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Investigation of Glucose 6-Phosphate Dehydrogenase (G6PD) Kinetics for Normal and G6PD-Deficient Persons and the Effects of Some Drugs

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In the present study, blood samples from 1183 children aged 0.5–6 years were taken. Three children were found with G6PD deficiency by examining the enzyme activity and hemoglobin ratio. Some kinetic properties of glucose 6-phosphate dehydrogenase enzyme (G6PD) were studied after the purification of the enzyme with ammonium fractionation, dialysis and 2′,5′ ADP-Sepharose 4B affinity chromatography from a healthy person and from three G6PD-deficient people. The purity of the enzymes was confirmed by SDS-PAGE electrophoresis. The effects of some drugs which are known inhibitors of G6PD activity were studied. Some of the drugs stimulated the activity of the enzyme in two of the three cases with G6PD deficiency. K_M values, V_{max} values for G6P and $NADP^+$, optimum pH and optimum temperature for the enzyme from the healthy person and the three G6PD-defficient people are reported.

Keywords: Glucose-6-phosphate dehydrogenase; Purification; Drug; Deficiency

INTRODUCTION

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oxidoreductase EC 1.1.1.49; G6PD) is the key enzyme catalyzing the first reaction of the pentose phosphate pathway(PPP), the only source of NADPH in erythrocytes, which involves the conversion of glucose into pentose sugars necessary for a variety of biosynthetic reactions.[1] G6PD has a vital function in many kinds of tissues and consequently is a housekeeping enzyme playing an important role in metabolism.[2] The NADPH produced in the PPP by both G6PD and 6-phosphogluconate dehydrogenase serves as an electron donor in reductive biosynthesis, notably of cholesterol and fatty acids.[3] NADPH is very important in the protection of cells from oxidative stress.[1]

G6PD deficiency was discovered in the mid-1950s as a result of researches conducted to discover why some people digesting primaquine were sensitive to the hemolytic effects.[4] G6PD deficiency is the most widespread red cell enzymopathy, affecting 400 million people throughout the world. It is an inherited disorder and causes neonatal haemolysis and jaundice.[1] G6PD deficiency is a X-linked trait; entirely expressed in males and homozygous females and is variably expressed in heterozygous females.[5] In normal erythrocytes, G6PD activity decreases with aging. In mild variants of this disease, the erythrocyte G6PD level is lower than in the normal person; in some variants even young erythrocytes do not endure stress.[6–8] G6PD deficiency is frequently seen in African, Mediterranean, Middle Eastern and Far Eastern nations and their lineages with a frequency ranging from 5%–40%.[5–7] In Turkey, cases of this disorder are present in the Çukurova region and Başkale district of Van and highest incidence is seen in the Jewish Kurd population (62% of males).[9]

Many drugs are being used in therapies and there are many reports related to changing of enzyme activities.[10] In the present study, the kinetic properties of glucose 6-phosphate dehydrogenase enzyme (G6PD) of subjects with G6PD-deficient and normal G6PD enzyme activity were studied after purification

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of the enzyme. The K_M and V_{max} of G6PD and $NADP^+$ for healthy and three G6PD-deficient people (cases 1, 2 and 3) were investigated on the purified G6PD enzyme. The *in vitro* effects of sodium ceftizoxime, sodium cefuroxime, streptomycin, netilmicin and metamizol with a known inhibitory effect on persons with normal G6PD activity was investigated on purified enzyme from G6PD-deficient human erythrocytes.

MATERIALS AND METHODS

Materials

2′,5′-ADP-Sepharose 4B was obtained from Pharmacia. $NADP^+$, glucose-6-phosphate, protein assay reagents and chemicals for electrophoresis were obtained from Sigma Chem. Co. All other chemicals used were analytical grade and obtained from either Sigma or Merck. Medical drugs were from the Hospital of Ataturk University.

Preparation of the Haemolysate

Fresh human blood collected in EDTA was centrifuged (15 min, 2500 × *g*). The red cells were isolated and washed three times with 0.16 M KCl, and haemolysed with five volumes of ice-cold water, and then centrifuged at 4°C, 10,000 × *g* for 20 min to remove the ghosts and intact cells.[11,12]

Ammonium Fractionation and Dialysis

Haemolysate was brought to 35–65% $(NH_4)_2SO_4$ saturation with solid $(NH_4)_2SO_4$. The precipitate was separated by centrifugation at 5000 × *g* for 15 min and dissolved in a small amount of 50 mM phosphate buffer (pH 7.0), and then dialysed at 4°C in 50 mM K-acetate/50 mM K-phosphate buffer (pH 7.0) for 2 h with two changes of buffer.[11]

Purification of G6PD by Affinity Chromatography

Dry 2′,5′-ADP-Sepharose 4B was resuspended in 0.1 M K-acetate/0.1 M K-phosphate buffer (pH 6.0), then used to pack a small column (1 × 10 cm) which was equilibrated with the same buffer. The dialysed enzyme solution obtained above was loaded onto the column (1 × 10 cm). The gel was then sequentially washed with 25 ml of 0.1 M K-acetate/0.1 M K-phosphate (pH 6.0), with 25 ml of 0.1 M K-acetate/0.1 M K-phosphate buffer (pH 7.85) and finally with 25 ml of 0.1 M KCl/0.1 M K-phosphate buffer (pH 7.85). Elution was carried out with 80 mM K-phosphate + 80 mM KCl + 0.5 mM $NADP^+$ + 10 mM EDTA (pH 7.85) solution. The flow rates of the washing and eluting steps were 50 ml h^{-1} and 20 ml h^{-1}, respectively. In the elutes of 2 ml volume, the activity of G6PD was determined on all fractions. Protein determination at 280 nm in elutes could not be determined since the $NADP^+$ absorbance masked the actual protein absorbance. Active fractions were collected. All procedures were performed at 4°C.[11,13]

Measurement of G6PD Activity

G6PD was measured spectrophotometrically at 37°C as described by Beutler.[14] Briefly, the enzyme sample was added to 2.5 ml (final volume) of incubation mixture containing 1 M Tris-HCl + 0.5 mM EDTA (pH 8.0), 10 mM $MgCl_2$, 0.2 mM $NADP^+$ and 0.6 mM glucose-6-phosphaten (G-6-P). The activity measurement at 37°C was conducted by monitoring the increase in absorption at 340 nm due to the reduction of $NADP^+$. One enzyme unit represents the reduction of 1 μmol of $NADP^+$ min^{-1} at 37°C, pH 8.0.

Protein Determination

Quantitative protein determination was conducted by absorbance measurement at 595 nm according to Bradford, with bovine serum albumin as a standard.[15]

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was conducted after purification of the enzyme in 10% and 4% acrylamide concentrations for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli.[16] To the sample and standard, 20 mg bovine serum albumin was applied to the electrophoresis medium. Gels were stained overnight in 0.1% Coomassie Brillant Blue R-250 in 50% methanol and 10% acetic acid, then destained with many changes of the same solvent without dye. The electrophoretic pattern is shown in Figure 1.

Optimal pH Determination

For the pH-optimal determination, the enzyme activity was measured in 1 M Tris-HCl buffers within the range pH 6.0–9.0.

Effect of Temperature on G6PD Activity

The enzyme activity was measured from 0–60°C at optimal pH for this purpose. The total volume of reaction mixture (without glucose-6-phosphate) was incubated for 10 min at each temperature. The activity was measured after addition of G-6-P.

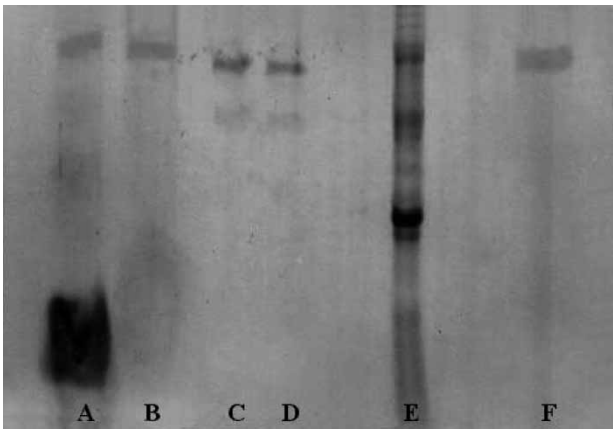

FIGURE 1 SDS-polyacrylamide gel electrophoresis of G6PD for a healthy person and G6PD-deficient persons 1,2 and 3. (A) band for person-3 (B) band for person-2, (C) and (D) band for healthy one, (F) band for person-1, (E) mixture of standard proteins. (Myosin, rabbit, M_r 205.000; β galactosidase, *E. coli*, 116.000; phosphorylase B, rabbit, 97.400; albumin, bovine, 66.000; ovalbumin, chicken, 45.000; carbonic anhydrase, bovine, 29.000).

Kinetic Studies

For K_M and V_{max} evaluation, Lineweaver-Burk curves were used.[17] These were obtained for $NAPD^+$ using five different concentrations of $NADP^+$ and a constant concentration of G6-P, and similarly for G6-P with a fixed $NADP^+$ concentration.[14] All kinetic studies were performed at 25°C and in optimal pH (1 M Tris-HCl, pH:8.0).

In Vitro Drugs Studies

Sodium ceftizoxime, sodium cefuroxime, streptomycin, netilmicin and metamizol were used as drugs. To determinate I_{50} values, percentage inhibition values were obtained with five different inhibitor concentrations using 0.6 mM constant substrate concentration. Regression analysis graphs were drawn using percentage inhibition values by a statistical package program. The inhibitor concentration causing 50% inhibition was determined from the graph.

RESULTS AND DISCUSSION

The most widespread red blood cell (RBC) enzyme defect world-wide is G6PD deficiency[6] which causes neonatal haemolysis and jaundice.[1] Although drug-induced haemolysis has attracted the most attention, it is probable that haemolysis induced by infection may be a more common cause of clinical haemolysis. Many reports attest to the importance of infection in causing hemolytic anemia[18–20] and it is difficult to be certain in some cases whether there is a relationship between ingestion of a drug and haemolysis.[7] Some studies have demonstrated a protective effect of G6PD-deficient genotypes.[21,22] G6PD-deficient alleles confer some resistance against severe malaria caused by infection with *Plasmodium Falciparum*. However, the genetic heterogeneity of G6PD deficiency means that a drug found to be safe in some deficient subjects may not be safe in all.[1]

Biochemical analysis has revealed great diversity in the properties of the G6PD enzyme in deficient subjects identified in different laboratories.[23]

In the present study, determination of G6PD-deficient was aimed at persons in the region of Erzurum, Turkey. The kinetic properties of the G6PD enzyme and effects of some drugs on erythrocyte G6PD were studied. Blood samples from 1183 children aged 0.5–6 years were taken. While the activity for G6PD enzyme was overall determined as 9.7 ± 2.27 EU/gHb, three children were found with G6PD deficiency by examining the enzyme activity and hemoglobin ratio.

Some kinetic properties of G6PD enzyme were studied after the purification of the enzyme with-ammonium fractionation, dialysis and 2′,5′-ADP Sepharose 4B affinity chromatography. Table I

TABLE I Purification scheme for glucose 6-phosphate dehydrogenase from normal, and persons 1, 2 and 3

Purification Step		Activity (EU/mL)	Total Volume (mL)	Protein (mg/mL)	Total Protein (mg)	Total Activity (EU)	Specific Activity (EU/mg)	Yield (%)	Purification factor
Haemolysate	Normal	0.36	350	78	27300	126	0.0046	100	1
	Person-1	0.043	236	57.3	13523	10.15	0.0075	100	1
	Person-2	0.07	44	56.6	2490.4	3.1	0.0012	100	1
	Person-3	0.32	173	47.8	8269.4	55.36	0.0067	100	1
Ammonium sulfate precipitation (35–65%)	Normal	0.51	150	37.8	5670	76.5	0.0135	60.7	2.9
	Person-1	0.1	84	20.4	1714	8.4	0.049	82.8	6.5
	Person-2	0.1	17	32.8	557.6	1.7	0.003	54.8	2.5
	Person-3	0.37	80	20.8	1664	29.6	0.017	53.5	2.54
2′,5′-ADP Sepharose-4B affinity chromatography	Normal	4.7	15	0.077	1.16	70.5	61.44	56	13,356
	Person-1	0.98	6	0.03	0.18	5.88	32.67	57.93	4,355
	Person-2	0.17	5	0.03	0.15	0.85	5.7	27.4	4,750
	Person-3	2.45	9	0.089	0.8	22.1	27.5	40	4,104

TABLE II Optimum pH, optimum temperature, K_M, V_{max} and SDS-PAGE data for G6PD from a healthy and deficient persons

			K_M		V_{max}		
	Optimum pH	Optimum temperature (°C)	G 6-P (μM)	$NADP^+$ (μM)	G-6-P (μmol/min.mg)	$NADP^+$ (μmol/min.mg)	SDS-PAGE (Kdalton)
Normal	8	37	500	210	415.6	545.5	59–60
Person-1	8.5	40	150	130	9.7	4.3	59–60
Person -2	9	45	48	70	1.2	2.3	59–60
Person -3	8.5	40	83	85	12.5	1	59–60

shows the purification step for persons 1, 2, 3 and a healthy one. The purification factor for normal and G6PD-deficient persons 1, 2 and 3 were 13,356; 4,355; 4,75; 4,104, respectively.

Figure 1 shows the SDS-PAGE gel for the determination of purity of the enzyme from the four persons under study. High purity was seen for the enzymes obtained.

The pH values for the healthy person and the G6PD-deficient persons 1,2 and 3 were 8, 8.5, 9, 8.5 using 1 M Tris-HCl respectively (Table II; Figure 2). It seems that these values are higher than for a normal person[13] and that deficient subjects are more basic than a healthy one. According to Figure 2 the pH-optimum for G6PD-deficient persons is monophase and different from that determined in previous studies for G6PD variants. The pH values for G6PD Adana, G6PD Samandağ, G6PD Balcali, G6PD Madrid, G6PD Antakya have been stated as 5–8 (biphasic), 7–9 (biphasic), 5–7 (biphasic), 7–9 (biphasic), heterogeneity, respectively.[24–26]

The enzymes for G6PD-deficient persons 1, 2 and 3 were seen to exhibit the highest activities at 40°C, 45°C and 40°C (Table II; Figure 3) in a temperature-study between 0–60°C respectively. It has been shown that enzymes purified from G6PD-deficient persons 1, 2 and 3 are more temperature stable than that from the healthy person.[24–27]

Kinetic parameters determined are shown in Table II. The K_M values of G6PD for G-6-P and

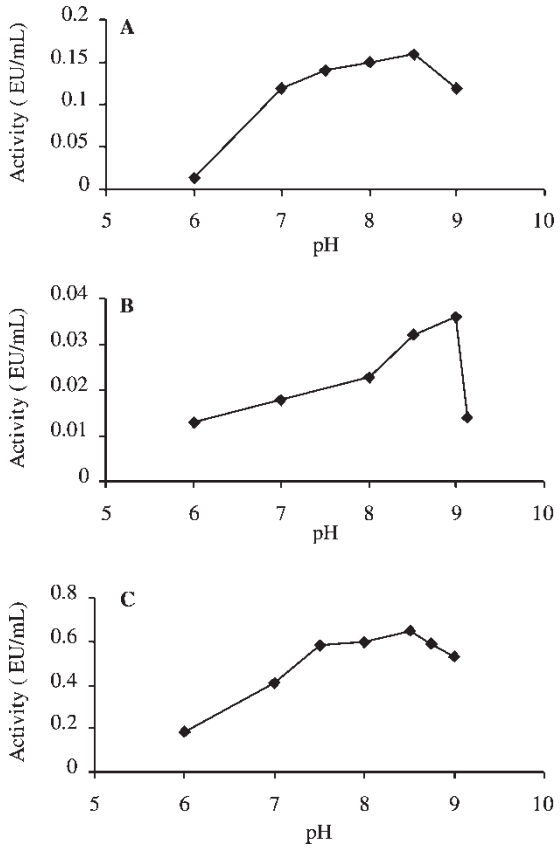

FIGURE 2 G6PD pH-activity graphs in 1M Tris-HCl for persons 1 (A), 2 (B) and 3 (C).

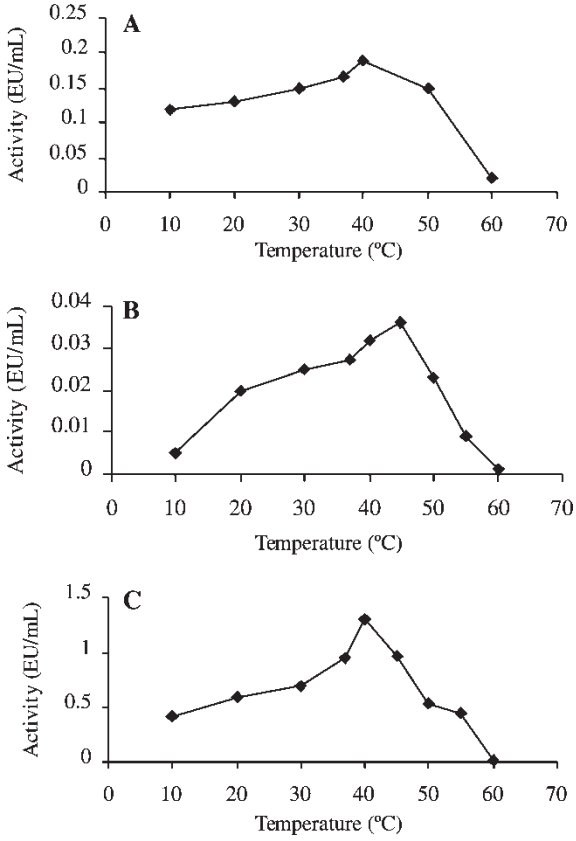

FIGURE 3 G6PD activity–temperature graphs in 1 M Tris-HCl for persons 1 (A), 2 (B) and 3 (C).

TABLE III I_{50} values for G6PD in the presence of different drugs

	Sodium ceftizoxime (mM)	Netilmicin (mM)	Sodium cefuroxime (mM)	Streptomycin (mM)	Metamizol (mM)
Normal[2,9]	2.9	9.8	6.7	25	17
Person-1	2.12	14.7	21.9	activation	activation
Person-2	1.36	15	11.1	activation	activation
Person-3	1.3	12.5	7.9	41.6	109.2

$NADP^+$ for the healthy person and G6PD-defficient persons 1, 2 and 3 were 500 μM, 150 μM, 48 μM, 83 μM and 210 μM, 130 μM, 70 μM, 85 μM, respectively. K_M values for both G6P and $NADP^+$ determined for G6PD-deficient persons are lower than for the healthy one. These results show that enzymes for deficient subjects have greater affinity for G6P and $NADP^+$. K_M values determined for G6PD-defficient persons 1, 2 and 3 is different from that reported in previous studies. The K_M values of G-6-P and $NADP^+$ for G6PD Adana, G6PD Samandağ, G6PD Balcali, G6PD Valladolid, G6PD Madrid, G6PD Antakya were as 210 μM (G-6-P), 13 μM ($NAPD^+$); 25 μM, 18 μM; 38 μM, 3 μM; 41 μM, 9 μM; 76 μM, 18 μM; 667 μM, 25 μM, respectively.[24–27]

V_{max} values of G6PD and $NADP^+$ for the healthy and G6PD-deficient persons 1, 2 and 3 were 415.5, 9.7, 1.2, 12.5 and 545.6, 4.3, 2.3, 1.0 (μmol/minxmg) respectively (Table II). V_{max} values, both G6P and $NADP^+$, for persons with G6PD deficiency are lower than that for the healthy one. It is clear that the catalytic activity of the enzyme in deficient subjects is lower than that for the normal person. This is the first reported attempt to study the V_{max} values of the G6PD enzyme in G6PD-deficient persons.

Interesting results were found from drug studies carried out on the enzyme from persons with G6PD-deficient (Table III). The I_{50} values for the enzyme belonging to the healthy person was 2.9 mM for sodium ceftizoxime, 9.8 mM for netilmicine, 6.7 mM for sodium cefuroxime, 25 mM for Streptomycin and 17 mM for metamizol.[2,9] The corresponding I_{50} values of sodium ceftizoxime, netilmicine and sodium cefuroxime for persons 1, 2 and 3 were 2.12 mM, 1.36 mM, 1.3 mM (sodium ceftizoxime) 14.7 mM, 15 mM, 12.5 mM (netilmicine) and 21.9 mM, 11.1 mM and 7.9 mM (sodium cefuroxime) respectively. I_{50} values of streptomycin and metamizol for person 3 were 41.6 mM and 109.2 mM, respectively; these drugs activated the G6PD enzyme of persons 1 and 2.

The kinetic properties of G6PD variants determined world-wide have been found different from each other. For example; sulfamethoxazole has been shown to produce shortening of the erythrocyte life span[28] in Asian subjects with G6PD deficiency, but in patients with G6PD A^- significant haemolysis did not appear when this combination was used.[29]

We conclude that G6PD-deficient persons may show persistence against the inhibitory effect of sodium cefuroxime, streptomycin, netilmicin and metamizol but not for sodium ceftizoxime. Since enzymes for G6PD-deficient persons have higher I_{50} values in comparison with that of the healthy person for sodium cefuroxime, streptomycin, netilmicin and metamizol. Moreover, the enzymes for persons 1 and 2 were activated by streptomycin sulfate and metamizol.

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