Rapid Determination of Erythrocyte Pyruvate Kinase Activity

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We report a new potentiometric method for determining pyruvate kinase (PK). Enzymatic activity is measured by monitoring the change in pH produced in the reaction buffer under International Committee for Standardization in Haematology (ICSH) standardized assay conditions, and the lactate dehydrogenase reaction is automatically subtracted in each measuring cycle. The analysis, performed at 37 °C, requires a 10-μL sample (isolated erythrocytes or whole blood) and is completed in 2.5 min. The intra-assay CV is <4% (PK between 3 and 35 U/g Hb); the interassay CV is 4.0% (PK 15 U/g Hb); results are linear from 3 to 30 U/g Hb. A good correlation with the ICSH reference method (x) was found: y = 1.011x − 0.5; n = 32; r = 0.9939; Syy = 0.75 (units: U/g Hb). The reference intervals of the PK activity in isolated erythrocytes (RBC-PK) were estimated in 89 normal subjects. We found that women possess a higher RBC-PK than do men (P <0.0001) and that the biological variability (CV,%) of RBC-PK is 13.5%. Applications of the proposed method to the hematological routine are reported.

Indexing Terms: potentiometry · pH · hemolytic anemia · enzyme activity · heritable disorders

Pyruvate kinase (PK; ATP:pyruvate phosphotransferase, EC 2.7.1.40) deficiency is the most common form of hereditary hemolytic anemia and is caused by enzymatic defects of erythrocytes.3 Subjects manifesting chronic hemolysis may be homozygotes for a PK variant or, more frequently, compound heterozygotes with PK containing two different mutant protein chains, each resulting in diminished expression (1). PK deficiency can also be an acquired disorder that occurs in various leukemic or preleukemic conditions (2) or can be induced after chemotherapy by many molecular mechanisms (3). The clinical manifestations associated with PK deficiency vary (4–6) but typically are characterized by lifelong hemolytic anemia with jaundice and splenomegaly.

For these reasons the determination of erythrocyte PK activity is frequently requested, often in association with that of glucose-6-phosphate dehydrogenase (G6PD). The "gold standard" method for erythrocyte PK assay is that proposed by the International Committee for Standardization in Haematology (ICSH) in 1977 (7).

This method is highly reproducible but not practicable for automatic analyzers, mainly because each sample has to be blanked separately without ADP.

Our objective was to develop a new rapid and accurate protocol for an erythrocyte PK assay. The method we propose is based on a potentiometric technology previously described (8) and already applied to determine several clinically useful indexes in whole blood and in isolated erythrocytes (9–14). The determination of G6PD in erythrocytes, the most recent protocol reported (15), is now performed routinely in several laboratories (16, 17).

Materials and Methods

Subjects and Samples

The study was performed on 189 blood samples anticoagulated with EDTA, kept in ice while transported to the laboratory, and analyzed within 24 h of venipuncture. Of these, 167 were collected from apparently healthy subjects during an epidemiology study on the prevalence of G6PD deficiency in the Region of Calabria (17).

From these samples a subgroup of 89 samples (from 41 women, ages 23 ± 15 years, and 48 men, ages 28 ± 14 years) was created and used to establish the reference intervals. All these samples were from subjects with normal erythrocyte, reticulocyte, leukocyte, and platelet counts (4.0–6.1 × 1012/L, 5–20 cells/1000 erythrocytes, 4.9–10.7 × 109/L, and 130–400 × 109/L, respectively) and normal hematological indexes [hemoglobin (Hb); 110–180 g/L; hematocrit: 0.37–0.52; mean cell volume: 80–99 fl; mean cell hemoglobin: 26–31 pg]. Pregnant women and subjects suspected of iron deficiency (transferrin iron saturation <10% and zinc protoporphyrin >50 μmol/mol heme) were excluded.

The remaining 22 subjects were all adult patients regularly monitored every 1–3 months. Six subjects (three women, three men; erythrocytes: 2.0–4.1 × 1012/L; Hb: 81–125 g/L; reticulocytes: 1.8–20.0%) were already known to be double heterozygotes of mutant PKs (one had PK Sioresina, one had PK Mantova, and the PK variants of the last four were not characterized). None received blood transfusions during the 2 months preceding the study; one was splenectomized. Three were members of the same family (two parents, one child), the child being affected by PK deficiency hemolytic anemia because of double heterozygosity for two unknown PK variants (later described as "PK def. heterozygotes").

Five subjects (three women, two men; erythrocytes: 3.5–4.7 × 1012/L; Hb: 101–162 g/L; reticulocytes: 3.2–13.3%) were affected by hereditary spherocytosis (HS) and the remaining eight subjects (three women, five men; erythrocytes: 3.1–6.4 × 1012/L; Hb: 71–119 g/L;
reticulocytes: 3.2–13.0%) had various syndromes (two with Hb S/β* thalassemia, two with thalassemia intermedia, two with Hb H disease, and two with an undefined hemolytic anemia).

Erythrocytes were isolated by filtering blood samples through a-cellulose and microcrystalline cellulose (7). Glycerin-stabilized hemolysates, prepared as previously reported (15), were used to assess between-day imprecision. Artificially aged erythrocytes were prepared by incubating the isolated erythrocytes in 0.156 mol/L NaCl at 37 °C for 48 h.

Instrumentation

The differential pH measurements were taken at 37 °C with a differential pH analyzer (DELPAS CL; Kontron AG, Analytical Division, Milano, Italy). Absorbance was measured with a double-beam spectrophotometer (Spectracomp 601; Advanced Products s.r.l., Milano, Italy). The total blood cell count was performed with a Coulter S Plus electronic counter (Kontron AG).

Principle of the Assay

PK activity was measured by monitoring the pH change produced in the reaction buffer during the conversion of phosphoenolpyruvate (PEP) to pyruvate. The pH change produced by the reduction of pyruvate to lactate, catalyzed by the lactate dehydrogenase present in erythrocytes, was automatically subtracted from each measurement. Pyruvate was added to the working buffer to keep the lactate dehydrogenase reaction from being rate limiting.

Working Solutions

The working buffer used for determining PK activity with the differential pH analyzer (standard system) was fundamentally the same as that proposed by the ICSH (7). It contained Tris (10 mmol/L), KCl (100 mmol/L), MgCl₂ (10 mmol/L), EDTA (0.5 mmol/L), NaN₃ (1 g/L), NADH (0.2 mmol/L), ADP (1.5 mmol/L), pyruvate (1.0 mmol/L), and Triton X-100 (1 g/L); pH was 8.0 at 25 °C. Differences from the buffer proposed by ICSH were a reduced Tris concentration (lowered from 100 to 10 mmol/L to decrease its buffer power), added pyruvate, and no lactate dehydrogenase.

The buffer’s stability, judged by its pH, buffer power, absorbance at 340 mm, and performance in assays with glycerin-stabilized hemolysates, was 3 days at room temperature and 5 days at 4 °C. The starter solution, freshly prepared each week, contained 252 mmol of PEP per liter, carefully titrated to pH 8.0 ± 0.1. The final PEP concentration was 5.0 mmol/L.

For the low-substrate system (Low S) and the Low S + fructose 1,6-diphosphate (Low S + FDP), two different working buffers were prepared according to the method of Beutler et al. (7). In both, the final ADP concentration was 0.6 mmol/L, but the second buffer also contained FDP at 0.5 μmol/L. For both determinations a freshly prepared 12.6 mmol/L PEP solution, titrated at pH 8.0, was used as a starter. The final PEP concentration was 0.25 mmol/L.

Differential pH Procedure

The assay was performed on 10 μL of blood or isolated erythrocytes. The sample was added to the mixing chamber, which was filled with 840 μL of the working buffer. After dilution and lysis of the erythrocyte membranes (10 s), part of the reaction mixture was automatically injected into each of the capillary electrodes, and 8 μL of the 252 mmol/L PEP solution was added to the mixing chamber, which contained the remaining 500 μL of the reaction mixture. After further mixing, this reaction mixture plus substrate was injected into only one electrode. The variation of pH between the two electrodes was recorded for 60 s after a lag phase of 60 s. PK activity was calculated as described for another enzymatic assay (18).

The following instrumental variables were set: MODE = kinetic, U/L; 2ND unit = nkat/L; CONV FACT = 16.65; DILUTION = 1; HEATING = 1; T0 = 60; MEASURE TIME = 60; and CALIB FACT = 285.

Reference Method

The PK assay on isolated erythrocytes was performed at 37 °C according to the method described by Beutler et al. (7) with ADP at 1.5 mmol/L. The within-run imprecision (CV) of this assay in our hands, estimated by the method of the differences of replicates, is 0.7% at a mean PK of 15.2 U/g Hb.

Statistical Analysis

Statistical analysis was performed on an Olivetti M380XP1 personal computer with the aid of LABSTAT, a statistical package offered by the Italian Society of Clinical Biochemistry (S.I.Bio.C.).

Results

Analytical Variables

The typical reaction kinetic plots (not reported) show that the rate of the pH change is constant during the measuring time. The stoichiometry, tested by carefully evaluating the titration curves of the reactants and of the reaction products, is 1 mol of H⁺ consumed per mole of PEP transformed to pyruvate.

Under these conditions the method showed a good reproducibility, as illustrated by the data reported in Figures 1 and 2. The CV measured in whole blood and in isolated erythrocytes never exceeded 4%, with typical within-run values of 1–3%. Only with one PK-deficient subject did the within-run imprecision reach 10% because of the very low residual PK activity. Regarding between-run imprecision, estimated by measuring the PK activity in a stabilized hemolysate over ~1 year, PK activity (mean ± SD) was 14.6 ± 0.6 U/g Hb (n = 20) and the CV was 4.0%.

The linearity test was performed by assaying several different dilutions of an erythrocyte suspension with hematocrit that varied from ~0.10 to 0.90. Dilutions were used because it was difficult to find specimens with
Fig. 1. Within-run imprecision of the proposed method
Whole-blood samples (○) and isolated erythrocytes (●) from normal subjects, β-thalassemia carriers, and PK-deficient subjects; n = 20 each. Broken lines depict the 1% and 3% CVs

Fig. 2. PK linearity test performed on serial dilutions of an erythrocyte suspension with isotonic saline
Mean of triplicate measurements (○) and the corresponding CV (A). From linear-regression analysis: y = 0.026x - 15; r = 0.9970

a very low PK activity. The concentration range tested corresponded to an interval of 3–30 U/g Hb, sufficiently representative of the range found in nondeficient (with or without microcytosis) and PK-deficient subjects. As can be seen from the results reported in Figure 2, the linearity can be considered satisfactory, even though the squared correlation coefficient is not equal to 0.999.

Accuracy was assessed by comparing the proposed method with the ICSH method. The study was performed on isolated erythrocytes collected from normal subjects, β-thalassemia carriers, and PK-deficient subjects and on aged erythrocytes. The results obtained from this comparison demonstrated that the two methods were equivalent (Figure 3).

Finally, the PK activity in erythrocytes from 10 normal subjects was determined with the proposed method and then compared with that measured under the Low S and Low S + FDP protocols. The results, shown in Table 1, agree with those obtained by other investigators (I9). This comparison, therefore, proves the feasibility of these other differential pH measurements, which represent an additional tool for diagnosing PK abnormalities.

Comparison between Erythrocyte and Whole-Blood PK Activity
PK activity determined in whole blood (wb-PK) is higher than that measured in isolated erythrocytes (RBC-PK) because of the contribution made by the PK present in leukocytes and platelets. This fact was investigated in detail by comparing the two activities in several samples (Figure 4). The statistical analysis of these results (see the significance of the correlation coefficients reported inside the figure) clearly indicates that the wb-PK is strongly correlated to the leukocyte count. However, even wide variations in the platelet count do not seem to influence the wb-PK activity. The wb-PK is also correlated to the RBC-PK through the following equation: y = 1.322x + 9.01; n = 167; r = 0.8177 (P < 0.0001); S_y|x = 3.27 (units: U/g Hb; graph not shown).

The descriptive statistics and the PK reference intervals, calculated on whole-blood samples and on isolated erythrocytes from a selected group of subjects shown in Figure 4 are reported in Table 2. The use of the parametric reference intervals is supported by the finding that no significant deviations from the gaussian distributions were evidenced for the wb-PK and RBC-PK

Table 1. PK Activity Determined with Three Different Systems

<table>
<thead>
<tr>
<th>Systems</th>
<th>Substrates, μmol/L</th>
<th>Percent activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>PEP</td>
<td>100</td>
</tr>
<tr>
<td>Low S</td>
<td>250</td>
<td>23.2 ± 7.2</td>
</tr>
<tr>
<td>Low S + FDP</td>
<td>250</td>
<td>51.9 ± 3.7</td>
</tr>
<tr>
<td>n = 10.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* With respect to the standard system (15.7 ± 2.0 U/g Hb).

Fig. 3. Comparison between the differential pH method and the ICSH method
Erythrocytes from ○, normal subjects; Δ, PK-deficient heterozygotes; and ●, β-thalassemia carriers. ○, aged erythrocytes. Each point is the mean of two determinations; y = 1.011x - 0.5; n = 32; r = 0.9938; S_y|x = 0.75

Fig. 4. Bias plots of the leukocytes and platelets' effect on whole blood vs erythrocyte PK activity
Bias is calculated as (PK_Whole - PK Isolated)/PK whole x 100. The lines symbolize the linear regression (-- --) and the linear regression ± 2S_y|x (--- ---)
activities; this finding is based on results of the Kolmogorov-Smirnov asymmetry and kurtosis tests and the pattern of the distribution histograms (not shown). The choice of the 90% limits for the reference values was necessary for PK activities of 100% ± 20% (with respect to the mean PK for normal values). This is the range most often used to detect PK-deficiency heterozygotes (20–22).

The subdivision of PK values by sex was supported by the discovery that significant differences existed between sexes with respect to wb-PK and RBC-PK (P < 0.0001 for both comparisons with the Student's t-test for unpaired data). Normalized dispersion indexes (CV) between RBC- and wb-PK values were not very different. Assuming that those CVs reflect the total variability [CV, comprising analytical (CVa) and biological variability (CVb)] and considering that CVt = [(CVa)2 + (CVb)2]0.5 (23), we calculated the biological variabilities of wb-PK and RBC-PK. With analytical variabilities of 2.3% and 1.8% (means of the within-run CVs on wb-PK and RBC-PK values between 8.6 and 34.9 U/g Hb, respectively), CVt values are 11.0% (wb-PK, women), 13.4% (RBC-PK, women), 13.8% (wb-men), and 13.6% (RBC-PK, men).

PK Activities in Subjects with Hemolytic Anemia

The comparison between the RBC-PK activities in normal subjects and in subjects with different types of hemolytic anemia is reported in Figure 5. The differences in the enzymatic activities are highly significant (P < 0.01) for the PK− and others groups and significant (P < 0.05) for the HS group. The PK activities are higher in the HS and other groups than in the controls group, probably because of the presence of a young erythrocyte population, as shown by the variable increase in the proportion of reticulocytes. The occurrence of the highest PK values in the other group is associated with microcytosis caused by ineffective hemoglobin synthesis. If the enzymatic activities were expressed in terms other than U/g Hb, such an increase would probably be less evident, as already reported (16).

Discussion

We describe a new potentiometric procedure for determining PK activity. Although it was demonstrated that the ICSH protocol could be modified, we decided not to optimize the protocol, as proposed by Lakomek et al. (24), but to present a new method that correlates well the ICSH standard assay.

Our method is reproducible and rapid (2.5 min/analysis) and is an adaptation of the ICSH procedure to the differential pH technique. Apart from the difference in the type of analytical signal measured (a difference in pH instead of a difference in the absorbance), the main distinction with respect to the reference method concerns the direct dilution of the sample in the working buffer. The ICSH procedure for preparing hemolysate is avoided, the lysis being quickly performed by the detergent present in the buffer. Therefore, our method should be specific for determining PK activity and should not be affected by the eventual deficiency of other enzymes of the Embden-Meyerhof pathway.

Our method is accurate, as demonstrated by the good correlation with the reference method and the RBC-PK reference intervals reported for normal assay conditions (standard system) and for the Low S and the Low S + FDP systems (7, 19). However, our reference intervals were slightly shifted toward higher values compared with those obtained more recently by others using the same ICSH standardized assay (5). This could be explained by a difference in the efficiency of the leukocyte-removal procedure or a variability in the activity assay protocol, which, on the basis of differences among commercial assay reagents, has been already advocated to explain the major source of variability in the between-laboratories measurements (7). We think that the efficiency of our leukocyte-removal procedure is the same as that described by Zanella et al. (5), and the number of leukocytes left after filtration (counted on several blood smears) was <0.003% of that present in whole blood. Therefore, there is only a negligible leukocyte contamination in the final erythrocyte suspension, similar to that noted by Zanella (<4 cells/10⁶ erythrocytes).

The procedure is also suitable for the direct measurement of PK activity in whole blood, because the color of the sample does not interfere with the differential pH measurements. However, because the leukocytes inter-

Table 2. PK Values in Isolated Erythrocytes and Whole Blood from Normal Subjects

<table>
<thead>
<tr>
<th>Erythrocytes</th>
<th>Whole blood</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Women (n = 41)</td>
</tr>
<tr>
<td>Minimum, U/g Hb</td>
<td>14.1</td>
</tr>
<tr>
<td>Maximum, U/g Hb</td>
<td>23.2</td>
</tr>
<tr>
<td>Mean, U/g Hb</td>
<td>17.5</td>
</tr>
<tr>
<td>SD, U/g Hb</td>
<td>2.4</td>
</tr>
<tr>
<td>CV, %</td>
<td>13.5</td>
</tr>
<tr>
<td>5.0 percentile</td>
<td>13.6</td>
</tr>
<tr>
<td>95.0 percentile</td>
<td>21.4</td>
</tr>
</tbody>
</table>

* Lower parametric reference limit, in U/g Hb.
* Upper parametric reference limit, in U/g Hb.
ference with the determination of RBC-PK activity if a whole-blood sample is analyzed, as clearly shown in Figure 4, the complete removal of leukocytes is mandatory.

In summary, we have modified the ICSH reference method to adapt it to the differential pH technique. The proposed method has an acceptable imprecision and good accuracy and, compared with the ICSH method, a much shorter turnaround time. The time-consuming chromatographic procedure for removing leukocytes cannot be avoided. The differential pH technique will be useful for determining the PK activity in subjects with persistent hemolytic anemia. The method can also be easily modified for studying the kinetic properties of PK variants.

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References