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(54) Title: METHODS FOR DETERMINING PLATELET ACTIVITY WITH ANTIPLATELET COMPOSITIONS

(57) Abstract: Methods, compositions and kits are disclosed for the determination of platelet activity on samples such as whole blood that have been affected by exposure to an antiplatelet therapeutic agent such as, *e.g.*, aspiring or a thienopyridine. A combination is provided in a reaction medium wherein the combination comprises the sample and a particle reagent comprising a GP IIb/IIIa receptor ligand such as fibrinogen covalently attached to particles. The medium further comprises a polyhydroxy aromatic compound and a metal cation. The combination is incubated under conditions for agglutinating the particles. The extent of agglutination of the particles is determined and is related to the platelet function activity of the sample. The methods and compositions have particular application to the detection of platelet sensitivity to therapy using aspirin or a thienopyridine and the assessment of the efficacy of such therapy.

METHODS FOR DETERMINING PLATELET ACTIVITY WITH ANTIPLATELET COMPOSITIONS

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to the field of diagnostic assays, and in particular to the determination of platelet function activity on blood samples to study effects of anti-platelet compositions. The invention has particular application to the determination of platelet function activity on blood samples from patients undergoing therapy for platelet anti-aggregation.

The role of platelets in mammalian physiology is extraordinarily diverse, but their primary role is in promoting hemostasis. In many situations, an evaluation of the ability of blood to clot is desired, a parameter that is frequently controlled by the ability of platelets to adhere and/or aggregate. Of interest, therefore, is the assessment of the adhesive functions of platelets. For example, questions of interest include whether to administer drugs that will block, or promote, clot formation, or whether to detect deficiencies in platelet function prior to surgical procedures. Also of interest is evaluating the effectiveness of a platelet inhibitor that is being tested as a new drug or is being used as approved clinical treatment in a patient.

Platelets are known to aggregate under a variety of conditions and in the presence of a number of different reagents. Platelet aggregation is a term used to describe the binding of platelets to one another. The phenomenon can be induced by adding aggregation-inducing agents to platelet-rich plasma (PRP) or to whole blood. Platelet aggregation *in vitro* depends upon the ability of platelets to bind fibrinogen to their surfaces after activation by an aggregation-inducing agent such as ADP or collagen.

Platelets play a critical role in the maintenance of normal hemostasis. When exposed to a damaged blood vessel, platelets will adhere to exposed sub-endothelial matrix. Following the initial adhesion, various factors released at the site of injury such as thrombin, ADP and collagen activate the platelets. Once platelets are activated, a conformational change occurs in the platelet glycoprotein GPIIb/IIIa receptor allowing it to bind fibrinogen and/or von Willebrand factor.

It is this binding of the multivalent fibrinogen and/or von Willebrand factor molecules by GPIIb/IIIa receptors on adjacent platelets that results in the recruitment of additional platelets to the site of injury and their aggregation to form a hemostatic plug or thrombus.

In vitro platelet aggregation is the laboratory method used to assess the *in vivo* ability of platelets to form the aggregates leading to a primary hemostatic plug. In this technique an aggregating agent such as ADP or collagen is added to whole blood or PRP and aggregation of platelets monitored. Platelet aggregometry is a diagnostic tool that can provide insights difficult to obtain by other techniques, thus aiding in patient diagnosis and selection of therapy. These methods of monitoring platelet aggregation require expensive, laboratory dedicated instruments that are not easily portable and require standardization to ensure accurate quantitative results. In addition, unless performed using whole blood, results are unlikely to be available for several hours.

Currently there are two detection methods used in instruments with FDA clearance for performing platelet aggregometry: optical and impedance measurements. The CHRONO-LOG Model 530 and Model 540 use the optical method for PRP and the impedance method for whole blood aggregometry. The impedance method has been shown to be substantially equivalent to the optical method for measuring platelet aggregation in PRP.

A rapid platelet function assay has recently been developed and is described in U.S. Patent No. 5,763,199 (Coller). The assay determines glycoprotein (GP) IIb/IIIa receptor blockade in whole blood. Agglutination of small polymeric beads coated with a GPIIb/IIIa ligand such as fibrinogen results when the beads are contacted with whole blood containing platelets with GPIIb/IIIa receptors that are not blocked. Failure to agglutinate indicates that blockade of the GPIIb/IIIa receptors has been achieved. In a preferred embodiment, the addition of a thrombin receptor activator results in an assay that is rapid and convenient enough to be performed at bedside and that results in agglutination of the small polymeric beads within a convenient, known period of time if the GPIIb/IIIa receptors are not blocked. The assay includes the ability to transfer blood to be tested from a collection container to an assay device without opening the collection container. This platelet aggregation assay can be conducted at the same time

as the activated clotting time (ACT), which is performed to assess the adequacy of heparinization.

The past decade has witnessed an evolution in the understanding of the role of platelets in cardiovascular disease. Platelet aggregation plays a key role in the pathogenesis of atherosclerosis, thrombosis and acute coronary artery disease. Platelets have been implicated in the formation of rapidly-progressing atherosclerotic lesions, and play a key role in acute arterial thrombosis. The importance of platelets is highlighted by the unrivalled therapeutic success of the anti-platelet drug, aspirin. This drug, available for over a century, has been shown in hundreds of trials to reduce the risk of serious ischaemic events in several cardiovascular disease states including stroke, myocardial infarction, and unstable angina and in patients following coronary artery bypass surgery. Although aspirin is a relatively weak platelet anti-aggregation drug, it has been used as one of the first-line therapeutic options in the treatment of cardiovascular diseases. Several clinical trials have proven its efficacy in primary and secondary prevention of occlusive cardiovascular events. Aspirin exerts its effect by blocking only one of the pathways that lead to platelet aggregation. It acts by producing an irreversible inactivation of the enzyme prostaglandin G/H synthase that is necessary for the conversion of arachidonic acid to thromboxane A2 that ultimately stimulates platelet aggregation and vascular constriction. Although aspirin has been the mainstay of anti-platelet therapy for several decades, it is acknowledged as a relatively weak inhibitor of platelet activity.

Different methods can be used to assess the effectiveness of aspirin on platelet activity or, the sensitivity of the platelets. Conventional aggregometry methods employing different agonists (*e.g.,* arachidonic acid, epinephrine) are not suitable in the clinical setting since they require bulky, expensive and complicated instruments as well as laborious separation of PRP (Platelet Rich Plasma) and PPP (Platelet Poor Plasma) from blood. Also, any possible prothrombotic effects of red cells may be lost due to this separation. Flow cytometry employing fluorescent-labeled monoclonal antibodies which binds specifically to activated platelet membrane glycoproteins (e.g. GPIIb/IIIa) after platelet activation is quite interesting (Valettas, *et al.*, Aspirin resistance using flow cytometry. *Thrombosis, Thrombolytic Therapy and Anticoagulants*. 1998; Abstract

3268), but again depends on very expensive instrumentation and very complicated procedures.

Evidence suggests that significant platelet function variability exists in the response to aspirin and to ADP antagonists among various groups of cardiac patients. Although several different methodologies were used to study the effects of aspirin on platelet aggregation, some studies estimate that approximately 15% of patients do not properly respond to aspirin and are, thus, aspirin resistant. Women were reported to be more prone to aspirin resistance than men. Other factors reported to be associated with aspirin resistance were cigarette smoking and stress/epinephrine.

Since the advent of newer rapid and easy-to-use methodologies to measure platelet aggregation, more is known about platelet function in the presence of aspirin or ADP antagonists. Preliminary data of an undergoing study designed to evaluate the prevalence of aspirin resistance in a cardiac population taking 325 mg of aspirin, requiring either diagnostic or therapeutic coronary catheterization, has demonstrated that 5-8% of these patients do not achieve the therapeutic benefit of aspirin inhibition. In the same study, a subset of patients representing about 25% of the group, fulfills partial criteria for aspirin resistance, and was called aspirin semi-responders. Another recent study using the same methodology and similar criteria showed that 24% of patients taking only 100 mg of aspirin daily were aspirin resistant and that this aspirin resistance could be overcome in the majority of patients by increasing the dose of aspirin.

Characterization of high risk populations for acute coronary events that demonstrate to have resistance to the platelet anti-aggregation effect of aspirin, *i.e.*, aspirin resistance, could provide useful information and help to evaluate whether patients with aspirin resistance have a worse outcome compared to an aspirin sensitive group. It has also been shown that a significant percentage of aspirin-treated patients are not protected from platelet-dependent vascular events. Studies performed in cerebrovascular (stroke) patients have confirmed the existence of such "aspirin resistance" of platelets in some patients. (Helgason, *et al.*, Development of aspirin resistance in persons with previous ischaemic stroke. *Stroke*. 1994; 25: 2331-2336.) With the recent development of the thienopyridines, including clopidogrel (Plavix) and ticlopidine, clinically proven antiplatelet therapies other than the "gold standard" (aspirin) are now available. Cardiac patients with aspirin resistance may not benefit

from aspirin administration as a preventive medication. However, such patients might benefit from other anti-aggregation drugs such as, for example, clopidogrel, ticlopidine and other ADP antagonists. It would make sense to be able to screen patients for aspirin sensitivity of their platelets and put those who are aspirin-resistant on alternative albeit more expensive antiplatelet drugs.

Clopidogrel and ticlopidine are thienopyridine derivatives that inhibit platelet aggregation. They are believed to inhibit the binding of adenosine-5-diphosphate (ADP) to its receptor. Clopidogrel and ticlopidine are FDA approved anti-platelet drugs and inhibit platelet activation by blocking the P2Y12 ADP receptor. The pharmacological activity of clopidogrel is very similar to the pharmacological activity of ticlopidine. However, the adverse effects of clopidogrel are far less severe than those of ticlopidine.

It has also been demonstrated that an inter-individual variability in platelet aggregation exists when the ADP antagonist clopidogrel is used for treatment of patients to achieve an anti-aggregation effect. The results of this study showed that 15% of patients receiving the drug did not achieve the expected platelet aggregation inhibition. The use of clopidogrel and other ADP antagonists are likely to increase significantly.

Current assays of platelet aggregation are cumbersome and time consuming. Conventional aggregometry method employing different agonists such as, for example, ADP, arachidonic acid, and epinephrine, are not suitable in the clinical setting since they require bulky, expensive and complicated instruments as well as laborious separation of platelet rich plasma (PRP) and/or platelet poor plasma (PPP) from whole blood. Also, any possible prothrombitic effects of red cells may be lost due to this separation. Flow cytometry employing fluorescent-labeled monoclonal antibodies that bind specifically to activated platelet membrane glycoproteins such as, *e.g.*, GPIIb/IIIa, after platelet activation is quite interesting, but again depends on very expensive instrumentation an very complicated procedures.

A novel platelet activator, namely, cationic propyl gallate (cPG) has been discovered (U.S. Patent Nos. 5,700,634 and 5,709,889); cPG was shown to exhibit, in optical aggregometry, a very wide separation of rates of platelet aggregation between baseline and aspirin-treated PRP samples. Propyl gallate/metal ion platelet activation has been used in various plasma assays to measure or assess platelet reserve, *i.e.*, the

qualitative and quantitative platelet excess necessary for normal coagulation, activated plasma clotting time (aPCT), and other plasma-based assays. A considerable disadvantage of these assays is that they require significant sample manipulation and take greater than about thirty minutes to perform.

Furthermore, since many patients with cardiovascular disease are currently taking one of the thienopyridine agents, a method for detection of aspirin resistance in these patients by standard platelet aggregation is desirable as well as a method for detection of resistance to a thienopyridine and assessment of the efficacy of thienopyridine treatment. Thus, there is a need for an assay the can evaluate platelet function that can quickly assess antiplatelet therapy. Also, there is a need to develop an assay that would provide information about aspirin sensitivity and thienopyridine, *e.g.*, clopidogrel or ticlopidine, sensitivity and efficacy of treatment in a given patient.

SUMMARY OF THE INVENTION

One embodiment of the present invention is a method for conducting an assay for platelet function activity on a blood sample. A combination is provided to form a reaction medium. The combination comprises the sample, a particle reagent comprising a GP IIb/IIIa receptor ligand covalently attached to particles, a polyhydroxy aromatic compound and a metal cation. The combination is incubated under conditions for agglutinating the particles. The extent of agglutination of the particles is determined where the extent thereof is related to the platelet function activity of the sample.

Another embodiment of the present invention is a composition comprising (i) a particle reagent comprising fibrinogen covalently attached to particles, (ii) a lower alkyl ester of trihydroxybenzoic acid, and (ii) a divalent transition metal ion. Preferably, the composition is in the form of a lyophilized pellet.

Another embodiment of the present invention is an apparatus comprising a container for receiving a sample and a composition as described above, preferably, in lyophilized form. The composition may comprise additional reagents such as, for example, buffers, lyophilization stabilizers and the like.

DETAILED DESCRIPTION OF THE INVENTION

We have discovered that activator compositions comprising certain trihydroxy aromatic compounds and a metal cation are useful agonists in measuring inhibition of

platelet aggregation by antiplatelet therapeutic agents such as aspirin, ADP antagonists, *e.g.*, thienopyridines, and so forth, in whole blood samples. Accordingly, the aforementioned compositions may be employed to determine the effectiveness of anti-platelet therapy involving treatment of patients with aspirin or an ADP agonist such as a thienopyridine. The above compositions may be employed in conjunction with particles coated with a GP IIb/IIIa receptor ligand and any other reagents necessary for conducting an assay for the efficacy of aspirin or thienopyridines. A lyophilized reagent composition may be used that comprises the aforementioned activator compositions and particles. In one approach, a metered volume of a sample to be measured such as whole blood is mechanically mixed with the lyophilized reagent. A change in light transmission is monitored and an index of platelet activity is calculated. In one aspect a whole blood sample is combined in a cuvette or a unitized cartridge with the aforementioned lyophilized reagent. An apparatus may be employed for carrying out the assay. The apparatus comprises a well for receiving the sample where the well contains the lyophilized reagent and other reagents for conducting the assay. The additional reagents may be various buffers and/or lyophilization stabilizers.

As mentioned above, in one aspect the present invention is directed to a method for conducting an assay for platelet function activity on a whole blood sample. In one embodiment, the sample is one that has been affected by an antiplatelet therapeutic agent. By the term "antiplatelet therapeutic agent" is meant agents or drugs that interfere with the ability of platelets to aggregate and form a platelet plug. Examples of such antiplatelet therapeutic agents include aspirin or an adenosine-5-phosphate (ADP) antagonist. For example, the sample may be from a patient undergoing treatment with aspirin or an adenosine-5-phosphate (ADP) antagonist. The character of the sample and the amounts employed in a method in accordance with the present invention is discussed in more detail hereinbelow.

In the present invention a combination is provided in an assay medium where the combination comprises the sample and a polyhydroxy aromatic compound in conjunction with a metal cation. Suitable polyhydroxy aromatic compounds include, by way of illustration and not limitation, lower alkyl esters of polyhydroxybenzoic acids, tannin, and the like. Lower alkyl means a straight or branched chain hydrocarbon moiety comprising from 1 to about 9, usually, about 2 to about 5, preferably, about 3

carbon atoms. Examples of lower alkyl groups include methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, and any appropriate iso-, tert-, neo-, sec-, and other forms thereof such as, for example, isopropyl, isobutyl, sec-butyl, tert-butyl, and so forth. Preferably, number of hydroxy groups on the benzoic acid is about 1 to about 4, preferably, about 3. A preferred polyhydroxy aromatic compound is propyl gallate.

The amount of the polyhydroxy aromatic compound employed in the reaction medium is that which is effective in achieving activation of the platelets so that an accurate assessment may be made of the activity of the platelets. The amount of the polyhydroxy compound is dependent on the type of sample. For example, the optimal propyl gallate concentration in platelet rich plasma (PRP) optical aggregometry was determined to be about 30 μM. The optimal propyl gallate concentration for whole blood RPFA-PG differs for at least two reasons: (i) the sample is different and red cells in whole blood might adsorb some propyl gallate, which is hydrophobic, and (ii) propyl gallate was specially prepared to remove extra unwanted salts (which is in the regular SPAT, Slide Platelet Aggregation Test, reagent) so as to not cause aggregation in a human fibrinogen-coated bead dispense reagent. Usually, the range of the amount of the polyhydroxy aromatic compound for all types of samples is about 20 to about 5000 μM, more usually, about 100 to about 1000 μM, preferably, about 200 to about 450 μM.

The metal cation employed in the present compositions is any suitable cation that in conjunction with the polyhydroxy aromatic compound achieves the desired platelet activation effect. A preferred metal cation is a divalent metal cation, usually, a divalent transition metal ion such as, for example, Ni(II), Cu(II), Co(II), and the like. The amount of the metal cation employed in the reaction medium is that which is effective, in conjunction with the polyhydroxy aromatic compound, in achieving activation of the platelets so that an accurate assessment may be made of the activity of the platelets. Usually, the amount of the metal cation is about 1 to about 5000 μM, more usually, about 5 to about 1000 μM, preferably, about 10 to about 40 μM.

Also employed in the present methods, is a reagent comprising particles coated with a compound that can result in the specific agglutination of platelets, *i.e.*, the agglutination of platelets by the specific interaction between a receptor on the platelets and the compound on the particles. Such compounds include, by way of illustration and

not limitation, antibodies to a platelet receptor and GPIIb/IIIa receptor ligands, which may be a small organic molecule, polypeptide, protein, monoclonal antibody or nucleic acid that binds, complexes or interacts with GPIIb/IIIa receptors on the platelet surface. Platelet mediated aggregation of the particles results when the GPIIb/IIIa receptors on the surface of platelets bind, complex or otherwise interact with the GPIIb/IIIa receptor ligands on the particles. Typical GPIIb/IIIa ligands include fibrinogen, monoclonal antibody 10E5 (Coller, *et al.*, J. Clin. Invest. 72:325 (1983)), monoclonal antibody c7E3 (The EPIC Investigators, N.E. Journal of Med., 330:956 (1994)), von Willebrand factor, fibronectin, vitronectin and other ligands that have an arginine glycine-aspartic acid (RGD) sequence or other peptides or peptidomimetics that mimic this sequence (Cook, *et al.*, Drugs of the Future 19:135 (1994)). Other compounds of interest include thrombin inhibitors, low molecular weight heparin, and so forth.

The particles to which the compound is attached are at least about 0.1 microns and not more than about 20 microns. In one embodiment the particles are about 0.1 microns to about 10 microns. In another embodiment the particles are at least about 1 micron and less than about 8 microns. The particles can be virtually any shape, but are generally spherical with uniform diameters. The particle may have any density, but preferably of a density approximating water, generally from about 0.7 to about 1.5g/ml. The particles may or may not have a charge on the surface, either positive or negative, preferably negative. The particles are functionalized or functionalizable so as to covalently bind or attach sbp members at their surface, either directly or indirectly.

The particles may be solid (*e.g.*, comprised of organic and inorganic polymers or latex), oil droplets (*e.g.*, hydrocarbon, fluorocarbon, silicon fluid), or vesicles (*e.g.*, synthetic such as phospholipid or natural such as cells and organelles). The solid particles are normally polymers, either addition or condensation polymers, which are readily dispersible in a liquid medium. Examples of suspendable particles are polymeric materials such as latex, lipid bilayers, oil droplets, cells and hydrogels. Other particle compositions include polymers, such as nitrocellulose, cellulose acetate, poly (vinyl chloride), polyacrylamide, polyacrylate, polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), nylon, poly(vinyl butyrate), polysaccharides such as dextrans and modified dextrans, etc.; either used by themselves or in conjunction with other materials. The solid

particles can be comprised of polystyrene, polyacrylamide, homopolymers and copolymers of derivatives of acrylate and methacrylate, particularly esters and amides, silicones and the like.

As mentioned above, the compound is coated on the particles. Usually, the compound is covalently attached to particles. Such covalent attachment can be accomplished by well-known techniques, commonly available in the literature. See, for example, "Immobilized Enzymes," Ichiro Chibata, Halsted Press, New York (1978) and Cuatrecasas, J. Biol. Chem., 245:3059 (1970). Briefly, as mentioned above, the surface of the particle may be polyfunctional or be capable of being polyfunctionalized. A wide variety of functional groups are available or can be incorporated. Functional groups include carboxylic acids, aldehydes, amino groups, cyano groups, ethylene groups, hydroxyl groups, mercapto groups and the like. The manner of linking a wide variety of compounds to surfaces is well known and is amply illustrated in the literature (see above). The attachment of the sbp member may be directly by a bond or indirectly through the intermediacy of a linking group. The length of a linking group may vary widely, depending upon the nature of the sbp member and of the particle.

The ratio of molecules of compound to particle is controlled in the attachment of the molecules of compound to the particle. In one approach the number of functionalized sites on the surface of the particle may be controlled by adjusting the number of such sites introduced on the surface of the particle. Alternatively, or in conjunction with the above, the ratio of molecules of compound to particle may be controlled by adjusting the concentration of the compound in the reaction medium for the attachment. Other approaches will be suggested to one skilled in the art in view of the above teaching.

The particle reagent employed in the present invention may be treated with a sufficient amount of material to block areas of adsorption on the particles. Such materials will not affect the functioning of the particles for their intended purpose in the present invention. The blocking materials include proteins such as bovine serum albumin, bovine gamma globulin, etc., polysaccharides such as dextran, etc., and the like. In another approach, which may be utilized in conjunction with the above, particles are employed wherein the number of functionalized sites for attachment substantially reduce the adsorption area on the surface of the particles.

The particles usually comprise a label, either attached thereto or incorporated therein. The label may be any moiety that may be used for the purpose of detection. The label is often a member of a signal producing system. The label is capable of being detected directly or indirectly. The label can be isotopic or nonisotopic, usually non-isotopic, and can be a dye, fluorescent molecule, chemiluminescent molecule, a catalyst, such as an enzyme, a polynucleotide coding for a catalyst, promoter, coenzyme, enzyme substrate, radioactive group, a small organic molecule, amplifiable polynucleotide sequence, and so forth.

In one specific embodiment of the present invention, the particles contain one or more dyes that absorb in the infrared. "Infrared" means electromagnetic radiation at wavelengths longer than the red end of visible light and shorter than microwaves. Visible light has wavelengths of about 400 to about 700 nm. Thus, light with a wavelength longer than about 700 nm and shorter than about 1000 nm is generally recognized as near infrared. In a preferred embodiment, the wavelength is about 800 nm. Such dyes include bacteriochlorin, bacteriochlorophytin, meropolymethine dyes, benzoannulenes, vinylogous porphorins, polymethine dyes, cyanines and merocyanines, and the like. Specific dyes of interest are Copper(II)-tetra-tert-butyl-tetrakis(dimethylamino)-29H-31H-phthalocyanine and Vanadyl-tetra-tert-butyl-tetrakis(dimethylamino)-29H-31H-phthalocyanine. The particular dye that is selected is one of convenience, availability, stability, compatibility with the particle and the like. These dyes may be incorporated directly into the particle itself, through polymerization or passive adsorption. The dyes may be loaded individually (*i.e.*, sequentially) or in combination (*i.e.*, simultaneously). Alternatively, the dyes may be linked to the bead in combination with the linking component, such that they do not leach from the surface. Irrespective of the loading method used, the conditions are such that the particle surface is unaffected with respect to the ability to agglutinate under appropriate conditions.

The dyes absorb light in the range of about 750 nm – 900 nm, particularly in the range of about 750 – 850 nm. For samples with high levels of red blood cells, the light is at about 800 nm ± 10 nm, which is the isobestic point for oxyhemoglobin and reduced hemoglobin. The amount of dye employed with the particles varies with the extinction coefficient of the dye in the light range of interest, the required sensitivity of the assay, the size of the particles, the mode of binding of the dye to the particles, compatibility of

the dye with the particle matrix, and the like. Usually, the amount of dye incorporated is in the range of about 1 to 20 weight percent, more usually 5 to 15 weight percent. Dyes which find a particular use in the present invention are phthalocyanines. Metal free phthalocyanines absorb at approximately 700 nm (e=162,000). The metal complexes shift the absorption to either shorter or longer wavelength, most metals shift the absorption to a much shorter wavelength, but some, such as lead absorb at much longer wavelength than the metal free phthalocyanines.

The complexes formed between transition metals and phthalocyanines (metollophthalocyanines and Metallonaphthalocyanines) are chemically very stable to light and heat. They are formed by condensation of opthalodinitriles in the presence of an appropriate metal. Some of the metals used in the formation of the metalophthalocyanines besides the copper (Cu) and the Vanadium (V) are magnesium (Mg), zinc (Zn), and cobalt (Co).

In one specific embodiment of the invention carboxylated microparticles with a flat absorption maximum are employed. These microparticles are prepared by incorporating multiple dyes that have distinct absorption maximum close to 805 nm. This results in a flat maximum absorption spectrum across a broad range wavelength from 780-820 nm.

The sample may be any solution, synthetic or natural, to be analyzed where the sample has been subject to an effect from an antiplatelet therapeutic agent. The term sample includes biological tissue, including body fluids, from a host, and so forth. The sample can be examined directly or may be pretreated, usually. The present invention has particular application to samples that comprise platelets, including body fluids such as, for example, whole blood, platelet-containing blood fractions such as plasma, and the like. In one embodiment the invention has particular application to whole blood samples. The amount of the sample depends on the nature of the sample. For fluid samples such as whole blood, the amount of the sample is usually about 30 µl to 5000 µl, preferably, about 100 to 300 µl. The term "sample" includes unprocessed samples directly from a patient or samples that have been pretreated and prepared in any convenient liquid medium, usually an aqueous medium.

Preferably, the medium for conducting the assays in accordance with the present invention is an aqueous medium. Other polar cosolvents may also be employed in the

medium, usually oxygenated organic solvents of from 1-6, more usually from 1-4 carbon atoms, including alcohols, ethers and the like. Usually, such cosolvents are present in less than about 70 weight percent, more usually, in less than about 30 weight percent. Additionally, various ancillary materials are frequently employed in the method in accordance with the present invention. For example, buffers are normally present in the assay medium, as well as stabilizers for the assay medium and the assay components; surfactants, particularly non-ionic surfactants; binding enhancers, *e.g.*, polyalkylene glycols; or the like.

The pH for the medium is usually in the range of about 2 to about 11, preferably, about 4 to about 9. Various buffers may be used to achieve the desired pH and maintain the pH during the method. Illustrative buffers include HEPES, borate, phosphate, carbonate, Tris, barbital, and the like. The particular buffer employed is not critical to the method but one buffer may be preferred over others in certain circumstances. In some circumstances HEPES is preferred and is present at a concentration of about 0.05M to about 0.001M but generally at a concentration of about 0.01M.

Usually, the volume of assay medium is about 25 to about 500 microliters, usually about 75 to about 250 microliters. The assays may be carried out in any suitable container. Conveniently, the container is a cuvette or cartridge that is used with the instrument for carrying out the assay and measuring the assay results. The reaction container usually contains the particle reagent in accordance with the present invention in dry lyophilized form together with other reagents such as an activation initiator and the like, stabilizers and so forth. The amount of the particle reagent is about 1×10^6 to about 5×10^9 particles/milliliter.

The combination of sample and particle reagent is incubated under conditions for agglutinating the particles. Moderate temperatures are normally employed for carrying out the method. The temperature may be constant or may vary. Usually, a constant temperature is employed during the reaction step. The temperature employed is usually about 10 to about 80°C, more usually, about 15 to about 45°C, preferably, the temperature should be at least 25°C, more preferably in the range of about 30 to about 40°C, usually about 37°C.

The extent of agglutination of the particles is determined and is related to the presence and/or amount of the member in the sample. The presence of agglutination

may be determined visually by observing clumping of the particles, which would indicate agglutination. Preferably, as mentioned above, the particles may be colored to aid in visualizing agglutination or clumping of the matrix. The extent of agglutination may be measured spectrophotometrically, turbidimetrically, nephelometrically, etc., by observing the rate of change of optical density of the medium, and so forth.

In a specific embodiment of the present invention an assay for platelet function activity is conducted on a whole blood sample from a patient undergoing treatment with an antiplatelet therapeutic agent. The sample is combined in a suitable container, *e.g.*, reaction cuvette, with fibrinogen coated particles, a lower alkyl ester of a trihydroxybenzoic acid, *e.g.*, propyl gallate, and a metal cation, *e.g.*, Ni(II) ion, to form an assay medium. The particles of the particle reagent have one or more infrared dyes incorporated therein. The combination is subjected to agglutination conditions. Then, the medium is irradiated with light in the infrared region. The transmission of infrared light from the assay mixture is determined where the level of transmission is related to platelet function activity.

The agglutination medium is selected to have high absorption at about 800 nm. The ratio between the agglutination medium absorption coefficient and whole blood absorption coefficient should preferably be greater than about 4:1 at about 800 nm. The absorption ratio for a particular assay is a function of both the absorption coefficient of the agglutination medium and the concentration of the agglutination medium in the assay sample.

After the sample has been combined with the reagents, desirably it will be heated to a temperature above room temperature, but below that which would interfere with the assay, so as to insure that the temperature can be controlled without adversely affecting the assay result. Desirably, the temperature should be at least 25°C, preferably in the range of 30-40°C, more preferably about 37°C. The reaction medium is usually gently agitated upon combining of the reagents with the sample and during the period of the reaction. Agitation is sufficient to achieve and maintain homogeneity in the assay samples. The total time of the readings from the zero time (time of mixing), may range from about 10 sec. to 5 min., more usually about 30 sec. to 5 min., and preferably about 30 sec. to 2 min. The data may be analyzed by any convenient means, particularly using an algorithm that can manipulate the data in relation to calibrators and/or controls.

The level of agglutination is an indication of the platelet function activity of the sample tested. The level of agglutination may be compared against a standard of known platelet function activity. Usually, the result will be compared to a calibrator, which may be performed concomitantly or have been performed previously or may be provided as a standard curve.

The method of the present invention may be employed in conjunction with an assay for platelet count such as that described in U.S. Patent Application Serial No. 09/177,884 filed October 23, 1998 (the '884 application), the relevant disclosures of which are incorporated herein by reference.

The above assays preferably may be conducted in a device, which allows the reactions in accordance with the present invention to occur and which measures the results thereof. The instrument should assess platelet function based upon the ability of activated platelets to bind fibrinogen. As activated platelets bind and agglutinate fibrinogen-coated particles, there is an increase in light transmittance. In general, an instrument to measure the result of the assay is one that can measure agglutination. Preferably, the instrument measures a change in optical signal due to agglutination. Suitable instruments include, by way of illustration and not limitation a kinetic spectrophotometer, ULTEGRA SYSTEM® instrument (commercially available from Accumetrics, San Diego, CA and employed for rapid platelet function activity measurements on normal samples), or the like.

The ULTEGRA SYSTEM® instrument is a turbidometric based optical detection system, which measures platelet induced aggregation as an increase in light transmittance. The system consists of an analyzer, disposable cartridge and controls. The cartridge contains reagents based on microparticle agglutination technology. The quality control system includes an electronic control, two levels of assayed "wet" controls (WQC), an in-cartridge humidity sensor, an in-packaging temperature indicator, and a test for concurrence of two assay channels. The analyzer controls assay sequencing, establishes the assay temperature, controls the reagent-sample mixing for the required duration, determines the degree of platelet function, displays the result and performs self-diagnostics. For use in the present methods, the test cartridge of the system contains a lyophilized preparation comprising particles with covalently attached GPIIa/IIIb receptor ligand, a polyhydroxy aromatic compound, a metal cation,

preservative and buffer. The patient sample is usually citrated whole blood, which is automatically dispensed from the blood collection tube into the cartridge by the analyzer, with no blood handling required by the user. The interaction is monitored by the infra red absorbency characteristics of the particles. As the particles interact with the platelets, the agglutination of the particles is measured through the optical system of the ULTEGRA SYSTEM® instrument. The agglutination is detected as an increase in the transmission of infrared light through the sample. The reaction kinetics are analyzed and translated into "Platelet Aggregation Units", PAU.

Another aspect of the present invention is a kit comprising in packaged combination a lyophilized preparation comprising particles with covalently attached fibrinogen, a lower alkyl ester of trihydroxybenzoic acid, a divalent transition metal ion, preservative and buffer. The lyophilized preparation may be present in a reaction container such as a cartridge used in the instrument of analysis. For the aforementioned ULTEGRA SYSTEM® instrument, the lyophilized preparation may be placed in the outer wells of the four-well cartridge used in the analyzer. The kit may also include a sample collection container and/or a device for carrying out the present method. The relative amounts of reagents may be varied widely to provide for concentrations in solution of the reagents that substantially optimize the sensitivity of a determination. Where appropriate, the reagents can be placed in an airtight package in order to maintain the activity of any reagents. The package may be, for example, a bag, pouch, or the like fabricated from a material that is substantially non-permeable to moisture. Such materials include, by way of example and not limitation, plastic, aluminum foil, and the like. For blood samples the kit may also include an article for piercing a person's skin, disinfectant or sterilizing pads and so forth. The kit may also include calibrators and standards. Furthermore, the kit may also include one or more reagents for conducting an assay for platelet count.

EXAMPLES

The following examples are offered by way of illustration and not limitation. Parts and percentages are by weight unless otherwise indicated. Temperatures are in degrees Centigrade (°C) unless indicated otherwise. Unless indicated otherwise, reagents are from Sigma Chemical Company or Aldrich Chemical Company.

The following examples and preparations are intended to illustrate the invention but are not intended to limit its scope.

With reference to the examples below and the disclosure herein "aspirin resistance" or "aspirin resistant" means that the rate of platelet aggregation in the presence of aspirin is substantially unaffected compared to the rate of aggregation in the absence of aspirin. When a blood sample is assayed by three methods (Aggregometry-PG, Aggregometry-EPI and RPFA-PG) in duplicate, if at least half of the test results (total of 6; 3 methods in duplicate) are positive, *i.e.,* above the cut-off for the respective assay, then the platelets in the sample are said to be "aspirin resistant."

With reference to the examples below and the disclosure herein "aspirin sensitivity," "aspirin sensitive" or "aspirin hypersensitivity" means that an extremely low steady state platelet activity is maintained in response to aspirin administration. Aspirin irreversibly inactivates cyclooxygenase (COX). The liver keeps making more of the enzyme to replenish it. Given a certain aspirin regimen, a steady state is reached in which the platelet activity is kept at a certain low level. For some people, due to the fact that either their livers can not make enough COX or they can not make more platelets to make up for the lost COX, this steady state platelet activity becomes extremely low, causing bleeding problems. These people are said to have aspirin hypersensitivity.

Example 1

Determination of Optimal Propyl Gallate Concentration for Whole Blood RPFA-PG

The optimal PG concentration for whole blood RPFA-PG was determined. Rates of agglutination were obtained from running baseline and aspirin-treated whole blood (3.2% citrate) samples with increasing concentrations of spiked PG using cartridges, cuvettes or the like containing all necessary ingredients but any activators. The optimal PG concentration (or range) should be where there is maximum differential in rates of agglutination between baseline (higher rates) and aspirin-treated samples (lower rates).

Typical time courses of PG-induced RPFA agglutination in pre- and post-aspirin blood samples at two PG concentrations are shown in Figure 1. As can be seen from Figure 2, the optimal PG concentration for RPFA in whole blood (around 250 μM) was significantly higher than that for aggregometry in PRP (about 30 μM). Also, the RPFA

PG-activation time – the time at which maximum slope is found – decreases with increasing PG concentration; lower PG concentration resulted in unnecessarily long activation time and, therefore, long assay time. Conversely, higher PG concentration caused higher rate of post-aspirin aggregation and, therefore, less differentiation between pre- and post-aspirin rates of platelet aggregation.

Example 2

Preparation of Reagent Cartridges for Ultegra RPFA-PG Assays

The reagent cartridges were prepared in a manner similar to that for preparation of reagent cartridges for RPFA-iso-TRAP assays on the ULTEGRA SYSTEM® instrument except PG was used instead of iso-TRAP and no HEPES saline was added to the dispense reagent.

Two lots of RPFA-PG cartridges were prepared: D10025 prepared to give a final PG concentration of 417 μM and D11006 prepared to give a final PG concentration of 250 μM. Both were used to study normal, healthy donors and only D11006 was used for the clinical feasibility study. Performance of these lots is discussed below in Example 4.

The detection well of the RPFA-PG cartridges contained PG, fibrogen-coated infrared dyed particles, buffers and lyophilization stabilizers. A preferred buffer was HEPES. HEPES was added to a final concentration of 10-20 mM after reconstitution with the whole blood sample.

Example 3

Effects of Acetyl Salicylic Acid (ASA) Concentration on RPFA-PG Rates of Agglutination, and Aggregometry-PG and Aggregometry-EPI (Epinephrine) Slopes

The algorithm used was a moving window linear regression (with a width of 40 seconds). The maximum rates of agglutination were obtained in three consecutive time zones: Zone I (40-180 seconds), Zone II (180-360 seconds) and Zone III (360-510 seconds). If either the maximum rate from Zone I was greater than 95 mOD/min or that from Zone II was greater than 70 mOD/min, then the maximum of the three maximum rates was obtained. If neither held, the maximum rate from Zone III was used. The results reported herein were obtained using the maximum rate from Zone III unless otherwise noted.

For purposes of comparison, two PRP aggregometry methods were studied. The study of the rates of agglutination or platelet aggregation as a function of increasing ASA concentrations provided the basis of comparison. A stock solution of 15 mM ASA in PBS was prepared. Appropriate amounts were then added to blood pools to final ASA concentrations at 0, 1.5, 3, 6, 15 and 150 μM. PRP samples were then prepared for the aggregometry assays: for aggregometry-PG, 44.4 μL of SPAT Reagent was added to 400 μL of PRP to attain a final [PG] of 30 μM; for aggregometry-EPI, 44.4 μL of a 500 μM epinephrine solution was added to 400 μL of PRP to attain a final [EPI] of 50 μM.

Increasing ASA concentration causesd RPFA-PG rates and Aggregometry-PG and Aggregometry-EPI slopes to go down. Aggregometry-PG slopes were apparently too fast to respond to the presence of low doses of ASA ([total ASA]< 5 μM). The difference between time courses of Aggregometry-PG and Aggregometry-EPI is self-evident in Figure 3: the latter was more sensitive to low doses of ASA than the former. Conversely, the former would then be more sensitive in revealing partial ASA inhibition. Time courses for RPFA-PG are shown in Figure 4.

It was determined that about 10 μM ASA in blood was needed to bring the aggregation rates down to minimal in every case. The RPFA-PG assay performed, similar to Aggregometry-EPI, seemed to be more sensitive to low doses of ASA than Aggregometry-PG, as can be seen in Figure 5.

The correlation between RPFA-PG rates and Aggregometry-PG slopes as well as that between RPFA-PG rates and Aggregometry-EPI slopes are shown in Figure 6. RPFA-PG correlated well with Aggregometry-EPI at lower doses of ASA and with Aggregometry-PG at higher doses. This study provided valuable information relating to what to expect when a comparison is made between data from a whole blood RPFA-PG assay and data from either PRP Aggregometry-PG or PRP Aggregometry-EPI.

Example 4

Studies on Pre- and Post-Aspirin Blood Samples from Normal, Healthy Donors

Blood was first drawn from a donor who had not taken aspirin or any NSAID medicines in the past 10 days; this was the pre-aspirin (or baseline) sample. A 500 mg

tablet of Bayer aspirin was then given to the donor and ingested. A minimum of 2 hours later, blood was drawn again; this was the post-aspirin sample. All three methods – aggregometry-PG, aggregometry-EPI and RPFA-PG – were used to assess platelet activities.

Subsequently, RPFA-PG rates, Aggregometry-PG and Aggregometry-EPI slopes were obtained for pre- and post-aspirin blood samples from normal, healthy donors (11 males, of which 3 were tested twice; 6 females, of which one was tested twice). Two kinds of RPFA-PG cartridges were used, with final PG concentration in blood at 250 and 417 μM, respectively (3 of the 11 male donors were not tested using the former). The latter did seem to increase the RPFA-PG rate of post-aspirin samples relative to that of pre-aspirin ones and, therefore, exhibited less specificity. The RPFA-PG rate differentiation between pre- and post-aspirin samples seemed to be somewhat better using cartridges with the lower PG concentration. Males in general tended to have lower RPFA-PG rates than females. There were still donors, almost exclusively males (6 out of the 11 male donors and only 1 out of 6 female donors), that exhibited longer PG-activation times, prolonging assay time to longer than 3 minutes, as well as lower rates of agglutination, even though their PRP's behaved the same way as the others in aggregometries. This didn't seem to be related to higher hematocrit (HCT), as males with high HCT (around 45) exhibited short PG-activation times and the converse was also true. Some slow RPFA-PG time courses can be found in Figure 7 together with some fast ones for comparison purposes. Bar graphs of pre- and post-aspirin RPFA-PG rates for the normal, healthy donors (some donated twice) are shown in Figures 8 and 9. Genders of donors can be found below the donor IDs of the top graph. Comparisons of distributions of pre- and post-aspirin RPFA-PG rates are shown in Figures 10-15 and those for Aggregometry-PG and Aggregometry-EPI slopes are shown in Figures 16.

Example 5

Effects of Blood Sample "Maturing" (or Aging) on RPFA-PG Assay Results

Blood was drawn, pooled and formed into aliquots as fast as possible. Aliquots were then run on an ULTEGRA SYSTEM® instrument.

The word "maturing" refers to the settling of PG-activation time to a minimum with concomitant settling of RPFA rate to a maximum. Frequently, when replicate

blood samples were run, the latter ones tended to have shorter PG activation times and higher RPFA-PG rates. A systematic investigation was carried out on two donors: Donor 16 was a female, only pre-aspirin blood was studied; Donor 4 was a male, both pre- and post-aspirin blood samples were studied. We found that there indeed was a shortening of PG-activation time with concomitant rise in RPFA-PG rate as blood samples age or, "mature." For example, the activation time was about 300 seconds when the 1st replicate of blood was run (5 minutes after drawing) and it dropped to about 200 seconds when the 2nd replicate from the same blood pool was run (15 minutes after drawing), 150 seconds for the 3rd replicate (25 minutes after drawing), finally settling between 50 and 100 seconds about 30 minutes after blood draw. Aspirin-inhibited samples didn't seem to exhibit this phenomenon. The RPFA-PG time courses are shown in Figure 17. Figure 18 shows the time courses of sample "maturing" of these two donors. Apparently, it is known that epinephrine-induced platelet aggregation is frequently depressed when analyzed immediately after blood drawing and may improve with increasing time of incubation. Multiple factors are probably involved, including ADP-induced refractoriness in response to low amounts of ADP being released, and perhaps the wearing off of the antiplatelet effects of NO as blood leaves contact with the endothelium.

Example 6

Clinical Feasibility Studies on CAD Patients That Are on Aspirin Regimen

IRB approval in a clinical setting was obtained to run pre- and post-aspirin studies on 30 normal donors and to run RPFA-PG on 270 CAD patients that are on aspirin regimen. Two 4.5 mL Becton Dickinson Vacutainer tubes and two 2 mL Greiner tubes were filled in each drawing. The 4.5 mL tubes were processed to obtain PRP and PPP for running the aggregometry-PG and -EPI assays and the 2 mL tubes were used directly to run RPFA-PG assays.

The goal was to collect data from 30 normal donors (pre- and post-aspirin) and 100 to 270 CAD patients that are on aspirin. In preliminary studies with 8 normal donors and 30 CAD patients, a pre- and post-aspirin RPFA-PG rate distribution was constructed from the data. In this way, a number of aspirin-resistant CAD patients were identified. It should be noted that this study was carried out in mid-December in Salt

Lake City with a mean altitude of around 4500 ft. In general, it was found that all rates of platelet aggregation were significantly slower there than in the warmer climates and lower elevations of San Diego using the same ULTEGRA SYSTEM® instrument (the Chronolog aggregometer was different) and the same lots of reagents. The mean pre-aspirin aggregometry slopes were 185 (agg.-PG) and 88 (agg.-EPI) and RPFA-PG rate was 195 in San Diego, whereas the corresponding numbers were 148 (agg.-PG), 62 (agg.-EPI) and 152 (RPFA-PG) in SLC. One subject, for example, had aggregometry slopes of 209 (agg.-PG) and 95 (agg.-EPI) and RPFA-PG rate of about 200 when tested in San Diego, but the slopes were 142 (agg.-PG) and 40 (agg.-EPI) and the RPFA-PG rate was about 100 when tested three days after arrival in Salt Lake City. Bar graphs of pre- and post-aspirin RPFA-PG rates for the normal donors are shown in Figures 19. Genders of donors can be found below the donor ID's of the top graph. Distributions of pre- and post-aspirin RPFA-PG rates along with those of patients are shown in Figures 20 and those for Aggregometry-PG and Aggregometry-EPI slopes are shown in Figures 21. If 60 was used as cutoff in RPFA-PG, the following aspirin-resistant patients were identified: # 1, 2, 3, 4, 5, 8, 9, 18, 21, 23, 25 and 28, of which # 8, 18 and 28 were resistant in one of two replicates. Using 55 as cutoff in Aggregometry-PG, the aspirin-resistant patients identified were # 1, 2, 3, 4, 6, 11, 12, 18, 20, 21, 24 and 25 as aspirin-resistant, of which # 21 was resistant in one of two replicates. Using 40 as cutoff in Aggregometry-EPI, the aspirin-resistant patients identified were # 1, 2, 3, 4, 5, 15, 21, 23, 25 and 28 as aspirin-resistant, of which # 1, 15, 23 and 25 were resistant in one of two replicates. Since there is no single reference assay at present for aspirin resistance, it is perhaps more reliable to identify a patient as aspirin-resistant if at least half of the test results (total of 6: 3 methods × 2 replicates) are positive. Application of the latter approach identified, as true aspirin-resistant, the following 10 patients: # 1, 2, 3, 4, 5, 18, 21, 23, 25 and 28. The sensitivities, specificities and accuracy of the three methods were then calculated and are shown in the following table (Table 1).

Table 1

method	Sensitivity (%)	Specificity (%)	Accuracy (%)
RPFA-PG	88.9	92.1	91.1
Aggregometry-PG	65.0	75.0	71.7
Aggregometry-EPI	73.7	97.4	89.5

RPFA-PG seemed to have the best sensitivity and accuracy with a respectable 92.1% specificity. All patient data can be found in the table below (Table 2).

"True" aspirin-resistant patients are those marked with an asterisk next to their Patient ID number. Assay results are marked with a "+" if they are above their respective cutoff values: 55 for Aggregometry-PG, 40 for Aggregometry-EPI and 60 for RPFA-PG.

Table 2

Patient ID	Aggregometry-PG		Aggregometry-EPI		RPFA-PG	
1*	90+	91+	40+	36	136.0+	
2*	126+	125+	66+	75+	149.0+	201.0+
3*	66+	73+	45+	47+	61.5+	108.5+
4*	60+	55+	45+		126.5+	
5*	49	53	44+	45+	118.0+	113.5+
6	93+	93+	34	36	25.8	35.6
7	40	42	38		26.9	24.2
8	42	44	36	38	64.0+	56.9
9	42	49	34	36	134.5+	138.0+
10	47	51	36	38	20.2	25.0
11	93+	95+	38	38	23.9	23.0
12	108+	123+	23	23	30.1	29.1
13	34	51	31	31	28.1	34.8
14	45	53	34	40+	23.0	58.8
15	40	42	27		23.9	23.3
16	44	47	33	34	21.4	19.4
17	36	40	33	34	27.2	21.0
18*	60+	60+	36	36	31.0	122.5+
19	44	45	12	14	19.9	
20	62+	66+	33	38	28.9	20.1
21*	47	64+	53+	57+	130.0+	104.6+
22	42	47	38	38	57.9	
23*	51	53	38	44+	130.5+	69.1+
24	110+	125+	12	18	43.4	39.3
25*	88+	101+	38	40+	155.5+	113.4+
26	49	51	5	7	24.5	20.8
27	29	33	16	20	31.2	25.6
28*	47	45	42+	45+	27.0	65.1+
29	5	5	5	9	24.3	21.7
30	20	18	9	11	19.3	22.1

Example 7

Reagent Preparation

Carboxylated particles (Interfacial Dynamics (IDC), Portland OR) with an absorbency spectrum of 780-830nm were covalently linked to fibrinogen to facilitate measurements of bead agglutination in whole blood. The conjugation procedure started with activation of the carboxylated particles. The particles were first reacted with an ethyl-carbodiimide (EDC) solution at 10mg/mL and an n-hydroxysuccinimide (NHS)

solution at 50mg/mL during a 30-minute incubation. Following the incubation, the particles underwent several centrifugation steps with 0.01M MES, pH 6.0 (Calbiochem-Novabiochem Corporation; La Jolla, CA) buffer, to remove excess EDC and NHS. The activated particles were incubated with fibrinogen for 2 hours. Fibrinogen became covalently linked to the particle through the attached EDC and produces a NHS-ester leaving group. The final fibrinogen conjugate was then washed several times with 20mmol/L HEPES, pH7.4

The fibrinogen conjugate was combined with 20mmol/L HEPES, pH 7.4, containing trehalose, bovine serum albumin (BSA), mannitol, sodium azide, and the propyl gallate reagent (American Control System, Fishers IN, containing 250 μM propyl gallate and corresponding metal cation) as an agonist to produce the pellet dispense reagent. The pellet dispense reagent was used to produce lyophilized pellets. These pellets were used as platelet function reagents to measure the activity of the platelets in whole blood samples from a patient being treated with clopidogrel. Pellets were produced by dispensing 0.0125mL of the pellet dispense reagent into liquid nitrogen. The pellets froze and sunk to a collecting tray in the bottom of a nitrogen reservoir. The frozen pellets were transferred to a frozen lyophilizer tray and freeze dried over a 24-hour cycle. Once the cycle was complete, the pellets were stored in a moisture-free environment at room temperature.

The pelletized reagent was transferred to the mixing chamber of the cartridge of the Ultegra® System together with one steel-mixing ball. The cartridge contained four mixing chambers. One pellet and one mixing ball were placed in the two outer mixing chambers of the cartridge. The mixing chamber was welded to the cartridge manifold and a needle was inserted onto the cartridge. Cartridges were sealed in a pouch containing one desiccant packet.

Assay

Blood samples from a patient under going anti-platelet therapy with clopidogrel (in anti-coagulant) were introduced into a cartridge for the Ultegra® System analyzer. The cartridge volume was 160 microliters and contained the particle reagents prepared as described above. The cartridges were agitated during reconstitution and during the assay. The Ultegra System analyzer performed the operations of incubating, agitating

and recording absorption readings according to its intended purpose. The apparatus measured the rate of change of light transmittance.

Testing PG reagents

Blood was drawn from normal, healthy subjects into blood collection tubes containing 3.2% citrate following a 3.0 mL discard tube. Baseline platelet function was tested using RPFA assay with Propyl Gallate as an agonist. An additional 2.0 mL EDTA tube was collected to determine baseline hematocrit and platelet count using Coulter Act-8 counter. After determining baseline platelet function, hematocrit and platelet count, the donor was given a 300 mg loading dose of clopidogrel. Blood was collected and processed in the same manner as above approximately 24 hours after ingestion of clopidogrel. Platelet function, hematocrit and platelet count was measured for post clopidogril sample. Percent inhibition of platelet function due to clopidogril was calculated as [(1-post clopidogril/baseline)*100]

Gender was evenly distributed, No abnormal hematology results were noted in this population. The mean exposure time to clopidogrel at the time of the post-clopidogrel blood samples was 23 hours.

Results

The above reagents and system successfully detects the efficacy of the treatment of the patient with clopidogrel.

To determine the interference effect, the difference in values is expressed as a percent of the baseline value:

Interference Effect =[(mean baseline ARU – mean treated ARU) / (mean baseline ARU)] *100

Data Summary

ID #	Baseline ARU #1	Baseline ARU #2	Baseline ARU Mean	Post ARU #1	Post ARU #2	Post ARU Mean	Interference Effect
001	625	657	641	376	355	366	43%
002	692	684	688	501	548	525	24%
003	573	525	549	495	500	498	9%
004	720	755	738	691	711	701	5%
005	686	697	692	648	581	615	11%
006	528	532	530	451	452	452	15%

It is evident from the above results that a simple, rapid method is provided by the present invention for conducting an assay for platelet activity on samples that have been affected by exposure to a thienopyridine.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims. Furthermore, the foregoing description, for purposes of explanation, used specific nomenclature to provide a thorough understanding of the invention. However, it will be apparent to one skilled in the art that the specific details are not required in order to practice the invention. Thus, the foregoing descriptions of specific embodiments of the present invention are presented for purposes of illustration and description; they are not intended to be exhaustive or to limit the invention to the precise forms disclosed. Many modifications and variations are possible in view of the above teachings. The embodiments were chosen and described in order to explain the principles of the invention and its practical applications and to thereby enable others skilled in the art to utilize the invention.

WHAT IS CLAIMED IS:

1. A method for conducting an assay for platelet function activity on a blood sample, said method comprising:

(a) providing in combination, to form a reaction medium, said sample, a particle reagent comprising a GP IIb/IIIa receptor ligand covalently attached to particles, a polyhydroxy aromatic compound and a metal cation,

(b) incubating said combination under conditions for agglutinating said particles,

(c) determining the extent of agglutination of said particles, the extent thereof being related to the platelet function activity of said sample.

2. A method according to Claim 1 wherein said sample is a whole blood sample from a patient undergoing treatment with an antiplatelet therapeutic agent.

3. A method according to Claim 2 wherein said antiplatelet therapeutic agent is a cyclooxygenase inhibitor or an adenosine-5-phosphate (ADP) antagonist.

4 A method according to Claim 3 wherein said ADP antagonist is a thienopyridine.

5. A method according to Claim 3 wherein said antiplatelet therapeutic agent is Cyclooxygenase-1 inhibitor

6. A method according to Claim 3 wherein said antiplatelet therapeutic agent is aspirin.

7. A method according to Claim 1 wherein said conditions comprise incubating said medium at a temperature and for a time to promote agglutination of said particles.

8. A method according to Claim 1 wherein the agglutination of said particles is determined by examining the optical density of said medium.

9. A method according to Claim 1 wherein the extent of agglutination of said particles is compared to a control standard.

10. A method according to Claim 1 wherein said ligand is selected from the group consisting of fibrinogen, von Willebrand factor, fibronectin, vitronectin, RGD peptides, and antibodies for GP IIb/IIIa receptor.

11. A method according to Claim 1 wherein said polyhydroxy aromatic compound is a lower alkyl ester of trihydroxybenzoic acid or tannin.

12. A method according to Claim 11 wherein said polyhydroxy aromatic compound is propyl gallate.

13. A method according to Claim 1 wherein the concentration of said polyhydroxy aromatic compound is an amount effective to promote agglutination of said particles.

14. A method according to Claim 12 wherein the concentration of said polyhydroxy aromatic compound is about 20 to about 5000 μM.

15. A method according to Claim 1 wherein said metal cation is a divalent transition metal ion.

16. A method according to Claim 15 wherein said metal cation is nickel (II) ion.

17. A method according to Claim 1 wherein the concentration of said metal cation is an amount effective to promote agglutination of said particles.

18. A method according to Claim 1 wherein the concentration of said metal cation is about 1 to about 5000 μM.

19. A method according to Claim 1 wherein said particles comprise a label.

20. A method according to Claim 19 wherein said label is a dye.

21. A composition comprising (i) a particle reagent comprising fibrinogen covalently attached to particles, (ii) a lower alkyl ester of trihydroxybenzoic acid, and (ii) a divalent transition metal ion.

22. A composition according to Claim 21, which is a lyophilized pellet.

23. A composition according to Claim 21 wherein said particles comprise a label.

24. A composition according to Claim 23 wherein said label is a dye.

25. An apparatus comprising a container for receiving a sample and a composition comprising (i) a particle reagent comprising fibrinogen covalently attached to particles, (ii) a lower alkyl ester of trihydroxybenzoic acid, and (ii) a divalent transition metal ion.

26. An apparatus according to Claim 25, which is a lyophilized pellet.

27. An apparatus according to Claim 25 wherein said particles comprise a label.

28. An apparatus according to Claim 27 wherein said label is a dye.