Guidelines on the laboratory aspects of assays used in haemostasis and thrombosis

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INTRODUCTION

Publications known to the writing group were supplemented with additional papers identified by searching Medline/PubMed for publications in the last 12 years using keywords: coagulation assays, amidolytic assays, haemostasis assays, and thrombophilia testing, in core clinical journals and English language. Additional relevant articles were identified by screening reference lists and by publications known to the writing group. The writing group produced the draft guideline which was subsequently revised by consensus by members

of the Haemostasis and Thrombosis Task Force of the British Committee for Standards in Haematology (BCSH). The guideline was then reviewed by a sounding board of approximately 50 UK haematologists, BCSH and the British Society for Haematology committee and comments incorporated where appropriate. The 'GRADE' system does not apply (because clinical trials are not available to support the 'best practice' recommendations that have been generated) and was therefore not used.

GENERAL INTRODUCTION

This guideline is intended to help clinical laboratories perform valid and reliable assays of pro- and anticoagulant factors in blood. Guidance on lupus anticoagulant (LA) testing, fibrinogen assays, D-dimer assays, platelet function tests, von Willebrand factor assays and anticoagulant monitoring is available in other BCSH and UKHCDO documents (www.bcshguidelines.com; www.ukhcdo.org/UKHCDOguidelines.htm). Clinical guidelines on thrombophilia, haemophilia and other bleeding disorders are also available from these bodies

and should be used in conjunction with these laboratory guidelines. Global assays of coagulation such as thrombelastography or thrombin generation will not be considered here.

PRE-ANALYTICAL VARIABLES

Blood sample collection and processing

The requirements for blood collection, handling and storage have been discussed in detail elsewhere [1–3], but key points are summarized as recommendations in Table 1. Particular attention is drawn to the correct filling of tubes containing the appropriate anticoagulant and the effects of high haematocrit.

Many tests used to assess haemostasis can be influenced by medication and this must be taken into account. Coagulation screening tests such as prothrombin time (PT) and activated partial thromboplastin time (APTT) may provide useful information (particularly when full clinical details are not available) which can help to avoid problems in the interpretation of coagulation assay results

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Table 1. Summary of recommendations for blood collection, handling and storage

Blood collection
    Perform clean venepuncture with minimal stasis
    Use a 21-gauge needle or butterfly (19 gauge may be used in adults with good veins; 23 gauge may be required for infants)
    Do not use heparin-contaminated venous lines. Where this is unavoidable because of poor venous access, flush the line with crystalloid and discard the first few millilitres of blood
    Use 0.105–0.109 M tri-sodium citrate (9 volumes blood to 1 volume anticoagulant)
    Use plastic or siliconized glass collection tubes
    Ensure correct filling of tube
    Blood and anticoagulant should be mixed immediately by gentle inversion 5–6 times
    If the haematocrit is >0.55, adjust volume of anticoagulant
Handling
    Transport blood samples rapidly (ideally within 1 h) at room temperature, or centrifuge, separate the plasma, freeze and ship on dry ice. Follow the institution's and courier's safety procedures (for biological and dry ice hazards)
    Centrifuge at 2000 g for at least 10 min at 18–25 °C (ensure platelet depletion)
    Inspect sample after centrifugation for clots, icterus, haemolysis and lipaemia (clotted or haemolysed samples should be rejected)
Storage and preparation
    Avoid prolonged storage at 4 °C; analyse within 4 h
    If plasma is to be stored frozen, use screw cap polypropylene tubes with an 'o'-ring
    Storage at −70 °C (or below) is recommended and essential for long periods of time. Storage at −20 °C should only be used for short periods (up to 2 weeks). Freezers with autodefrost cycles must not be used for storing haemostasis samples
    Prior to analysis, thaw rapidly to 37 °C in a water bath (this usually takes at least 3–5 min for a 1–2 mL sample) and mix gently to resuspend any cryoprecipitate

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(e.g. anticoagulant effects or the presence of coagulopathy).

Haemolysis can affect some tests of haemostasis, either because of the presence of thromboplastic substances or interference of haemoglobin pigment with photo-optical systems. For example, the APTT can be shortened and antithrombin (AT) decreased by *in vitro* haemolysis [4]. Where *in vitro* haemolysis has occurred, such samples should not be tested. Gross icterus and lipaemia may also affect analysers, by interfering with optical absorbance or impeding light transmittance. Rheumatoid factor and anti-mouse antibodies may produce erroneous results in immunoassays [5, 6].

Reference intervals and cut-off values

Whenever possible, normal reference ranges should be established locally with an appropriate number of healthy normal subjects; apparently normal patients should be excluded, as they may be subject to stress which influences certain haemostasis proteins. A minimum number of 120 subjects have been recommended for establishing reference intervals [7]. Some authors suggest that a close approximation can be obtained by testing at least 30 normal subjects [8], but with groups <50, the values defining the reference interval have a confidence interval wider than the standard deviation of the observations [9]. The reference interval may vary for different reagent and analyser combinations and for some analytes, age, sex and blood group should be considered in the interpretation. Specific and separate intervals are required for paediatric populations [10, 11].

The manufacturer quoted or other, existing normal range may be revalidated in a sample population (20–40 normal subjects, depending on the required accuracy) by investigating whether the range of values is similar [7]. When calculating a reference interval, the data should be statistically evaluated to see whether they fit a normal distribution (in which case the mean plus or minus two standard deviations may be calculated) or whether data are skewed, in which case it might be possible to normalize it by log transformation before calculating the interval and converting back. However, it is important to note that being outside the reference interval is not the same as being clinically significant. For some analytes, it is

more appropriate to establish cut-off values using normal or patient populations, in which case receiver operating characteristic (ROC) analysis is usually required, for example when studying D-dimer for DVT exclusion [12].

Recommendations

- **Coagulation screening tests should be performed prior to haemostasis assays to provide background information.**
- **The presence of haemolysis, lipaemia or icterus should be noted as these may interfere with analytical procedures.**
- **Clotted or haemolysed samples should not be analysed (unless *in vivo* haemolysis is confirmed).**
- **Reference ranges or clinical cut-off values should be locally validated for the reagents and analyser in use.**

ASSAY TECHNIQUES AND REAGENTS

Coagulation end-point assays

Coagulation end-point assays are bioassays used for estimating the concentration of individual blood coagulation factors within a test sample. The concentration is determined relative to a standard or reference preparation (that has itself been calibrated against a standard), by assessing the degree to which dilutions of those preparations (i.e. the standard and the test) correct the coagulation time of a substrate plasma specifically deficient in the factor, in a suitable test system (e.g. PT or APTT).

A linear portion of the dose response curve should be determined for each set of reagents by testing a wide range of normal plasma dilutions (e.g. serial doubling dilutions 1/2–1/1024, plus a buffer blank to determine the maximal coagulation time). At least two dilution sets should be made independently to avoid systematic error. This process produces a classical sigmoid dose–response curve with a central linear component (Figure 1a). The differences between plasma dilutions in the series selected for an assay should be as large as possible (i.e. twofold, threefold, fourfold, etc. dilutions), to enable the assay system to discriminate between potency values. However, the coagulation times must remain within the linear por-
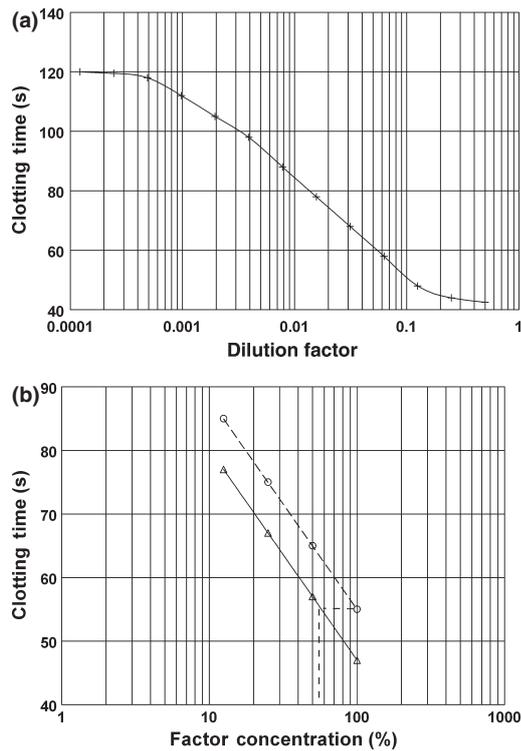
Figure 1. One- and two-stage coagulation assays. (a) Schematic representation of a coagulation assay dose–response curve; (b) Graphical solution of a coagulation assay. Solid line = Coagulation times of reference preparation; broken line = Coagulation times of test sample.

presence of antibodies to coagulation factors, LA, unfractionated heparin (UFH), direct factor Xa (FXa) or thrombin inhibitor), or test sample activation (e.g. because of poor venesection, or the presence of activated coagulation factors) (Figure 2). Determining the relative potency from a single dilution of test sample can give misleading results because linearity and parallelism cannot be determined. Parallelism of the test and standard results is essential for validation of an assay.

Although originally assessed subjectively from manually plotted graphs, computer programs [14–16] improved the reliability and accuracy of bioassays. The programs assess variance ratios and statistically significant deviation from linearity (if a minimum of
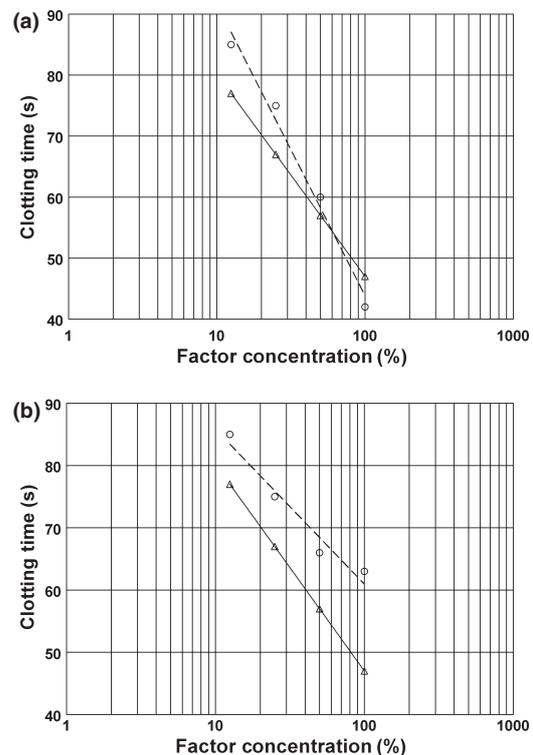

Figure 2. Detection of activated samples and samples containing inhibitory activity in a parallel-line bioassay system. (a) Activated test samples tend to show an apparent decrease in relative potency as the sample is diluted; (b) Test samples containing inhibitory activity show an apparent increase in relative potency as the sample is diluted. Solid line = Coagulation times of reference preparation; broken line = Coagulation times of test sample.

tion of the dose–response curve. The selection of dilutions must also be balanced for their ability to allow the determination of relative potencies over a wide range of concentrations.

Coagulation assay data may be analysed by the parallel-line method [13], where the dose–response curve of serially diluted test plasma is plotted against the corresponding dilutions of a standard (or reference preparation). With coagulation times on the *Y*-axis and log concentration (or dilution) on the *X*-axis, the line of best fit is constructed through the data points of the standard and then the best parallel straight line is constructed for the test sample (Figure 1b). In some cases, coagulation times may need to be log transformed to achieve linearity. This process allows not only quantification of relative potency, but also reveals any evidence of inhibition (e.g. because of the

three dilutions of test sample and standard are used) or parallelism (if a minimum of two dilutions are tested). By testing the reference preparation at the beginning and end of the run, the effect of temporal drift during the assay can be assessed and removed from the potency estimate. These programs have been incorporated into modern, automated coagulation analysers so that a calibration curve is processed, assessed for linearity and then subsequently either validated or rejected by the operator. If accepted, the curve is saved and used to determine the relative potencies of test samples. Validation of a linear calibration curve is usually achieved by measuring the correlation coefficient (r), which should generally be 0.998–1.000. Deviation from the usual r-value for a given assay may indicate contamination or deterioration of reagents, such as pH drift in buffer or reagent. A few coagulation analysers perform a statistical evaluation of linearity and parallelism of the calibrator and test sample(s). From the regression model, a slope ratio (calibrator slope/test sample slope) can be calculated (to estimate parallelism) and this should normally be in the range 0.9–1.1; when the ratio falls outside this range, it may indicate an activated sample or the presence of an inhibitor.

The use of stored calibration curves on analysers is widely practiced and can give acceptable results for a single reagent batch [17], but has not been recommended [18] because test samples and standards should be treated in exactly the same way to avoid analyser or operator errors, the effects of different buffer batches, changes in ambient temperature, and other differences.

Analyser calibration curves are usually composed of four to six user definable dilutions covering factor levels that are commonly encountered in the laboratory (e.g. 10–150 U/dL activity), although it is necessary to have different standard and/or test sample dilution series for samples with very low or high activity. The practice of extrapolating relative potencies from data points outside the standard curve range is unsafe and should be avoided. Low values should be reported as being less than the sensitivity limit of the assay, while samples with high values should be diluted and tested again.

Recommendations

- **Stored calibration curves generated in a different assay batch should not be used.**

- **At least three test dilutions should be used to determine linearity and parallelism.**
- **Test samples must have coagulation times within the range of times for the standard curve.**

Amidolytic substrate assays

Synthetic amidolytic (chromogenic) substrates contain three or four amino acids selected to give appropriate specificity for the given coagulation serine protease [19]. A chromophore, usually para-nitroaniline (pNA) is cleaved from the C-terminus amide bond by the protease and free pNA exhibits a yellow colour with absorbance at 405–410 nm, which can be detected by most modern coagulation analysers. The absorbance is proportional to the protease concentration (or inversely proportional to the concentration of natural inhibitors such as AT). Assay specificity may be improved by the inclusion of specific protease inhibitors or direct activators. Fluorogenic substrates have also been employed, but are generally less automated, time-consuming and require careful set-up of individual fluorometers. However, fluorescence is less affected by sample turbidity and these assays have greater sensitivity.

Amidolytic assays are performed as end-point assays (absorbance is measured after a fixed incubation time), or kinetic assays (the reaction rate is continuously monitored). End-point assays may be unreliable if sample opacity adds to the absorbance. Some analysers record initial absorbance and subtract it from the end-point value, which reduces this problem. When the analyte level is above the upper level of the calibration curve, substrate depletion can occur and causes underestimation. Kinetic assays are recommended for reliability, but they must be designed to operate within the linear portion of the reaction rate curve and if a nonlinear curve is observed, the results will not be reliable (a different plasma dilution may rectify the problem). If serine proteases other than the one being assayed (or proteases complexed to alpha-2-macroglobulin (A2M)) are present in the test sample, they may cleave the substrate and cause erroneous values [20]. High bilirubin levels may cause problems in amidolytic assays where there is little dilution of the test sample [e.g. protein C (PC) assays], and this may be corrected by employing a reagent blank.

Quantitative immunoassays

A variety of immunoassays are available for haemostasis proteins, including radial immunodiffusion (RID), immunoelectrophoresis (IEP), ELISA and immunoturbidimetric (ITB) methods. The latter two are generally recommended for their ease of use, precision and sensitivity. They are usually employed to identify type II defects (where a dysfunctional protein is present, i.e. antigen exceeds activity) and to detect molecular variants where activity assays are inappropriate or unavailable.

Assay detection limits

It is important to know the lower limit of detection for most analytes [21]. In coagulation assays, an indication is obtained from the buffer blank time. However, to establish an accurate detection limit in coagulation, amidolytic and immunoassays, the minimum dilution of the analyte that reliably gives a value statistically significant from zero may be determined. When clinical samples are tested, results below the detection limit should be reported as being less than this value.

Calibrators and quality control samples

World Health Organization (WHO) International Standards (IS) are available from the National Institute for Biological Standards and Control (NIBSC, Potters Bar, Hertfordshire, UK) for most plasma coagulation factors. However, these are only intended for calibrating working standards. The International Society for Thrombosis and Haemostasis/Scientific and Standardization Committee Secondary Coagulation Standard (distributed by NIBSC) has been calibrated against the WHO IS and is available to manufacturers for the calibration of commercial reference preparations. Lyophilized or frozen commercial calibrators are also available with assigned potencies for most coagulation factors, relative to either pooled normal plasma, or the WHO IS. For factors where there is an IS available, potencies should be assigned in IU.

Most reagent manufacturers produce normal and abnormal QC preparations, although the latter are usually diluted normal plasma and may have different properties to true, pathological patient samples.

Commercial QC preparations are assigned potencies for most coagulation factors either relative to a pool of normal plasma or relative to the WHO IS. QC preparations should be run with every batch of tests to ensure that adequate precision is maintained. The slope of the assay calibration curve may also be recorded and monitored for QC purposes. Clinical laboratories should also take part in accredited external quality assurance (EQA) schemes for each analyte, to ensure that there is no long-term drift in results and that they are comparable with those achieved by peer group laboratories.

Recommendations

- **Only standards and calibrators with potencies traceable to an IS should be used (and the results expressed as IU/mL or IU/dL), unless there is no established IS preparation for the analyte.**
- **At least two levels of internal quality control samples (normal and pathological) should be used.**
- **Clinical laboratories should participate in EQA for each analyte and assay type that they routinely use.**

'In house' methods

When 'in house' methods are used in clinical laboratories for the purpose of diagnosis or patient management, they should be validated to the same standard as 'CE' marked products to meet the requirements of the EU In Vitro Diagnostic Medical Devices Directive [22]. This also applies to the use of commercial assays on analysers for which the product does not have a CE mark.

ASSAYS OF PROCOAGULANT COAGULATION FACTORS

One-stage coagulation assays

In clinical laboratories, coagulation factor levels are predominantly measured by one-stage assays based on the ability of test samples to correct either the PT (for factors VII, X, V, II), or the APTT (for factors XII, XI, VIII, IX), of a factor-deficient substrate plasma. PT reagents vary in their source of tissue factor (e.g. rabbit brain, human placenta and relipidated recombinant

human tissue factor). Human tissue factor reagents usually give the best sensitivity in coagulation factor assays (see Bolton-Maggs *et al.* [2] for further discussion in relation to diagnosis of rare bleeding disorders). Some of these preparations also contain a heparin neutralizing agent. APTT reagents vary in type of contact activator (e.g. ellagic acid, kaolin, or micronized silica) and phospholipid composition (e.g. rabbit brain cephalin, soy bean phospholipid extract, or synthetic phospholipid mixtures). Some preparations are relatively insensitive to LA [23–25], and these may be advantageous, making the assay more specific. Factor-deficient substrate plasmas are available from congenitally deficient human donors or after specific depletion (chemical inactivation or immunoadsorption) of normal plasma and are available lyophilized or frozen. Wherever possible, the specificity of the deficient plasma should be checked to ensure that there is a single factor deficiency. The deficient plasma should have normal levels of the nondeficient factors and <1 U/dL of the deficient factor. It has been reported that some FVIII immune-depleted plasmas (also deficient in VWF) can give anomalous results in the Nijmegen modification of the Bethesda assay of FVIII inhibitors [26]. When cooled reagent positions on coagulometers are used for deficient plasma, the plasmas should be allowed to equilibrate before use to avoid temporal drift.

Snake venoms have been used as activators instead of PT and APTT reagents to improve specificity [27, 28] and some forms of factor X deficiency not detected by the conventional assays have been identified using Russell's viper venom as activator [29].

Amidolytic and two-stage assays of factor VIII:C

Factor VIII:C is most commonly assayed by one-stage APTT-based assay (reviewed by [30]), but two-stage coagulation and amidolytic methods are sometimes used. Amidolytic assays are available for many coagulation factors [19], but their use other than for FVIII assay is usually limited to prekallikrein (where one-stage assays have poor sensitivity) and factor X (used for monitoring oral anticoagulant therapy, particularly in patients with LA [31–33].

The results obtained by the different types of FVIII assay are concordant in most instances, but in certain situations may differ significantly [34], particularly in DIC and to a lesser extent in pregnancy. In mild and moderate haemophilia A, the assays usually, but not always, yield concordant results [35, 36]. There are reports of a twofold discrepancy between results of one-stage and other assays in up to 40% of mild haemophilia A cases [37, 38]. Importantly, up to 10% of mild/moderate haemophilia A cases have a normal one-stage activity and APTT, but a reduced activity determined by the two-stage coagulation [39] or amidolytic assay [40]. In these circumstances, the bleeding symptoms are usually in accord with the two-stage or amidolytic assay result and the discrepancy is associated with FVIII gene mutations destabilizing the interaction between the A1 and A2 domains.

The opposite discrepancy (one-stage activity is reduced and two-stage coagulation or amidolytic activity is normal) has also been described. At least some of these cases [41] are not associated with a bleeding disorder, but have been identified when investigating chance findings of an isolated prolonged APTT [39, 42], although other such cases with different genetic defects may have a mild bleeding disorder.

Some FVIII concentrates display potency discrepancies when different assay methods are used and this can cause problems when assessing postinfusion recovery. For such products, the use of the one-stage method can produce unexpected and confusing results when the labelled potency of the concentrate has been determined using the amidolytic assay. This discrepancy can be avoided by using a concentrate-specific standard [43, 44].

Recommendation

- **APTT reagents with low LA sensitivity should be used in one-stage assays to improve specificity, unless the presence of LA has been excluded.**
- **Two-stage or amidolytic FVIII assays should be performed in addition to a one-stage assay, to ensure detection of all mild/moderate haemophilia A cases and to correctly assess the severity.**
- **Concentrate-specific standards should be used when recommended by the manufacturer for FVIII assays in patients treated for haemophilia A.**

Factor XIII assays

The most widely used procedure for the detection of factor XIII (FXIII) deficiency is the clot solubility test. Patient plasma is incubated with thrombin in the presence and in the absence (abnormal control) of calcium ions until a clot is formed. The clots are then suspended in 5 M urea, 2% acetic acid, or 1% monochloroacetic acid. A survey conducted by UK NEQAS suggested that the most sensitive combination was thrombin and acetic acid. The test is poorly standardized; relatively insensitive, detecting only severe deficiency (i.e. <5% FXIII) [45]; and may be misleading. Specific assays [46] are required to detect mild deficiency and should be used in preference.

Chromogenic FXIII assays based on quantitative ammonia release offer good precision and sensitivity and are readily automated on coagulation analysers capable of monitoring a reaction at 340 nm. An iodo-acetamide blanking procedure is recommended for samples with < 10% FXIII activity to avoid overestimation of the FXIII level, which may be clinically significant [47]. A fluorogenic FXIII assay [48] is also available. Immunoassays using automated ITB or ELISA methods may be used to distinguish between FXIII-A and FXIII-B subunit deficiency, which is important if therapy with recombinant FXIII-A subunit is planned.

Recommendations

- **Clot solubility screening tests for FXIII deficiency have poor sensitivity and may be unreliable; therefore, specific assays of activity should be used.**
- **Immunoassays of FXIII-A and FXIII-B subunits should be performed to confirm and further categorize any deficiency.**

ASSAYS OF NATURAL ANTICOAGULANTS

Antithrombin assays

Amidolytic assays of antithrombin

Functional assays of AT are recommended in thrombophilia testing [49]. Amidolytic AT assays are simple and precise and are recommended. Thrombin or FXa is incubated with diluted plasma and heparin, and then residual enzyme is measured using an amidolytic substrate; absorbance being inversely proportional to AT concentration. Heparin cofactor II influences human thrombin-based AT assays and therefore these are not recommended [50, 51]. Bovine thrombin and human or bovine FXa-based AT assays are considered specific.

AT function may be decreased due to defects in the heparin-binding site (HBS), reactive site or mutations with pleiotropic effects [52, 53]. In type II deficiency, AT level may be reduced when measured by bovine thrombin–based assays, whereas FXa-based assays may be normal [54]. Shortening the incubation time of the sample, dilution with the enzyme may increase the sensitivity to type II AT defects [55, 56]. It may be that bovine thrombin–based assays are most sensitive, however no single assay can be guaranteed to detect all type II AT defects.

Antithrombin antigen assays

AT antigen may be measured by RID, although ELISA and ITB assays have greater precision, but its measurement is only necessary to identify type II AT deficiency. This may be clinically relevant; in particular the HBS defect has a low thrombotic risk in the heterozygous form [52, 57]. A reference range for AT activity/antigen ratio can help identify type II defects. Further investigations might include an AT assay with a different enzyme or crossed IEP with heparin in the first dimension to detect type II HBS defects. The 'progressive AT assay' (AT assay in the absence of heparin) will be normal when the defect is limited to the HBS, but these assays are not readily available and lack specificity and sensitivity. Some laboratories proceed directly to genetic analysis of the AT gene (*SERPINC1*) which is now relatively easy to perform. Frequently, but not always, the type and location of the mutation defines the defect and accurately predicts the phenotype.

Recommendations

- **The initial assay for AT deficiency should use FXa or bovine thrombin in an amidolytic, heparin cofactor AT assay.**
- **When AT deficiency is identified, further tests should be performed to distinguish type II HBS defects from type I and other forms of type II deficiency.**

Protein C assays

Coagulation end-point assays

To measure its activity, PC in test plasma is usually converted to activated PC (APC) by an enzyme from Southern Copperhead snake venom [58], known as Protac® (Pentapharm, Basle, Switzerland). APC prolongs the coagulation time of PC-depleted plasma through destruction of factor FVa or FVa and FVIIIa (depending on assay type). APTT-based assays are not influenced by the type of contact activator, but the phospholipid composition is important, as it influences sensitivity to the anticoagulant effect of APC, which in turn is related to the reagent sensitivity to FV and FVIII. There is also variability according to the protein S (PS) dependence of the assay [59, 60]. Different graphical transforms are required, depending on the APTT reagent, to achieve a linear dose–response curve [60].

APTT-based assays may underestimate the PC level in samples with high FVIII, while FV Leiden impairs the destruction of both FVa and FVIIIa and may reduce PC levels measured in coagulation assays [61, 62]. Predilution of patient plasma in PC-depleted plasma may reduce or remove the influence of FV Leiden [62], although this increases costs and may reduce sensitivity to low levels of PC. Commercial coagulation-based PC assays generally recommend testing a single patient plasma dilution; however, we recommend testing three dilutions of standard and test plasma so that parallelism can be assessed. LA may affect the assay depending on the reagent phospholipid concentration and composition. Coagulation assays may detect functional defects that are missed by amidolytic and antigenic assays. Approximately 75% of PC-deficient patients have type I deficiency (low antigen and function); 95% of the remainder have type IIa deficiency, with low PC levels by both coagulation and amidolytic assays; deficiency detected solely by the coagulation assay (type IIb) is rare. The consequences of failing to detect a rare functional defect by amidolytic assay must be balanced against the poorer specificity and generally poorer precision of coagulation assays. The latter may be required for diagnosis of severe homozygous PC deficiency, where a type IIb PC defect is undetected using the amidolytic assay [63].

Amidolytic protein C assays

The amidolytic PC assay is recommended for routine screening for PC deficiency because the assays are functional and are more specific than coagulation assays [49]. APC generated in patient plasma following addition of an activator (see above) is measured using an amidolytic substrate. The APC substrates have relatively poor specificity, being cleaved by various other serine proteases (e.g. factor XIa), either free or complexed to A2M. Over estimation can be avoided in end-point assays by including a blank, where the activator is replaced by buffer. The absorbance value for this blank is subtracted from the test absorbance and the PC level is calculated. In kinetic assays, this may be more problematic, as it is difficult to determine the contribution of other proteases to the rate of cleavage of the substrate, although this may sometimes be indicated by poor linearity of the reaction rate. Activator blanks should always be performed where increased effects of nonspecific proteases are anticipated, for example with paediatric samples, and in patients with disseminated intravascular coagulation or pathological fibrinolysis. In this situation, a blank must also be performed for calibrants and QC samples. Partially clotted samples and serum give a false high PC level with substrate depletion that cannot be corrected using a blank.

PC may be over estimated by amidolytic assay in patients receiving vitamin K antagonists (VKA), because of the presence of acarboxy PC, which has similar reaction kinetics to fully carboxylated PC and when activated, cleaves the substrate. In a study of patients stabilized on oral anticoagulants, mean PC values were 28%, 29% and 57%, by coagulation assays based on the PT, APTT and an amidolytic assay, respectively [64].

Protein C antigen assays

PC antigen is generally assayed by ELISA or IEP, the latter being time-consuming with poor sensitivity and precision. EDTA must be included in the IEP assay to ensure that both carboxylated and acarboxy forms migrate at the same rate [65]. ELISA is relatively precise and sensitive to low levels of PC. Antigen assays differentiate type I and II deficiency and help identify carriers of type II deficiency when the PC activity lies

within the normal range. However, there is no accepted difference in thrombotic risk between type I and II deficiencies [53].

Protein C – physiological and pathological factors

PC levels are significantly lower in adolescents than adults and even lower levels are seen in children and neonates [10, 11, 66]. PC levels increase by more than 20% in pregnancy and remain elevated in the post-partum period [67, 68].

> **Recommendations**
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> • **Amidolytic assays of PC activity are less subject to interference than coagulation assays and are preferable for PC deficiency screening.**
> • **When interpreting PC levels, patient age should be taken into account.**
> • **PC deficiency should not be diagnosed or excluded on the basis of assays performed when the patient is taking VKA.**

Protein S assays

Functional assays of protein S (PS)

These are reliant on the ability of PS to act as cofactor for APC-mediated inactivation of FVa or FVa and FVIIIa. This activity may be detected using PT, APTT or FXa coagulation time [69]. Functional assays may detect type II defects which are missed by antigenic assay. However, in the past, many patients were incorrectly diagnosed as having type II PS deficiency when APC-resistance (due to FV Leiden) resulted in underestimation of PS by functional assay [70]. In the presence of FV Leiden, increased plasma dilution will not always fully normalize spurious low PS levels [71]. PS can be over or under-estimated by functional assay in the presence of LA. If coagulation-based PS assays are used, patient samples should be tested at three dilutions to assess parallelism. UK NEQAS have identified marked differences in PS results between different commercial kits [72], and this complicates the interpretation of quality assurance assessments for these assays. Coagulation-based PS assays are therefore not recommended for routine use and

immunoassays of free PS are preferred for initial investigations [49].

Protein S antigen assays

A total of 60–70% of plasma PS is bound to C4b-binding protein (C4bBP), the remaining free PS acts as a cofactor for APC-mediated inactivation of FVa and FVIIIa. Total PS antigen can be measured by IEP, ELISA and by ITB assays. The IEP assay is time-consuming and complicated because C4bBP must be dissociated from PS, to avoid double precipitin peaks [73]; this assay is therefore not considered suitable for routine use. In some types of total PS ELISA assays, antibodies bind with different affinity to free and bound PS, and C4bBP must be dissociated from PS by high plasma dilution (e. g. ≥1/500 plasma dilution) as well as prolonged incubation (18 h) of plasma dilution with capture antibody [74]. In certain commercial methods using monoclonal antibodies with the same affinity for free and bound PS, such stringent conditions are not required.

The free PS antigen level reflects functional PS (APC cofactor) activity in plasma and can be measured by RID, ELISA or ITB assays. ELISA for free PS utilizes either specific monoclonal antibodies or C4bBP to capture free PS from plasma. Once bound, suitably labelled antibodies can be used in the detection stage of the ELISA. It has been reported that delay in addition of diluted test sample to the microplate, or prolonged incubation times, can result in overestimation of free PS in deficient subjects. Therefore, dilutions should be tested without delay, incubation times must not exceed the manufacturer's validated limits and incubation temperatures must be controlled appropriately [75]. Latex-based ITB assays of free PS utilize either two monoclonal antibodies specific for free PS; or latex particles coated with C4bBP to capture free PS, then monoclonal antibody coated particles agglutinate in the presence of this captured PS. These ITB assays are designed for specific coagulation analysers, but may be adapted for alternative instruments. Both latex methods are precise and correlate well with ELISA. The major advantage of ITB methods is that they are fast, simple to perform and easily automated.

Type I PS deficiency is defined by reduced levels of total and free PS antigen as well as PS activity. Type II

deficiency is characterized by isolated deficiency of PS activity, while in type III deficiency total PS is normal, but free PS antigen and PS activity are reduced.

Measurement of free PS was shown to be better at detecting PS deficiency in subjects with *PROS1* gene defects than measurement of total PS [76]. It has been reported that anti-mouse antibodies and the rheumatoid factor in plasma can occasionally result in significant overestimation of analyte in ELISA and latex-based immunoassays.

Protein S – physiological and pathological modifiers

PS is produced mainly in the liver and levels are affected by liver disease and VKA. Reference ranges should be sex specific as PS levels are significantly lower in premenopausal women than men. Levels are also low in pregnancy and may be reduced by oral contraceptives or hormone replacement therapy [67, 68, 77, 78]. PS is reduced in the newborn and levels should be interpreted against suitable reference ranges [10, 11].

Recommendations

• Immunoassays for free PS should be used because of the wide variability in PS activity assays.

• Interpretation of PS level must take age, sex, pregnancy and use of hormone preparations into account.
• PS deficiency should not be diagnosed on the basis of assays performed when the patient is taking VKA.

DISCLAIMER

While the advice and information in these guidelines is believed to be true and accurate at the time of going to press, neither the authors, the British Society for Haematology nor the publishers accept any legal responsibility for the content of these guidelines.

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