Guideline

GUIDELINES ON FIBRINOGEN ASSAYS

Haematology departments in the UK have traditionally performed fibrinogen assays to detect decreased levels and abnormalities of fibrinogen, and to assess haemorrhagic risk. It has also been shown that elevated fibrinogen levels are a predictor of a variety of arterial cardiovascular events, and fibrinogen assays are sometimes recommended with this in mind.

The Clauss fibrinogen assay (based on the thrombin clotting time) is the most popular technique in UK hospital laboratories, although many other methods are also in use. There appears to be great variability in both the source of reagents and the exact method used for the Clauss assay. Most laboratories are now equipped with automated coagulation analysers, and many of these perform a fibrinogen estimation derived from the degree of change of light scatter or optical density during the prothrombin time (PT-Fg). A number of problems have been described in the use of the PT-Fg method; it generally gives higher values than the Clauss technique, but the exact degree of discrepancy seems to depend on a number of different variables. International and National standards are available for fibrinogen, but do not appear to be universally used.

These guidelines have been prepared against this background and recommend which methods should be used in various clinical settings, as well as highlighting a variety of problems with fibrinogen assays.

INTRODUCTION

Fibrinogen is a major plasma protein (normal concentration 1·5–4·0 g/l), which is synthesized in the hepatocyte. It comprises two each of three different polypeptide chains (Aα, Bβ and γ) linked by disulphide bridges. Thrombin cleaves the Aα and Bβ chains to release fibrinopeptides A and B, respectively, from the amino-terminal ends. After the fibrinopeptides are released, the resulting fibrin monomers undergo polymerization to form an insoluble fibrin clot (Hantgan *et al*, 1994). Fibrinogen also shows heterogeneity due to cleavage in the circulation, at the carboxyl termini of the polypeptide chains, giving rise to a series of molecules of varying size (de Maat *et al*, 1999). Fibrinogen and fibrin can be degraded to progressively smaller fragments by proteolytic enzymes, including plasmin and neutrophil elastase.

Fibrinogen is an acute phase reactant (Whicher & Dieppe, 1985; Hantgan *et al*, 1994) and levels may be raised in relation to a variety of physiological variables and inflammatory conditions (Table I). The variation of fibrinogen

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levels within the same normal subject has been observed to be approximately 1·00 g/l in a study of 14 normal subjects over a 3-year period, although in some of the subjects fibrinogen levels varied by more than 2·5 g/l (Thompson *et al*, 1987). Such variation must be taken into account when fibrinogen is being considered as a risk factor for ischaemic heart disease. One further pre-analytical variable that has been reported to affect fibrinogen measurements, which is relevant to epidemiological studies, is the season of the year; fibrinogen levels are considerably higher in winter (van der Bom *et al*, 1997). A seasonal difference of 0·34 g/l (95% CI 0·29–0·30 g/l) was present in subjects aged 55–75 years which was more pronounced at age > 75 years.

A polymorphsim of the beta fibrinogen gene promoter (G-455A) has been associated with increased levels of fibrinogen (Humphries *et al*, 1999). Carriers of the A-allele (about 20% of the population) have 7–10% higher fibrinogen levels than those of the GG genotype. Reduced fibrinogen levels may occur as a result of inherited gene defects, resulting in abnormal, reduced or absent fibrinogen protein, or as a result of liver disease or consumptive coagulopathies.

THE ROLE OF FIBRINOGEN ASSAYS

Traditionally, fibrinogen assays have been required in the investigation of haemorrhagic states or in laboratory testing for haemorrhagic disorders (Table II). Some centres use a fibrinogen assay along with the prothrombin time (PT) and activated partial thromboplastin time (APTT), as part of a general haemostatic screen. In this situation, the fibrinogen level replaces the use of a thrombin clotting time, but the information provided may differ depending on the type of assay used. Whereas a sensitive thrombin time will detect low fibrinogen levels, decreased fibrin monomer polymerization, abnormal fibrinopeptide release, and may be influenced by acquired dysfibrinogens, heparin or fibrin/fibrinogen degradation products (FDP's), different fibrinogen assays show varying sensitivity to each of these factors.

Inherited fibrinogen defects

The rare conditions of congenital afibrinogenaemia, hypofibrinogenaemia and dysfibrinogenaemia are due to gene defects, resulting in reduced fibrinogen synthesis and release and/or abnormal molecular species. In afibrinogenaemia, there is a grossly decreased hepatic synthesis of fibrinogen, with very low or undetectable plasma fibrinogen, which results in a haemorrhagic diathesis, with prolonged clotting times and abnormal platelet function. In hypofibrinogenaemia, circulating fibrinogen levels show a mild-to-moderate

Table I. The physiological, pathological and lifestyle factors influencing fibrinogen level.*

Elevated fibrinogen
  Increasing age and female sex
  Season
  Pregnancy and oral contraception
  Post-menopausal women
  Acute phase reaction
  Smoking
  Acute exercise
  Disseminated malignancy

Reduced fibrinogen
  Afibrinogenaemia, hypofibrinogenaemia
  Decompensated liver disease
  Viral hepatitis
  DIC
  Haemodilution

*(Eliasson *et al*, 1993; Hantgan *et al*, 1994; Lowe *et al*, 1997; Kluft & Lansink, 1997; van der Bom *et al*, 1997; Humphries *et al*, 1999).

Table II. The clinical utility of fibrinogen assays.

Investigation of a haemorrhagic state (afibrinogenaemia, hypofibrinogenaemia, acquired or congenital dysfibrinogenaemia)
Investigation of unexpected prolonged/abnormal coagulation tests
Detection of DIC
The establishment and monitoring of prolonged thrombolytic therapy
During Arvin therapy
Risk assessment profiling for arterial disease
Commercial use (diagnostics industry, pharmaceutical industry, blood transfusion service) for quality monitoring and potency assignment (e.g. factor VIII and fibrinogen concentrates)

reduction, and patients may be asymptomatic or have haemorrhagic problems. Dysfibrinogenaemia is characterized by abnormal fibrinogen function, therefore, immunoassays tend to give disproportionately higher levels than clottable protein measurements. In some patients, hypodysfibrinogenaemia is present (reduced levels of fibrinogen protein and abnormal function). Approximately 250 patients with dysfibrinogenaemia have been reported in the literature, 55% of whom were asymptomatic, 25% of whom had a haemorrhagic tendency and 20% of whom had thrombophilia (Haverkate & Samama, 1995). The precise incidence of inherited dysfibrinogenaemia is unclear, as patients are usually detected because of a prolonged thrombin clotting time and obviously this will only be sensitive to certain types of fibrinogen abnormality.

Acquired fibrinogen defects
Acquired dysfibrinogens are usually due to excessive glycosylation of the fibrinogen molecule and typically occur in liver disease. In addition, in hepatocellular failure,

fibrinogen levels may be markedly reduced and fibrinogen function may be influenced by the presence of elevated levels of FDPs. In disseminated intravascular coagulation (DIC), there is gross consumption of fibrinogen due to generalized fibrin formation throughout the microvasculature, and the presence of a variety of degraded forms of fibrinogen and FDPs. Massive blood transfusion or the use of colloids may also result in decreased fibrinogen levels as a result of haemodilution.

Systemic thrombolytic therapy results in a gross reduction in fibrinogen levels and there is a high risk of haemorrhage. Prior to starting a prolonged infusion (over more than 1 d) of a plasminogen activator, and serially after starting therapy, the fibrinogen level may be monitored as an indication of appropriate therapy (target 0·5–1·0 g/l; Ludlam *et al*, 1995). In some patients receiving thrombolytic therapy, a fall in fibrinogen does not occur and an adjustment of therapy is necessary to prevent venous thrombotic extension (Marder & Sherry, 1988).

Increased fibrinogen
Elevated fibrinogen levels are also clinically relevant. Fibrinogen is a major plasma protein and, therefore, a small elevation in fibrinogen level will have a significant impact on plasma viscosity, and thereby modify blood rheology. Increased plasma viscosity (e.g. in hyperviscosity syndrome) has been associated with an increased risk of thromboembolism. In recent years, a number of large prospective studies have shown that the fibrinogen level is a predictor of a variety of cardiovascular events, including stroke, myocardial infarction, leg ischaemia (in arterial disease) and postsurgical arterial re-occlusion (Lowe & Rumley, 1999). This statistical association has remained significant regardless of the type of fibrinogen assay employed, although some authors have suggested that immunological assays may be better predictors of cardiovascular disease than functional assays (Cremer *et al*, 1994; Sweetnam *et al*, 1996, 1998). However, the interpretation of a fibrinogen value at the upper end of the normal range, or of an elevated fibrinogen measurement, in an individual patient is often difficult because of the genetic, physiological and other factors outlined above (Machin & Mackie, 1993). It is not known whether all types of fibrinogen assay are equally sensitive to the increased fibrinogen levels resulting from these different conditions. The various fibrinogen assays may have a quite different sensitivity and clinical utility when used on an individual patient basis.

THE MEASUREMENT OF FIBRINOGEN

A variety of different tests and assays of fibrinogen have been used by clinical laboratories (for review see de Maat *et al*, 1999). The assays vary in the degree of expertise and time required, and the equipment available. Some tests are designed for emergency situations, where an exact fibrinogen level may not be required but merely an estimate of whether the levels are normal or grossly decreased. The expanded range of coagulation automation and reagents has had a varying impact on the performance of laboratory

tests and their precision. Automated coagulometers utilize different technologies to detect fibrin clot end-points. Some employ mechanical principles involving the movement of a metal hook or clot impedance of a ball bearing in a magnetic field; others use photo-optical devices to measure changes in light transmission or light scatter through 90°. Coagulation analysers that use a photo-optical detection principle continuously monitor the change in optical density or light scatter and the degree of change is proportional to the fibrinogen concentration: hence the derived fibrinogen assay. The most reliable derived fibrinogen methods are based on the prothrombin time (PT-Fg), where there is rapid thrombin generation and fibrin formation.

TYPES OF FIBRINOGEN ASSAY

Clauss assays

A high concentration of thrombin (ranging from 35 to 200 U/ml, but typically about 100 U/ml) is added to dilute test plasma and the clotting time is measured (Clauss, 1957). The test result is compared with a calibration curve prepared by clotting a series of dilutions of a reference plasma sample of known fibrinogen concentration, and a result in g/l is obtained. The technique is relatively time consuming and requires a high degree of technical expertise if performed by manual methods, as the end-point is very difficult to determine at the higher dilutions of standard plasma, in test samples with low fibrinogen content, or in samples forming friable clots. Mechanical end-point techniques depend on the tensile strength of the clot and, although this makes them sensitive at low fibrinogen concentrations, they are particularly affected by heparin therapy. Photo-optical systems depend on the change in optical density resulting from fibrin formation, which decreases light transmission and increases scattered light. These methods are usually suitable for detecting weak fibrin formation, but may give erroneous values when fibrin monomer polymerization is slow. They are often affected by turbid or lipaemic plasmas, while the light transmission may be affected by bile pigment and free haemoglobin, depending on the wavelength used by the optical system.

Most laboratories now use commercial reagents and an automated method, and a multiplicity of products are available, varying in thrombin strength, buffer composition (inclusion of inhibitors of heparin and FDP's), calibration method and dilution range. The reagents in common use in the UK have been evaluated (MDA, 2000; Mackie *et al*, 2002) and only small differences in fibrinogen levels were observed. There were significant differences in Clauss estimations depending on the analyser used, but these are unlikely to have a clinical impact ($\sim$0·1 g/l). A reasonable degree of precision was obtained with normal plasma [coefficient of variation (CV) typically 3–7% by optical methods and 6–9% by mechanical]. The performance of Clauss fibrinogen assays by fully automated coagulometers in routine clinical laboratories may decrease the throughput for samples requiring only PT and APTT, and many laboratories only perform them when there is a special

indication. There is also the potential on some analysers for reagent carry-over, as the Clauss assay uses a high concentration of thrombin, which has the propensity of sticking to foreign surfaces and may contaminate subsequent tests (e.g. PT, APTT), resulting in a false shortening of clotting times.

The standard plasma or calibrant must be carefully selected, as very turbid reference plasmas may cause poor linearity on photo-optical coagulometers and poor parallelism with serial dilutions of test plasmas (MDA, 1999). This may lead to incorrect potency assignment, particularly for samples with low fibrinogen levels, when single-point assays are performed. Some commercial reference plasmas designed for use in Clauss assays, appear to be erroneously calibrated when compared with the International Standard. This may reflect the calibration method used by the manufacturer or the use of calibrants other than the International Standard.

Recommendations

Although there is generally little impact of reagent system or analyser on the Clauss assay, certain clinical subgroups (e.g. DIC, thrombolytic therapy) may show greater discrepancies, probably as a result of the thrombin concentration and buffer composition (which will influence the impact of heparin and FDP's). **Where possible, assays should not be performed on samples collected within 4 h of administration of therapeutic doses of unfractionated heparin, or on samples collected from heparin-contaminated venous or arterial lines.** This may be a particular problem during cardiopulmonary bypass or renal dialysis procedures, and in these situations it may be necessary to remove the heparin using suitable ion exchange resins or heparinase enzymes.

The reagent must only be used during its stated period of stability, and care should be taken that pH changes do not occur as a result of the reagent vial remaining open to the air for prolonged periods. If single calibration curves are used for the lifespan of the reagent, ensure that the reagent is still stable/viable for the entire period. **Quality control samples should be used to check for temporal drift.** As most Clauss reagents contain high concentrations of thrombin, **appropriate cleaning methods should be employed on the reagent delivery system of the analyser to avoid carry-over.**

An optically clear fibrinogen standard is desirable to avoid loss of linearity on some analysers. **A suitable dilution range must be used** (this will vary depending on the calibrant, reagent and analyser) and **the calibration curve must be linear** (this normally requires logarithmic transforms). **The calibration curve must include at least three (but preferably five) dilutions** of the plasma, and duplicate clotting times are advised. **Test samples should be re-diluted if they are outside the linear range of the assay. Use a single type of reagent kit, standard and analyser within the laboratory, as the results may not be easily interchangeable between methods.**

PT-derived (PT-Fg) tests

Fibrinogen estimations derived from the prothrombin time (PT-Fg) have been widely used in recent years (Vermylen

et al, 1963; Natelson & Dooley, 1974; Becker *et al*, 1984; Cambas *et al*, 1985; Chantarangkul *et al*, 1987; de Metz & van Wersch, 1987; Hoffmann & Verhappen, 1988; Rossi *et al*, 1988). The analyser is calibrated by performing a prothrombin time on a plasma (or series of plasma dilutions) of known fibrinogen concentration and by plotting a graph of the optical change against fibrinogen concentration. The optical change in each test sample is then converted to a fibrinogen value. As the technique is an indirect measurement of fibrinogen, the choice of calibrant or standard is very important. The attraction of the PT-Fg method is that it rapidly provides a fibrinogen value at no extra cost (other than that of the PT reagents), and it is currently used in approximately 50% of UK Haematology laboratories [UK National External Quality Assessment Scheme (NEQAS) for Blood Coagulation, 2000], although there is controversy regarding the suitability for clinical use. PT-Fg assays are really a collection of associated methods that vary depending on the method of calibration, type of analyser and reagent, and optical clarity of calibrant and test plasmas, as well as the nature of any coagulopathy present.

Calibration methods recommended by manufacturers differ; they may involve single- or multiple-point calibration, and the use of fresh normal pooled plasma of good optical quality or lyophilized plasma with a PT-Fg-specific calibration value. It is difficult to achieve calibration in some PT-Fg methods if the reference plasma has a high fibrinogen level or excess turbidity (MDA, 1999). Reference and test plasmas with high turbidity tend to show an increased discrepancy between PT-Fg and Clauss assays (Mackie *et al*, 2002).

Some, if not all, PT-Fg methods give higher values than the Clauss technique (Palareti *et al*, 1991; Chantarangkul *et al*, 1994; Chitolie *et al*, 1994, 1998; Kitchen *et al*, 1995; Lawrie *et al*, 1998; MDA, 2000; Mackie *et al*, 2002). This phenomenon has been observed in samples from patients with DIC, liver disease, renal disease, dysfibrinogenaemia, high fibrinogen levels and in those receiving anticoagulants, or thrombolytic therapy. In clinical samples, the discrepancy is not consistent over the entire range of observed fibrinogen values and may depend on the nature of the clinical group as well as the thromboplastin reagent used. Some laboratories have found apparently normal fibrinogen levels by PT-Fg, but reduced results by the Clauss assay in patients with dysfibrinogenaemia or those receiving thrombolytic therapy. In these groups, it is unclear which method correlates best with *in vivo* haemostasis. Other workers have found a good comparability between Clauss and particular types of PT-Fg assays (Rossi *et al*, 1988; de Cristofaro & Landolfi, 1998); however, the latter stated that precision was inadequate in PT-Fg assays if either the PT was prolonged (e.g. as a result of anticoagulants) or the fibrinogen level was high.

Recent multicentre studies (MDA, 1999, 2000; Mackie *et al*, 2002) have shown that PT-Fg results vary according to the reagent and analyser (even between different models from the same manufacturer). A higher level of imprecision (CV typically 6–12% for normal plasma) was seen compared with Clauss assays, particularly if turbid reagents and reference plasmas were used. In thrombolytic therapy,

fibrinogen degradation products (which were formed in excess of fibrin split products) interfered in the Clauss assay, but not in PT-Fg. Degraded fibrinogen molecules may reduce fibrin polymerization by termination of the fibrin chain, as they retain at least one fibrinopeptide A domain (Nieuwenhuizen & Bos, 1999). The resulting weak, diffuse clot formation and delayed clot detection would affect the Clauss technique, while a large opacity change would still eventually occur, giving a higher PT-Fg level. This effect would be accentuated as the fibrinogen level fell, and by the dilution effect seen in Clauss assays. Intermediate and late FDPs cause a dose-dependent decrease in Clauss fibrinogen and an increase in PT-Fg (Oosting & Hoffmann, 1997). More turbid fibrin gels are generated at lower thrombin concentrations (de Cristofaro & Landolfi, 1998). As the thrombin concentration generated in the PT-Fg method is substantially lower (on a U/ml basis) than that typically used in the Clauss assay, while the fibrinogen concentration is 2·5–5-fold higher, the fibrin gels generated by the PT-Fg method will be more turbid. This may particularly influence the results under conditions of abnormal fibrin polymerization.

PT-Fg results on clinical samples, particularly those with abnormal fibrinogen molecules and either high or low fibrinogen levels, show a sigmoid relationship with Clauss and other functional assays (Chitolie *et al*, 1998; MDA, 1999, 2000; Mackie *et al*, 2002). The plateau of the sigmoid curve differs depending on the PT-Fg thromboplastin reagent, reducing the upper limit of detection by as much as 2 g/l (at equivalent Clauss values of 5–6 g/l). It is not known whether the upper cut-off limits vary between all reagents or whether there is a significant batch dependency. This problem precludes the use of simple data correction (algorithm) equations to normalize the PT-Fg results to Clauss values.

Recommendations

PT-Fg assays are not recommended for general use in Haematology laboratories. As both the analyser and reagent influence PT-Fg values, each reagent/instrument combination must be regarded as a separate assay type. The results from different instruments or thromboplastin reagents should not be mixed and separate reference ranges are required. PT-Fg assays are only suitable for samples with good optical clarity, where a normal fibrinogen value can be predicted, such as large epidemiological studies of normal populations, where an accurate measurement of either normal or elevated fibrinogen might not be required. In this setting, as well as external quality assessment exercises, it is important that data from different PT-Fg assays are not pooled. Where PT-Fg assays have been used, there may be difficulty in the subsequent interpretation of fibrinogen results from patient records, as the results are not interchangeable between laboratories or hospitals because of the differences between instrumentation and reagents. The reference plasma must be carefully selected for optical clarity, and calibration values must be available in relation to the International Standard (either directly or indirectly). Where necessary, local plasma pools should be calibrated

against the International Standard in a Clauss assay and the observed value should be used in the PT-Fg calibration procedure.

Clottable protein

These assays are very accurate and have been used as reference assays for fibrinogen. Thrombin is added to plasma, in the absence of calcium ions, the clot is washed and then dissolved in alkaline urea or other reagents, followed by a spectrophotometric protein assay or estimation (Ratnoff & Menzie, 1951; Jacobsson, 1955; Blomback & Blomback, 1956; Swain & Feders, 1967; Gaffney & Wong, 1992). As the majority of the protein in the clot will be fibrin, the protein concentration will be approximately equivalent to the fibrinogen concentration. One convenient method (Blomback & Blomback, 1956) is to measure the absorbance at 282 nm and to calculate the fibrinogen concentration using the extinction coefficient. Clottable protein assays are time consuming, labour intensive and technically difficult, making them **unsuitable for routine use in coagulopathy screening. However, they can be of occasional use in the investigation of congenital fibrinogen defects** (Table III).

Immunological assay

A variety of immunological assays are available: enzyme-linked immunosorbent assays (ELISAs), radial immunodiffusion and electrophoretic techniques, take many hours to perform, the former giving the best accuracy and precision. Some clinical analysers (e.g. BN-100; Dade Behring, Marburg, Germany) have a facility for fibrinogen assay using immunonephelometry. All available immunological assays have the disadvantage of measuring protein concentration rather than functional activity. False estimates may be obtained in some methods where degraded forms of fibrinogen are present, as these have a different antigenicity and may migrate at different rates in immunodiffusion and electrophoresis techniques. These effects are likely to explain the discrepancies between fibrinogen antigen and either Clauss or PT-Fg results observed in certain clinical groups.

Some commercial assays utilize monoclonal antibodies directed against terminal regions of the fibrinogen molecule; these allow the detection of intact (non-proteolysed) fibrinogen or various specific types of degraded fibrinogen molecule. Such assays are sometimes useful in research studies or specialized investigations of individual patients.

Table III. The recommended types of fibrinogen assay in different clinical situations.

Bleeding diathesis	Clauss
Congenital fibrinogen defects	Clauss + clottable protein + immunoassay
DIC, acquired defects	Clauss
High fibrinogen levels	Clauss or immunoassay
Thrombolytic therapy	Clauss

Recommendations

ELISAs and immunonephelometric assays give comparable results overall, although the latter has a high degree of variability.

The Second International Standard for fibrinogen has been calibrated by functional rather than immunological methods. **Until a directly calibrated standard becomes available, the same standard should be used for both types of fibrinogen assay and similar potency assumed. It is essential that the same standard material be used for different fibrinogen assays within an individual laboratory, to avoid systematic error.**

Fibrinogen antigen measurement is suitable for studies assessing fibrinogen as a risk factor for cardiovascular disease (Cremer *et al*, 1994; Sweetnam *et al*, 1996, 1998), **where normal or elevated fibrinogen levels are anticipated. It also has utility in the investigation and differentiation of congenital fibrinogen defects** (Table III).

Other methods

The fibrinogen titre is an emergency test where plasma (and, therefore, fibrinogen) is diluted in different types of buffer, before the addition of thrombin (Mackie & Machin, 1989). The titre is reported as the last dilution to display a clot after a given incubation time. The test can be performed in the presence of various inhibitors, such as epsilon-amino-N-caproic acid (EACA), to block plasmin, and protamine to block heparin and FDP's. However, the results are very inaccurate, and the test is time consuming and does not lend itself to automation. **Owing to the widespread availability of automated analysers, the test has virtually disappeared from clinical laboratories and it is not recommended.**

In gravimetric assays (Mackie & Machin, 1989), fibrinogen is clotted from dilute plasma, using a strong thrombin solution and calcium chloride. The clot is compressed to express solution and non-clotted proteins, washed in saline, and then dried and weighed on an accurate microbalance (the number of available decimal places on the microbalance obviously affects accuracy). The assay demands a high degree of technical ability to obtain reproducible results and is time consuming, as the clot must be completely dry before weighing. It is not, therefore, of clinical utility when fibrinogen assays are required urgently. **The method does not lend itself to large numbers of tests, cannot be automated and is not recommended for routine use.**

Sulphite precipitation techniques (Rampling & Gaffney, 1976) have been described, but are unreliable in some clinical situations (particularly in patients with acute phase reaction), and are not normally used in hospital haematology laboratories.

PRE-TEST VARIABLES

The main pretest variables affecting fibrinogen assays are summarized in Table IV. The recommended anticoagulant for fibrinogen assay is tri-sodium citrate (World Health Organization, 1999), at a strength of 0·105–0·109 mol/l

Table IV. Pre-test variables.

Anticoagulant	Tri-sodium citrate (0·105–0·109 mol/l)
Correct filling	9 parts blood to 1 part citrate
Haematocrit	Adjust citrate for polycythaemia
Venepuncture	Clean, fast, minimal stasis
Blood and plasma inspection	Clots, haemolysis, lipaemia, icterus
Platelet depletion	Centrifuge > 1700 g, 10 min, < 10×10^9/l
Storage	4°C for 48 h (unless DIC); −70°C for ≥18 months
Freezing and thawing	Thaw at 37°C for ≥5 min, mix well
Correct dilution	Dilute concentrates and plasmas with high fibrinogen, so that values are within standard curve limits

(1 part anticoagulant: 9 parts blood), as used for other blood coagulation tests (NCCLS, 1998). Inadequate filling of the collection tube affects this ratio and leads to inaccurate results. For haematocrits greater than 0·55 l/l, the final blood citrate concentration requires adjustment and charts are available which indicate the amounts of anticoagulant and blood to be mixed (NCCLS, 1998). It is essential that blood is collected by clean venepuncture, with minimal stasis, and as rapidly as possible (Rosenson *et al*, 1998). Prior to fibrinogen determination, blood samples should be inspected by gentle inversion for the presence of clots. Samples with any evidence of clots or marked haemolysis should be rejected.

Plasma should be prepared so that platelets are removed (to a count of $< 10 \times 10^9$/l) by centrifugation at a minimum of 1700 g for 10 min; this can be performed at room temperature. Severely lipaemic or icteric samples may cause analysis difficulties in some photo-optical methods of fibrinogen measurement. Samples can be stored at room temperature prior to fibrinogen determination, for at least 4 h and probably longer. If more prolonged storage is required, then 4°C is a suitable temperature for Clauss fibrinogen determination, and storage at 4°C for up to 48 h did not affect assay results in one study (Rosenson *et al*, 1998). Plasma can be stored deep frozen for longer periods (Cushman *et al*, 1995), preferably at −70°C, where it is stable for at least 18 months (Blanco *et al*, 2000). Storage of samples from patients with DIC or those receiving thrombolytic therapy may lead to *in vitro* proteolysis of fibrinogen unless suitable protease inhibitors are included in the anticoagulant. If plasma is frozen prior to analysis, it should be immediately transferred to a 37°C water bath, thawed for 5 min at 37°C and mixed by inversion prior to analysis. Any slow or partial thaw can lead to the formation of cryo-precipitate, which contains fibrinogen, and thus may affect the results. For assay of fibrinogen in therapeutic cryo-precipitate products and other fibrinogen concentrates, a full thaw at 37°C is necessary followed by careful mixing to re-suspend the fibrinogen. This material will normally require a greater dilution than normal plasma in order to achieve a concentration that can be successfully assayed by the Clauss technique.

STANDARDS, REFERENCE PREPARATIONS AND REFERENCE RANGES

Local reference plasmas may be prepared by pooling plasmas from at least six healthy normal donors and calibrating against National (British Standard for Blood Coagulation Factors, Plasma, NIBSC) or International (Second International Standard for Fibrinogen, Plasma) standards. These pools should ideally be stored in aliquots at −70°C and completely thawed at 37°C before use. **If commercial reference preparations are used, they should be suitably calibrated;** some have shown significant deviations from the International Standard in their potency estimates (MDA, 1999; Mackie *et al*, 2002). If commercial plasmas are used, it is advisable to select preparations with a good optical clarity wherever possible.

In order to provide reference materials for widespread use, lyophilization is required to prevent the deterioration of fibrinogen. Reference plasmas should not be turbid, as this creates practical problems with the calibration of photo-optical coagulometers for Clauss and PT-Fg assays. High fibrinogen concentrations may be incompatible with PT-Fg calibrations when using certain analysers and reagents. Purified fibrinogen is generally unsuitable in the preparation of standard materials for assay in clinical laboratories, as after its lyophilization there are often problems with solubility, specific activity, degradation and optical clarity.

Reference ranges should be established in each laboratory, using at least 40 healthy normal subjects, covering a wide age range and both sexes. Blood samples from patients should not be used, even if they are free from any major illness, as it is likely that they will be stressed or may have an acute phase reaction, which would skew the range of values.

QUALITY CONTROL

It is recommended that all laboratories participate in an accredited external quality assessment scheme and investigate any evidence of local problems. For participants in larger groups, it may be possible to identify methodological bias related to particular components of the assay (such as reference plasma or source of reagent) and these should be

taken into account when selecting a suitably standardized technique.

An internal quality control (QC) sample, with a normal fibrinogen level, should be analysed with each group of fibrinogen determinations (or at intervals of 2–3 h for continuous processing systems). A QC sample with reduced or raised fibrinogen may be useful, depending on the purpose for which fibrinogen assays are being performed, or if unexpected results are obtained from test samples. A written record of internal QC results should be maintained to include the result, time of analysis and reagent batch/lot numbers. It is useful to maintain a cumulative record, preferably as a graph, because this can identify any gradual drift in QC results, which could indicate that the method is moving out of the range of the control. If a QC result lies outside acceptable limits (frequently provided by manufacturers as a target range), then patient testing must be suspended until the problem has been identified and resolved.

AUDIT

Routine audit of the laboratory measurement and the clinical utility of fibrinogen assays should be incorporated into routine practice. The potential variability of laboratory methodology, involving both reagents and end-point detection systems, should be regularly examined by participation in an accredited external quality assessment scheme. Clinical audit is more complex and should involve the diagnostic value of a low/normal fibrinogen level in the investigation of acquired and inherited bleeding disorders, and also the value of normal/high levels in the risk assessment of thrombotic cardiovascular disease. For the investigation of bleeding disorders, a comparison of fibrinogen levels with the thrombin time, the variation during thrombolytic therapy and the correlation with replacement therapy (i.e. fibrinogen concentrates) should be studied. Specific levels of fibrinogen should be defined for further action, as part of clinical protocols (i.e. the levels following massive transfusion or acute DIC when specific replacement therapy must be initiated). Risk assessment of arterial thrombosis is more controversial, but levels of risk in specific diseases (i.e. myocardial infarction) need to be defined with actual fibrinogen levels divided into quartiles (or even quintiles) of the overall normal range. These ranges could then be discussed with the relevant clinicians, so that patients may be counselled as to their overall risk, specific interventions advised (such as stopping smoking), and the timing of follow-up measurements decided. To differentiate high levels from an acute phase response, comparisons with other acute phase proteins, particularly C-reactive protein and von Willebrand factor antigen, can be studied.

CONCLUSIONS

The Clauss fibrinogen assay appears to be the most reliable method for general use in clinical laboratories. In the UK, most clinical experience with fibrinogen levels in coagulo-

pathies relates to the Clauss assay and most educational material is also based on this technique. The PT-Fg method and immunological techniques may be useful in large population studies where elevated fibrinogen is sought, but may not always provide accurate fibrinogen measurements. For the investigation of congenital dysfibrinogenaemias, both functional (Clauss or clottable protein assays) and immunological fibrinogen assays should be performed (Haverkate & Samama, 1995) to identify any discrepancy between results.

PT-Fg levels appear to show a false elevation in a variety of clinical settings and, if the assay is used, it must be supported by a different functional assay before either diagnosis or management decisions are made. **In light of the available literature, which shows that the PT-Fg measurement is significantly influenced by the choice of calibrant, reagent and analyser, the test is not recommended for routine clinical use.**

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TASK FORCE OF THE
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IN HAEMATOLOGY

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