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SUPPLEMENTAL TECHNICAL BULLETIN
ST – 2009 – 02

Title: Platelet Aggregation Sample Processing and Handling

Bulletin No: ST-2009-02

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Platelet Aggregation Sample Processing and Handling

A. Specimen Collection and Processing

Inattentiveness to variables in the pre-examination phase of platelet function testing can be readily implicated in producing abnormal results for platelet function tests. Variability can be introduced in two ways: 1) endogenous biological issues as they relate to the patient; and 2) exogenous variability resulting from specimen collection, transport and processing and sample preparation and handling.

1. Patient Requirements Before Collection

Platelet function testing is used to ascertain if a patient has a congenital/acquired disorder of platelet function or to evaluate the efficacy of antiplatelet agents. It is critical that care providers undertake a thorough clinical and family bleeding history before requesting laboratory evaluation for a platelet function defect. There must be detailed knowledge of medication and dietary history, which pharmacologic agent(s) require laboratory monitoring, and which inhibitory endpoints are acceptable for determining treatment efficacy.

2. Patient Preparation for Specimen Collection

Knowledge of the pharmacologic state of the patient before testing is important. Unless the aim of testing is to demonstrate platelet inhibition resulting from pharmacologic intervention, a patient must be drug-free before undergoing specimen collection. Though not comprehensive, the Appendix A lists drugs that inhibit platelet aggregation, prescription drugs and over-the-counter (OTC) drugs that contain aspirin, and food and herb supplements that adversely affect platelet function. The half-life of a platelet is seven to ten days. If medically possible, patients should ideally avoid any substances affecting platelet function for at least 14 days, should be fasting, and should also avoid fatty meals prior to collection. Patients should consult their physician before discontinuing any medication.

The platelet concentration of the patient's blood should be known before testing. Though dependent on instrument type used for assessing platelet function, sufficient platelets (as suggested by the instrument manufacturer) must be present to yield a functional response that falls within the threshold limitations of the instrument. For above studies, fibrinogen and blood type are useful information.

B. Specimen Collection

This section relates specifically to the specimen obtained directly from the patient. Issues as they relate to sample preparation and storage are addressed in the following areas of the document:

1. Systems and Techniques

Evacuated tube systems or plastic syringes may be used in combination with approved winged needle collection sets for platelet function testing and the needle gauge should be between 19 and 21. For evacuated tubes and syringes, specimens should be gently mixed by hand with end-over-end inversion for three to six times. Specific specimen collection requirements should follow guidelines presented in this document.

Concerns have been raised that use of evacuated tubes for collection of the specimen may lead to platelet activation. Comparison studies between the use of syringes or evacuated tubes showed minimal differences. Likewise, studies have shown little difference in platelet function test results when using either a one- or two-syringe technique for blood collection. Depending on technique and proficiency, syringe draws are susceptible to variability in the shear forces to which platelets are exposed. Therefore, blood collected in evacuated tubes may be used for platelet function testing. Several references suggest that an initial discard tube is unnecessary. It should be noted that if a syringe is used for blood collection, the needle must be removed before gently adding blood down the side wall of an opened evacuated tube or polypropylene tube containing anticoagulant, and that the ratio of blood to anticoagulant be adhered to strictly. If this technique is used, extreme caution should be exercised when removing the needle from the syringe. An alternate technique may be to draw the blood into a syringe containing the anticoagulant. Again, the blood-to-anticoagulant ratio should be adhered to strictly.

A needle with a diameter greater than 1 mm (19-gauge) may be traumatic for a vein and adversely affect hemostasis. Needles with diameters under 0.7 mm (22-gauge or higher) prolong blood collection and increase the pressure gradient in the needle, which could lead to hemolysis and platelet activation. However, Carcao and colleagues noted that blood specimens obtained from children using 23- vs 21- gauge needle sizes showed no significant differences. Cannulae reduce blood flow and consequently may increase the risk of platelet activation. However, Mani and colleagues demonstrated that differences could not be shown with either PRP maximal aggregation responses or high shear systems when

specimens were collected in the same subjects using 21-gauge ordinary needle systems or 21-gauge winged needle cannulae systems.

Stasis due to a cuff or tourniquet should be minimized; however, studies have shown no differences in PRP aggregation responses to ADP or epinephrine when stasis was produced by a cuff at 60 mmHg pressure for ten minutes. Recommendations for routine blood collection are to release the tourniquet as soon as blood begins to flow.

C. Anticoagulants

1. Sodium Citrate

Sodium citrate is the recommended anticoagulant for platelet function testing as it is defined in this document. Buffered Trisodium citrate is the sodium salt of citric acid with the chemical formula of $Na_3C_6H_5O_7$. Using citric acid to buffer blood and/or plasma maintains a physiologic pH. The recommended concentration of the dihydrate form of trisodium citrate is 105 to 109 mmol/L (commonly described as 3.2%).

The anticoagulant effect of trisodium citrate depends on its ability to chelate calcium. However, some unbound calcium must be present in whole blood (WB) and PRP for platelet aggregation to occur. Physiological calcium concentrations are lowered by sodium citrate to the micromolar range (40 µmol with 109 mmol/L citrate[i] and <5 µM with 129 mmol/L citrate). These low concentrations of ionized calcium affect platelet responses to agonists. For example, the primary reversible response to ADP is lower than when physiological calcium concentrations are available; as trisodium citrate concentrations increase, platelet response decreases, since less calcium ions are available. Also, at higher concentrations of ADP, the primary phase of aggregation is followed by an irreversible secondary wave of aggregation.

The anticoagulant-to-blood ratio is one part trisodium citrate to nine parts blood with a hematocrit value of 0.45 L/L (45%). Chelation of calcium ions will be impacted if there is no adherence to this nominal ratio. If the anticoagulant is present in excess resulting from either an elevated hematocrit or underfilling of the specimen tube, then more than the nominal amount of calcium ions will be chelated resulting in a blunted aggregation response. On the other hand, if less anticoagulant is available resulting from a decreased hematocrit (value not defined) or overfilling of the specimen tube, then fewer calcium ions are chelated (more available in the test system), which could lead to either a normal response (could be false-normal) or one that is enhanced. T'sao and colleagues clearly showed that even very small incremental increases in citrate concentrations had a significant impact on platelet responses to ADP, epinephrine, and collagen (blood collected in ACD). The concern they raised was the impact that their findings have on the underfilling of specimen tubes. Possibly this could be extrapolated to high hematocrits but certainly this topic brings forth numerous opportunities for experiments in the clinical laboratory.

PPACK is an inhibitor of alpha-thrombin. Blood anticoagulated with PPACK contains physiological concentrations of calcium. PPACK anticoagulant is used in pharmacologic studies that determine efficacy of glycoprotein IIb-IIIa (GPIIb-IIIa or $\alpha_{IIb}\beta_3$) inhibitors. Clinical trials using eptifibatide showed that sodium citrate removed calcium ions from GPIIb-IIIa and falsely enhanced the inhibitory activity of the drug, whereas blood specimens collected in PPACK did not show this phenomenon.

2. Specimen Hematocrit

Individuals with higher hematocrit values have an increased mean threshold value for aggregating agents (require higher concentrations of an agonist to elicit a response). This occurs because a direct relationship exists between citrate concentration and the amount of free calcium that is available. Several authors suggest that the amount of citrate in a collection tube be adjusted to compensate for hematocrit values greater or less than 0.45 L/L (45%). This would permit sufficient quantities of free calcium to be available to achieve maximal aggregation responses for a particular individual.Two methods can be used to correct for high hematocrits. One method can be found in Appendix B (nomogram with an equation) the other is to use the following equation: 5/(1-.hct) = amount of whole blood to add to 1 ml of citrate.

D. Specimen Transport

The way in which a whole blood specimen is transported from the patient to the testing site can significantly impact the specimen's integrity.

Issues to consider are as follows:

- Specimen temperature during transport must be maintained at ambient air or "room temperature," which is generally defined as 20 to 25 °C (68 to 77 °F). There must be no exposure to severe cold (refrigeration, ice packs, or winter temperatures) or heat (summer temperatures).

- Mode of transportation (hand-carried is preferred; avoid pneumatic tube systems; vehicular transport may be unavoidable due to physical location of the referring blood collection site). Specimens should not be left in closed vehicles where they may be exposed to very high or low temperatures.

- Duration of transport (subsequent to transport sufficient time must be allowed for testing.

- Position of tubes (upright, which is preferable to lying on the side).

- No traumatic handling, such as vibration, shaking, or agitation (all can lead to hemolysis and subsequent platelet activation).

E. Pre-examination Information

For LTA, the recommended concentration of anticoagulant (dihydrate form of trisodium citrate) for specimen collection is 105 to 109 mmol/L (3.2%). Evacuated tube systems or syringe systems can be used and the needle gauge should be between 19 and 21. For evacuated tubes, specimens should be gently mixed by hand with end-over-end inversion for three to six times. The amount of citrate in the collection tube may be adjusted (decreased) for individuals with hematocrit values greater than 0.55 L/L (55%). The specimen should be transported to the laboratory at room temperature.

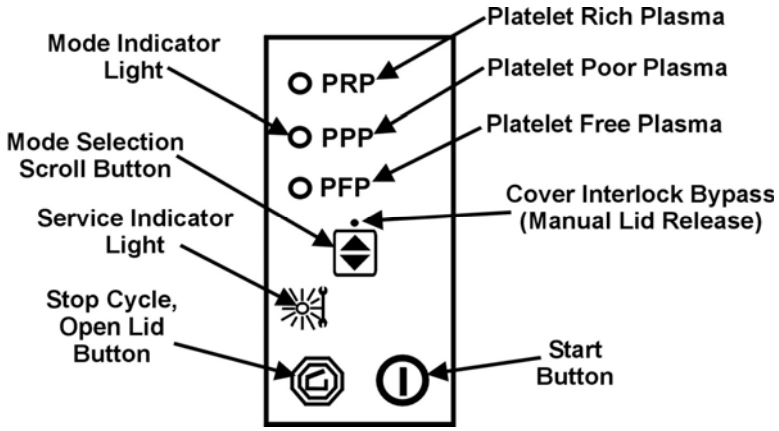
F. Standard Method - Sample Preparation

PRP and platelet-poor plasma (PPP) are required for performing LTA studies. Both can be obtained by centrifugation of the whole blood specimen at room temperature. The goal is to obtain PRP in which only platelets are retained and wherein red and white blood cells are removed. Centrifugation speeds should be denoted in relative centrifugal force (RCF), also known as g-force. RCF is calculated by knowing the rotating radius of a centrifuge and its rotational speed (revolutions per minute [RPM]). To minimize remixing of plasma and red cells, a swing-out bucket rotor should be used and the brake not applied at the end of centrifugation. The recommended speed and length of time for centrifugation is 170 g for 15 minutes. This recommendation is based upon representative centrifugation speeds and times denoted in the literature for preparation of PRP (100 g for ten minutes, 135 g for 15 minutes, 150 g for 30 minutes, 150 to 200 g for ten to 15 minutes, 180 g for ten minutes, 180 g for 15 minutes, 250 g for ten minutes). Deviations are potentially acceptable if prior experience and certain circumstances, e.g. large platelets or low platelet counts, warrant altering these parameters.

PDQ, Platelet Function Centrifuge – Sample Preparation

PDQ Operation

a. review the Control Panel functions

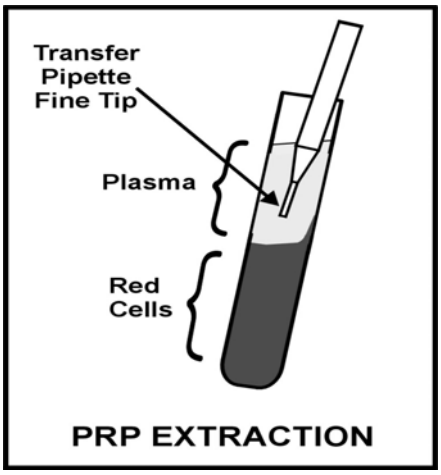


b. the PDQ has no “on-off” switch, therefore it is normally left plugged in and “on”.
c. cover interlock by-pass. The cover interlock can be released manually by inserting the end of a large paper clip into the small hole in the center of the panel.
d. ensure the appropriate tube inserts are installed.
e. the rotor must be properly balanced
   - use the same size and type tubes in opposite positions
   - balance liquid in tubes to within 0.5mL
   - replace balance tube every 10 cycles
f. if after loading, the PDQ will not spin, the lid may not be completely latched.
g. use only large bulb, fine tip transfer pipettes that come with the system
h. squeeze the transfer pipette bulb before inserting the tip into plasma to avoid introducing air into the sample and foaming.

1. Preparation of PRP, Platelet Rich Plasma

a. invert tube three times to mix
b. place tube cap up into PDQ
c. allow tubes to rest one minute, this will prevent scaling
d. close lid and use select button to scroll to PRP
e. press green button to start – will spin for 30 seconds
f. pull up latch and open lid, remove tubes – re-spin if RBC’s are present in plasma layer.

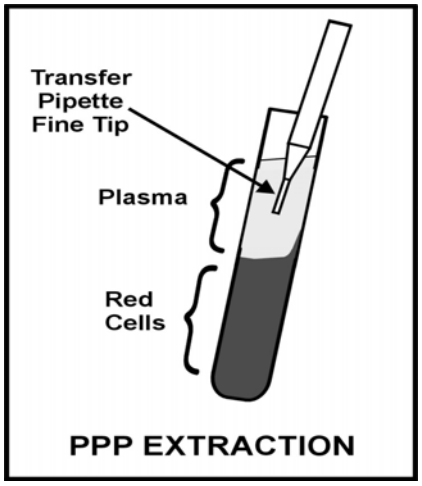
g. Tilt tube and insert large bulb, fine tip transfer pipette ¾ of the way down the center of the tube into the plasma layer, avoiding the sides of the specimen tube.



h. slowly extract and transfer the PRP into a plastic sample tube labeled PRP.
i. cap plastic sample tube labeled PRP and keep at room temperature
j. repeat process for all specimen tubes

2. Preparation of PPP, Platelet Poor Plasma

a. place the specimen tube back into PDQ, do not remix.
b. close lid and use select button to scroll to PPP
c. press green button to start – will spin for 120 seconds
d. pull up latch and open lid, remove tubes – re-spin if RBC's are present in plasma layer.
e. Tilt tube and insert large bulb, fine tip transfer pipette just below the surface of the plasma layer, avoiding the sides of the specimen tube avoiding the sides of the specimen tube.



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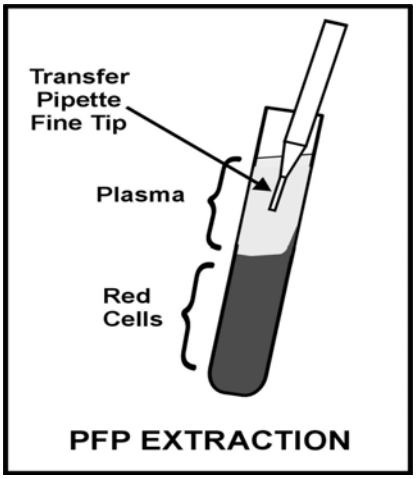
f. slowly extract and transfer the PPP into a plastic sample tube labeled PPP.
g. cap plastic sample tube labeled PPP and keep at room temperature.
h. repeat process for all specimen tubes.

3. Preparation of PFP, Platelet Free Plasma

a. place the specimen tube back into PDQ, do not remix.
b. close lid and use select button to scroll to PFP
c. press green button to start – will spin for 180 seconds
d. pull up latch and open lid, remove tubes – re-spin if RBC's are present in plasma layer.
e. Tilt tube and insert large bulb, fine tip transfer pipette just below the surface of the plasma layer, avoiding the sides of the specimen tube avoiding the sides of the specimen tube.



PFP EXTRACTION

f. slowly extract and transfer the PFP into a plastic sample tube labeled PFP.
g. cap plastic sample tube labeled PFP and keep at room temperature.
h. repeat process for all specimen tubes.

4. Platelet Counts on PRP/PPP/PFP

As a general rule, it is not necessary to perform a platelet count on the PRP/PPP and PFP. If a patient is known to have a substantially reduced count, or you are following a protocol requiring adjusted counts, you may have to do a platelet count on the PRP.

The optical density of PRP is directly proportional to the concentration of platelets. As the number of platelets increases in a sample, the opportunities for platelet collisions in the test cuvette increase, which result in 1) an increasing rate of aggregation; and 2) a relative greater change in optical density. It is for this reason that the platelet concentration of PRP is "standardized" (PRP adjusted to a target value with PPP). Although individual platelet function studies may not need platelet count adjustments to be examined under LTA, standardized performance of platelet function testing with the development of normal

values and interpretation guidelines requires that clinical samples be examined with a prescribed platelet count. It is recommended that the platelet concentration of PRP be adjusted, with autologous PPP. However, it is possible that preparation of PPP results in platelet granule release. Therefore, adjusting PRP counts with a physiologic buffer is an acceptable alternative until such time as there are clarification data. Subsequent to centrifugation, PRP is removed using a plastic pipette, then placed in a plastic tube with limited surface area-to-volume ratio, and capped. The remainder of residual blood is centrifuged at a higher RCF to obtain PPP. PPP should be platelet "free" (residual platelet concentration of less than 10 x 10^9/L [(10 000/µL]). The recommended speed and length of time for centrifugation is 1500 g for no less than 15 minutes at room temperature. Likewise, the platelet concentration of the PRP is determined to calculate how much PPP will be needed to dilute the initial PRP to a target PRP concentration for testing. PRP with concentrations below the target value should be used undiluted. For platelet concentrations in excess of 400 x 10^9/L, the recommended target PRP concentration subsequent to dilution with PPP is 200 to 250 x 10^9/L. This is based upon representative values denoted in the literature: 200 x 10^9/L, and 200 to 350 x 10^9/L. It is important to note that the option to adjust or not to adjust the platelet concentration of PRP to a target value must be done consistently (establishment of reference intervals, control samples, and patient samples).

NOTE: Some authors and manufacturers suggest that adjustment of the PRP by PPP is not necessary. Others suggest that PRP should be adjusted with physiologic buffer, since release material from the centrifugation to make PPP could adversely affect platelet function testing.

The PRP and PPP should be examined for interfering substances. Lipemia impacts the baseline turbidity of the sample. Though the PPP blank, in relation to the PRP, may compensate for the presence of lipids to some extent (this varies by instrument manufacturer), testing on lipemic samples should be avoided. **Note:** follow the aggregometer manufacturer's instructions for preparing the PPP blank; inappropriate blanks may invalidate test results or prevent the instrument from properly setting the 0 and 100% aggregation baselines. Hemolysis, due to an improper venipuncture or resulting from exposure to excessive heat or agitation for example, will result in release of nucleotides from the disrupted red cells and subsequent activation/desensitization (particularly to ADP) of platelets. Red cell contamination of PRP can occur due to improper centrifugation, braking of centrifuge, or disturbing the cellular component of the centrifuged specimen when attempting to pipette PRP. Red cells, due to their large size, can absorb more of the transmitted light in an aggregometer and by that result in a falsely depressed aggregation response. Icteric specimens also may not be acceptable. Like lipemic samples, the degree of icterus that can be corrected for by the blank is limited.

G. Sample Storage Before Testing

1. pH

Commercial trisodium citrate blood collection tubes are buffered with citric acid, to a pH of 5.1 to 5.3, which maintains the pH of a plasma sample between 7.3 and 7.45 (near the physiologic pH of 7.36 for venous blood). In unbuffered citrate, the pH of a plasma sample will rapidly increase because plasma has lost the buffering capacity of hemoglobin—found in red blood cells—which was removed by centrifugation to prepare PRP and PPP. Immediately upon preparation, the pH of PRP is approximately 7.5. For PRP stored at room temperature, maximum aggregation occurs at pH ~8.0 for ADP, pH 7.7 for epinephrine, and pH 7.6 for ristocetin-induced platelet aggregation (RIPA). PRP exposed to air (uncapped) undergoes a rise in pH due to diffusion of CO_2 from plasma into the ambient atmosphere. Appropriate pH can be maintained by: 1) capping the test tube containing PRP; 2) limiting surface area-to-volume ratio (use large volume of PRP in a small size test tube); 3) avoiding frequent mixing/agitation of PRP; and 4) introducing PRP directly into the tube and not allowing it to flow down the side. PPP used for PRP platelet concentration adjustment should be capped to minimize pH changes.

2. Temperature of Sample

The preferred temperature for maintaining a PRP sample is at room temperature (~ 25 °C).

Room temperature (~25 ^{o}C)

Platelets stored at room temperature are more sensitive to various aggregating agents, especially ADP, than platelets stored at 37 ^{o}C. Platelets stored at room temperature show little change in responsiveness for the first two hours (phlebotomy to sample preparation to testing).

Cold temperature (0 to 4 ^{o}C)

Cold causes platelets to become contracted, rounded, granular, and to lose their microtubular system. These changes are partly reversible when platelets are chilled for less than one hour and then restored to 37 ^{o}C. Platelets undergo spontaneous aggregation with cold storage. However, if chilled platelets are warmed for one hour at 37 ^{o}C, no spontaneous aggregation occurs and the subsequent response to some agonists is significantly higher than in samples stored at room temperature or 37 ^{o}C.

Elevated temperature (37 ^{o}C)

O'Brien showed that PRP capped and stored at 37 °C (elevated temperature) for 90 minutes failed to respond to epinephrine. After two hours of storage at 37 ^{o}C, some platelets samples begin to lose responsiveness to some agonists. Silver and colleagues compared PRP samples stored at 25 ^{o}C to those stored at 37 ^{o}C. Subsequent to storage (capped) for two hours at these respective temperatures, the authors showed that the effect on peak aggregation responses was not significantly different for ADP, but substantially lower for both epinephrine and collagen when stored at 37 ^{o}C.

3. Age of Sample

Use PRP within four hours after platelet donation. Various interpretations exist in the literature concerning effects of time on PRP samples. Most studies examining this issue were performed in the mid-1970s. These studies were done either before or at approximately the same time as studies by Han and Ardlie, and Coller and Gralnick. Work by these two groups showed that the effects of time were related to changes in pH and that those changes were directly related to the escape of CO_2 from the PRP sample tube. A comprehensive study performed by Roper and colleagues in 1977 showed that PRP samples processed 90 minutes after incubation at room temperature consistently showed values lower than those processed within 30 minutes of PRP/PPP dilution. However, they did not clearly state whether or not their sample tubes had been capped.

In 1975, Rossi and Louis clearly showed refractoriness by platelets to epinephrine when using PRP samples tested within 30 minutes of venipuncture. This platelet refractoriness was verified by Warlow and colleagues and subsequently cited by others as the rationale for not testing PRP within the first 30 minutes after phlebotomy. Zucker suggested that this initial platelet refractoriness and subsequent gain of function may occur because centrifugation releases ADP from red blood cells and platelets.

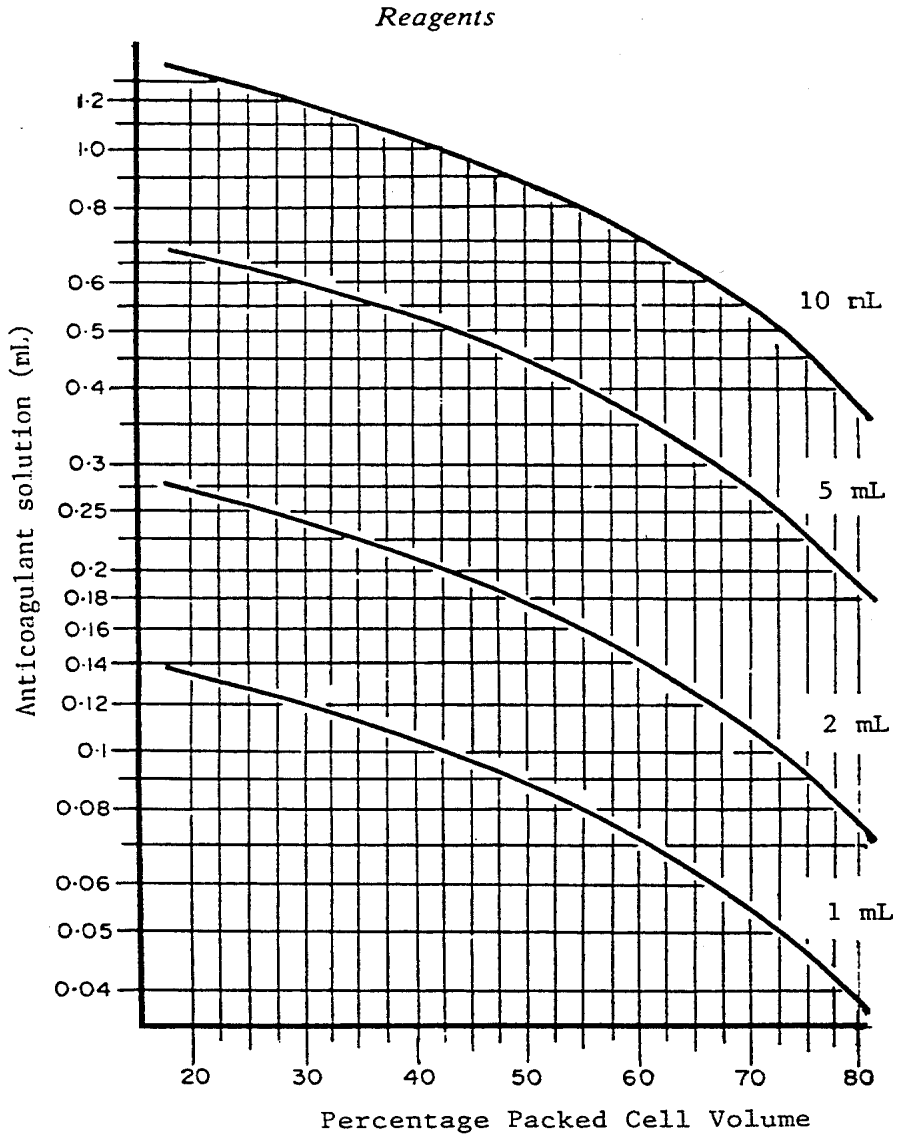
Studies seem to reach a degree of agreement as to maximal time intervals between venipuncture and testing. Based on their findings, Silver and colleagues recommended that a PRP sample be maintained at room temperature and used between two and four hours after platelet donation. Their data showed that responses for most agonists decline between two and four hours (120 to 240 minutes), and these changes are not as significant as the substantial decreases noted between four and six hours.

Appendix A. Products Affecting Platelet Function

COX-1 Inhibitors		**Antimicrobials**
Aspirin and all proprietary or over-the-counter (OTC) preparations containing acetylsalicylic acid		β-Lactams (penicillins, cephalosporins)
COX-1 and COX-2 Inhibitors (Nonsteroidal anti-inflammatory drugs [NSAIDs])		Amphotericin (antifungal)
Ibuprofen		Hydroxychloroquine (antimalarial)
Indomethacin, Naproxen		Nitrofurantoin
Mefenamic acid		**Chemotherapeutic Agents**
COX-2 Inhibitors (Coxibs)		Asparaginase
Celecoxib		Plicamycin
Inhibitors of Platelet Receptors		Vincristine
Abciximab ($\alpha_{IIb}\beta_3$)		**Psychotropics and Anesthetics**
Clopidogrel ($P2Y_{12}$)		Tricyclic antidepressants (imipramine)
RGD Peptomimetics		Phenothiazines (chlorpromazine)
Eptifibatide		Local and General Anesthesia (fluothane)
Tirofiban		**Miscellaneous Agents**
Phosphodiesterase Inhibitors		Clofibrate
Dipyridamole		Dextrans
Cilostazole		Guaifenesin (expectorant)
Anticoagulants		Radiographic Contrast
Heparin		**Foods/Herbals**
Warfarin		Alcohol
Direct Thrombin Inhibitors (lepirudin, argatroban, bivalirudin)		Caffeine (methylxanthine)
Cardiovascular Agents		Garlic, Onion, Ginger
β-Adrenergic Blockers (propranolol)		Fish Oil
Vasodilators (nitroprusside, nitroglycerin)		Vitamins C and E
Diuretics (furosemide)		**Adapted from George JN, Shattil SJ. The clinical importance of acquired abnormalities of platelet function. *N Engl J Med.* 1991; 324(1):27-39; and Kottke-Marchant K, Corcoran G. The laboratory diagnosis of platelet disorders. *Arch Pathol Lab Med.* 2002;126(2):133-146. Reprinted with permission from *Archives of Pathology & Laboratory Medicine.* Copyright 2002. College of American Pathologists.**
Calcium Channel Blockers		

NOTE: This is only a partial list; many other agents not shown also affect platelet function. Product information should always be reviewed for comments pertaining to platelet inhibiting activity.

Appendix B. Amount of Anticoagulant Solution/Volume of Blood at Different Packed Cell Volume Values



Select the curve for the total volume of anticoagulated blood required (10, 5, 2, or 1 mL). Enter the chart at the patient's packed cell volume (hematocrit [HCT]), on the horizontal axis and read off the corresponding volume of anticoagulant solution on the vertical axis. Place the volume in a collection tube and add blood up to the required total volume. The chart assumes a normal HCT of 43%; in that case, 1 volume of anticoagulant is made up to 10 volumes with blood.

Alternatively, the anticoagulant volume may be calculated using the formula:

$C = (1.85 \times 10^{-3})(100\text{-}HCT)(V_{Blood})$

where: C is the volume of citrate remaining in the tube; HCT is the hematocrit of the patient; V is the volume of blood added (if a 5 mL tube is used then the volume is 4.5 mL); and 1.85×10^{-3} is constant (taking into account the citrate volume, blood volume, and citrate concentration).

Reference:

CLIS Platelet Function Testing Aggregometry; Approved Guideline – H58-A
June 2007