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BLOOD TESTING METHOD

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This invention relates to a blood testing method for early detection of diseases.

Biochemical analysis of a body fluid such as blood is currently increasingly conducted for diagnosing various sicknesses in many hospitals. In the case of diagnosis of diabetes, for example, blood samples before and after meal are generally analyzed for the concentration of glucose therein.

Glucose is a source of energy for human body's cells and is released from the liver into blood. In a healthy individual, the release of glucose from the liver and the utilization of glucose in the cells are balanced so that the blood glucose level is maintained at about 110 mg/dL or less. Insulin is a hormone secreted from pancreas and necessary for the human body to properly convert sugar, starches and other food into a cellular energy source. When starch is digested in the small intestine, the level of glucose present in the blood increases. Such an increase in the blood glucose level stimulates increased secretion of insulin so that the liver stops releasing the glucose. Upon intracellular utilization of the glucose, the blood glucose decreases to the normal level. Such increase and decrease of the blood glucose level are repeated every time a healthy individual takes foods.

Some individuals are unable to quickly or sufficiently secrete insulin even when their blood glucose level rises after meal. When insufficient exercise and excess ingestion are added to this condition, glucose remains excessively in blood so that the blood glucose level does not decrease even after several hours from the intake of meals.

In the conventional biochemical analysis, the blood glucose level is measured by isolating plasma from blood samples taken before and after meals. When the glucose concentration in a plasma sample obtained in an empty

stomach stage is greater than 140 mg/dL or when the glucose concentration in a plasma sample taken just after meal is greater than 200 mg/dL, then he or she is diagnosed as being diabetic (type-2 diabetes). A high glucose level when continued for a long time will cause heart, circulation, eye and kidney problems.

There are recent reports concerning symptoms of cardiac infarction in individuals whose blood glucose levels were found to be not significantly high when measured with the conventional plasma test method. Namely, it has been found that arteriosclerosis proceeds even in prediabetics. Especially, individuals whose insulin secretion is slow are likely to cause arteriosclerosis when they meet with one or more conditions of mild type-2 diabetes, slight overweight, mild hyperlipidemia and light hyperinsulinemia. The conventional blood test methods in which plasma of a blood sample is measured for the concentration of glucose is not effective for the early detection of diabetes. Thus, there is an urgent demand for a diagnostic method capable of precisely detecting diabetes and other diseases even at their early stages.

With the conventional blood test methods in which plasma of a blood sample is measured for components such as glucose and lactic acid, the measured values merely represent those of the components exuded and released from the blood cells into the plasma. It has been found that in order to detect diabetes and other diseases at their early stages it is important that components contained in red blood cells should be measured. Namely, since red blood cells have metabolic functions to decompose glucose and to precedently control the glucose concentration in the plasma, it is effective to know the glucose level in the red blood cells for the early detection of diabetes. Further, the oxidation-reduction potential in the blood cells and the concentrations of pyruvic and lactic acid

which are metabolites of glucose have been found to be important parameters for early detection of various diseases.

The present invention has been made in view of the problems of the conventional blood testing method.

In accordance with the present invention, there is provided a blood test method comprising the steps of:

(a) centrifuging a mammalian blood sample into a plasma and blood cells;

(b) removing buffy coats from said blood cells to obtain red blood cells containing solutes confined therewithin;

(c) washing said red blood cells with a buffered physiological saline solution and isolating said washed blood cells;

(d) mixing said washed red blood cells with a buffered physiological saline solution to obtain a suspended liquid;

(e) centrifuging said suspended liquid to remove a supernatant and to obtain a red blood layer;

(f) mixing said red blood layer with a hypertonic solution and maintaining the resulting suspension at a temperature of 25 to 40°C for a period of time sufficient for the solutes confined in the red blood cells to penetrate into the hypertonic solution;

(g) centrifuging the suspension to obtain a supernatant containing the solutes; and

(h) measuring the supernatant for at least one factor selected from a glucose concentration, a pyruvic acid concentration, a lactic acid concentration and an oxidation-reduction potential.

The present invention will be described in more detail below with reference to the accompanying drawing, in which:

FIG. 1 is an example of a working curve for

determining an oxidation-reduction potential.

A blood test method according to the present invention comprises the following steps (a)-(h).

Step (a):

A mammalian blood is sampled and centrifuged into a plasma and blood cells. A conventional intravenous blood sampling tube containing a suitable amount of an anticoagulation agent such as hepalin or EDTA may be used for sampling. The blood sample (in an amount of 2 ml, for example) is preferably centrifuged at a force of 130×g to 200×g for 5 to 10 minutes into a plasma and blood cells including red blood cells, white blood cells and platelets.

Step (b):

The blood cells from Step (a) are separated into buffy coats including white blood cells and platelets and red blood cells containing solutes confined therewithin. Preferably, the blood cells from Step (a) are mixed with a sedimentation agent such as metrizamide, and the mixture is centrifuged at a force of 800×g to 1,200×g for 7 to 12 minutes to separate red blood cells from the buffy coats.

Step (c):

The red blood cells obtained in Step (b) are washed with a buffered physiological saline solution and the washed blood cells are isolated. Preferably, the red blood cells are mixed with a phosphate buffered physiological saline solution, and the resulting mixture is centrifuged at a force of 130×g to 200×g for 5 to 10 minutes. As a result of the washing, impurities such as white blood cells, platelets and plasma which deposit on the red blood cells are removed.

Step (d):

The washed red blood cells are mixed with a buffered physiological saline solution to obtain a suspended liquid. Preferably, the washed red blood cells are mixed with a phosphate buffered physiological saline solution in an

amount so that the suspended liquid has a hematcrit value of 40 to 50 %. More preferably, the phosphate buffered physiological saline solution is added so that 2 ml of the suspended liquid has a hematcrit value of 50 %. Alternatively, the washed red blood cells may be mixed with a phosphate buffered physiological saline solution so that the suspended liquid has a number of red blood cells in the range of 4,000,000/μL (4×10^6 microliter) to 5,000,000/μL (5×10^6 microliter). A customarily employed automatic blood cell counter is suitably used to count the red blood cell number.

Step (e):

The suspended liquid obtained in step (d) is centrifuged to remove a supernatant and to obtain a red blood layer. Preferably, the suspended liquid is centrifuged at a force of 130×g to 200×g for 5 to 10 minutes.

Step (f):

The red blood layer is then mixed with a hypertonic solution and the resulting suspension is maintained at a temperature of 25 to 40°C for a period of time sufficient for the solutes confined in the red blood cells to penetrate into the hypertonic solution. Preferably, the red blood layer is mixed with a 5 to 10 % by weight saline solution in a predetermined amount and the resulting mixture is maintained at a temperature of 35 to 38°C for 7 to 15 minutes. Because of osmotic pressure, the solution in the blood cells containing glucose, lactic acid, pyruvic acid and other solutes passes through the cell wall and diffuses into the hypertonic solution. In order to efficiently and completely recover the intracellular components while preventing denaturation thereof, it is necessary that the suspension be maintained at 25-40°C.

Step (g):

The suspension obtained in step (f) is centrifuged

to obtain a supernatant containing the intracellular components transferred from the red blood cells. Preferably, the suspension is centrifuged at a force of 1,500×g to 2,000×g for 7 to 12 minutes.

Step (h):

The supernatant obtained in Step (g) is measured for at least one factor selected from a glucose concentration, a pyruvic acid concentration, a lactic acid concentration and an oxidation-reduction potential.

The glucose concentration, pyruvic acid concentration and lactic acid concentration may be performed using any customarily suitably employed automatic biochemical analyzer. When 2 ml of a suspended liquid having a hematcrit of 50 % is prepared in Step (d), the glucose concentration (mg/dL), lactic acid concentration (mg/dL) and pyruvic acid concentration (mg/dL) are the values obtained by multiplying 2 by the measured values. When a suspended liquid having a specific number of red blood cells is prepared in Step (d), the glucose concentration (mg/dL), lactic acid concentration (mg/dL) and pyruvic acid concentration (mg/dL) may be easily calculated from the measured value, mean red blood cell volume, red blood cell number and the volume of the suspended liquid.

An oxidation-reduction potentiometer is suitably used for measuring the oxidation-reduction potential of the red blood cells (ORP-RBC). In this case, ORP-RBC is determined using a working curve prepared by measuring the oxidation-reduction potential of the serum (ORP-srm) obtained from the same blood sample. When 2 ml of a suspended liquid having a hematcrit of 50 % is prepared using a 5 % by weight saline solution in Step (d), the working curve is typically prepared by the method described below.

From 4 ml of the venous blood sample, a serum is

separated by centrifuging the sample at 3,000 rpm for 10 minutes. The oxidation-reduction potential of the serum (Ax) is measured with a potentiometer. The serum is diluted with the same volume of a 5 % by weight saline solution and the oxidation-reduction potential of the diluted serum (Ay) is measured. Separately, the serum is diluted with the same volume of a phosphate buffered saline solution (PBS) and the oxidation-reduction potential of the diluted serum (Bx) is measured. The PBS-diluted serum is further diluted with the same amount of a 5 % by weight saline solution and the oxidation-reduction potential of the diluted serum (By) is measured. Additionally, the original serum is diluted with three times the volume of PBS and the oxidation-reduction potential of the diluted serum (Cx) is measured. The PBS-diluted serum is further diluted with the same amount of a 5 % by weight saline solution and the oxidation-reduction potential of the diluted serum (Cy) is measured. As shown in FIG. 1, the values Ax, Bx and Cx in the X-axis are plotted against the values Ay, By and Cy in the Y-axis, respectively. A curve 1 passing the point A (Ax, Ay), point B (Bx, By) and the point C (Cx, Cy) represents the working curve. The coordinates of the points A (Ax, Ay), B (Bx, By) and C(Cx, Cy) are summarized in Table 1 below.

Table 1

Point / Axis	A	B	C
X	Ax: ORP of original serum (non-diluted)	Bx: ORP of 2-fold dilution of original serum with PBS	Cx: ORP of 4-fold dilution of original serum with PBS
Y	Ay: ORP of 2-fold dilution of above serum with 5 % saline solution	By: ORP of 2-fold dilution of above serum with 5 % saline solution	Cy: ORP of 2-fold dilution of above serum with 5 % saline solution

A point P in the working curve having the Y-axis value Y1 which equals the oxidation-reduction potential of the red blood cells as measured in Step (h) is then determined. The value of the X-axis (X1) of the point P represents the oxidation-reduction potential of the red blood cells (ORP-RBC).

Incidentally, the oxidation-reduction potential using a potentiometer, a potential of the reference electrode must be added to the measured value. The reference electrode potential varies with the temperature of the supernatant. Temperature dependency of the potential of the reference electrode PTS-2019C is as shown in Table 2.

Table 2

Temperature (°C)	Potential (mV)
0	224
5	221
10	217
15	214
20	210
25	206
30	203
35	199
40	196
45	192
50	188
55	185
60	181

The following examples will further illustrate the present invention.

Example 1

37 patients with type-2 Non-Insulin Dependent Diabetes Mellitus (NIDDM) and 52 healthy persons were each tested for D-glucose concentration in red blood cells according to the following method.

Venous blood (2 ml) was sampled (hepalin was used as an anticoagulation agent) and centrifuged at a force of 150×g for 5 minutes into a plasma and blood cells. The blood cells were mixed with 0.2 ml of metrizamide, and the mixture was centrifuged at a force of 1,000×g for 10 minutes to separate red blood cells from the buffy coats. The red blood cells were washed with a phosphate buffered physiological saline solution, and the resulting mixture was centrifuged at a force of 150×g for 5 minutes. The washed red blood cells were mixed with a phosphate buffered physiological saline solution in an amount so that 2 ml of a suspended liquid having a hematcrit value of 50 % was obtained. An automatic blood cell counter was

employed to control the hematcrit. The suspended liquid obtained was centrifuged at a force of 150×g for 5 minutes to obtain a red blood layer. The red blood layer was then mixed with a 5 % by weight saline solution and the resulting mixture (2 ml) was maintained at a temperature of 37°C for 10 minutes to obtain a suspension. The suspension was centrifuged at a force of 1,750×g for 10 minutes to obtain a supernatant containing the intracellular components transferred from the red blood cells. The supernatant was measured for its glucose concentration using a biochemical analyzer.

A blood glucose level in plasma was also measured in the conventional method.

In the case of the NIDDM patients, it was found that the higher the blood glucose level in the plasma, the higher was the glucose concentration in the red blood cells. The patient having 350 mg/dL of a blood glucose level in the plasma had 50 mg/dL of a glucose concentration in the red blood cells. In the case of the healthy individuals, too, it was found that the higher the blood glucose level in the plasma, the higher was the glucose concentration in the red blood cells. The individual having 150 mg/dL of a blood glucose level in the plasma had 10 mg/dL of a glucose concentration in the red blood cells. From the above results, it is evident that there is a correlation between the blood glucose level in the plasma and the glucose concentration in the red blood cells.

Example 2

A blood cell test according to the present invention was carried out for a diabetes mellitus patient (male, 70 years old) to determine oxidation-reduction potential (ORP), lactic acid concentration, pyruvic acid concentration and pH in the red blood cells in the same

manner as described in Example 2. Also measured were oxidation-reduction potential (ORP), lactic acid concentration, pyruvic acid concentration and pH in the plasma in the conventional manner. CPK, myoglobin, LDH and aldolase values were also measured in the conventional manner. The results are summarized in Table 3.

Table 3

	Measurement 1	Measurement 2
In red blood cell		
ORP	248 mV	221 mV
Lactic acid	10.6 mg/dL	18.4 mg/dL
Pyruvic acid	0.68 mg/dL	1.2 mg/dL
pH	7.13	7.24
In plasma		
ORP	136 mV	121 mV
Lactic acid	16.8 mg/dL	18.2 mg/dL
Pyruvic acid	0.8 mg/dL	0.9 mg/dL
pH	7.39	7.44
CPK	29475 U/L	15854 U/L
Myoglobin	1700 ng/mL	840 ng/mL
LDH	1550 IU/L	1177 IU/L
Aldolase	169.2 IU/L	96.2 IU/L

Measurement 1 and Measurement 2 were performed before and after minus ion irradiation treatment of the patient, respectively.

Before the minus ion irradiation treatment (Measurement 1), the intracellular ORP is very high (248 mV), indicating significant oxidation in the cells. For this reason, the driving power of Na^+/H^+ channel is reduced to cause a reduction of pH (7.13). Generally, the lactic acid concentration in the cell is slightly higher than

that in the blood. In the present case, however, the lactic acid concentration (10.6 mg/dL) in the cell is much lower than that in the blood (16.8 mg/dL). In the red blood cells, there is no TCA circuit, i.e. only a glycolysis system exists. Therefore, in the red blood cells, glucose is decomposed to produce pyruvic acid, which in turn is converted into lactic acid. Thus, the fact that the the lactic acid concentration in the cell is much lower than that in the blood indicates that the metabolism in the cells is low. Namely, the low pH and high ORP in the cells indicate an increase of acidity and oxidation which causes reduction of metabolism. The CPK, myoglobin, LDH and aldolase values are thus high, indicative of bad conditions of the patient.

As a result of the minus ion treatment (Measurement 2), the acidity and oxidation in the cells are in normal values. The lactic acid concentration in the cell is slightly higher than that in the blood, though the value per se is greater than the normal value. Although the CPK, myoglobin, LDH and aldolase values are still higher than the normal values, they decrease as a result of the minus ion irradiation treatment, indicating that the condition of the patient is improved.

Example 3

A blood cell test according to the present invention was carried out for a patient (male, 4 years old) to determine oxidation-reduction potential (ORP), lactic acid concentration, pyruvic acid concentration and pH in the red blood cells in the same manner as described in Example 2. Also measured were oxidation-reduction potential (ORP), lactic acid concentration, pyruvic acid concentration and pH in the plasma in the conventional manner. The results are summarized in Table 4.

Table 4

	Measurement 1	Measurement 2
In red blood cell ORP Lactic acid Pyruvic acid pH	 229 mV 17.5 mg/dL 0.61 mg/dL 7.11	 250 mV 23.8 mg/dL 0.4 mg/dL 7.28
In plasma ORP Lactic acid Pyruvic acid pH	 132 mV 15.9 mg/dL 0.5 mg/dL 7.33	 121 mV 18.2 mg/dL 0.6 mg/dL 7.37
Blood glucose level	380 mg/dL	309 mg/dL

Measurement 1 and Measurement 2 were performed before and after minus ion irradiation treatment of the patient, respectively. Before the minus ion irradiation treatment (Measurement 1), the lactic acid concentration in the cell is 17.5 mg/dL which is slightly higher than that in the blood (15.9) and that of the normal range (8 to 16 mg/dL). Further, the intracellular ORP is not high (229 mV), indicating oxidation in the cells is not in a high level. However, the pH is very low (7.11), indicating that the glycolysis in the cells does not proceed. After the minus ion irradiation treatment, the lactic acid concentration is high (23.8 mg/dL) and ORP is high (250 mV), suggesting that the oxidation in the cells occurs and the metabolism will decrease. However, the pH is in a good range, indicating that the glycolysis in the cells proceeds satisfactory. In fact, the blood glucose level is improved.

From the data of concentrations of organic acids (pyruvic acid and lactic acid), ORP and pH in the red blood cells and in the plasma, possibility of onset and

conditions of diseases may be diagnosed. As a disease gets worse, the blood data changes in the following order:
(1) First stage: values of concentrations of organic acids in the in the red blood cells and in the plasma do not fall in standard range, or the balance between the concentrations of organic acids in the red blood cells and those in the plasma is not normal;
(2) Second stage: values of ORP in the in the red blood cells and in the plasma do not fall in standard range, or the balance between the ORP in the red blood cells and that in the plasma is not normal;
(3) Third stage: pH values in the red blood cells and in the plasma do not fall in standard range, or the balance between the pH value in the red blood cells and that in the plasma is not normal.

Table 5 shows standard values.

Table 5

	Reference value
In red blood cell	
ORP	235 mV or less
Lactic acid	8-16 mg/dL
Pyruvic acid	0.5-1.2 mg/dL
pH	7.24-7.30
In plasma	
ORP	130 mV or less
Lactic acid	6-14 mg/dL
Pyruvic acid	0.3-0.9 mg/dL
pH	7.36-7.42
CPK	35-200 U/L
Myoglobin	60 ng/mL
LDH	106-211 IU/L
Aldolase	0-6 IU/L

A blood test method according to the present invention can detect changes of glucose, pyruvic acid and lactic acid concentrations and oxidation-reduction potential which changes have not yet significantly occurred in plasma. Further, the blood test method can precisely diagnose various diseases such as diabetes, cardiac insufficiency, renal insufficiency and chronic respiratory disease, at early stages thereof.

WHAT IS CLAIMED IS:

1. A blood test method comprising the steps of:

(a) centrifuging a mammalian blood sample into a plasma and blood cells;

(b) removing buffy coats from said blood cells to obtain red blood cells containing solutes confined therewithin;

(c) washing said red blood cells with a buffered physiological saline solution and isolating said washed blood cells;

(d) mixing said washed red blood cells with a buffered physiological saline solution to obtain a suspended liquid;

(e) centrifuging said suspended liquid to remove a supernatant and to obtain a red blood layer;

(f) mixing said red blood layer with a hypertonic solution and maintaining the resulting suspension at a temperature of 25 to 40°C for a period of time sufficient for the solutes confined in the red blood cells to penetrate into the hypertonic solution;

(g) centrifuging the suspension to obtain a supernatant containing the solutes; and

(h) measuring the supernatant for at least one factor selected from a glucose concentration, a pyruvic acid concentration, a lactic acid concentration and an oxidation-reduction potential.

2. A blood test method as claimed in claim 1, wherein step (a) comprises centrifuging the venous blood sample at a force of 130×g to 200×g for 5 to 10 minutes.

3. A blood test method as claimed in claim 1 or 2, wherein step (b) comprises mixing the blood cells with a sedimentation agent, and centrifuging the resulting

mixture at a force of 800×g to 1,200×g for 7 to 12 minutes.

4. A blood test method as claimed in any preceding claim, wherein step (c) comprises mixing said red blood cells with a phosphate buffered physiological saline solution, and centrifuging the resulting mixture at a force of 130×g to 200×g for 5 to 10 minutes.

5. A blood test method as claimed in any preceding claim, wherein step (d) comprises mixing said washed red blood cells with a phosphate buffered physiological saline solution in an amount so that the suspended liquid has a hematcrit value of 40 to 50 %.

6. A blood test method as claimed in any preceding claim, wherein step (e) comprises centrifuging the suspended liquid at a force of 130×g to 200×g for 5 to 10 minutes, and removing the supernatant.

7. A blood test method as claimed in any preceding claim, wherein step (f) comprises mixing the red blood layer with a 5 to 10 % by weight saline solution and maintaining the resulting mixture at a temperature of 35 to 38°C for 7 to 15 minutes.

8. A blood test method as claimed in any preceding claim, wherein step (g) comprises centrifuging the suspension at a force of 1,500×g to 2,000×g for 7 to 12 minutes.

9. A blood test method as claimed in any preceding claim, wherein step (h) comprises measuring the supernatant for at least one factor selected from a glucose concentration, a pyruvic acid concentration and a lactic acid concentration using an automatic biochemical

analyzer.

10. A blood test method as claimed in any one of claims 1-8, wherein step (h) comprises measuring the supernatant for an oxidation-reduction potential using a potentiometer.