THE ACTIVATED PARTIAL THROMBOPLASTIN TIME

Prepared on behalf of the World Health Organization

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1. INTRODUCTION

The designation, partial thromboplastin time, distinguishes the procedure from the prothrombin time and derives from the fact that the partial thromboplastin reagent lacks the apoprotein component of the complete tissue thromboplastin. The aPTT is the main screening test for the intrinsic clotting system and is the second commonest coagulation test being exceeded in frequency only by the prothrombin time. An American College of Pathologists survey in 1995 indicated that 95% of participants relied principally on the aPTT for the monitoring of heparin. The original unactivated test (PTT) has been replaced by the activated partial thromboplastin time (aPTT), which incorporates an activator. The presence of the activator in the test system shortens the test by effecting maximum activation. This increases the precision and reproducibility of the results by eliminating the variable effects of contact from glass surfaces.

2. APTT PHOSPHOLIPIDS

The partial thromboplastin of the aPTT is a phospholipid extract of animal tissue or from vegetable sources. The phospholipids act as a platelet substitute in the intrinsic system. The lipid composition of different aPTT reagents, however, varies considerably. The concentrations of phospholipids and fatty acids in some widely used aPTT reagents have been shown to differ by as much as 300 times (1). These discrepancies markedly affect responses to coagulation defects and inhibitors of coagulation. The requirements for the phospholipid component of the test system may also vary according to the nature of the clotting defect being measured. For example, the concentration of negatively charged phospholipids, e.g. phosphatidyl serine, has been shown to be critical (2).

3. OTHER COMPONENTS OF THE APTT TEST

Components which affect the clotting response include the type of activator, length of incubation time with the plasma and the composition of buffers (3,4). Particulate activators include kaolin, celite and micronized silica, whereas other activators, e.g. ellagic acid, are non-particulate. The amount of activator present in the various commercial techniques, and the length of the incubation time, show considerable variation. It is the combination of the activator with other components which appears to determine the reliability of the test (5). The trend is to utilise less opaque activators to avoid interference with newer types of coagulometer. Different types of coagulometer can also have a considerable effect on the clotting time (6).

4. PREPARATION OF A PARTIAL THROMBOPLASTIN EXTRACT FOR THE APTT TEST

4.1 Source of APTT extract

The partial thromboplastin extract may be an in-house (see section 4.2) or commercial preparation. Various current commercial aPTT reagents may be considered satisfactory for the different clinical purposes but details of their production and content are not available as they are proprietary information.
4.2 Method of preparation of aPTT extract

The following is suitable for preparation by a routine laboratory. The principles of production of the reagent described are those established for the non-proprietary Manchester aPTT reagent used in many clinical studies including the European Concerted Action on Thrombosis (7). It is derived from rabbit brain tissue and is an example of a type of aPTT reagent showing high sensitivity to depression of intrinsic clotting factors V, VIII, IX, X, XI and XII, good sensitivity to the effect of heparin and comparative reliability in screening for lupus anticoagulants. It is compatible with most coagulometers but some coagulometers may not record an end-point because of the opacity of the kaolin activator. The use of a non-opaque activator e.g. ellagic acid, micronised silica, could if necessary be considered as alternatives. These have a shorter incubation period than the kaolin activator but require careful standardization to maintain clotting factor sensitivity.

4.2.1 Procedure

- Strip the meninges from freshly obtained rabbit brain tissue. Cut the tissue into small pieces.

- Place in a large mortar, cover well with cold acetone. Pound with a pestle for 2 minutes then filter through Whatman GP filter paper. Pour on fresh, cold (4°C) acetone and pound again for 2 minutes. Repeat the process until the material has a "sandy" consistency (up to 10 times). The neutral lipids and water are removed by the acetone, leaving the phospholipids.

- Leave overnight, on filter paper, in a fume cupboard to complete the drying process, or preferably vacuum desiccate for several hours.

- To 10 g dried material add 250 mL ether. These measurements may have to be varied according to the source. Leave overnight in a tightly stoppered flask or reagent bottle with a ground glass stopper, in a fume cupboard.

- Filter through Whatman GP filter paper. Discard the residue.

- Evaporate the filtrate to dryness at 37°C. Use a Buchner flask connected to a filter pump to facilitate the process. Rotary evaporation is the method of choice.

- Resuspend the dried filtrate in 100 mL Owren's buffer at 45°C. (Owren's buffer pH 7.35; Sodium diethylbarbiturate 11.75 g; Sodium Chloride (NaCl) 14.67 g; 0.1 mol/L Hydrochloric Acid (HCl)430 mL; Distilled water 1570 mL).

- Add buffer gradually, shaking between each addition (glass beads may facilitate the process). Shake continuously on a mechanical shaker until the precipitate is completely broken up and a fine suspension is obtained.

- Store in small aliquots in plastic containers at -40°C or below. The frozen aliquots of the reagent are stable for at least 3 months.
4.3 **Standardization of the extract**

Prepare dilutions (1:500 to 1:10,000) from an aliquot of the deep-frozen extract in Owren's buffer and test against fresh normal plasmas to determine the dilution which gives the desired normal range. Further testing against different levels of many known coagulation defects and inhibitors is required to establish the optimum sensitivity of the extract to individual clotting defects and inhibitors. The concentration adopted will be based on the balance of results of the data from normals and the multiplicity of coagulation defects in which the aPTT is used for screening. The reliability of the test in the detection of abnormalities of intrinsic clotting depends not only on the composition of the phospholipid extract but also on the choice of activator if an alternative to kaolin is preferred.

5. **BLOOD SPECIMENS**

5.1 **Containers**

Collection into capped plastic containers with a measured line for filling or into evacuated tubes is optional. The trisodium citrate content in the containers should be 3.2% (mmol/L)

5.2 **Storage**

Testing should be performed on fresh plasma, as soon as possible after collection and ideally within 2 hours from venipuncture. Delay in testing for more than 2 hours may result in unpredictable changes in the aPTT result. The test plasma is to be kept in a stoppered polystyrene container at room temperature. Care should be taken in specimen collection to avoid unnecessary trauma and thus prevent platelet activation which releases platelet factor 4 (PF4) from the α-granules of the platelets which has anti-heparin activity. The blood should be centrifuged to give platelet-poor plasma with a platelet count of less than 15,000/μL, particularly if specimens are frozen prior to testing.

6. **TECHNIQUE OF THE TEST**

6.1 **Thawing of aPTT reagent**

An aliquot of the diluted suspension should be thawed out rapidly by placing in a water bath at 37 °C for 1-2 min immediately before use. This is maintained in crushed ice prior to use. The reagent is stable for at least 2 hours under these conditions. Any remaining reagent is discarded after use and is not refrozen.

6.2 **Preparation of activator**

Light kaolin (US Pharmacopeal or British Pharmacopeal preparations suitable 2.5 g/L) is suspended in Owren's buffer (store at +2 to 8 °C). It is mixed well before use. An aliquot is decanted when required into a separate container and any remainder is discarded. Ellagic acid, micronised silica and sulphatides alone or in combination may be substituted as in some commercial aPTT reagents.

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1The range of dilutions will depend on the source of the phospholipid extract and the potency of the extraction.
6.3 Testing procedure

The kaolin suspension and calcium chloride should be warmed in separate test tubes in a water bath at 37°C. The following reagents are added without delay in the order indicated into a glass tube, pre-warmed in a water bath:

- 0.1 mL test plasma
- 0.1 mL aPTT reagent
- 0.1 mL warmed kaolin suspension (after resuspension) and start stop watch.

The test tube is tilted three times immediately, and subsequently at approximately 1 min intervals in order to resuspend the kaolin.

At exactly 10 minutes, 0.1 mL warmed calcium chloride (0.025 mol/L) is added. The tube is tilted gently three times and left undisturbed in a water bath for exactly 20 seconds from the addition of the calcium chloride. It is then tilted gently until a solid clot forms. The clotting time (seconds) is recorded and the test is performed in duplicate. With some types of activator including ellagic acid, micronised silica and sulphatides the appropriate incubation period is usually much shorter, e.g. 3 to 5 minutes.

7. NORMAL (REFERENCE) RANGE

The reference range should be based on results from a large healthy population of both sexes spanning the adult age range (minimum 20). This should be based on the mean value ± 2 standard deviations. Correction for non-Gaussian distribution is required with some reagents (1).

8. PATHOPHYSIOLOGICAL VARIATION IN THE APTT

Stress, exercise, pregnancy, the post-partum state and surgical operations result in acceleration of the test. Prothrombotic changes associated with recent deep vein thrombosis, thromboembolic disorders and oestrogen administration may result in accelerated clotting times. Acquired pathological states such as liver disease, disseminated intravascular coagulation (DIC), and drug toxicity cause prolongation. Certain drugs, including oral anticoagulants, heparin and thrombolytic agents, prolong the aPTT. Oral anticoagulants depress factor IX. The aPTT is sometimes used as an adjunct to prothrombin time control during the stabilisation period or in the event of unexpected bleeding at therapeutic INR. Heparin is discussed in section 12. The test is also prolonged in the newborn (8) and is also relatively prolonged during childhood and adolescence.

9. SOURCES OF ERROR

Although it is a relatively simple test to perform, the aPTT is subject to a number of possible sources of variables. These include faulty blood collection, haemolysis, the use of siliconised or plastic instead of borosilicate test tubes, speed of centrifugation, the length and bore of glass tubes and the angle and speed of the manual tube tilting. Further causes of erroneous results include incorrect citrate anticoagulant concentration, presence of heparin, addition of an incorrect volume of blood to anticoagulant (overfilling = undercitration, underfilling = overcitration), partial clotting during venesection or blood collection and the contamination of a blood sample direct from indwelling catheters or intravenous lines. With continuous infusion, blood samples should be taken from the extremity opposite from the infusion site.
Contamination by tissue fluids causing acceleration of the test can result from traumatic and/or difficult venipuncture. The presence of heparin in the circulation or by local contamination from indwelling catheters or lines prolongs the test. In addition, delay in mixing with the anticoagulant may cause partial clotting, which is not always obvious. This possibility can be minimised by the use of siliconised evacuated tubes. The use of test tubes which are not chemically clean for the performance of the test can also have a deleterious effect. With the manual technique it is important to control contact activation in order to facilitate the reading of the endpoint. During the incubation period, the kaolin should be kept suspended in the reaction mixture by gentle mixing at regular intervals.

10. COAGULOMETER TESTING

The manufacturer's recommended method which may incorporate larger or smaller volumes than in the manual technique should be carefully followed.

11. SENSITIVITY OF THE APTT TO CLOTTING FACTORS

A reliable aPTT reagent should be sufficiently sensitive to record an abnormal result when the level of any single or combined intrinsic clotting factor deficiency is reduced to that which may cause spontaneous bleeding, or haemorrhage following a haemostatic challenge. The test should be able to detect factor deficiencies of 30% or less (9). With the method described here, depression of factor VIII below levels of 40% should be detectable.

12. RESPONSIVENESS TO HEPARIN

It is now recommended that the test should also be sufficiently responsive to low concentrations of heparin whilst giving a linear response to graded concentrations of conventional heparin, spanning a clinically relevant range of 0.05 - 0.5 units/mL. The importance of assessing heparin response to patients' heparin-treated samples rather than normal plasma "spiked" with heparin was emphasised in the ISTH/ICSH Study as the response to heparin-treated patients may differ markedly with some aPTT reagents compared with the same concentration of heparin added in vitro (10). This is partly due to differing responses of the various aPTT reagents to the pro-coagulant components of plasma in patients with recent clinical thrombotic episodes ("anti-heparin activities"). The variable responses reagents in the US and UK demonstrated by national external quality assessment surveys are illustrated in figure 1 (11,12). Variable effects on the heparin response of aPTT reagents from coagulometers used to perform the test were observed in the ISTH/ICSH study (see figure 2) and a UK survey from the National External Quality Assessment Scheme (13). A calibration constant was devised in the ISTH/ICSH Study based on the orthogonal regression slope (as in prothrombin time standardization) of the plots of the log aPTT results of the same two reagents at different centres but substituting different coagulometers (10). The coagulometer effect is therefore seen to be appreciable in ratio terms.
Fig. 1 - APTT ratios from surveys from the College of American Pathologists and UK external quality assessment showing responses to *in vitro* heparinized samples. The dotted line indicates the Manchester aPTT response on fresh plasma from heparinized patients. The concentration of heparin given a 1.5 aPTT ratio with individual commercial aPTT reagents (D, G, O) is indicated by vertical arrows: M = Manchester.
Fig. 2 - Orthogonal regression calibration slopes (calibration constants) of a commercial reagent used on a variety of coagulometers against the manual reference reagent, based on 20 normal subjects and 20 heparin treated patients with acute thrombotic disorders at each centre. This demonstrates the coagulometer effects on the orthogonal regression slopes (calibration constants).

Other factors influencing the clinical response of the aPTT to heparin include conditions affecting the blood volume such as obesity and ageing, conditions which affect production of heparin binding plasmas proteins, disseminated intravascular coagulation, acute inflammation, hepatic and renal disorders, which affect the half-life of heparin in the circulation. The relative heparin resistance of patients with recent thrombotic episodes is discussed in section 15. Specific coagulation factors which affect the aPTT response to heparin include factor VIII and fibrinogen (acute phase reactants) thrombin and antithrombin levels.

13. RESPONSE TO LUPUS ANTICOAGULANTS

The sensitivity to lupus-like anticoagulant of an aPTT system should be established in comparison with a responsive formulation of the viper venom test. The responsiveness of aPTT reagents varies considerably. Reagents with a high concentration of phospholipids, particularly phosphatidyl serine, shorten the time of activation and therefore make the aPTT test less sensitive(2).

14. CLINICAL USES

The aPTT remains the main test for screening for intrinsic clotting defects including haemophilia, detection of lupus anticoagulants and for laboratory monitoring of heparin administration. The test is prolonged by deficiencies of factors VIII, IX, X, XI and XII and defects of the contact phase, e.g. prekallikrein, high molecular weight kininogen. It also may be prolonged by gross defects of factors II, V and fibrinogen. With a reliable aPTT system, specific and non-specific inhibitors of intrinsic clotting
factors are detected. The degree of abnormality depends upon the responsiveness of a particular aPTT method to a specific defect. When used as a screening test for lupus anticoagulants (LA) the detection rate is greatly influenced by the concentration and type of phospholipid content of the reagent. Reagents containing the highest concentrations of phospholipid tend to be less responsive to LA. The aPTT is also the most widely used method for the laboratory monitoring of heparin administration (6, 11, 12). Although other more specific techniques have been advocated, e.g. anti-Xa assays, viper venom assays, and thrombin clotting times the aPTT is still universally used as it is regarded as a global test of coagulation which assesses the overall effect of heparin on clotting. The aPTT may also be of value in case of unexpected bleeding during oral anticoagulant therapy as there might be a disproportionate depression of factor IX. Accelerated aPTT have been reported after operations, oral contraceptive administration and withdrawal of oral anticoagulation ("rebound hypercoagulability").

In aPTT monitoring for heparin, minimal levels should be measured e.g. just before the next dose with intermittent injections. With continuous infusion, heparin monitoring should be performed at six hour intervals until the stable therapeutic level is achieved.

15. NEED FOR STANDARDIZATION

Because aPTT results are influenced by changes in various different stages of the coagulation mechanism they are subject to more variables than specific clotting assays. The need for standardization of the aPTT with relation to factor VIII and factor IX has been demonstrated in several reports. The greatest need has been in laboratory monitoring of heparin administration (3, 14-19). The difficulty of employing these alternatives for standardization is that the vast majority of centres prefer to use the aPTT for heparin control. As far as the heparin response is concerned, anti Xa levels of 0.3 to 0.7 u/mL have been recommended as corresponding to the ½ to 1½ ratio prolongation of the aPTT (20). A problem with attempts to standardise heparin monitoring by aPTT is that aPTT test systems differ in response to other components of the clotting mechanism than heparin, e.g. anti-heparin activities. The difficulties of standardization of aPTT heparin monitoring were highlighted by the recent ISTH/ICSH international collaborative study (10). APTT responses of patients with recent thrombosis were much less to the same concentrations of heparin. A calibration constant was determined for each local aPTT system from comparison of the orthogonal regression slope of plasmas from patients and healthy subjects with the reference manual aPTT technique. This follows the example of prothrombin time standardization where determination of the international sensitivity index (ISI) in thromboplastin calibration is the method used to derive INR. It was found that the use of a single calibration value for a brand of reagent for all laboratories for use with all coagulometers was not advisable. Each laboratory should calibrate its own test system. Originally Basu et al had recommended that the therapeutic range should be given by an aPTT ratio between 1.5 and 2.5 (21). The intensity of treatment corresponding to this range varies however with the local aPTT test system as shown previously. Hirsh et al gave the equivalents for an aPTT ratio of 1.5 for the protamine titration as 0.2 units/mL and for the anti-factor X assay as 0.3 units/mL (20). A major difficulty with attempts to standardise heparin monitoring by aPTT is that aPTT test systems vary in response to other components of the clotting mechanism than heparin, e.g. anti-heparin activities. The ISTH/ICSH study (10) recommended the adoption of a reference aPTT reagent.
In the absence of an international reference preparation for thromboplastin and recommended reference method for performance of the test, laboratories have the problem of establishing their own therapeutic range with their local technique. They should check their therapeutic range in aPTT ratios with the dose response to heparin by the anti-Xa assay over a range of concentrations from 0.2 to 0.7 heparin units/mL rather than simply depending on the 1.5 to 2.5 APTT ratio.
REFERENCES


AKNOWLEDGEMENTS

Gratitude is expressed to the following members of the Co-ordinating Group WHO IEQAS in Blood Coagulation for their advice and assistance:
