Validating the Assessment of Glucose-6-Phosphate Dehydrogenase (G6PD)

CHARLES ASOWATA MAJOR, LESTER PRETLOW, EMILY FINCHER, VERNON KOZIATEK

ABBREVIATIONS: CAP = College of American Pathologists; E_A = allowable error; G6PD = Glucose-6-phosphate dehydrogenase; MDC = medical decision chart; MDL = medical decision level; NADP = nicotinamide adenine dinucleotide phosphate; R_E = random error; SD = standard deviation; S_E = systemic error.

INDEX TERMS: enzyme deficiency; Glucose-6-phosphate dehydrogenase; hemolytic anemia; medical decision chart; Roche Cobas Mira Plus.

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Charles Asowata Major MS MT(AMT) CLS(NCA), Emily Fincher MS MT(ASCP), and Vernon Koziatek BS CLS(NCA) *are of the Dwight D Eisenhower Army Medical Center, Fort Gordon GA.*

Lester Pretlow PhD CLS(C) NRCC(CC) *is of the Department of Biomedical and Radiological Technologies, Medical College of Georgia, Augusta GA.*

Address for correspondence: *Lester G Pretlow PhD CLS(C) NRCC(CC), Assistant Professor, Department of Biomedical and Radiological Technologies, AL-106, Medical College of Georgia, Augusta GA 30912-0500. (706) 721-7629, (706) 721-7631 (fax). lpretlow@mail.mcg.edu*

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme of clinical significance to the Armed Services. The ability to determine accurate erythrocyte concentrations of G6PD is imperative for the prophylaxis and treatment of service members against a variety of opportunistic hemolytic infectious diseases, such as malaria, which might be encountered during deployment.[1] G6PD catalyzes the first oxidation reaction in the pentose phosphate shunt, in which glucose-6-phosphate is dehydrogenated to 6-phosphogluconolactone with the concurrent reduction of nicotinamide adenine dinucleotide phosphate (NADP).[2] This reaction is the only source of the reduced form of NADP, NADPH, which provides the reducing power to change oxidized-glutathione into reduced-glutathione.[3] Reduced glutathione acts as a reducing agent against oxidative insults to the erythrocyte.

G6PD deficiency is expressed when individuals with decreased reduced-glutathione concentrations are administered oxidant drugs such as quinine, analgesics, and sulfonamides.[3] The decrease in reduced-glutathione leads to the inability of erythrocytes of these individuals to endure the oxidative assault of these drugs. As a consequence, erythrocytes lose their structural integrity, resulting in hemolytic anemia that can be moderate to life threatening. G6PD deficiency is the most widely distributed enzyme defect of erythrocytes in humans, therefore, it is important to screen all service members for the enzyme deficiency before they are prophylaxed in preparation for deployment.[4] This potential for drug induced hemolytic complications demands screening and for that reason, the analytical procedures for assessing G6PD concentrations must be rigorously validated to ensure the best quantitative determinations.[5] Most methods for the analysis of G6PD are based on the assessment of the enzymatic activity of erythrocyte G6PD.

In analyzing erythrocyte G6PD in whole blood specimen on the Roche Cobas Mira Plus™, it was noticed that the enzyme activity (U/g Hgb) for a group of specimens decreased concomitantly as the number of specimens per run was increased. The purpose of this study was to discover the reason for the decrease in erythrocyte G6PD activity seen in the sequential assessment of whole blood specimens. To accomplish this, four objectives were targeted: (1) The G6PD assay by Sigma Diagnostics was validated for precision, linearity, and correlation on the Cobas Mira Plus instrument. (2) Since College of American Pathologists (CAP) survey material was not available, proficiency testing was performed using whole blood specimens by correlating the assay periodically

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with the clinical laboratory at Fort Sam Houston, Texas. Fort Sam Houston's laboratory used the Cobas Mira Plus for the determination of erythrocyte G6PD activity. (3) The stability of erythrocyte G6PD specimens was tested by storing whole blood specimens for different time intervals and then retesting the specimens to determine any difference from the original assessment. (4) Within-batch stability of the assay was tested by varying the number of specimens assessed per analytical run.

The Cobas Mira Plus was chosen because it has onboard lyses of red cells and because of its ability to deliver the hemoglobin concentration as well as the enzyme activity in a single analysis. Comparable instruments required that the hemosylate be prepared offline before specimens were placed on the instrument. The Cobas Mira Plus was refurbished and distributed by Spectron (Burlington, WA) and was acquired specifically for the analysis of G6PD.

MATERIALS AND METHODS

Validation of G6PD assay on the Cobas Mira Plus

Precision, linearity, and correlation determinations were performed on the Sigma Diagnostics G6PD reagent adapted to the Cobas Mira Plus. Within-run precision was tested by combining several previously analyzed specimens and then allocating the mixture into forty separate samples. Varying the number of specimens per run, the specimens were then analyzed on the Cobas Mira Plus and the resulting data were evaluated by entering it into the EP-5 Evaluator™ software (Rhoads, Kennett Square, PA) version 5.0 for simple precision to obtain a mean, standard deviation, and coefficient of variance.[6] Varying the number of specimens per run assessed the length of analytical run that had the best within-run precision.

To test for linearity, seven pre-assigned controls were run. The Accumark controls were purchased from Sigma Diagnostics. Using the EP-5 Evaluator software, assigned values were plotted against measured values.[6] Regression statistics with slope and Y-intercept were calculated. [6]

A correlation study was conducted by split sample comparison with the assay performed at a large reference laboratory that also utilized the Sigma reagent to assay G6PD. Seventy whole blood specimens were aliquotted and analyzed by each laboratory. The set of specimens sent to the large reference laboratory was processed through the laboratory's shipping and receiving department where the set was wrapped in plastic, packed on ice in a Styrofoam container, placed in a cardboard box, and overnighted to the shipping and receiving department of the large reference laboratory for analysis. Data from these two assessments of G6PD were analyzed using the EP-5 Evaluator software for alternative method comparison. The EP-5 Evaluator software calculated the correlation coefficient, slope, Y-intercept, and standard error estimate. [6]

Error analysis for the assay was calculated using appropriate statistical analysis. The statistical Allowable Error (E_A) was determined as fifteen percent of the medical decision level (MDL) of G6PD deficiency, 10.0 U/g Hb. Fifteen percent was half of the acceptable performance for several enzymes of clinical significance which had a performance criterion of ten to thirty percent.[7] Random Error (R_E) was calculated as four times the standard deviation (SD) as determined from the simple precision for the assay.[7] Systemic Error (S_E) was calculated around the MDL as the absolute value of Y minus X for the MDL.[7] The regression equation obtained from the split sample comparison with the large reference laboratory was used to calculate the Y-value for our assay as compared to the MDL. The SD as a percentage of E_A was then plotted against the S_E as a percentage of E_A on a medical decision chart (MDC).

> The medical decision chart is a graphical tool that considers a method's random and systemic error simultaneously. It classifies a method by how easily its errors can be controlled to keep it below the allowable error. A MDC is constructed by labeling the x-axis "SD as % of Allowable Error" and scaling the x-axis from zero to fifty in increments of ten. The y-axis is labeled "Bias as % of Allowable Error" and scaled from zero to one-hundred in increments of ten. A line is drawn from the one-hundred percent on the y-axis to the 50% on the x-axis, labeling the region as unacceptable; additional lines are drawn from the 100% on the y-axis to the thirty-three percent, twenty-five percent, and sixteen-point seven percent on the x-axis and labeling the regions marginal, fair, good, and Six Sigma, respectively. The labeled regions judge the performance of a method based on the location of the operating point.[7]

Periodic correlation of G6PD enzyme activity with another facility

Due to the unavailability of proficiency survey material, our G6PD method was periodically (as required by CAP)

validated against a similar methodology to access the laboratory's technical competency, instrument, and reagent accuracy/precision. For this study, only the enzyme activity was validated and not the enzyme activity per grams of hemoglobin. Twenty-two specimens were collected in EDTA tubes. The specimens were aliquotted. One set of specimens was then sent to the laboratory shipping and receiving department where it was wrapped in plastic, packed on ice in a Styrofoam container, placed in a cardboard box, and sent overnight to the shipping and receiving department of Fort Sam Houston facility for analysis. Once specimens arrived at Fort Sam, they were unpacked and refrigerated overnight before being analyzed the next day. Both laboratories utilized the Sigma Diagnostic G6PD reagent and the Cobas Mira Plus instrument for the quantitative determination of G6PD in whole blood at 340 nm. Data from this study was evaluated using the EP-5 Evaluator software utilizing the alternate method comparison to determine the correlation coefficient, slope, Y-intercept, and standard error.[6]

Stability of G6PD in whole blood specimens

To look at specimen integrity overtime, six whole blood specimens were collected in EDTA tubes and then aliquotted. An initial G6PD activity was determined at the time of collection for one of the aliquotted specimens. The other specimens were then refrigerated at 4 °C – 8 °C for seven, nine, ten, 11, 14, and 23 days. At the end of a given time period, the stored specimens were retested with the Sigma Diagnostic reagent adapted to the Cobas Mira Plus instrument.

The analytical run length was observed by visual inspection during run lengths of four to 12 specimens. The purpose of this activity was to documents any problems that could be visually noted during the analytical phase of G6PD assessment. Additionally, a group of specimens was combined and then allocated into 16 specimens. Observations of any problems seen during these analytical runs were noted.

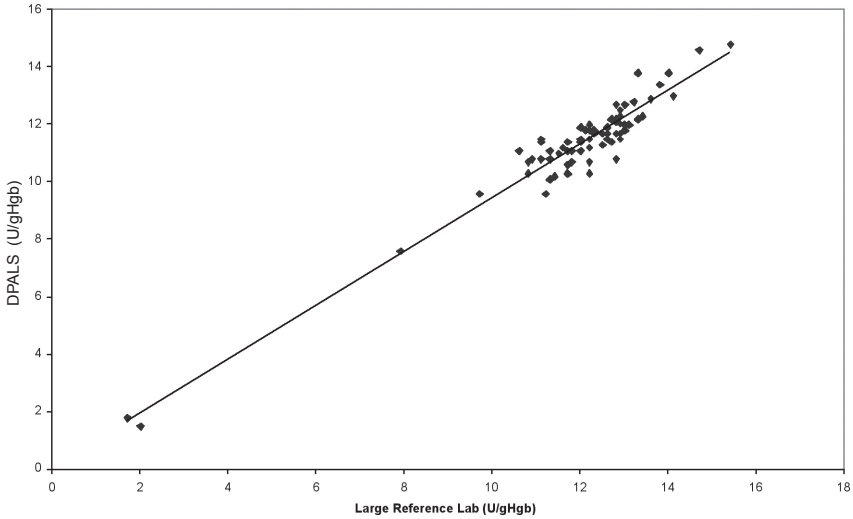
RESULTS

The precision of the Sigma Diagnostic reagent when adapted to the Cobas Mira Plus was determined by measuring or calculating the mean, SD, and CV from the simple precision experiment. It was determined that analytical runs of four specimens had the best precision. The mean was determined to be 14.3 U/g Hgb, with an SD of 0.15 U/g Hgb, and a CV of 1.0%. The assay was linear with a slope of 1.087 and a Y-intercept of 0.320. The assay correlated with a reference assay at a large reference laboratory with a correlation coefficient of 0.9667, a slope of the regression line of 0.968, a Y-intercept of -0.291, and a standard error estimate of 0.521. Figure 1 shows the scatter plot from the method comparison.

Allowable error was calculated as 15.0% of the medical decision level of 10.1 U/g Hgb and was determined to be 1.5 U/g Hgb. The S_E was 0.60 U/g Hgb and the T_E was 1.2 U/g Hgb. S_E and R_E expressed as percentage of the allowable error were 40.0% for S_E and 10.0% for R_E. These results were plotted on a medical decision chart with random error (R_E) on the X-axis and the bias (S_E) on the Y-axis (see Figure 2).

Periodic correlation of the assay with the laboratory at Fort Sam Houston obtained the following data. The correlation coefficient was 0.9483; the slope of the regression line was 0.990; the Y-intercept was 104.8; and the standard error estimate was 66.6. Figure 3 shows the regression line for the two facilities.

Figure 1. Scatter plot for the method comparison for our facility and large reference lab, showing acceptable correlation between the two facilities



A minimal difference was found between specimens retested after being refrigerated for one to three weeks, showing little change in G6PD activity. The difference was close to one enzyme activity unit for any specimen in the study. Table 1 shows the change in activity over time.

Two observations were seen as the number of specimens in the analytical run was increased. In the experiment with four to 12 specimens, when the run length was greater than four specimens, the enzyme activity decreased. In the experiment with 16 aliquots, the enzyme activity began to decrease at the fifth specimen. As the run was increased, red cells appeared to settle out of solution while on the instrument.

DISCUSSION

In validating the G6PD assay, the precision, linearity, and correlation are acceptable. A CV of 1.0% shows that the R_E of the reagent and instrument system is very precise with only a small difference in error between replicate samples when the length of the analytical run is optimized. The linearity experiment for the system shows that the difference between assigned and measured values is small and closely approximates a 1:1 ratio with a slope of 1.087. The bias between the assigned and measured values, 0.60 U/g Hgb, is well with in the E_A of 1.5 U/g Hgb. The correlation of the assay with the assay as performed at a large reference laboratory is also acceptable.

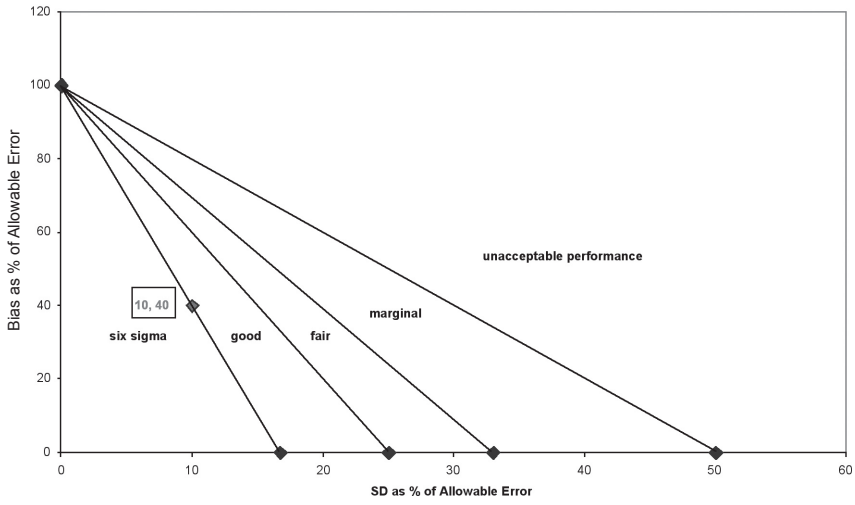
The R_E, S_E, and T_E for the G6PD assay are less than the E_A and therefore are acceptable. Plotting the S_E and the R_E as a percentage of the E_A on an MDC shows that the system is likely to perform well and that errors can be controlled within the allowable margin. The data plotted on the border of the Six Sigma and good range. The means that the method meets quality requirements and can be well managed in routine service with a reasonable amount of quality control.[9]

Periodic correlation of the assay with the laboratory at Fort Sam Houston shows that the system is performing acceptably. The regression statistics are acceptable.

Whole blood specimens appear to be very stable. Our results show that whole blood specimens collected in EDTA tubes are stable up to three weeks with a maximum lost of G6PD activity of approximately 1U. Changes in G6PD enzyme or whole blood specimen integrity appear to be noncontributory in the assessment of enzyme activity.

Analytical processing appears to be a major contributor to problems experienced in our facility. The observation of decreasing enzyme activity in batch runs of greater than four specimens is significant and is likely due to red cells settling out of solution while specimens are on the instrument. As always, in any reagent/specimen driven analysis, the analytical procedure of mixing cannot be underemphasized. Our standard operating procedure includes the rocking of specimens for ten minutes before they are placed on the instrument. However, the Cobas Mira Plus, with its onboard hemolysis feature, does not remix specimens before they are sampled. We observed, in case of large batches, that erythrocytes consistently settled out of solution, causing the observation of decreasing G6PD activity (U/g Hemoglobin) as the number of specimens in the run was increased. This observation affects the qualitative and quantitative use of the Cobas Mira Plus for G6PD screening.

Figure 2. Evaluation of Sigma Diagnostics reagent on the Cobas Mira Plus analyzer



The MCD shows that the reagent and system meet reasonable quality requirements for allowable error.

This observation clearly impacts the number of specimens that can be run per day and thus the workload for the test. In our case, this means that only 120 specimens can be run in an eight hour day. Furthermore, it requires that a technician be dedicated to the instrument, since our facility receives several hundred specimens per week. With this system, onboard lysis of erythrocytes is clearly a trade off for the number of specimens that can be assayed per day. Preparing the hemosylate offline and then placing the specimens on the instrument would likely prevent the observations we have seen with our system. The question that needs to be answered is which method is really the most time-management friendly…preparing the hemosylate offline or using this system with onboard red cell lysis? This will require further investigation.

However, our findings confirm that as long as the number of specimens is optimized the Cobas Mira Plus using the G6PD reagent by Sigma Diagnostics is accurate, user friendly, and easily maintained. We recommend the system as a good method for the qualitative and quantitative assessment of G6PD activity and effective in keeping soldiers who may have a G6PD deficiency out of harm's way.

Figure 3. Scatter plot for the method comparison for the Eisenhower Army Medical Center and Fort Sam Houston, showing acceptable correlation between the two facilities

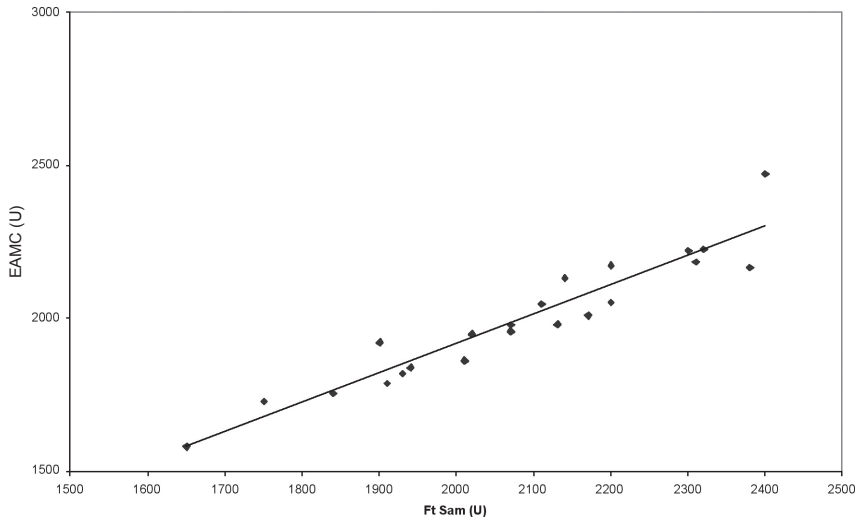


Table 1. G6PD activity when whole blood specimens were collected in EDTA tubes, refrigerated, and retested after one to three weeks

Specimen	Age (days)	Initial G6PD activity	Retest G6PD activity	Unit change
GPD689	23	16.0	16.1	0.1
GPD1034	14	17.8	16.2	1.6
GPD1077	11	17.7	16.8	0.9
GPD1103	10	2.8	1.5	1.3
GPD1181	9	1.2	0.4	0.8
GPD1127	7	17.1	18.0	0.9

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