Activated Partial Thromboplastin Time (APTT) Testing

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Topics for Discussion

- APTT Test Basics
- Monitoring Antithrombotic Agents Using the APTT
- APTT Mixing Studies & Factor Assays
- Inhibitors and the APTT
Coagulation in the Laboratory

Intrinsic Pathway
- XII
- XI
- IX
- VIII

Extrinsic Pathway
- VII
- Tissue Factor

APTT
Intrinsic + Common

PT
Extrinsic + Common

Fibrinogen → Fibrin Clot

Common Pathway
- X
- V
- II

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APTT Test Basics
The Specimen

- Blood is collected into a tube containing 3.2% sodium citrate as the anticoagulant
- Blood fluidity is maintained because sodium citrate binds calcium ions, which are critical to the coagulation process
- Tube is centrifuged in order to separate plasma from buffy coat and red blood cells
  - Plasma is “platelet poor” (PPP)

- Plasma is used for testing
  - PLASMA contains FIBRINOGEN
  - Serum does not contain Fibrinogen
**APTT Test**

- **“Partial thromboplastin”**
  - Tissue Factor not used to initiate (activate) coagulation

- **Two-stage assay**
  - Activation & re-calcification

- **Reagent composition**
  - **Activator**
    - Converts FXII to FXIIa
  - **Phospholipid**
    - Replaces *in vivo* platelet phospholipid surface on which coagulation reactions occur
  - **Buffer** (minimizes pH changes in plasma reaction mixture)
  - **CaCl₂**
    - Re-introduces calcium ions that were chelated by sodium citrate anticoagulant

- Time for clot formation: ~30 seconds

- **Reagent Composition**
  - 0.1 ml Activator
  - 0.1 ml Plasma
  - 0.1 ml CaCl₂
  - Incubate at 37 °C for ~5 minutes
Pre-analytical variables
- Phlebotomy, specimen transport, specimen examination, centrifugation & aliquoting, time to testing & storage
  - Please refer to Focus Article entitled *Garbage In—Garbage Out: Talking Trash About Pre-Analytical Variables (Part 1)* for more details

Analytical variables
- Reagent sensitivity to coagulation factors, heparin, Direct Thrombin Inhibitors (DTI), and Lupus Anticoagulant (LA)
  - Types of activators
  - Source, concentration, and types of phospholipid
- Instrument clot detection method
  - Mechanical
  - Photo-optic
  - Nephelometric
APTT Reagent Activators

- **Types of activators**
  - Particulate activators
    - Kaolin (diatomatacious earth)
    - Micronized or colloidal silica
    - Celite
  - Non-particulate activator
    - Ellagic acid (liquid activator)

- **Rationale for using activators**
  - Provide large surface areas for reactions to occur
  - Influence time required for test incubation
    - Replace glass surfaces originally used to perform Partial Thromboplastin Time (PTT)
    - Shorten clotting times that historically were seen with PTT thereby reducing test imprecision
Phospholipids in APTT Reagent

- **Source**
  - Human placenta, rabbit brain, bovine brain, soybean (purified), or synthetic lipidation in liposomes

- **Concentration**
  - High levels
    - Insensitive to the Lupus Anticoagulant (overwhelm or mask the inhibitor)
  - Low levels
    - Sensitive to the Lupus Anticoagulant (accentuate inhibitor effect)

- **Types of phospholipids**
  - Reagents with lower levels of phosphatidylderine are more sensitive to the Lupus Anticoagulant
Intended Use for APTT

- **Screening test**
  - APTT reagent should be sensitive to a reduction in coagulation factors that are associated with bleeding
    - Intrinsic (and severe common pathway) factor deficiencies

- **Laboratory monitoring**
  - Unfractionated heparin
  - Direct Thrombin Inhibitors

- **Laboratory detection of inhibitors**
  - Lupus Anticoagulant is most common
What is Normal?

- Reference intervals provide a range of normalcy.
- Establish using minimum of 20-30 normal healthy individuals.
- Desired test (APTT, Factor VIII) is performed with instrumentation and reagents used for patient testing.
- Determine mean and ± 2 SD (standard deviation or sigma [σ]).
- A 2 SD range reflects normalcy for 95% of population whereas a 3 SD range includes 99.7%.
Causes for Prolonged APTT

- **Most common causes**
  - Heparin (contamination from lines or therapeutic)
  - Lupus Anticoagulant
  - Normal after retesting (pre-analytical issues)

- **Other causes**
  - Reduction in or deficiencies of coagulation factors
    - FVIII, FIX, FXI, FXII, Contact Factors, FV, FVIII Inhibitor (↓ FVIII)
  - Liver disease (site for production of most coagulation factors)
  - Consumption of coagulation factors as seen in Disseminated Intravascular Coagulation (DIC)
  - Vitamin K deficiency (warfarin: affects FII, FIX, FX)
  - Hypo and Dys-Fibrinogenemias
APTT Monitoring of Antithrombotic Agents
Tests to detect unfractionated heparin
- APTT
- Thrombin Time (TT)
- Reptilase Time
- Hepzyme® (Dade Behring®)
  - Heparinase I enzyme
  - Neutralizes up to 2.0 Units of unfractionated heparin in 1.0 ml of citrated plasma

If heparin is present, is it a contaminant or a therapeutic?
APTT Monitoring of Heparin

Assumes antithrombotic (anti-IIa) effect parallels anticoagulant effect

Limitations

- Pre-treatment APTT of patient
  - Baseline APTT of patient prolonged due to Lupus Anticoagulant
  - Baseline APTT of patient sample below or at low end of reference interval (-2 to –3 SD) due to high levels of FVIII (apparent “heparin resistance”)

- APTT reagents vary in sensitivity to heparin
  - Laboratories must determine responsiveness of their APTT reagent to unfractionated heparin
  - Determine APTT therapeutic interval (seconds) for reagent used to monitor heparin therapy
Response curve: comparing APTT to Anti-Xa levels
- Perform APTT and Anti-Xa assays simultaneously on plasma samples from patients receiving heparin (*ex vivo* samples)
- Note APTT values that correspond to low (0.3 U/ml) and high (0.7 U/ml) ends of therapeutic interval when using Anti-Xa assay (see next slide for example)

Comparing APTT reagents (current lot number to new)
- Perform APTT on minimum of 30 *ex vivo* samples using both reagents
- Sum data for each reagent, determine means, and compare difference between means
- Repeat process for each new reagent change (yearly) and determine cumulative change
- Difference of more than 7 seconds between 1) reagent means or 2) cumulative means, indicates an unacceptable level of variation that can adversely affect therapeutic interval currently in use
Heparin Response Curve

Anti-Xa Reference versus APTT

\[ y = 109.08x + 21.228 \]

\[ R^2 = 0.8176 \]

Therapeutic Range
53.9 – 97.6 seconds
APTT Ratio = 1.9 – 3.4
(using mean normal APTT of 29 seconds)

Therapeutic Range
0.30 – 0.70 Anti-Xa U/ml
Direct Thrombin Inhibitors

- Hirudin [Lepirudin (rDNA)—trade name: Refludan®]
  - Approved in USA, Canada, and EU for Heparin Induced Thrombocytopenia (HIT) complicated by thrombosis
  - Target APTT is 1.5-2.5 x patient baseline APTT
  - In absence of severe thrombosis, some experts recommend a target APTT of 1.5-2.0 x patient baseline APTT and monitor every 4 hours

- Argatroban [non-US trade name: Novastan]
  - Approved for HIT with or without thrombosis and also for anticoagulation during percutaneous coronary intervention (PCI) in patients with, or at risk for, HIT
  - Target APTT 1.5-3.0 x patient baseline APTT (maximum 100 seconds)

- Hirulog [Bivalirudin-trade name: Angiomax™]
  - Undergoing evaluation for use as an anticoagulant for “on-pump” and “off-pump” cardiac surgery in patients with HIT
  - Target APTT is 1.5-2.5 x patient baseline APTT
APTT Mixing Studies
and
APTT Factor Assays
Use normal pooled plasma (NPP)
- Mix with patient plasma
- Perform APTT
- Controversy as to what constitutes a “correction”
  - Value within APTT reference interval?
  - Value within 5 seconds of upper limit (+2SD) of reference interval?

**IF baseline APTT is prolonged:**
- Mix patient platelet poor plasma (PPP) with normal pooled plasma (NPP) and repeat APTT
  - Most common mix is “1:1” Mix (50/50 Mix)
- Failure to “correct” (upper limit of reference interval) indicates presence of an inhibitor
- “Correction” indicates a factor deficiency
Mixing Study Patterns

% NPP = Percent Normal Pooled Plasma; % PAT = Percent Patient Plasma

Upper Limit of Reference Interval

Factor Deficiency
APTT Factor Disorders

- Reagent must prolong APTT above upper limit (+2SD) of reference interval if factor level is below ~40% activity

- Deficiency of Factor VIII
  - Associated with Hemophilia A and Von Willebrand Disease

- Deficiency of Factor IX (Hemophilia B)

- Deficiency of Factor XI (Hemophilia C)
  - Found predominantly in individuals of Ashkenazi Jewish descent

- Contact factors
  - FXII, Prekallikrein, HMW Kininogen
  - Reagents are generally relatively insensitive to these factors
  - Patients do not have a bleeding history
Factor Assays

- Based on the APTT
- Test performed in a dilute system (1:10, ie 1 part plasma to 9 parts buffer)
- Assays are “mixing studies” that use diluted patient plasma to correct specific deficiency in a “deficient substrate plasma”
  - For example: diluted patient plasma is used to “correct” the deficiency of FVIII (complete absence) in FVIII deficient substrate plasma
- Quantify by interpolating “mixing study” clotting time from a standard curve
**Example**

Patient value is determined by interpolating time, 50.1 seconds using a 1:10 dilution, from the reference curve to yield a FVIII activity of 38%. This indicates that the patient’s FVIII activity level is only 38% that of normal activity.
Inhibitors and the APTT
“Circulating Anticoagulants”

- Inhibitors to specific factors
  - Neutralizing antibodies
  - Non-neutralizing antibodies

- Non-specific inhibitors
  - Lupus Anticoagulant, Fibrin Degradation Products, paraproteins (Dysproteinemias)

- Global inhibitors
  - Heparin-like activity (glycosaminoglycans)
  - Heparin (unfractionated) and Direct Thrombin Inhibitors (hirudin, argatroban, hirulog)
Specific Inhibitors

Immunoglobulins (generally IgG4) with epitope specificity for a single protein/site that interfere with reaction(s) involved in generating Thrombin and formation of a stable fibrin clot

- Neutralizing (completely neutralize factor)
  - FVIII
  - FV, FIX, FXI, FXII, VWF, Thrombin, Fibrinogen

- Non-neutralizing
  - FVIII (acquired)
  - FIX (cleared in nephrotic syndrome)
  - FX (amyloidosis)
  - FII
  - VWF
FVIII Inhibitors

- Alloantibodies develop in ~15-35% of Hemophilia A (HA) patients who have received exogenous (non-self) FVIII through replacement therapy.
- Autoantibodies are found in “acquired hemophilia” (antibodies develop against self FVIII).
- Antibodies lead to neutralization of exogenous (transfused) or endogenous FVIII.
- Time (and temperature) dependent.
- Quantify by Bethesda assay.

**Graph:**
- APTT (seconds) vs. % NPP and % PAT.
- Legend:
  - Incubated Mix: 2 hrs @ 37°C
  - Immediate Mix: at room temperature
  - Upper Limit of Reference Interval

**Legend:**
- % NPP = Percent Normal Pooled Plasma
- % PAT = Percent Patient Plasma
Bethesda Assay

- Prepare serial dilutions of patient plasma in buffer
- Mix patient plasma dilutions with equal part normal pooled plasma (NPP)
  - NPP is source for FVIII
  - NPP + buffer (NPP Mix) is control for FVIII lability
- Incubate for 2 hours at 37 °C
- Prepare 1:10 dilution of each mixture and perform FVIII assay
- Patient mix / NPP mix = corrected residual FVIII activity (RA)
  - (20% “FVIII” patient/40% “FVIII” NPP) x 100 = 50% RA
- RA utilized to quantify inhibitor activity from curve
  - 50% RA = 1.0 x 2 (1:2 mix) = 2 BU
Patient FVIII / Control FVIII = Corrected Residual FVIII Activity (RA); Read RA from Graph; (RA x Dilution = BU)

(20% “FVIII” patient / 40% “FVIII” NPP) x 100 = 50% RA
From Curve: 50% RA = 1.0 x 2 (1:2 mix) = 2 BU

1 BU = amount of inhibitor that inactivates 50% of FVIII in NPP
2 BU = amount of inhibitor that inactivates 75% of FVIII in NPP
Non-specific Inhibitors

- **Lupus Anticoagulant (LA)**
  - Please refer to Slide Presentation entitled *Laboratory Diagnosis of the Lupus Anticoagulant* for more details

- **Paraproteins**
  - Bind to coagulation proteins
  - Complexes readily cleared

- **Fibrin(ogen) Degradation Products (FDP or FSP)**
  - “Natural anticoagulants”
  - FDPs truncate Fibrinogen protofibril formation
LA Effect: APTT Based Assays

- Factor assays
  - Non-parallelism of patient curve to reference (calibrator) curve
  - Factor activity increases with increasing dilution of plasma

- Protein C & Protein S assays based on APTT
  - False prolongation of clotting times leads to over-estimation of Protein C and Protein S activities
Non-Parallelism Due to LA Effect

Reference Curve

<table>
<thead>
<tr>
<th>Reference Dilution</th>
<th>% FVIII (log)</th>
<th>Seconds (lin)</th>
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</thead>
<tbody>
<tr>
<td>1:10</td>
<td>92</td>
<td>70</td>
</tr>
<tr>
<td>1:20</td>
<td>46</td>
<td>41.4</td>
</tr>
<tr>
<td>1:40</td>
<td>23</td>
<td>53.8</td>
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<td>1:80</td>
<td>11.5</td>
<td>60.6</td>
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<tr>
<td>1:160</td>
<td>5.7</td>
<td>65.7</td>
</tr>
<tr>
<td>1:320</td>
<td>2.9</td>
<td>71.9</td>
</tr>
<tr>
<td>1:640</td>
<td>1.4</td>
<td>76.2</td>
</tr>
</tbody>
</table>

LA Sample showing inhibitor effect

Factor activity 1:40 > 1:20 > 1:10 dilution
- 11% x 1 (1:10) = 11%
- 18% x 2 (1:20) = 36%
- 15.8 x 4 (1:40) = 63%
- 12.4% x 8 (1:80) = 99%

Inhibitor effect

Inhibitor diluted

Normal Sample (parallel to reference curve)

<table>
<thead>
<tr>
<th>Sample Dilution</th>
<th>% FVIII (log)</th>
<th>Seconds (lin)</th>
<th>Interpolated Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>11</td>
<td>60</td>
<td>11</td>
</tr>
<tr>
<td>1:20</td>
<td>17.7</td>
<td>55</td>
<td>36</td>
</tr>
<tr>
<td>1:40</td>
<td>15.7</td>
<td>57</td>
<td>63</td>
</tr>
<tr>
<td>1:80</td>
<td>12.4</td>
<td>59</td>
<td>99</td>
</tr>
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</table>
APTT test is a global assay that must be sufficiently sensitive and robust to fulfill three requirements:

- Screen for intrinsic coagulation factor defects that may lead to bleeding and to monitor replacement therapy used to correct these deficiencies
- Monitor unfractionated heparin and Direct Thrombin Inhibitors
- Detect the Lupus Anticoagulant

Types and concentrations of activators and phospholipids define an APTT reagent and its ability to meet the above stated objectives

Assays for quantification of intrinsic coagulation factors and specific inhibitors are based on the APTT and are subject to its limitations
References

- Clinical and Laboratory Standards Institute (CLSI). One-stage Prothrombin Time (PT) test and Activated Partial Thromboplastin Time (APTT) test; Approved Guideline H47-A, 1996.