Pre-analytical Variables in the Hemostasis Laboratory

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Learning Objectives

At the completion of this session, the participant will be able to:

1. List the most common reasons a sodium citrate sample should be rejected
2. Describe the ideal manner a sodium citrate sample should be collected, processed and stored
3. Explain why whole blood sodium citrate samples should not be stored at cold temperatures

Pre-analytical Variables

- **Variables that occur prior to the time the sample is analyzed which include:**
  - Conditions associated with the patient
    - Medications, lipemia, icterus
  - Conditions associated with specimen collection, transport, processing, and/or storage
- ***The* most important source of laboratory error**
  - Exceeds analytical error
- **Coagulation samples - *especially* susceptible**
  - Sample procurement initiates clotting
  - Complex nature of APTT and PT reactions
  - *In vitro* lability of both platelets and clotting factors

Pre-analytical Variables

- Not only are coagulation samples ***especially*** susceptible but the effect on results can be:

- As laboratory results lead to clinical action, compromise of sample integrity leading to erroneous results may cause:

Mistaken Diagnosis and Serious Patient Mis-management

Minimizing Variables – Improves Quality

- **It is not always clear when a sample referred to the laboratory is unsuitable or compromised**
  - Pre-analytical phase is often out of the laboratory's control
  - Analytical errors can be avoided by applying proper assay methodology and QC; controlled by the laboratory
- **When a sample is compromised, the test result might accurately reflect the status of the sample *but not accurately reflect the clinical status of the patient***

Minimizing Variables – Improves Quality

Guidelines for sample collection, transport, processing and storage must be ***strictly followed and deviations avoided,*** unless their impact, or lack thereof, on coagulation testing is known

Phlebotomists must be properly educated regarding appropriate pre-analytical sample handling

Collection, Transport, and Processing of Blood Specimens for Testing Plasma-Based Coagulation Assays and Molecular Hemostasis Assays; Approved Guideline — Fifth Edition H21-A5; 2008

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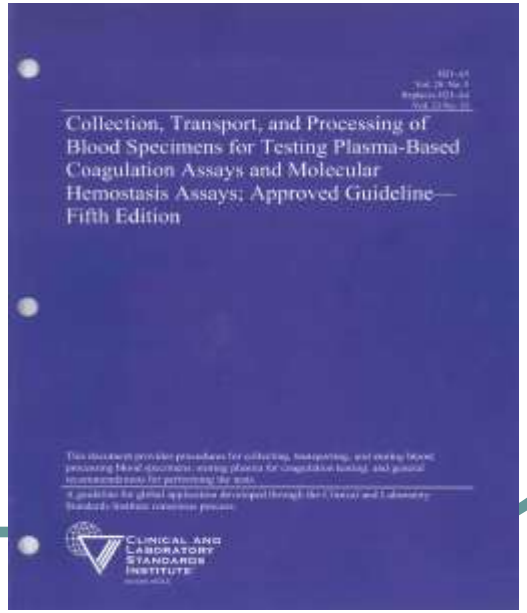


Blood Collection

- **Venipuncture from peripheral vein using evacuated tube system - preferred method**
  - Recommended needle size: 19 – 21 gauge
- **Syringe draw with straight needle acceptable**
  - Greater potential for hemolysis, platelet activation
  - ≤ 20 mL size to avoid clot formation
  - Add blood to anticoagulant ≤ one minute
- **Collection from vascular access device**
  - Potential for sample dilution or contamination
    - Flush with 5 ml saline and discard first 5 ml or discard 6 dead space volumes
    - Saline lock – discard 2 dead space volumes

Blood Collection - Discard Tube

- ***Not necessary*** **for routine* and many special coagulation assays****
  - Blue top tube can be the first tube drawn or
  - Blue top tube should be collected after a non-additive (***not clot activator***) tube
- ***Recommended***
  - Winged (butterfly) blood collection system with tubing
  - Platelet function studies

*Adcock D, et al. Lab Med 1997 28:530 ** AJCP 2010:133:331-335

Prevent *in vitro* clot formation

- **Avoid prolonged tourniquet use**
- **Avoid probing the vein with needle**
- **Encourage blood to flow freely and briskly**
- **Promptly and thoroughly mix anticoagulant with whole blood**
  - Three to six end over end inversions
    - Avoid vigorous shaking (hemolysis)

Clot formation is cause for specimen rejection!!!

Prevent *in vitro* clot formation

- **Impact of clot formation may cause**
  - ***Consumption*** of clotting factors
    - Loss of fibrinogen and other clotting factors such as FVIII and FV
  - ***Activation*** of clotting
    - Shortening of the APTT and PT, elevation of FVII and FIX

A fibrin clot is pale and may be evident after a frozen sample is thawed

Components of the Collection System

- **Anticoagulant**
  - Sodium citrate: Light Blue Top Tube

    - 105 to 109 mmol/L = 3.13% to 3.2% (commonly described as 3.2%) preferred
    - 129 mmol/L or 3.8% is also acceptable
    - Standardize to one concentration within system
    - Clotting times may be longer in 3.8% vs 3.2%*
      - Excess calcium binding in 3.8%
  - EDTA (purple top) and heparin (green top) plasma, serum **not acceptable**

***Adcock D, et al. AJCP 1997; 107:105**

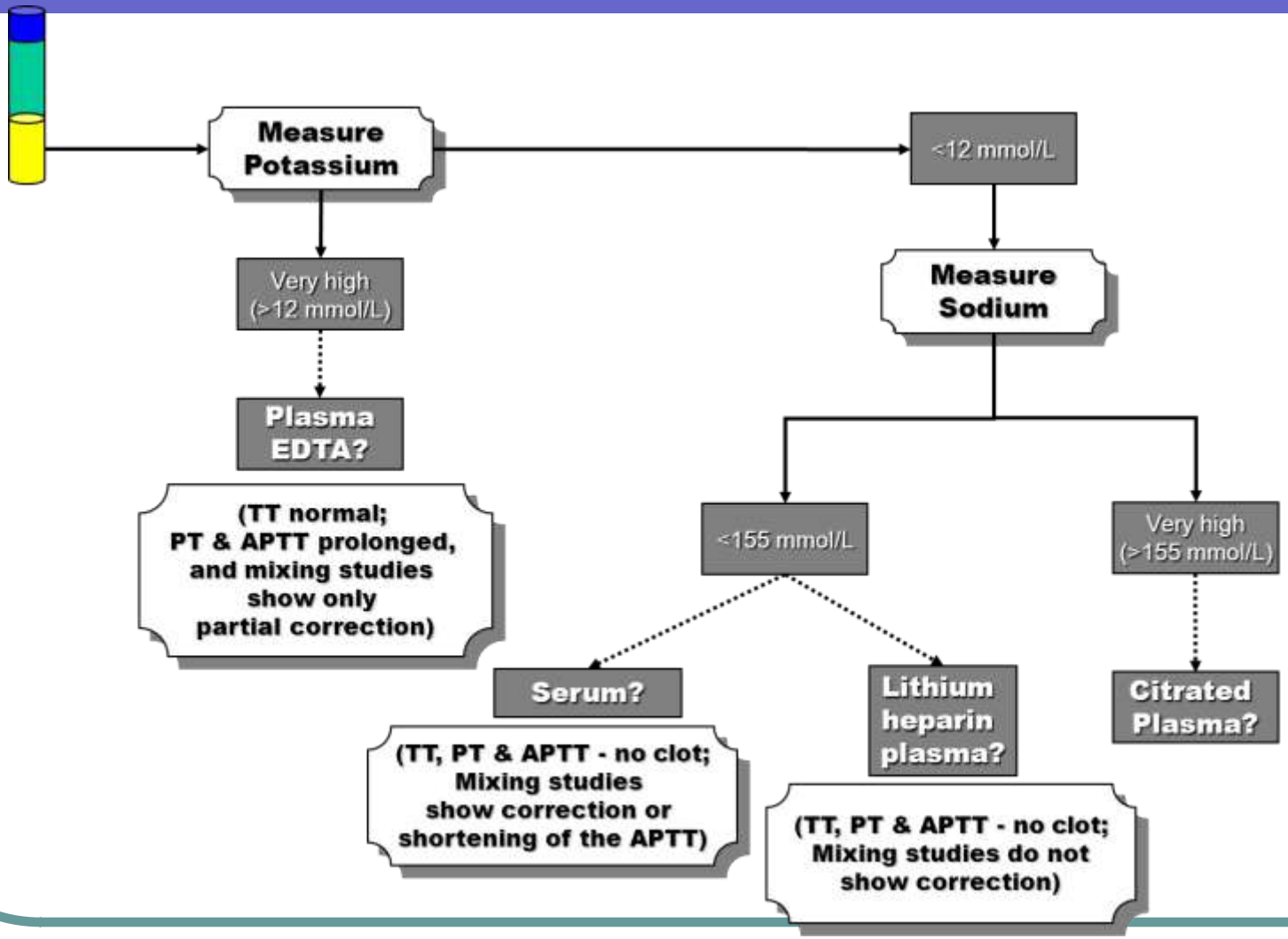
Evacuated Tube Effect

Tube Type Assay	3.2% Citrate Mean	EDTA Mean	Heparin Mean	Serum Mean
APTT (sec	29	68	>180	>180
PT (sec)	12.4	23	>60	>60
FVII Act (%)	115	116	77	308
FVIII Act (%)	141	4.5	< 1	4.5
FIX Act (%)	122	115	< 1	350
VWF:Ag (%)	122	143	70	101
VWF:RCo (%)	114	131	37	74
PC Act (%)	111	152	< 1	15.3
PS Act (%)	96	30	< 1	21.6

EDTA Plasma

- **PT, APTT –** Prolonged but measurable
- **Mixing studies** - Lack of correction - Mimics a time-dependent inhibitor
- **Select factors decreased** ↓↓FVIII,↓FV, ↓PS

Algorithm for detecting incorrect sample type



Lippi G, Favaloro E, Adcock D. Int J Lab Hematol 2010;23(1):132-137

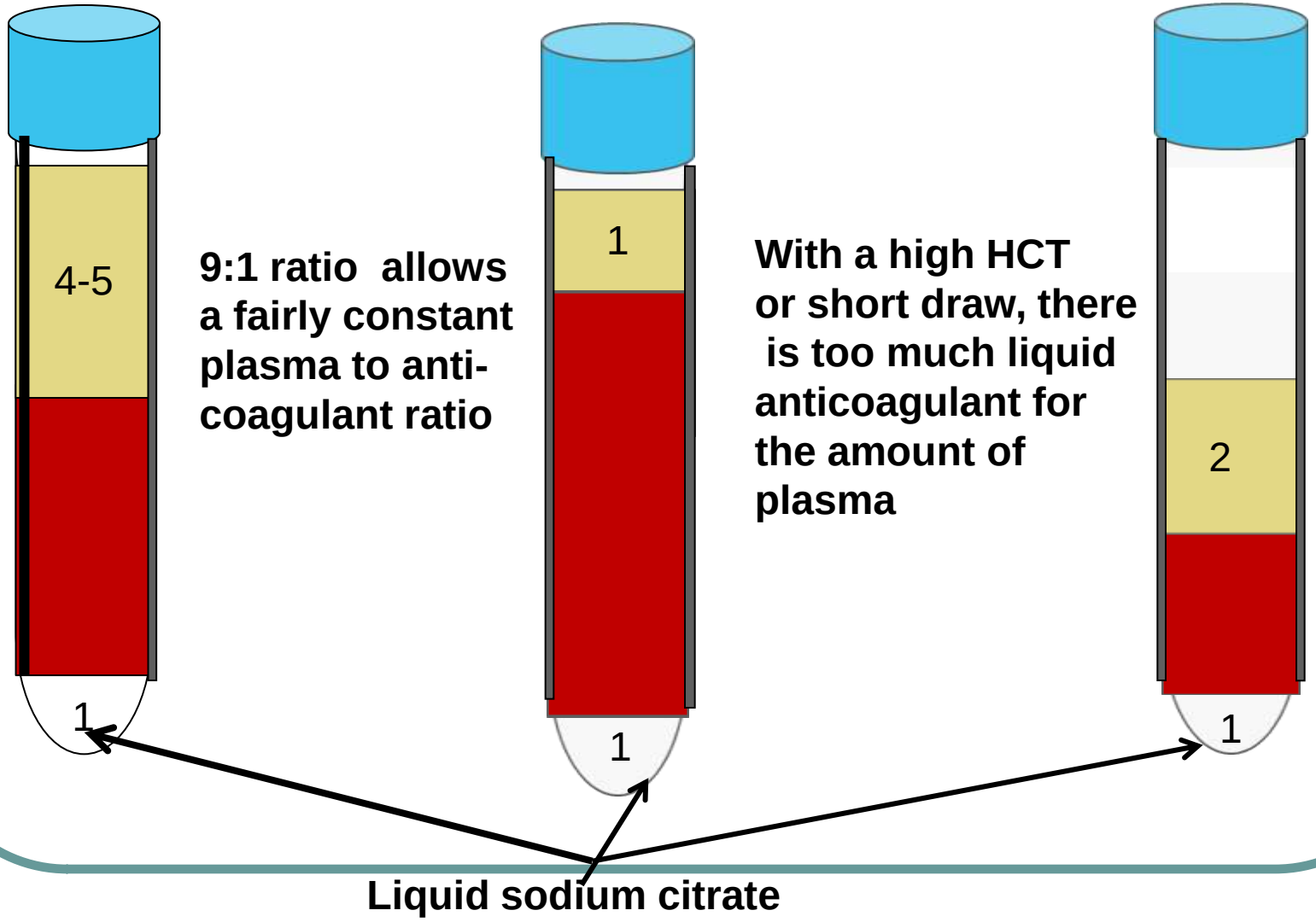
Components of the Collection System

- **Blood to anticoagulant ratio (fill volume)**
  - 9 parts blood to 1 part anticoagulant
    - Under-filled tubes - prolonged clotting times
      - Prolongation may be reagent dependent
    - < 90% fill is unacceptable unless locally validated
      - More forgiving with 3.2 vs 3.8% sodium citrate*
      - Small volume tubes are less forgiving**
  - **Samples with hematocrit > 55% require adjustment of citrate concentration**
    - To avoid spuriously prolonged clotting times
  - **Samples with hematocrit < 25% do not require citrate adjustment[#]**

***Adcock D, et al. AJCP;109:595**
**** Chuang et al. Chest 2004;126:1262**

[#] Siegel JE. AJCP1998;110:106-110.

9:1 Blood to Anticoagulant Ratio



Collection System Components

- **Under-filled blue stoppered tubes are a cause for specimen rejection**
  - Excess calcium binding plus dilutional effect of the liquid citrate
    - Essentially same as Hct >55%
- ***Never*** **transfer blood from one primary tube to another to provide required fill volume**
  - *Even if combining two blue stoppered tubes!!!*

Sample Processing, Transportation and Storage

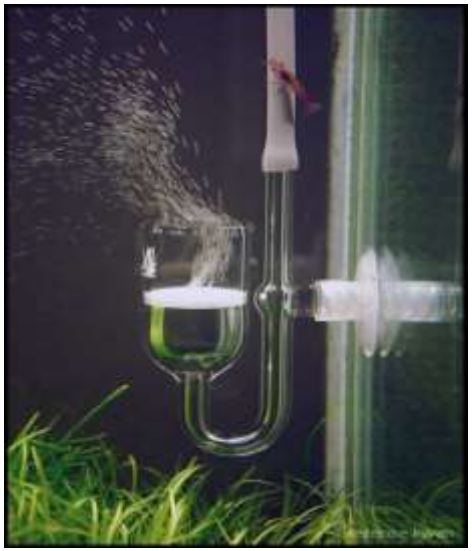
- Method of transport
  - Pneumatic tube
- Maintenance of proper pH
- Time between collection and analysis
- Temperature of transport and storage
- Conditions of centrifugation

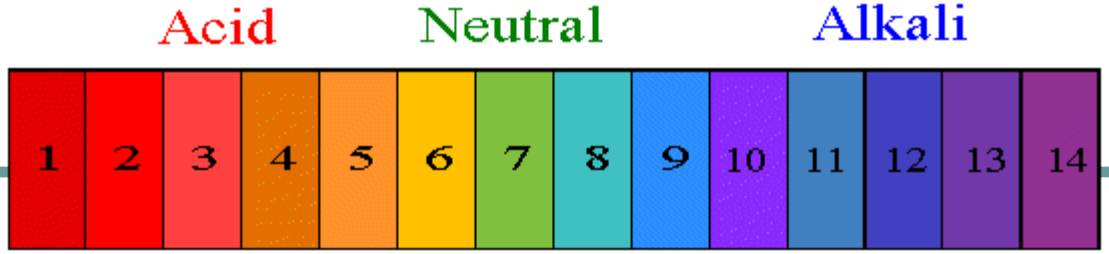
Specimen Transport

- **Specimens must arrive in the testing facility allowing sufficient time to be processed and analyzed**
  - According to sample stability guidelines
- **Use of a pneumatic tube system allowable for most plasma based assays**
  - Not recommended for platelet function studies or samples for thromboelastography

Specimen pH

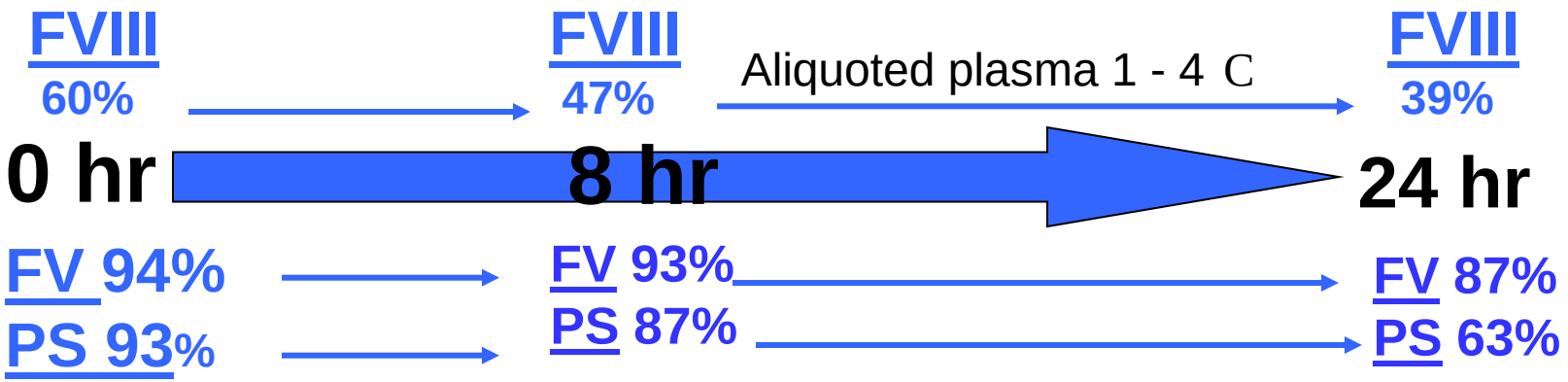
- **Sodium citrate has buffering capacity and maintains sample pH 7.30 – 7.45**
- **Uncapped samples lose CO_2 which elevates pH**
- **Elevated pH**
  - Prolongs the APTT and PT
- **Whole blood more stable due to buffering capacity of Hgb**





Time and Temperature

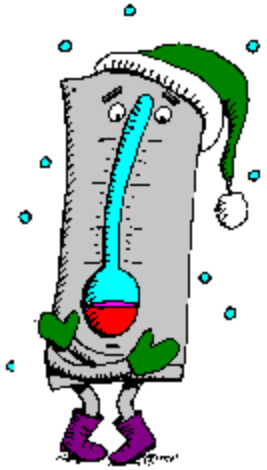
- **Platelets & coagulation factors are subject to time & temperature dependent activation or degradation**



- **FVIII, FV and PS are labile factors**
  - Accelerated loss occurs at warmer temperatures
  - Factors will lose all activity if maintained at 58ºC for a period of time

Temperature and Time

- **Cold activation of whole blood samples may result in**:
  - Clinically significant loss of cryoprecipitable proteins: **VWF, FVIII, Fibrinogen and FXIII**
    - As much as 50% from baseline
    - May lose activity > antigen
  - Spontaneous platelet aggregation and activation
  - Elevation of FVII activity by ≥150%



Favaloro E. Thromb Haem 2001;86:1589 *Favalaro E. Am J Clin Path 2004;122:686

Transportation of Sample

- **Transport at room temperature, ideally within one hour of collection**
- **Transport/Storage of *Whole Blood* at *2- 4° C* is *Not Recommended***
  - Potential Cause for Rejection!!
  - Potential for mistaken diagnosis of VWD or FVIII deficiency in a normal individual

Favaloro E. Thromb Haem 2001;86:1589-90.

Sample Processing

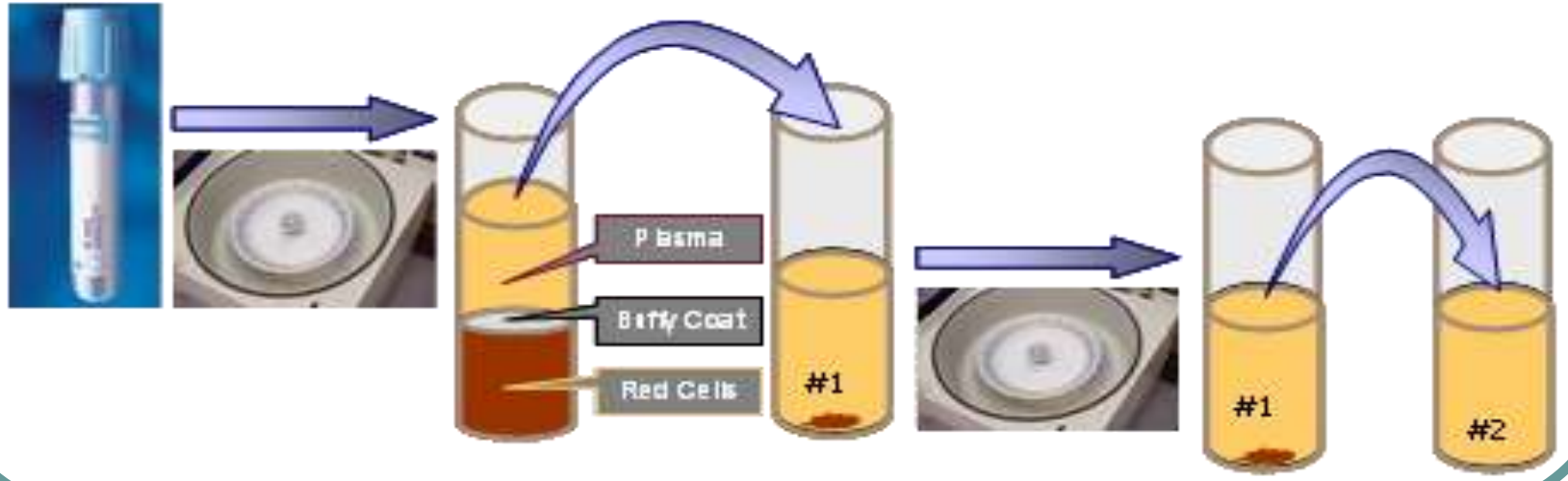
- **Centrifuge to obtain platelet poor plasma**
  - Post centrifugation plasma plt ct $\leq 10 \times 10^9/L$
    - Confirm every 6 months or after modification of centrifuge
    - Critical for frozen but not fresh plasma:
      - APTT, PT/INR and TT performed on fresh plasma samples not affected by platelet counts $\leq$ 200 x 109/L (200,000/μL)*
- **Other methods to obtain plt poor plasma**
  - ***Double centrifugation*** - recommended
  - Filtration using a 0.2 μm Millipore filter ** - ***NO!***
    - Can result in spurious prolongation of APTT and PT results due to selective removal of factors V, VIII, IX, XII and VWF

***Carroll WE (2001) J Med 32:83-96 ** Favaloro E. Bl Coag Fibrin 2007;18:86**

Sample Processing

Double Centrifugation

- Centrifuge the capped specimen tube
- Remove plasma layer and transfer to an aliquot tube being careful not to disturb the buffy coat (white blood cells & platelets)
- Cap and centrifuge the first aliquot tube
- Remove plasma leaving a small amount at the bottom of the tube and using care not to aspirate the small pellet of red blood cells & platelets at the bottom
- Transfer this platelet poor plasma to a second clean polypropylene aliquot tube, cap, and either use or freeze



Hemolysis

- **Visible hemolysis- reject sample**
  - Potential for activation of clotting factors*
  - Controversial
- **May impair end point detection using optical system of clot detection**
- **Samples that appear hemolyzed due to hemoglobin substitutes are not a cause of rejection**
  - Test using mechanical end point detection

***Lippi G, et al. Arch Pathol Lab Med 2006; 130:181**

Specimen Storage

- **The allowable time interval between collection of the specimen and testing of the sample depends on:**
  - Whether the sample is stored as whole blood or plasma
  - Assay to be performed
  - Temperature
- **Specimens should always be stored capped**

Sample Stability – PT Testing

- Stored as whole blood or processed into plasma, room temp ≤ 24 hrs*
- Sample integrity enhanced if samples are centrifuged immediately after blood collection
- 24 hour stability of vitamin K dependent factors reported**

0 hr  24 hr

* Adcock D, et al. Blood Coag Fibrinolysis 1998;9:463

**Awad MA, Selim TE, Al-Sabbagh FA. (2004) Hematology 9:333-337.

Sample Stability – APTT*

Non-Heparin Sample

- Whole blood or processed, in an unopened tube at room temperature ≤ 4 hours
  - APTT and specialty testing dependent on APTT
- Local validation of longer storage acceptable - normal and abnormal samples should be evaluated

*** Adcock D, et al. Blood Coag Fibrinolysis 1998;9:463**

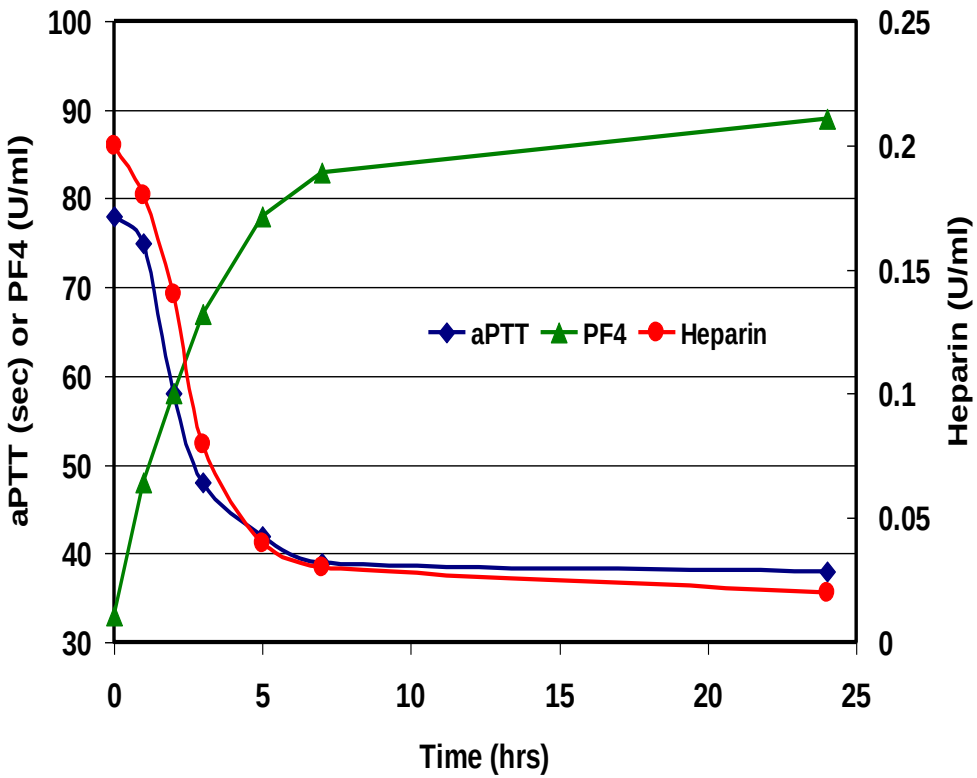
Sample Stability – APTT

UFH Sample

- Centrifuge within ***one hour*** of collection, test within four hours from time of collection*
- CTAD tubes may enhance stability – 4 hours

LMWH Sample

- Stable 24 hrs for anti-Xa testing**



*Adcock D, et al. Blood Coag Fibrinolysis 1998;9:463

**Birri N, et al. Br J Haematol 2011;155:629-631

Sample Stability – Other assays

- **Maintain whole blood or plasma at room temperature and test ≤ 4 hours from time of collection**
- **PS/FVIII/FV activity – limited stability***
- **PC/fibrinogen/AT activity – stable up to 7 days***

Heil W. (1998) Clin Chem Lab Med 36:459-452

Published Sample Stabilities

Clin Chem Lab Med 1998; 36:459-452.

Thromb Haemost 2008;99:416-426.

Am J Clin Pathol 1998;109:758-763.

Br J Haematol 2011;155:620 – 641.

Haemophilia 1996; 2: 218-223.

Blood Coagul Fibrinolysis. 1998; 9:463-470.

ASSAY	STABILITY WHOLE BLOOD SAMPLE	
	CLSI H21 A5	OTHER
APTT	4 hr	18-24 hr
PT	24 hr	24-72 hr
APTT or Anti-Xa assay for sample containing UFH	1 hr	
APTT or Anti-Xa assay for sample containing LMWH	4 hr	24 hr
Factors II, VII, IX, X & XI Activities	4 hr	48 hr
Factors V & VIII	4 hr	24 hr
VWF:Ag & VWF:RCo	4 hr	24-48 hr
Fibrinogen	4 hr	48 hr - 7 d
D-Dimer	4 hr	48 hr
Antithrombin Activity	4 hr	48 hr - 7 d
Protein C Activity	4 hr	48 hr
Protein S Activity	4 hr	4-6 hr
Free Protein S	4 hr	24 hr

Long Term Stability

- **If the testing is not completed within 24 hours for PT specimens and 4 hours for APTT and other assay(s), plasma should be removed without disturbing the sedimented cells and frozen at -20°C or colder for short-term storage (up to two weeks) or -70 C or colder for long-term storage**

- **Do not use frost-free (automatic defrost) freezers for sample storage**

Frozen Samples

- **Frozen plasma specimens should be rapidly thawed at 37°C then gently mixed and tested immediately**
  - Thorough mixing immediately after thawing prior to testing mandatory
    - Consider using **purple top** rocker

  - Excessive time or temperature in water bath may lead to significant loss of sample integrity

Patient Variables - Medications

- **Daptomycin and Telavancin (anti MRSA drugs)**
  - Dose dependent prolongation PT and APTT*
    - Degree of interference reagent dependent
- **Some Pegylated Compounds (e.g. PEG-hemoglobin and PEG-TNF alpha)****
  - Dose dependent prolongation of APTT using cephalin/silica reagent

Adcock D, et al. Bl Coag Fibrin. in press** * Adcock D, et al ISTH abstract 2007**

Common Sources of Error

- Collection tube other than sodium citrate
- Incomplete filling of evacuated tube
- Inadequate mixing of evacuated tube
- Cold-activation of the whole blood sample
- Inadequate thawing and mixing of previously frozen samples

Causes for Specimen Rejection

- **Plasma collected into anticoagulant other than sodium citrate**
- **Other than 9:1 ratio**
  - Evacuated tubes under or over-filled
  - Hematocrit > 55%
- **Clot evident in tube**
- **Hemolysis (in an ideal world)**
- **Improper specimen storage**

The Ideal Hemostasis Sample

- Draw blue top first or following a non-additive tube
- Atraumatic phlebotomy with minimal tourniquet use
- Drawn into 3.2% sodium citrate with no less than 90% fill (whole blood to citrate ratio of 9:1)
- Promptly and thoroughly mix with anticoagulant
- Transported at room temperature
- Centrifuged within one hour of phlebotomy to obtain platelet poor plasma
- Test or aliquot into a non-activating secondary tube immediately following centrifugation

Thank you!

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