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PYRIDAZINE REAGENTS AND MEANS FOR STABILIZING BLOOD PLATELETS THEREWITH
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11 Claims

ABSTRACT OF THE DISCLOSURE

A way to stabilize blood platelets and thereby facilitate their counting for blood diagnosis is achieved by the addition of a measured amount of pyridazine to a sample of whole blood or platelet rich plasma prior to counting and while the sample is fresh.

This present invention relates to a diagnostic aid and more particularly to a compound which when added to a sample of whole blood or platelet rich blood plasma provides a stabilizing effect on the platelets in the sample and prevents the platelets from aggregating while permitting an accurate counting of their number.

In disorders of blood coagulation, it is important to be able to accurately detect the number of platelets found in a patient's blood. However, heretofore when blood is drawn and left standing, the platelets have exercised their natural tendency to aggregate and come together and thereby prevent an accurate counting of their true number.

The present invention is based upon my discovery that the addition of a measured amount of pyridazine to a sample of whole blood shortly after drawing will produce a striking reduction of surface activation and aggregation of the platelets whereupon the platelet behavior can be easily and carefully monitored. A similar effect is obtained when pyridazine is added to platelet rich plasma. Thus, by preventing the aggregation of the platelets, the technician is allowed accurately count the blood platelets found in the sample. The technique thus developed is highly important in a number of diseases but is particularly useful in myocardial infarction wherein it is essential to know the extent of platelet surface activation because of its critical relationship to thrombosis. This activation is reflected by a structural change in the platelets. Round, spread, dendritic, abortive dendritic platelet types can be visibly counted on an electron microscope provided that the natural tendency of the platelets to activate and aggregate can be arrested.

Accordingly, a prime object of the present invention is to provide a reagent which can readily be used to reduce surface activation and aggregation of blood platelets and thereby greatly facilitate blood diagnosis involving the counting of blood platelets.

Another object of the present invention is to provide a blood platelet aggregation inhibitor which when added to fresh blood or platelet rich plasma will prevent aggregation of the platelets and permit platelet behavior to be easily and carefully monitored.

Still another object of the present invention is to provide a reagent and methods of using the same whereby the status of the blood of myocardial infarction patients can be readily determined and in which the aggregation of blood platelets is substantially avoided.

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Still a further object of the present invention is to provide a diagnostic reagent which when employed with blood count techniques will eliminate platelet aggregation and thereby provide a more accurate count of individual platelets and a more precise determination of platelet structure.

A still further object of the present invention is to provide methods and reagents for the counting of blood platelets in blood specimens whereby the need for immediate platelet counting is eliminated and greater efficiency in laboratory and diagnostic procedures is obtained.

Still a further object of the present invention is to provide a reagent for use in the routine blood count of blood platelets which is liquid and therefore easily measured and readily handled.

These and still further objects as shall hereinafter appear are fulfilled by the present invention in a remarkably unexpected fashion as can be discerned from a careful consideration of the following description of exemplary embodiments of this invention.

In one practice of the present invention, a sufficient concentration of my platelet aggregation inhibitor, namely pyridazine, is added to whole blood or platelet rich plasma to control the surface activation and aggregation of the platelets in normal and pathological blood samples. It is believed that the observed effect is accomplished by the ability of pyridazine to inhibit platelet activating and aggregating substances such as ADP, thrombin, collagen, serotonin, epinephrine and platelet Factor III whereby a mechanism for the stabilization of the platelet is provided, and subsequently, ease and accuracy of platelet count is obtained.

By the addition of my platelet aggregation inhibitor in sufficient concentration, as shall be hereinafter defined, I am able to control platelet surface activity and aggregation in normal and pathological samples of whole blood or platelet rich plasma and thereby provide a total and accurate count of what can be called "stabilized platelets."

It should be understood that there are several known techniques for counting blood platelets such as, phase microscopy counting, electronic cellular sizing, and electron microscopy.

In each technique, the use of pyridazine is found to provide the aggregation inhibition necessary to assure an accurate and reproducible account. As will appear, however, different carriers will be suggested for the different techniques although the effective concentration of pyridazine will be essentially the same irrespective of the technique selected.

Thus, when the phase microscope technique is used, my carrier will be 1% ammonium oxalate whereas when the electronic sizing or electron microscope is the technique used, my carrier will be 0.1M tris (hydroxymethyl) aminomethane (tris buffer) pH 7.6.

To perform the invention when an electronic cellular sizing or an electron microscopic technique is to be used, a blood sample is collected from the patient in a routine manner using conventional techniques and equipment. The sample is then anticoagulated, as by the addition of sodium citrate, heparin, or the like. Next, pyridazine in a tris buffer solution is added to the treated sample to provide 9 ml. of blood to 1 ml. of the tris buffer solution (0.1 molar pH 7.6) which solution contains 8 mg. of pyridazine. With the sample prepared in this fashion, it can be set aside. Later the blood platelets in the stabilized

sample can be routinely counted at the technician's convenience by electronic sizing or an electron microscope since the threat of activation and aggregation of platelets in the blood sample has been obviated.

While the ratios described above are believed to provide optimum results, the measured amount of pyridazine can vary from about 4 mg. up to about 10 mg. per ml. of the buffer solution.

In a similar vein, the ratio of 1 part pyridazine containing-buffer solution to 9 parts blood has been found to provide optimum results although the weight of pyridazine (mg.) to the volume of buffered blood solution (in ml.) can vary from 400 to 1000 with some benefits being obtained.

To perform the invention when phase microscopy is to be used, the blood sample is taken and anticoagulated as before. The inhibitor reagent is, however, prepared using 1% ammonium oxalate pH 6.3 as the carrier for pyridazine instead of tris buffer. The quantity of pyridazine mixed into the ammonium oxalate can vary from 4 mg. to about 10 mg. per ml. of the oxalate with about 8 mg. being preferred.

The inhibitor reagent is added to the anticoagulated blood specimen which may be set aside for later measurement on the phase microscope.

By virtue of this invention, I have provided means and methods for accurately counting blood platelets while eliminating the need for immediate platelet counting. I further provide a novel diagnostic reagent which can be easily handled and quickly used.

To further assist in the understanding of the present invention, and not by way of limitation, the following examples are presented.

EXAMPLE I

A platelet inhibiting reagent for use with samples of whole blood or platelet rich plasma which are to be counted on either an electron microscope or by electronic cellular counting, using a Coulter Electronic Counter, is prepared by mixing 80 milligrams of pyridazine with 10 ml. of 0.1 molar, pH=7.6, of tris (hydroxymethyl) aminomethane (tris buffer) and stirred to uniformly disperse the pyridazine throughout the buffer solution.

EXAMPLE II

A platelet inhibiting reagent for use with samples of whole blood or platelet rich plasma which are to be counted by phase microscopy is prepared by mixing 800 mg. of pyridazine in 100 ml. of 1% ammonium oxalate, stirring to assure the dispersion of pyridazine throughout the oxalate solution, and adjusting the pH of the resulting solution to 6.3 as by the addition of hydrochloric acid thereto.

EXAMPLE III

A laboratory protocol was developed to evaluate the new reagents and determine the concentration necessary to inhibit in vitro platelet aggregation when known aggregating agents, such as, collagen or adenosine diphosphate are added to the assay system.

The system as developed comprised the use of 1.0 ml. of animal or human platelet rich plasma of known platelet count, 0.3 ml. of 0.1 M tris buffer pH 7.6; 0.1 ml. of either the platelet aggregating agents collagen or adenosine diphosphate; and 0.1 ml. of tris buffered pyridazine (8 mg./0.1 ml.). The reagent was checked to see that it was optically free of turbidity by making a spectrophotometric comparison of it with control samples of 1% ammonium oxalate or 0.1 M tris buffer pH 7.6.

The quantitation of the assay system was achieved by determining the changes in optical density, associated with induction of platelet aggregation, between a sample to which an addition of aggregation agents, such as collagen or ADP was added, and a sample to which both the aggregating agent and the inhibitor has been added. The degree of inhibition of platelet aggregation is deter-

mined by noting the difference in optical density between the samples.

EXAMPLE IV

In collecting blood samples for counting platelets by phase microscopy, care must be exercised in the collection because improper collection can cause clumping which will distort accuracy regardless of the reagent used.

One proper technique for obtaining a blood sample comprises taking the sample by venipuncture without difficulty and thereafter anticoagulate the sample, keeping the time lapse between withdrawal and anticoagulation to a minimum. Excellent results are obtained when the blood is collected with a 20 gauge needle and a siliconized or disposable syringe. The blood sample is then transferred to a siliconized test tube, maintained at 25° C. and immediately anticoagulated by the addition to the sample of ethylenediamine tetraacetate (EDTA) at a concentration of 1 mg. per 1 ml. of blood. The tube is then covered with parafilm and inverted ten times.

Another accepted technique for obtaining the blood sample comprises taking the blood from a capillary finger prick after a clean finger puncture. In this instance, the blood may be taken up directly in a red cell pipette.

Continuing, the blood is thoroughly mixed with a diluent formed by admixing it with the reagent prepared in Example II. The contents of each pipette is then discharged to fill a hemocytometer chamber where it is held for about 15 minutes while a piece of wet filter paper is laid thereacross to maintain moisture.

The platelets are thereafter counted by phase contrast microscopy using a long working condenser having a 43× annulus, a 43× phase objective (medium dark contrast) and 10× oculars.

The platelets stand out as individual round or oval bodies having a pink or purple sheen. On focusing, the platelets are seen to have one or more fine processes. The platelets in the four corner squares and the central square of the control large square, as for red cell counts, are counted in each chamber.

If the number of platelets counted in the total of ten squares is less than 100, additional squares should be counted until a total of at least 100 platelets have been counted. The number of platelets per cubic millimeter of blood is then found by multiplying the number of cells counted by the dilution and dividing this figure by 0.004 times the number of square counted.

EXAMPLE V

Using finger prick samples and phase microscopy, as described in Example IV, two samples were prepared from each of five patients. In one sample of each patient, no inhibitor was added to the sample and in the other sample, 8 mg. of pyridazine per ml. of sample was added. The results of platelet counts per cubic millimeter for each specimen performed immediately, after 4 hours and after 24 hours, are reported in the table below.

Patient No.	Inhibitor added	Immediate	Post 4 hours	Post 24 hours
1.....	{N	252,500	215,000	212,500
	{Y	248,000	280,000	252,500
2.....	{N	140,000	142,500	110,000
	{Y	140,000	147,000	142,500
3.....	{N	287,500	272,500	247,500
	{Y	280,000	270,000	265,000
4.....	{N	307,500	247,500	287,500
	{Y	300,000	300,000	290,000
5.....	{N	262,500	197,500	182,500
	{Y	275,000	272,500	280,000

NOTE.—N=No; Y=Yes.

EXAMPLE VI

Five myocardial infarction patients had blood specimens taken from them using the venipuncture technique described in Example IV. The platelet count per cubic millimeter was accomplished by phase microscopy. Platelet counts were completed immediately, after 4 hours and after 24 hours on samples to which no pyridazine was

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added (denote "—A"), to which 1.6 mg./pyridazine per ml. sample was added (denoted "—B") and which 4.0 mg. pyridazine per ml. sample was added (denoted "—C").

The results for each patient sample are reported in the table below.

Patient sample	Immediate	Post 4 hours	Post 24 hours
1-A-----	185,000	175,000	159,000
1-B-----	245,000	247,000	250,000
1-C-----	245,000	240,000	240,000
2-A-----	82,500	60,000	85,000
2-B-----	142,500	130,000	112,500
2-C-----	140,000	132,000	145,000
3-A-----	230,000	175,000	200,000
3-B-----	212,500	222,500	220,000
3-C-----	225,000	225,000	225,000
4-A-----	197,500	162,500	182,500
4-B-----	255,000	175,000	195,000
4-C-----	247,500	237,500	262,500
5-A-----	265,000	195,000	180,000
5-B-----	295,000	275,000	262,500
5-C-----	282,000	275,000	267,500

EXAMPLE VII

Another accepted means for counting platelets involves the use of a Coulter Electronic Counter.

The principle of such counting is based on the proposition that if particles or cells are passed through a very small aperture along with an electric current, each particle or cell will produce a temporary resistance to the flow of current as it transiently occupies the aperture. The longer the volume of the cell, the greater the resistance. This resistance can then be converted to a proportional signal which is detectable electronically. Therefore, if a known volume of a suspension of particles or cells is passed through the aperture, it is possible to count electronically the number of cells or particles in the volume. Large cells, such as red cells and white cells, produce a relatively large signal and are counted by this method using an aperture of 100μ in diameter. As the volume of platelets varies from $2-30\mu$ or $\frac{1}{40}$ to $\frac{1}{3}$ of a red cell, the resistance they provide is smaller. Thus, to enhance the signal produced by the platelet, it is necessary both to reduce the size of the aperture to 50μ and to increase the current flowing through the aperture current-setting.

A sample of platelet rich plasma is obtained by the two syringe technique, whereupon 9 ml. of venous blood are collected by careful venipuncture. The blood sample is quickly placed into a siliconized test tube and mixed with 1 ml. of 3.8% sodium citrate. Following anticoagulation with the citrate, the sample is split into two 10 ml. portions. To one portion is added 0.1 ml. of tris buffer (tris hydroxymethyl aminomethane, 0.1 M pH 7.6) and to the other is added 0.1 ml. of tris buffer containing 8 mg. of pyridazine. The blood is centrifuged at 900 r.p.m. (60 g.) for 15 minutes and the supernatant, that is, platelet rich plasma, is separated from the packed red cells.

The platelet rich plasma is then diluted for electronic counting by adding 20 lambdas of platelet rich plasma to 100 ml. particle-free 0.85% saline, thereby giving a $\frac{1}{5000}$ dilution.

The $\frac{1}{5000}$ dilution is then counted using the Coulter counter with a 50μ orifice, an aperture current setting of 7, and a threshold of 25. Platelet free saline is also counted to determine the background count due to electronic noise and debris and this value subtracted from the counts obtained for the platelet-containing sample.

The platelet count for platelet rich plasma equals the electronic count times $10^3/\text{cu. mm.}$ This value, for platelet rich plasma, may be converted to whole blood counts by the following formula:

$$\text{Whole blood counts} = \frac{\text{platelet rich plasma count} \times (100\text{-packed cell volume of whole blood})}{100}$$

Table A below reports plate counts made of the first portion of the platelet rich plasma samples at 0, 6 and 24 hours and Table B reports like readings made on the second portions of the platelet rich plasma samples (to which

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8 mg. of pyridazine were added per ml. of specimen) at the same intervals.

TABLE A.—PLATELET RICH PLASMA

Time in hours-----	Platelet count/mm. ³		
	0	6	24
Number:			
1-----	133,000	115,000	84,000
2-----	198,000	156,000	88,000
3-----	155,000	100,000	58,000
4-----	218,000	190,000	141,000
5-----	260,000	216,000	140,000
6-----	282,000	217,000	130,000
Average-----	207,000	165,000	106,000

TABLE B.—DIAGNOSTIC REAGENT ADDED

Time in hours-----	Platelet count/mm. ³		
	0	6	24
Number:			
1-----	214,000	217,000	221,000
2-----	252,000	244,000	247,000
3-----	200,000	193,000	202,000
4-----	260,000	264,000	269,000
5-----	305,000	314,000	300,000
6-----	292,000	303,000	293,000
Average-----	253,000	255,000	255,000

EXAMPLE VIII

An additional in vitro study was conducted in which an electron microscope method was used to assess both the quantitative activation and aggregation capacity of blood platelets in normal subjects and in patients with clinically diagnosed myocardial infarction.

In the study, a sample of whole blood was drawn using a 2-syringe hemorepellant technique. The samples were then anticoagulated by the addition of heparin or sodium citrate. The anticoagulated sample next was divided into equal parts with the control sample consisting of 9 parts of whole blood to 1 part of 0.1 tris buffer pH 7.6. The test sample consisted of 9 parts of whole blood to 1 part of tris buffer solution containing a measured amount of pyridazine. The concentrations of pyridazine ranged from 0.075 mg./ml. of whole blood to 1.0 mg./ml. of whole blood. The samples, both control and test, were subjected to surface activation under standardized conditions. The blood platelets in the samples undergo a series of morphological activations which can be monitored and quantitatively differentially classified with the electron microscope. Quantitative differential classification of blood platelet types is based on the counting of 100 individual platelets in an electron microscope grid field. Platelet aggregates are also counted and recorded as numbers of aggregates per 100 platelets counted.

In the normal control subjects, the platelets exhibited little surface activation and showed only a slight tendency to aggregate. The average individual had approximately 5% in the round form, 9% in the abortive dendritic form, 75% in the dendritic form, 3% in the intermediate form and 6% in the spread form. Approximately 5% of the platelets were classified in the aggregate form.

In contrast, platelets from those patients who had experienced a clinically confirmed myocardial infarction, exhibited extreme surface activation with development of large and numerous platelet aggregates. The most prominent platelet type, percentagewise in contrast to the normal, were the intermediate and spread platelets. There also was a substantial decrease in the number of platelets classified as abortive dendritic, dendritic and round forms which are the more normal platelet types.

When either heparin or short term warfarin was given to these latter patients, the activated state of their platelets remained unaltered and intermediate and spread platelets continued to dominate the differential counts. However, it was observed that heparin therapy did partly block the capacity of the platelets to aggregate while warfarin had no effect in reducing the number of platelet aggregates.

When pyridazine was added in vitro to whole blood platelets from normal subjects, the degree of surface activation was decreased and the number of platelet aggregates which formed also decreased markedly.

When pyridazine was added to the whole blood platelets from those patients with myocardial infarction, the platelet differential count was reversed and could not be distinguished from the count of a normal subject. Dendritic rather than intermediate and spread platelets were dominant in the differential count.

In addition, pyridazine also markedly inhibited the formation of platelet aggregates and far surpassed heparin in the ability to interfere with platelet aggregate formation. Pyridazine also reduced the tendency of red cells and leucocytes to adhere to the activating surface.

From the foregoing, it becomes apparent that I have herein described and illustrated methods and reagents for use in the normal clinical evaluation of patients, particularly presurgical patients and those who are experiencing coagulation problems, which stabilizes the platelets and thereby facilitates the accuracy and the ease of platelet counting in normal blood or platelet rich plasma. As such, my invention also aids in the diagnosis of those disease states in which platelet aggregation critically influences platelet counting, and it fulfills all of the aforementioned objectives in a remarkably unexpected fashion. It is, of course, understood that such applications, alterations or modifications as may readily occur to the artisan when confronted with this disclosure are intended within the spirit of this invention especially as it is defined by the scope of the claims appended hereto.

What is claimed is:

1. A method for stabilizing the blood platelets in a fresh sample of whole blood or platelet rich plasma whereupon the number of platelets in the sample may be determined by a platelet counting technique, said method comprising anticoagulating the sample and thereafter adding to the anticoagulated sample a platelet stabilizing amount of pyridazine.

2. The method of claim 1 in which said pyridazine is employed in a carrier selected from the group consisting of a 1% solution of ammonium oxalate, pH 6.3 and a 0.1

molar solution of tris-(hydroxymethyl)aminomethane, pH 7.6.

3. The method of claim 2 wherein said carrier includes from 4 to 10 mg. of pyridazine per ml.

4. The method of claim 3 in which said technique is phase microscopy and said carrier consists of a 1% solution of ammonium oxalate, pH 6.3.

5. The method of claim 3 in which said technique is electronic cellular sizing or electron microscopy and said carrier consists of a 0.1 molar solution of tris-(hydroxymethyl)aminomethane, pH 7.6.

6. The method of claim 3 in which the ratio of the pyridazine carrier composition to said blood sample is, by volume, about 1:9.

7. A reagent for stabilizing blood platelets in a sample of fresh anticoagulated blood or platelet rich plasma consisting, on a per ml. basis, of about 4 to about 10 mg. of pyridazine and the remainder a liquid carrier selected from the group consisting of a 1% solution of ammonium oxalate, pH 6.3 and 0.1 molar solution of tris-(hydroxymethyl)aminomethane, pH 7.6.

8. The reagent of claim 7 in which said carrier is a 1% solution of ammonium oxalate, pH 6.3.

9. The reagent of claim 7 in which said carrier is 0.1 molar solution of tris-(hydroxymethyl)aminomethane, pH 7.6.

10. The reagent of claim 8 containing about 8 mg. pyridazine.

11. The reagent of claim 9 containing about 8 mg. pyridazine.

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