstances the unconjugated portion represents the largest portion of the total bilirubin and is termed pre-hepatic or *indirect* bilirubin. Conjugation is accomplished in the liver with uridyl diphosphate glucuronyl transferase, rendering bilirubin water soluble, and is termed *direct*, which is then transported in bile.

- In the intestine bilirubin is degraded to stercobilinogen and a portion reabsorbed into the liver via enterohepatic circulation as urobilinogen to be excreted in urine. As the conjugated bilirubin is water soluble it can, therefore, be excreted by the kidneys, but in normal subjects the concentration in urine is extremely small, but it may be detected by dipsticks if the urine sample is concentrated.

- As the secretion of conjugated bilirubin into the bile is very rapid in comparison with the conjugation stage, healthy persons have almost no detectable plasma conjugated bilirubin.

- Most clinical laboratories define the upper limit of normal for total bilirubin as about 20 μmol/l, with no difference in the sexes. In our own laboratory we have isolated a database of subjects participating in phase 1 studies and found a significant difference between males and females; whereas the value of 20 μmol/l is acceptable for females, this range requires extension to 28 μmol/l for normal healthy fasting males. It has been suggested that this may be due to a partial defect in the conjugation enzyme, and be classified as Gilbert syndrome. However, without adequate proof of a conjugation defect this may simply be a difference between the sexes and can be classified as unexplained mild hyperbilirubinaemia.

**Reference range**: Males, 2–28 μmol/l; females, 2–20 μmol/l.

**High values** of bilirubin may be encountered in three circumstances:

- Pre-hepatic (haemolytic) – increased breakdown of RBCs, in excess of the conjugation limit, as in haemolytic anaemia, pernicious anaemia, neonatal jaundice and haemoglobinopathies.
Hepatic – liver cell damage (acute, chronic, fatty liver hepatitis), intrahepatic obstruction, infective mononucleosis, chemical toxicity and Gilbert’s disease.

Post-hepatic – obstruction of the main bile ducts, whether due to stone, tumours, cancer at the head of the pancreas or biliary inflammation.

**Reference range:** urinary bilirubin, $<3.5$ mmol/l.

**Reference range:** urinary urobilinogen $<17$ mmol/L.

For an up to date assessment on the evaluation of liver chemistry tests, the American Gastroenterological Association Clinical Practice Committee report is well worth reviewing (Green and Flamm, 2002).

**Total protein and albumin**

- Plasma proteins are formed mainly in the liver, and albumin is quantitatively the most important, representing about 50 per cent of the total protein.

- Plasma proteins consist of a complex mixture of transportation agents, enzymes, coagulation factors, immunoglobulins, glycoproteins and lipoproteins, and there are over 100 individual plasma proteins, many at extremely small levels of concentration.

- As such, therefore, the measurement of total protein and albumin may be consider as a screening test.

- Further investigation usually commences with the application of protein electrophoresis, which separates the protein into the following fractions: Albumin, $\alpha_1$ globulins (predominantly $\alpha_1$ antitrypsin), $\alpha_2$ globulins (predominantly $\alpha_2$ macroglobulin and haptoglobin), $\beta$ globulins (mainly complement and transferring), $\gamma$ globulins (predominantly immunoglobulins).

**Reference range:** total protein, 64–82 g/l; albumin, 36–50 g/l. There is a slight but significant difference between the sexes (mean male value 45 g/l; mean female value 42.2 g/l). There is also a decline in albumin with increasing age, a point worthy of note with compounds which bind to albumin.

Low values may be found in nephrotic syndrome, glomerulonephritis, malabsorption and malnutrition.

High values may be found in dehydration, chronic liver disease, certain neoplasia, collagen disease and many other conditions.

**Prothrombin time**

- The hepatocytes synthesize most of the coagulation factors, of which factor II is PT.

- PT and factors VII, IX and X are vitamin K dependent.

- Therefore, measurement of PT may be considered as a liver function test; however, in this respect, other biochemical tests are considerably more sensitive.
Reference range: the results of PT should be reported in relation to the International Normalized Ratio (INR). This is the ratio of the patient’s PT to mean PT reference range for the particular laboratory raised to the power of the International Sensitivity Index (ISI) provided by the reagent manufacturer.

A prolonged PT value may be found in

- inadequate vitamin K intake
- poor fat absorption
- liver damage due to impaired synthesis of PT complex factors
- hypofibrinogenemia
- drug therapy, such as aspirin, anti-histamines and non-steroidal anti-inflammatory drugs.

In endeavouring to differentiate liver hepatocellular damage from cholestasis, the changes in various measurements shown in Table 5.9 may be of help.

Table 5.9 The different responses of liver function tests in hepatocellular damage and cholestasis. The ULRR factors (upper limit of the reference range) are for guidance only, as these will vary considerable during the progress of the diseases

<table>
<thead>
<tr>
<th></th>
<th>Cholestatic</th>
<th>Hepatocellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>Normal to slight increase, usually ≤×5 ULRR</td>
<td>Increased, usually &gt;×5 ULRR</td>
</tr>
<tr>
<td>AST</td>
<td>Normal to slight increase, usually ≤×3 ULRR</td>
<td>Increased, usually &gt;×3 ULRR</td>
</tr>
<tr>
<td>AP</td>
<td>&gt;3× ULRR</td>
<td>&lt;3× ULRR</td>
</tr>
<tr>
<td>GGT</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Slight increase</td>
<td>Normal or lowered</td>
</tr>
<tr>
<td>PT after vitamin K</td>
<td>Response</td>
<td>Nil</td>
</tr>
</tbody>
</table>

Lipids and lipoprotein

In a recent assessment in our laboratory of cholesterol values from volunteers accepted into phase 1 studies there were 336 males with a median age of 31 years (18–50) and 222 female subjects with a median age of 49 years (32–62). The calculated reference range employed in the laboratory is 2.9–7.3 mmol/l.

The males showed nine of the subjects with a cholesterol level greater than 7.3 in the range of 7.4–10.4 mmol/l. However, when the earlier desirable upper limit of cholesterol at 6.2 mmol/l was employed, 43 (13 per cent) of the males showed raised values. It is now being recommended that the desirable limit should be set at 5.6 mmol/l, if not lower.

The females showed 17 (7.6 per cent) of the subjects with values greater than 7.3 mmol/l in the range of 7.4–9.6 mmol/l. On using the desirable level of 6.2 mmol/l, 60 (27 per cent) of the volunteers demonstrated raised values. As the age range for the female population covered the peri- and post-menopausal period, these results were not unexpected.

Screening of the population will yield a number of asymptomatic subjects with markedly
elevated total cholesterol values worthy of further investigation, and thus the inclusion of this measurement in pre-study safety profiles is essential.

- Dietary lipids are absorbed from the small intestine after emulsification with bile salts synthesized from cholesterol. The emulsification renders the lipids accessible to pancreatic lipase, liberating free fatty acids.

- Triglycerides are formed by esterification of three different fatty acids with glycerol and a portion of the fatty acids remains free as NEFAs.

- Phospholipids are also formed, containing phosphate in a nitrogenous base in place of one of the fatty acid residues.

- Cholesterol derived from the mevalinate pathway is the precursor of bile acids, steroids, lipoproteins and vitamin D. In plasma about 60–70 per cent of the cholesterol is esterified with fatty acids to form cholesterol esters.

- The major lipoproteins are complex molecules of triglycerides and cholesterol surrounded by phospholipids, cholesterol esters and different proteins, termed apolipoprotein.

- The content of the various lipoproteins varies considerably, as shown in Table 5.10.

### Table 5.10 The varying compositions of lipids in lipoprotein

<table>
<thead>
<tr>
<th>Complex</th>
<th>Source</th>
<th>Electro-phoresis mobility</th>
<th>Protein (%)</th>
<th>Major protein</th>
<th>Triglyceride (%)</th>
<th>Phospholipid (%)</th>
<th>Cholesterol ester (%)</th>
<th>Cholesterol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>Intestine</td>
<td>Origin 1–2</td>
<td>A, B</td>
<td>85–88</td>
<td>8</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td>Liver</td>
<td>Pre beta 7–10</td>
<td>B, C, E</td>
<td>50–55</td>
<td>18–20</td>
<td>12–15</td>
<td>8–10</td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>VLDL</td>
<td>Beta 20–22</td>
<td>B</td>
<td>10–15</td>
<td>20–28</td>
<td>37–48</td>
<td>8–10</td>
<td></td>
</tr>
<tr>
<td>HDL2</td>
<td>Liver and</td>
<td>Alpha 33–35</td>
<td>A, C, E</td>
<td>5–13</td>
<td>26–43</td>
<td>20–30</td>
<td>5–10</td>
<td></td>
</tr>
</tbody>
</table>

- **Chylomicrons** are the transporters of lipids from the intestine to all cells. In the capillaries and adipose tissues the triglyceride component is removed by the action of the lipoprotein lipase enzyme.

- **Very low density lipoprotein** (VLDL) transports lipids from the liver to the cells and the fatty acid component is released for re-use, similar to the chylomicrons.

- **Intermediate density lipoprotein** (IDL) in the absence of metabolic disease is found in plasma at very low concentration and is considered a precursor of LDL.

- **Low density lipoprotein** (LDL) is the primary carrier protein for cholesterol for delivery to all tissues. Insulin and triiodothyronine (T3) increase the binding of LDL to cells,
whereas glucocorticoids have the opposite effect. This may be an explanation for the hypercholesterolaemia associated with the uncontrolled diabetic and in subjects with hypothyroidism.

- **High density lipoprotein** (HDL) is the transporter of cholesterol from the cells to the liver. There are sub-fractions of HDL classified as 1, 2 and 3, which vary in composition. It has been suggested that HDL3 is static and HDL2 a dynamic parameter and that up until the menopause females tend to show higher HDL2 levels than males of similar ages.

- The measurements undertaken tend to be limited to triglycerides, total cholesterol, HDL and LDL fractions and in specific cases the inclusion of apolipoprotein A and B. Total cholesterol is of limited value in the assessment of coronary risk.

- Serum cholesterol levels are not altered significantly after a fatty meal, whereas triglycerides demand that the patient fasts for 12 h prior to sample collection.

- Both LDL and HDL show a reduction in levels during the day, but with post-prandial measurements using modern technology the changes during the day are unlikely to alter the clinical diagnosis.

*Reference ranges:* for all lipoproteins these need to be confirmed with the analytical laboratory.

**Total cholesterol (mmol/l)**

<table>
<thead>
<tr>
<th>Age</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;25–35</td>
<td>3.7–7.4</td>
<td>3.3–6.5</td>
</tr>
<tr>
<td>36–50</td>
<td>4.0–8.0</td>
<td>3.8–7.5</td>
</tr>
<tr>
<td>51–70</td>
<td>4.2–8.6</td>
<td>4.7–9.0</td>
</tr>
<tr>
<td>&gt;70</td>
<td>3.7–8.8</td>
<td>4.5–9.0</td>
</tr>
</tbody>
</table>

**LDL (mmol/l):** males, 2.2–5.4; females, 2.3–5.8.

**HDL (mmol/l):** males, 0.7–1.9; females, 0.9–2.5.

**Triglycerides (mmol/l):** males, 0.3–3.6; females, 0.2–2.9.

*Low total cholesterol levels* may be found in

- severe liver damage
- hyperthyroidism
- malnutrition
- chronic anaemia.

*Raised total cholesterol levels* are used for the assessment of coronary risk and also in secondary hypercholesterolaemia, including
• hyperlipoproteinaemia
• biliary obstruction
• nephrotic syndrome
• hypothyroidism
• diabetes mellitus.

Low levels of HDL may be seen in
• obesity
• starvation
• lack of exercise
• smoking
• diabetes mellitus
• and a number of familial lipid disorders.

Raised levels of HDL may be seen in
• vigorous exercise
• moderate alcohol consumption
• insulin therapy
• a number of genetic abnormalities.

The risk of atherosclerosis and coronary heart disease is associated with high levels of LDL, whereas HDL is inversely correlated to risk, and high concentrations may be considered as cardio-protective. A number of factors must be given attention in the collection of samples for lipoprotein assessment, including avoidance of alcohol for 4–5 days prior to sample collection, standardized posture at phlebotomy by sitting for 15 min, minimal use of a tourniquet and treatment must not be based on a single result.

Thyroid

The incidence of hypothyroidism in the world population is high, with estimates from the USA of 6–7 million. This figure refers to overt hypothyroidism and does not include the fact that the condition develops slowly. Screening to assess sub-clinical hypothyroidism will demonstrate a significant increase in this incidence figure. It has been suggested that worldwide some 200 million people may be affected by thyroid abnormalities.

Elderly people, especially women, experience the highest incidence. The Merck Manual of Geriatrics quotes a prevalence of 2–5 per cent of the population over the age of 65 years with overt disease, increasing to 5–10 per cent with the inclusion of the sub-clinical condition assessed by thyroid test measurements.

With the life span of the worldwide population increasing and more clinical trials being
carried out on the elderly the inclusion of thyroid tests in pre-screen safety profiles is essential to avoid on-study clinical issues due to this endocrine abnormality.

The inclusion of endocrine topics is outside the scope of this chapter, but some comment on thyroid is considered to illustrate feedback mechanisms:

- The thyroid cubical epithelium cells trap iodide from the circulation, which is initially linked to the amino acid tyrosine. In the thyroid gland this is converted to *triiodothyronine* (T3) and *tetraiodothyronine* (thyroxine; T4) and stored in a colloid with protein as *thyroglobulin*.

- The thyroid gland is part of the hypothalamic–anterior pituitary–thyroid axis and the control of the thyroid hormone secretion is exerted by the classical negative feedback system.

- Thyroid-releasing hormone (TRH) from the hypothalamus stimulates the release of the thyroid-stimulating hormone (TSH) from the anterior pituitary, which stimulates thyroid hormone release. As the blood concentration of the T4 from the thyroid increases, this inhibits both TSH and TRH, leading to a shutdown of thyroid activity, as shown in Figure 5.10.

- Thyroid hormones (T4 and T3) are poorly soluble in water, and more than 99 per cent of the circulating hormones in blood are bound to a carrier proteins, particularly the thyroid-binding globulin and to a lesser extent the thyroid-binding pre-albumin, albumin and lipoproteins.

- T3 and T4 are carried by the blood to all body tissues. T4 is converted in the cells to T3, which binds to receptors in the nuclei to increase messenger RNA and hence protein synthesis. This results in an increase in oxygen consumption, heat production and increased metabolism.

![Figure 5.10](image-url)
A normal thyroid output is required for normal growth.

Owing to the high binding of the hormone to the thyroid-binding globulin, which is increased during pregnancy, hepatitis and with oestrogen-related drugs, the value of the total T4 is limited. Thus, the measurement of free T4 is preferred.

TSH has a major benefit in hypothyroidism, in that it is increased usually prior to any obvious clinical signs of the disorder and hence is a valuable screening procedure.

Reference range: TSH, 0.3–5.0 mIU/l; free T4, 9.0–25.0 pmol/l; Free T3, 3.0–8.5 pmol/l.

Interpretation

In hypothyroidism, the TSH results may be markedly elevated (>50 mIU/l) and the free T4 value is low. In borderline cases the free T4 may be normal, but the TSH is usually raised.

On thyroid replacement therapy TSH should be the measure employed to monitor success of therapy.

In hyperthyroidism the TSH is low and, although sensitive methods are available, the diagnosis is usually confirmed by an elevated free T4.

In hypopituitarism, both of the hormones will be reduced.

Considerable care is required in the interpretation of thyroid function tests due to changes in the concentration of the thyroid-binding globulin. Increased levels of the protein may be congenital, due to high concentrations of oestrogen, hepatitis and influence by a number of drugs. Low levels of thyroid-binding globulin may be encountered in subjects on androgens, glucocorticoids and in clinical nephrotic syndrome and cirrhosis.

Haematology

Haematology is the study of the blood cells to determine normal haemopoiesis and/or the diagnosis of blood diseases. However, as is characteristic of most laboratory measurements the basic haematology profile is merely a screening procedure. In the event of any abnormality being identified, then much more rigorous testing of the bone marrow and associated biochemical markers is needed.

Blood is a specialist fluid tissue concerned predominantly with the transport system. The average volume is 5.6 l, representing about 8 per cent of body weight. Some 45 per cent of whole blood is cellular and 55 per cent plasma. Plasma is 93 per cent water, with the remaining solid content being mainly albumin.

The cellular content consists:

- red blood cells (4.1–6.5 \times 10^{12}/l)
platelets \((250 - 500 \times 10^9/l)\)

white blood cells \((4 - 11 \times 10^9/l)\).

For normal functional haemopoiesis, numerous substances are necessary. The kidney must secrete erythropoietin, which stimulates bone marrow cell production but also requires vitamin B12, thyroid and adrenal hormones, iron, copper, manganese, vitamin C, folic acid and the intrinsic factor secreted from the gastric mucosa.

The normal full blood count is accomplished with specialist analytical systems and includes the following measurements:

- **Haemoglobin (Hb, g/l)**
- **Haematocrit (HCT or PCV, fl)**
- **Mean cell volume (MCR, pg)**
- **Red cell distribution width (RDW, %)**
- **Mean cell haemoglobin (MCH, pg)**
- **Mean cell haemoglobin concentration (MCHC, g/l)**
- **Red cell count (RBC, 10^{12}/l)**
- **White cell count (WBC, 10^{9}/l)**
- **Differential white cell count (Diff WBC, 10^{9}/l)**

**Haemoglobin**

Haemoglobin is the protein that carries oxygen from the lungs to the tissues and in exchange carries carbon dioxide from the tissues back to the lungs. The molecule consists of four polypeptide chains, two \(\alpha\) chains of 141 amino acids and two \(\beta\) chains of 146 amino acids. Each chain is conjugated with a haem group that binds the oxygen to ferrous ions.

Females have lower values than males, and although the molecular weight of haemoglobin is known, the SI unit has not been universally accepted.

Strictly, anaemia is defined as a decrease in total body red cell mass, but for practical purposes anaemia is usually defined by a haemoglobin value of <100 g/l.

*Reference range*: males, 135–180 g/l; females, 115–164 g/l.

**Haematocrit**

In the original technology the haematocrit was measured in a graduated tube filled with blood, which was centrifuged to pack the red blood cells. The percentage of packed cells is expressed relative to the total whole blood volume. With the analytical instrumentation now in common use the haemoglobin, red cell count and mean cell volume are measured and the other parameters calculated. In the case of the haematocrit this is obtained by multiplying the red cell count by the mean cell volume with results expressed in femtolitres.

*Reference range*: males, 0.40–0.54 fl; females, 0.37–0.47 fl.
**Erythrocytes: red cell count**

The erythrocytes are disc-shaped cells with a thick rim, and their major function is gas transportation and the maintenance of pH. Some of the 35 per cent cytoplasm is haemoglobin, and it also contains the enzyme carbonic anhydrase to convert carbon dioxide and water reversibly to carbonic acid.

Erythrocytes progress from blast cells in the marrow over a period of several days, then are released into peripheral circulation as reticulocytes. Within 24 h the reticulocytes change to erythrocytes and remain in circulation for about 120 days. Using special stains the reticulocytes can be determined; normally they represent only 1.5 per cent of the total RBC. The reticulocyte count can be considered as an index of bone marrow production. Increases are encountered in increased red cell turnover due to bleeding or haemolysis and in response to successful therapy in marrow deficiencies. A reduction in reticulocytes may be due to impairment in erythrocyte production, such as aplastic anaemia.

A drop in the RBC number causes hypoxaemia in the kidney, resulting in stimulation of erythropoietin which acts on the marrow to increase RBC production. Stimulus for erythropoiesis may also result from low levels of atmospheric oxygen, increased exercise activity and haemorrhaging.

*Reference range:* females (3.9–5.6) × 10¹²/l; males (4.6–6.5) × 10¹²/l.

**Red cell indices**

The red cell indices are sometimes referred to as absolute values and include MCV, MCH and MCHC, but the red cell distribution width should also be considered as an index.

**Mean cell volume** The MCV defines the average volume of all the erythrocytes counted in an examination with the results expressed in femtolitres. After phlebotomy with the whole blood sample collected into EDTA containers the MCV will show an increase within 6–10 h. This is singly the most useful measurement, as it clearly classifies anaemias as microcytic and macrocytic (small and large volume cells) from normocytic cells.

*Reference range:* both sexes, 81–95 fl.

As the MCV expresses information on the average of the cell volume it is important to bear in mind that a normal MCV could be obtained but there might be an abnormal variation in the cell sizes. This is referred to as anisocytosis. The red cell distribution width RDW provides a measure of the dispersion around the mean cell population expressed as a percentage. The percentage is normally 11.5–14 per cent, and values greater than this indicate the degree of anisocytosis.

*Reference range:* both sexes, 11.5–14.5 per cent.

**Mean cell haemoglobin** The MCH defines the weight of haemoglobin in the average red blood cell expressed in picograms. Since small cells have less haemoglobin than larger cells, variation in the MCH tends to follow the MCV.

*Reference range:* 26–32 pg.
Mean cell haemoglobin concentration  The MCHC defines the amount of haemoglobin in the average RBC compared with its size, with results expressed in g/l. As the red cells constitute about one-half of the whole blood and all the haemoglobin is located in the RBC, it would be expected that the MCHC value would be about twice the haemoglobin concentration.

Cells with low and high levels of MCHC are referred to as hypochromic and hyperchromic respectively.

Reference range: 300–350 g/l.

Anaemia  Anaemias are generally classified as microcytic or macrocytic (Figure 5.11).

![Normal blood smear, Iron deficiency, Megaloblastic anaemia](image)

Figure 5.11  Typical stained blood smears showing normal, microcytic and macrocytic anaemias

The most common cause of microcytic anaemia is iron-deficiency due to blood loss from the stomach, intestine or excess menstruation. Defects in nutrition and or a lack of absorption, chronic inflammation, malignancy and thalassaemia may also cause an iron-deficiency anaemia.

Macrocytic anaemia causes are usually vitamin B12 deficiency, folate deficiency, alcohol abuse and liver disease. Of these the most important is pernicious anaemia, which results from the inability of the stomach to produce the intrinsic factor for the absorption of vitamin B12.

Haemolytic anaemia is due to a loss of RBC caused by an abnormally high rate of destruction due to inherited abnormalities, including red cell membrane defects, haemoglobin abnormalities as in thalassaemia and sickle disease, and metabolic disorders, such as glucose-6-phosphate dehydrogenase deficiency.

Aplastic anaemia is suppression of the blood cell formation in the bone marrow with a failure in the production of all types of cell. Most often aplastic anaemia occurs without any prior history and is termed idiopathic, but it may also be due to certain poisons, drugs, radiation and chemicals. There are a number of pharmaceutical products that have an association with aplastic anaemia in a minority of patients. These include chloramphenicol, non-steroidal anti-inflammatories, thyrostatics, psychotropics and some cardio-vascular drugs. However, it is sometimes difficult to confidently implicate medical drugs as the causative agent due to the idiopathic nature of the condition.

Polycythaemia is defined as an increase in total body erythrocyte mass – the opposite of anaemia. The blood volume is increased with hyperviscosity. It may occur due to abnormally low fluid intake or marked loss of fluid, secondary to defective saturation of arterial blood with oxygen at high altitudes or to a myeloproliferative disorder.
White blood cells

To consider the leucocytes as a group is of limited value, as each type of white cell has its own individual function; therefore, much greater emphasis should be placed on the absolute count for each of the different leucocytes. Traditionally, the differential white cell count expressed the values as a percentage, a practice that still continues and which is requested in many clinical studies. Fortunately, laboratories express the individual leucocytes in absolute values and, as an addition, the percentage figure may also be defined in the clinical laboratory report if required.

*Reference range*: both sexes (3.5–11.0) × 10⁹/l.

Granulocytes

The granulocytes include neutrophils, eosinophils and basophils, although it is questionable whether they should be grouped together.

Neutrophils  In normal blood the neutrophils represent the largest population of the leucocytes. They have a short life span of several days. They increase in response to bacterial infection, infarction, inflammation and acute body stress. Marked neutrophilia may indicate haematological malignancy. Smokers tend to have higher neutrophils than non-smokers, and corticosteroid therapy may increase the neutrophil count. Neutropenia is common in different ethnic groups, particularly Afro-Caribbean and Indian populations, and may be present in subjects on anticonvulsant and antithyroid therapy.

*Reference range*: (2.5–7.5) × 10⁹/l.

Eosinophils  Increased eosinophils (eosinophilia) are found in allergic disorders and parasitic infestations. Other causes include rheumatoid arthritis, sarcoidosis, and acute rheumatic fever, and they may also increase in the resolution phase of infections.

Eosinopenia may occur in shock, major pyogenic infections, trauma, post-surgery and in response to corticosteroids, niacin (vitamin B3) and a number of other drugs.

*Reference range*: (0.1–0.4) × 10⁹/l.

Basophils  The basophils represent a small concentration of the total leucocytes. If markedly increased then this may suggest myeloproliferative disease.

Other causes of basophilia include allergic reactions, ulcerative colitis, myxoedema, the therapeutic use of oestrogens, desipramine and antithyroid medication.

*Reference range*: (0.0–0.2) × 10⁹/l.

Lymphocytes  Normally the lymphocytes represent about 35 per cent of the total leucocyte count. They originate from bone narrow, germ cell centres of lymph nodes and spleen. There are large and small varieties in peripheral blood and they have a life span of several months.

There are two varieties: T-lymphocytes react against foreign proteins, viruses, tissue transplants etc.; B-lymphocytes develop into immunoglobulins.

Lymphocytosis occurs in viral infection, infective mononucleosis, infective hepatitis, chronic infections and thyrotoxicosis. Severe lymphocytosis may indicate lymphocytic leukaemia. A number of drugs can result in lymphocytosis, and these include haloperidol, phenytoin and niacinamide.
Lymphopenia is characteristic of AIDS patients, and can also occur in systemic lupus, renal failure and carcinomatosis. Corticosteroids, lithium, niacin and ionizing radiation may result in lymphopenia. 

Reference range: \((1.5–3.5) \times 10^9/l\).

**Monocytes** Monocytes share the same stem cell as neutrophils; they migrate into inflammatory sites and act as phagocytes under the influence of cytokines.

Monocytosis may be caused by chronic inflammatory processes, rheumatoid arthritis, Crohn’s disease, tuberculosis and haematological neoplasms.

Monocytopenia is generally not a clinical problem. 

Reference range: \((0.2–1.3) \times 10^9/l\).

**Platelets (thrombocytes)**

Thrombocytes are required for the maintenance of normal vascular endothelium. When vessels are damaged they act as a mechanical plug and by releasing serotonin, cause local vasoconstriction. They are essential for thrombin generation and release accessory factors for normal coagulation while also acting as phagocytes.

Thrombocytosis is seen in many inflammatory disorders, as well as in acute and chronic blood loss, after recent surgery, myeloproliferative disorders, haemolytic anaemia and post-exercise.

The most common cause of thrombocytopenia is small clots in samples for haematological assessments, but it is also found in megaloblastic and severe iron-deficiency anaemias, viral infections and aplastic anaemia. Non-steroidal anti-inflammatory drugs, diuretics and alcohol may also cause thrombocytopenia.

Screening for proper platelet function is best accomplished in conjunction with the bleeding time test.

Some drugs suppress the bone marrow, resulting in thrombocytopenia; however, in these circumstances there is usually a corresponding reduction in both white and red cells. Other drugs cause a production of antibodies, which attach to platelets leading to an isolated destruction of the platelet and is termed immune thrombocytopenia. The most common compounds related to thrombocytopenia are heparin, but quinidine, procainamide, carbamazepine and cimetidine have also been implicated.

Reference range: \((140–340) \times 10^9/l\).

**Erythrocyte sedimentation rate and C-reactive protein**

The erythrocyte sedimentation rate (ESR) measurement is carried out routinely on most hospital admissions. This simple laboratory test offers a measure of inflammation as an acute phase reactant. Elevated values are found in most inflammatory conditions, including bacterial and viral infections, tissue damage as a result of surgery, myocardial infarction, rheumatoid arthritis and certain malignancies.

ESR must be measured within hours, and thus is an unsuitable test in drug studies when samples are forwarded to central laboratories. Furthermore, ESR measurements are multifactorial and may be influenced by hypoalbuninaemia, hyperlipidaemia and severe anaemia. Also, there is a relatively slow response to clinical improvement.

C-reactive protein (CRP) is an unspecific marker of inflammation. As an acute phase
reactant it responds similarly to the ESR test, but with a much more rapid response to inflammatory stimulation. The serum CRP levels will begin to increase within 6–12 h after the onset of inflammation. Also, an increase or decrease in the serum CRP concentration follows the changes in the clinical status of the patient much more rapidly than ESR changes; therefore, in monitoring the response to therapy CRP it has a major benefit. In certain inflammatory reactions a 1000-fold increase in CRP will be noted, offering much greater clinical sensitivity. CRP will also show increases in acute infection and in certain malignancies.

Reference range: in healthy non-smoking individuals the mean value is usually less than 1 mg/l, with an upper limit of 6 mg/l.

Urinalysis

Urinalysis performed by dipstick generally consists of the following assessments: pH, protein, blood, bilirubin, urobilinogen, glucose, ketones, nitrite and leucocytes.

pH

The pH of urine in healthy subjects is usually in the range of 4.5–7.8, but generally a much closer interval of pH 5.0–6.0 is found.

In the stomach, gastric acid is secreted in anticipation of a meal; however, there is an increase in blood pH known as the ‘alkaline tide’ after a meal, caused by bicarbonate ions being secreted into the extracellular fluid and then into venous blood that may result in post-prandial alkaline urine.

Acidic pH will be encountered in diabetic acidosis, in subjects on a high protein diet and in kidney failure, whereas alkaline urine may be found in severe potassium deficiency, diuretic treatment and vomiting.

Protein

The sensitivity of dipsticks is set to show negative values at concentration of <30 mg/dl.

Proteinuria can result normally during periods of stress and after physical exercise. Haemoglobin and vaginal secretion could well give positive readings.

In pathological conditions, raised urine protein levels may be found in renal disease, myocardial infarction, cardiac insufficiency, paraproteinanaemia and many other conditions.

In screening for diabetic microalbuminuria, even the trace readings on dipsticks do not provide adequate sensitivity.

Blood

Dipsticks will detect greater than 5 RBCs/μl and the test is specific for haemoglobin and myoglobin.

The principal causes of haematuria relate to the kidney or urogenital tract.

Haematuria is an important sign in glomerulonephritis, stone formation, tumours, intravascular haemolysis and during infections.

Myoglobulinuria is generally due to muscle injury or muscle necrosis and can be found in crush injuries.
Physical exercise can also give positive results.

**Bilirubin**

Normally bilirubin is undetectable, with adults excreting less than 0.1 mg/dl. Values as low as 0.3 mg/dl will give a positive result. The presence of bilirubin provides an early sign of jaundice, biliary stasis, or hepatocellular damage, as in acute, chronic and alcohol hepatitis, liver cirrhosis and liver toxicity.

**Urobilinogen**

Normally, small amounts of urobilinogen are present and the dipstick test will detect concentrations greater than 0.4 mg/dl. False negative results can occur in old samples and in samples exposed to direct sunlight. Drugs that colour urine can give false positive results. Urobilinogen excess may be found when hepatitis, liver congestion, toxicity and liver tumours are present. In situations of overload, such as in haemolytic anaemia, pernicious anaemia, and intravascular haemolysis, increases in urobilinogenuria will be found.

**Glucose**

The assessment of glycosuria probably led to the advent of urinary dipsticks, and these provide an inexpensive method for diabetic screening and self-monitoring. Blood glucose values reaching the renal reabsorption threshold overspill into urine. In fasting subjects’ urine samples the levels are normally less than 20 mg/dl, and on random samples less than 30 mg/dl. The detection limit on dipsticks is normally set at 40 mg/dl. Renal glycosuria may occur during pregnancy and when kidney function falls to 30 per cent or less of normal renal performance.

**Ketones**

The test detects acetoacetic acid and acetone, but does not detect β-hydroxybutyric acid. Values are normally less than 5 mg/dl and the detection limit of the dipstick is set at the 5 mg level. Raised levels are found in diabetic ketoacidosis due to fatty acid metabolism acceleration in insulin deficiency. Ketonuria of non-diabetic origin may be found in hunger states, patients on slimming diets, fever and in vomiting.

**Nitrite**

Most pathogenic bacteria have the ability to reduce nitrate (normally present in urine) to nitrite, and this forms the basis of the test. Urinary tract infections are common and the spread increases with advancing years, particularly in the female.
False negatives may be found in strong diuresis, vegetable-free diets and in subjects being administered antibacterial therapy. This must be discontinued for at least 3 days prior to urinalysis for the test to be valid.

False positive results can occur when samples are left standing at room temperature for lengthy periods.

**Leucocytes**

The limit of detection is set at 10 leucocytes/µl for granulocyte esterase activity, and includes both intact and lysed cells. White cells in the range of 10–20/µl are classified as suspicious and levels greater than 20/µl are considered pathological.

False positive results may occur in excessive protein excretion.

Leucocyturia indicates the presence of inflammatory disease of the kidney and the urinary tract.

The great majority of positive findings are due to urinary tract infections, cystitis, pyelonephritis, glomerular disease and parasitic infestations.

All of the dipstick tests must be considered as preliminary screening procedures, and any abnormal result must be followed by microscopy of urine sediment and other appropriate blood measurements.

**Test selection**

Selection of the most appropriate clinical laboratory tests in drug studies is difficult to define, and that will vary dependent on the compound, data from pre-clinical toxicology studies and the phase of clinical development. In respect of the latter, it is important to remember that the most stringent testing should be undertaken at phase 1 to support early administration of the drug to humans. Thus, the most sensitive tests should be carried out as early as possible in the drug development to determine potential toxicities and so avoid substantially increased cost at the later phases of the drug programme. It is questionable, as to whether sufficient attention is devoted to the chemical structure of the compound and the pre-clinical data in clinical laboratory safety testing that could provide the necessary emphasis towards the most appropriate laboratory measurements.

Supplementing the above criteria will be the need for special testing dependent on the function of the new compound, and this will differ markedly from drug to drug. A definition of special measurements is beyond consideration in this section, but it is strongly recommended that these non-routine tests be discussed with the laboratory to ensure that the pre-analytical conditions are clearly met and the limitation of the procedure known.

If the range of laboratory investigations undertaken is the minimum possible for reasons of economy or endeavouring to minimize results outside the reference interval, then there will always be a possibility of a laboratory adverse event not being detected. It is well worth considering taking an additional sample of blood on all occasions and having this correctly stored by the analytical laboratory, which may then be used at a later date for other tests or for the confirmation of clinically significant abnormal results.

For general safety screening the tests shown in Table 5.11 are recommended as the minimum requirements, and these can be carried out on non-fasting patients.

In phase 1, additional sensitive tests of renal function should be given priority, including...
cystatin C as a measure of the GFR rate and NAG to assess renal tubular function, especially if there was any evidence of renal toxicity from animal studies.

Exclusion criteria and ‘Panic levels’

To define pre-trial patient acceptance criteria is extremely difficult. At phases 1 and 2 the aim is to protect the safety of the patient by the exclusion of sub-clinical disease that might expose them to unnecessary risk. The aim is also to protect the drug by excluding patients with sub-clinical disease that might progress to overt disease during the study and be mistaken for a drug-induced effect. In phase 3 the patients are likely to have other diseases, and owing to the nature of the disease these patients may demonstrate clinical laboratory abnormal results. Furthermore, at phase 3 the monitoring of laboratory tests is undertaken to

<table>
<thead>
<tr>
<th>Table 5.11</th>
<th>The recommended biochemical and haematological tests to be included in a general safety testing profile</th>
</tr>
</thead>
</table>
| Renal function and electrolytes | Urea  
Creatinine  
Uric acid  
Sodium  
Potassium |
| Glucose metabolism | Glucose |
| Bone and Protein metabolism | Calcium  
Total protein  
Albumin |
| Liver Function | Gamma glutamyltranspeptidase (GGT)  
Aspartate aminotransferase (AST)  
Alanine aminotransferase (ALT)  
Alkaline phosphatase (AP)  
Bilirubin |
| Lipid metabolism | Cholesterol |
| Thyroid function (pre-study) | Free thyroxine  
Thyroid-stimulating hormone (TSH) |
| Haematology | Haemoglobin  
Haematocrit  
MCH, MCHC and MCV  
Red cell count  
White cell count  
Differential white cell count  
Platelets  
C-reactive protein |
| Urinalysis | General screening dipstick |
ensure that the drug is not affecting organ functions other than the target organ. Compiling these factors, to define values of acceptance is not an easy exercise.

Ideally, at phase 1 all results should be within the laboratory quoted reference ranges, particularly the measurements related to haematology, renal and liver function. As volunteers tend to be screened without any consideration being given to pre-analytical factors, any abnormality requires further sampling with attention being given to subject preparation and timing of phlebotomy.

In clinical laboratories, study coordinators and project managers need guidance on the interpretation of patient results. Table 5.12 lists the tests of a standard safety profile with detail of the reference ranges, a grey area of results requiring further investigation and ‘panic’ levels. These numerical values are based on clinical experience. The grey area defines test values marginally outside the reference range that demand confirmation for the subject to take part in phase 1 studies. The panic values assume that normal results were expected in the particular phase 3 study and refer to isolated single results, whereas the grey area values need to be examined with respect to organ-related tests. The information can be incorporated into the specific study database as alert ranges and used by study coordinators to contact the investigator immediately or, in his absence, the clinical project manager.

Table 5.12  A numerical guide to the necessary action when test results fall into the grey or panic areas

<table>
<thead>
<tr>
<th></th>
<th>Units</th>
<th>Ref. range</th>
<th>Grey area</th>
<th>Panic levels</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Haematology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC</td>
<td>$10^9/l$</td>
<td>4.0–11.0</td>
<td>2.8</td>
<td>14.5</td>
</tr>
<tr>
<td>RBC</td>
<td>$10^{12}/l$</td>
<td>4.5–6.5</td>
<td>3.5</td>
<td>7.5</td>
</tr>
<tr>
<td>HB</td>
<td>g/dl</td>
<td>13.0–18.0</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td>PCV</td>
<td>fl</td>
<td>40.0–54.0</td>
<td>32</td>
<td>60</td>
</tr>
<tr>
<td>MCV</td>
<td>pg</td>
<td>80.0–100.0</td>
<td>70</td>
<td>120</td>
</tr>
<tr>
<td>Platelets</td>
<td>$10^9/l$</td>
<td>150–400</td>
<td>130</td>
<td>500</td>
</tr>
<tr>
<td><strong>Clinical chemistry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST</td>
<td>IU/l</td>
<td>13–40</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>ALT</td>
<td>IU/l</td>
<td>7–40</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>AP</td>
<td>IU/l</td>
<td>97–240</td>
<td>300</td>
<td>350</td>
</tr>
<tr>
<td>GGT</td>
<td>IU/l</td>
<td>3–38</td>
<td>80</td>
<td>120</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>μmol/l</td>
<td>4.5–20</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>Glucose</td>
<td>mmol/l</td>
<td>3.8–6.0</td>
<td>3.0</td>
<td>8.5</td>
</tr>
<tr>
<td>Urea</td>
<td>mmol/l</td>
<td>2.9–7.3</td>
<td>8.5</td>
<td>11</td>
</tr>
<tr>
<td>Sodium</td>
<td>mmol/l</td>
<td>135–147</td>
<td>125</td>
<td>155</td>
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<tr>
<td>Potassium</td>
<td>mmol/l</td>
<td>3.8–5.4</td>
<td>3.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Creatinine</td>
<td>μmol/l</td>
<td>83–127</td>
<td>170</td>
<td>170</td>
</tr>
<tr>
<td>Uric acid</td>
<td>μmol/l</td>
<td>220–450</td>
<td>150</td>
<td>650</td>
</tr>
<tr>
<td>Calcium</td>
<td>mmol/l</td>
<td>2.24–2.64</td>
<td>2.2</td>
<td>2.75</td>
</tr>
<tr>
<td>Total protein</td>
<td>g/l</td>
<td>59–79</td>
<td>50</td>
<td>85</td>
</tr>
<tr>
<td>Albumin</td>
<td>g/l</td>
<td>38–50</td>
<td>28</td>
<td>58</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>mmol/l</td>
<td>3.1–7.5</td>
<td>2.3</td>
<td>8.0</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>mmol/l</td>
<td>60–150</td>
<td>40</td>
<td>180</td>
</tr>
</tbody>
</table>
Harmonization of data from different laboratories

As mentioned previously, there are many occasions when local laboratories are used to support clinical studies. This is an obvious requirement when drug trials are being carried out on acutely ill subjects, such as patients with myocardial infarction, cerebral vascular accident, etc., but also a number of companies accept the resistance of investigators towards central facilities and use local-area laboratories. There is then a need to adjust the laboratory patient results from the various participating laboratories to attain a comparable structure of results in a single database.

There have been a number of reported procedures for ‘normalizing’ data (Abt and Krupps, 1986; Sogliero-Gilbert et al., 1986; Chuang-Stein, 1992), all based on manipulating factors on the individual laboratory’s lower and upper limits of the reference ranges being employed.

At this point it is important to appreciate that although the reference ranges used in central laboratories are accurately determined on a normal healthy population, and regularly reviewed and governed by standard operating procedures, this may not be the case with local laboratories, whose main interest is diagnostic pathology. Therefore, mathematical manipulation of patients’ results based on lower and upper limits of reference ranges may not always be valid. Further, Groth and de Verdier (1993) commented on the transferability of clinical laboratory data, ‘even with instruments of the same type and from the same manufacturer, and with identical reference materials for calibration, there may well be an inter-instrument difference that jeopardises the analytical goals’.

We were requested to review prolactin measurements undertaken as an efficacy marker and analysed by 14 different European laboratories. All the laboratories used their standard routine diagnostic kit for prolactin measurement. Five reported their results in ng/ml and the remaining laboratories reported in International Units as mIU/ml. However, different diagnostic manufacturer’s kits were used and different international reference preparations were employed as calibrators and so the reference ranges varied dramatically. No amount of manipulation could ever harmonize these data, and as an efficacy marker, the results had to be discarded.

Dijkman et al. (2000) detailed the Virtual Central Laboratory (VCL) harmonization approach developed in the Netherlands, where samples are analysed in local laboratories and the patient results transferred electronically to the VCL data-management system. All participating local laboratories, irrespective of the instruments and technology employed, are provided with calibrators at the outset to determine conversion factors based on linear regression analyses to be incorporated into the data-management system. Concurrently, during the studies calibrator checks are included to ensure no deviations occur.

In our own laboratory, although a dedicated central facility, harmonizing data with different laboratories has had to be carried out in complex studies based in distant locations, including both efficacy and safety markers. The approach used in the Pivotal structure is carried out on randomly selected patient samples, usually 40 in number, shipped by courier immediately after collection and analysed in each laboratory at identical times on the same day. The statistical assessment includes calculation of the mean, median, confidence limits, paired t-test, the Passing and Bablok (1983) regression analyses and the Bland and Altman (1986) bias assessment. The regression equation is used to calculate any necessary correction factors to be applied to the participating laboratories, and the bias assessment used for confirmation.
To illustrate this structure, Figure 5.12 shows the results from two laboratories for serum creatinine determinations. Lab A, the reference laboratory, shows a mean value of 76.8 µmol/l and Lab B a mean value of 90.2 µmol/l. Application of the Passing and Bablok (1993) regression equation converts the results of Lab B to a mean value of 76.2 µmol/l.

The data from the Altman and Bland (1986) bias plots before and after the application of the regression equation are illustrated in Figure 5.13. The uncorrected data in the left-hand

<table>
<thead>
<tr>
<th>CREATININE</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>SE</th>
<th>95% CI of Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAB A</td>
<td>40</td>
<td>76.8</td>
<td>18.02</td>
<td>2.85</td>
<td>71.0 to 82.5</td>
</tr>
<tr>
<td>LAB B</td>
<td>40</td>
<td>90.2</td>
<td>17.15</td>
<td>2.71</td>
<td>84.7 to 95.7</td>
</tr>
<tr>
<td>regressed</td>
<td>40</td>
<td>76.19</td>
<td>18.054</td>
<td>2.855</td>
<td>70.4 to 82.0</td>
</tr>
</tbody>
</table>

Figure 5.12 The descriptive statistics of serum creatinine measurements performed in two laboratories, before and after the application of the Passing and Bablok (1983) regression equation

Figure 5.13 Shows the Altman and Bland (1986) plots of the serum creatinine measurements before and after the application of the regression equation
panel show a 13 per cent positive bias in the results from Lab B. After the application of the regression equation (right panel) all the results surround the zero line, demonstrating satisfactory correction. The maximum difference found after correction represented 6 μmol/l, which for creatinine is considered to be of little clinical significance.

The regression conversion of the data shown in Figure 5.13 can be applied to harmonize results for laboratory measurements carried out in different laboratories. It must be appreciated that this type of correlation and correction study applies at a single point in time; therefore, to ensure that the parameters are maintained during the course of a study, meticulous IQC is mandatory.

It must be apparent from this section that harmonization of data requires cooperation between the analytical laboratories, the coordinating statistician, and the industry’s biometric department if successful standardization of clinical laboratory results between different laboratories can be accurately accomplished.

Data analysis and presentation

The interpretation of efficacy data is generally not too large a problem, as it is usually directly compared with a control group and a definition of the efficacy is predefined. However, a full appreciation of the analytical limitations is an essential requirement, and although population-based statistics are mandatory and helpful it is also important to examine the change in individual subjects. Conversely, safety data present an extremely complex situation, as the change in measurement from normal to abnormal may occur in only a few subjects; thus, population-based statistics are of very limited value.

Two major points of confusion emerge. The first relates to the reference range, where it cannot be assumed that all subjects will fall within the ‘normal’ population and that the crossover between health and disease at the upper limit of the reference range has to be treated as a significant grey area. Also, the more subjects and tests that are included in a study the greater will be the likelihood of finding so-called abnormal results, and in new drug applications vast volumes of clinical laboratory data are included. The second issue relates to the fact that rarely do we find a single test result showing a clinically significant change as, in safety profiles, more that one test will usually be associated with a diseased state or an adverse drug reaction. Unfortunately, multivariate analyses of laboratory data using advanced software technology do not appear to help in this direction. For example, to combine the liver function tests of the aminotransferases, AP and bilirubin into a single database for interpretation only adds to the complications. Interpretation of laboratory reports and data still demands professional observation, which requires a combination of science, medicine, statistics and experience to derive any clinical significance on the testing.

A further complication lies in the fact that perhaps one investigator may find only one or two patients demonstrating minor changes in laboratory reports and considers these to be of little significance. However, there may be over 100 investigators all finding similar levels of the same abnormal results. Unless either the clinical laboratory with its total database or the sponsor is constantly monitoring these, early warning of possible toxicity may be missed.

With adverse events being the fifth major cause of death in hospitalized patients (Lazarou et al. 1998) and laboratory data playing an integral part in this process, it is important that laboratory safety analyses are given considerable attention at all stages of a trial. Generally, the clinical laboratory is unaware of who is on active drug and who is on placebo. Also, the
laboratory has little patient case history information. Therefore, for the laboratory to help in interpretation will demand much closer involvement in the study. Nevertheless, the laboratory does have data on all patients in a particular study or centre and perhaps should be looking at providing interim interpretation on purely the laboratory data.

On many occasions I have been requested to evaluate clinical laboratory data, which is usually a mixture of both efficacy and safety measurements. As mentioned previously, efficacy is not a major problem and interpretation of the derived information is comparatively easy.

The structure below is a simplified approach using Microsoft Excel with Analyse-it (Leeds, UK; http://www.analyse-it.com) clinical statistical software as an example for formatting data, which can no doubt be transferred into more elaborate statistical packages.

Overall, clinical trial safety data is transferred into Excel electronically and using the pivot table facilities separate sheets are cut and pasted for each test using the test name, patient identifiers, occasions and results. These separate result sheets are sorted in increasing test result order in respect of the baseline sample and displayed as graphical presentations with high/low bars for the subsequent occasions. An example is shown in Figure 5.14, which provides a visual impression of the change in data over the study period. If required, the chart may be modified to include error bars based on the range of the SSD, which in the case of calcium is 7.2 per cent and thus highlight results outside this SSD parameter. From this the change from baseline is easily identified and any bias in the on-treatment samples noted.

With the test results in Excel, it is a simple procedure to incorporate ‘if functions’ to highlight results above and below the reference interval and also results showing a significant change from baseline based on the SSD value.

An example of this is shown in Figure 5.15. The left-hand panel detailing the calcium values at baseline and two subsequent occasions. This panel may contain several hundred results, but by the application of ‘if functions’ and filtering, only those results outside the reference range can be viewed for assessment, as shown in the right-hand panel. Similarly, the application of the

![Figure 5.14](image.png)
SSD to highlight reference value changes (RVCs) can also be abstracted from the overall data to demonstrate changes of significance. There is then the opportunity to combine, by cutting and pasting, organ-related tests such as calcium, phosphate, albumin and AP, etc. Also, shift tables can be produced from the spreadsheet. A simple shift table is shown in Table 5.13. This defines the number of test results below, within and above the reference range for each occasion in a study. These shift tables can be developed to a more complex form when there are a significant number of abnormal results, by illustrating the number of test results 25 per cent, 50 per cent and 100 per cent or greater than the upper limit of the

**Figure 5.15** The employment of ‘if functions’ to extract data with values outside the reference range and the SSD

<table>
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<tr>
<th>Subject ID</th>
<th>V01</th>
<th>V13</th>
<th>V16</th>
<th>V01 RR</th>
<th>V13RR</th>
<th>V16RR</th>
<th>% change</th>
<th>RVC</th>
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**Table 5.13** A basic shift table of the calcium data showing the number of subjects in and outside the reference range on the different occasions

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<th>‘Normal’</th>
<th>High</th>
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</tr>
<tr>
<td>Visit 13 (V13)</td>
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<td>64</td>
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</tr>
<tr>
<td>Visit 16 (V16)</td>
<td>8</td>
<td>66</td>
<td>0</td>
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</table>
reference range. It is also of help in interpretation to examine the number of subjects showing a change from within to outside the reference range between the baseline and the last visit, as demonstrated in Table 5.14, when the concern is the number of subjects with a change from normal to high.

**Table 5.14** An example of a shift table to demonstrate the number of subjects showing a reference range change from baseline to final study visit

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<th>High to ‘normal’</th>
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<td>7</td>
</tr>
<tr>
<td>‘Normal’ to ‘normal’</td>
<td>80</td>
<td>4</td>
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</table>

The basic statistical data can be obtained using Analyse-it and the information on the above calcium data is shown in Figure 5.16.

![Figure 5.16](image-url)  

**Figure 5.16** Basic statistical data obtained from Analyse-it software

Outliers can easily be identified from this, and both parametric and non-parametric information provided. Analyse-it can also be used to determine significance in these data, but in general these statistics can give misleading information. We have seen many
occasions when the statistical difference is highly significant, yet from a clinical viewpoint is of dubious significance. Conversely, it is not uncommon for the statistics to show no significance in the data and yet reviewing individual patients may show major clinical differences.

Finally, Analyse-it offers a facility to determine agreement (or disagreement) between the baseline sample and subsequent occasions.

Figure 5.17 illustrates an example of the calcium data as both an $x/y$ plot and a bias plot between baseline and visit 16. The bias data shows a mean difference of 0.037 mmol/l with the upper and lower dotted lines being the 95 per cent confidence limits. The calcium data used in the example are uncorrected for albumin concentration.

This approach merely provides a tool to examine results outside of the reference range and the SSD and to obtain compiled statistical information on the laboratory test data. What is then required is a clinical assessment of the abnormal highlighted results. There is really no substitute for biochemists and haematologists experienced in the field of drug studies to help in the interpretation of laboratory safety data.

**Conclusion**

The views expressed in this chapter are those of the author and may contain some opinions not totally supported either by colleagues in Pivotal Laboratories or other central laboratories offering services to the pharmaceutical industry.

It must be remembered that laboratory medicine is not a static discipline. It is constantly changing with improvement in analytical procedures and technology. The pharmaceutical industry generally does not employ specialists in laboratory medicine and, therefore, there is a need for greater collaboration between sponsoring companies and the central laboratory to ensure success in the laboratory aspects of drug studies.
References


