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Investigation of the Mutation Points and Effects of Some Drugs on Glucose-6-phosphate Dehydrogenase-deficient People in the Erzurum Region

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We have carried out a systematic study of the molecular basis of glucose-6-phosphate dehydrogenase (G6PD) deficiency on three samples of 1,183 children aged 0.5–6 years from Erzurum, in eastern Anatolia. Total genomic DNAs were isolated from the blood samples of a healthy person and the three persons determined with G6PD deficiency by examining the enzyme activity and hemoglobin ratio. Then PCR amplification of the entire coding region in eight fragments was carried out followed by Agarose gel electrophoresis. The 540-bp PCR fragment containing exons VI–VII and the 550 bp PCR fragment containing exons XI–XIII were digested with EcoRI and with NIaIII, respectively. SSCP techniques for eight fragments (exons II, III–IV, V, VI–VII, VIII, IX, X, and XI–XIII) were employed to determine the mutations on the exons of the G6PD gene. A mutation occurred on the region of the exons 6 and 7 of one person (person-1) and exon 5 of two G6PD-deficient persons (person 2 and 3) examined. The sequential approach described is fast and efficient and could be applied to other populations.

Effects of analgesic drugs on G6PD were studied on the purified enzyme (ammonium fractionation, dialysis and 2′,5′ ADP-Sepharose 4B affinity chromatography) for the healthy person and G6PD-deficient persons 1, 2 and 3. The effects of remifentanil hydrochloride, fentanyl citrate, alfentanil hydrochloride and pethidine hydrochloride, as analgesic drugs, on G6PD activity were tested. Although remifentanil hydrochloride, fentanyl citrate (I_{50} values; 1.45 mM and 6.1 mM, respectively) inhibited the activity of the enzyme belonging to the healthy person, they did not alter enzyme activity on two of the three persons with G6PD deficiency. Other drugs (alfentanil hydrochloride and pethidine hydrochloride) did not effect the enzyme activity of the healthy or G6PD-deficient children.

Keywords: Glucose-6-phosphate dehydrogenase; Deficiency; Mutation; SSCP; Drugs

INTRODUCTION

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oxidoreductase EC 1.1.1.49; G6PD) catalyzes the first reaction of the pentose phosphate pathway (PPP) which involves the conversion of glucose into pentose sugars, necessary for a variety of biosynthetic reactions. The principal source of cytoplasmic NADPH catalyzed by G6PD and 6-phosphogluconate dehydrogenase is the PPP.[1,2]

G6PD deficiency is the most widespread red cell enzymopathy, affecting 400 million people throughout the world. It is an inherited disorder of mankind and causes neonatal haemolysis and jaundice.[1,2] Most G6PD-deficient individuals are asymptomatic and develop symptoms only in response to oxidant stress but the deficiency does not affect the life expectancy.[1,2] G6PD deficiency is an X-linked trait; entirely expressed in males and homozygous females and is variably expressed in heterozygous females.[3] In normal erythrocytes, G6PD activity decreases with age. 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.[4–6] G6PD deficiency is frequently seen in African, Mediterranean, Middle Eastern and Far Eastern nations and their lineage with a frequency ranging from 5%–40%.[3,4,7]

G6PD was sequenced by Persico *et al.*[8,9] and then independently by Takizawa and Yoshida.[10] The gene contains 13 exons and it is over 20 Kb in

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length. The first exon includes no coding sequence. The intron between exons 2 and 3 is excessively long, extending for 9,857 bp. At the 5′ end of the gene is a cytidine-guanine dinucleotide (CpG)-Rich Island.[11] Differential demethylation of some of the CpG's is accountable for expression of the gene on the active X chromosome[12] and this island has been preserved between man and mouse.[13] The human G6PD monomer comprises 515 amino acids with a molecular weight of 59 kDa.[14]

Since first description of SSCP by Orita *et al.*,[15] it has been recognized as a strong tool for detecting small mutations. SSCP analysis has been applied successfully for many genetic diseases including G6PD deficiency.[16] It can detect DNA polymorphism and point mutations at a variety of positions in DNA fragments.

The G6PD deficiency shows substantial regional variation in Turkey. Previous studies have revealed frequencies of less than 1% in Ankara (central Anatolia)[17] 2–9% in the Aegean region, 7.9–11.4% in Adana (south Anatolia, among Eti Turks)[17,18] and 5–20% percent in Antalya and Alanya (southwest Anatolia).[19,20] Highest incidence is seen in the Jewish Kurd population (62% of males).[21] These reports have indicated a high prevalence in the Mediterranean region of the country.[22]

The relationship between drugs with G6PD inhibitory activity has attracted much interest and there have been numerous reports related to drugs changing G6PD and other enzyme activities.[23] In the present study, initial studies on DNA isolation, PCR and SSCP were performed. The *in vitro* effects of the analgesics remifentanil hydrochloride, fentanyl citrate, alfentanil hydrochloride and pethidine hydrochloride on the purified enzyme from three G6PD-deficient persons and one with normal enzyme activity were studied.

MATERIALS AND METHODS

Materials

2′,5′ ADP-Sepharose 4B was obtained from Pharmacia. Materials for extraction of genomic DNA and the polymerase chain reaction (PCR), $NADP^+$, glucose-6-phosphate, protein assay reagents, chemicals for electrophoresis and other materials were purchased from Sigma Chem. Co. or Merck. Primers for amplification of each exon were purchased from Iontek. All reagents were of acceptable or analytical grade. Medical drugs were from the Hospital of Ataturk University. Blood samples were obtained from the village clinic at the Erzurum region.

Methods

Preparation of the Haemolysate and Measurement of G6PD Activity

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.[24,25] G6PD and G6PD deficiency was measured as described by Beutler.[6] In this spectrophotometric measurement, the reaction medium contained 0.1 mM Tris-HCl (pH 8.0) with 0.5 mM EDTA, 10 mM $MgCl_2$, 0.2 mM $NADP^+$, and 0.6 mM glucose-6-phosphate (G6P) in a total volume of 1 ml. NADPH produced in the reaction mixture was measured at 340 nm. One unit of enzyme (EU) activity was defined as the enzyme amount reducing 1 μ mol $NADP^+$ per 1 min at 25°C, pH 8.0. The activity measurement was done by monitoring the increase in absorption at 340 nm due to the reduction of 1 μmol of $NADP^+$ min^{-1} at 25°C, pH 8. G6PD deficiency was diagnosed by measuring the red blood cell (RBC) enzyme activity according to the International Committee for Standardization in Haemotology.[5,6]

Genomic DNA Isolation

Genomic DNA was extracted from peripheral blood leukocytes of normal and affected individuals, using lysis buffer (131 mM NH_4Cl/0.9 mM NH_4HCO_3) and phenol/chloroform extraction.[26]

PCR Assays

G6PD-coding regions were selectively amplified in eight fragments (exons II, III–IV, V, VI–VII, VIII, IX, X, and XI–XIII) from genomic DNA by PCR, using specific oligonücleotide primers as described elsewhere.[27] Amplification was performed in 10 × PCR buffer with a final $MgCl_2$ concentration of 1.5 mM, with 30 cycles at 94°C for 45 s, 56°C for 45 s, 72°C for 45 s.

Agarose Gel Electrophoresis and Digestion with Restriction Endonuclease Enzymes

Products of the PCR reactions were then run on 2% agarose gel electrophoresis stained with ethidium bromide at 1.5 V/cm and photographed under UV light. Exons 6–7 and exons 11–13 were digested with EcoR I and NIaIII, respectively.

SSCP Assays

SSCP assays were examined according to SSCPs.[15,28] A portion of each amplified sample (6 μl) was withdrawn and mixed with an equal volume of 95%

formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol and then heated at 95°C for 5 min and snap-frozen in an ethanol-dry ice bath. 6 μl of each sample, per lane, were applied to 8% polyacrylamide gel containing 90 mM Tris-Borate buffer (pH 8.3), using a compact gel system (8 × 8 × 0.1 cm). Electrophoresis was performed at 120 V for 2 h at 20°C.

Procedures for Enzyme Purification

Protein Determination

Quantitative protein determination was done by absorbance measurement at 595 nm according to Bradford, with bovine serum albumin as a standard.[31] Protein determination and enzyme activity was measured for each purification step to give a specific activity and purification factor.

Ammonium Fractionation and Dialysis

After the haemolysate was centrifuged at 4°C, 10,000 × *g* for 20 min to remove the ghosts and intact cells it was brought up to 35–65% $(NH_4)_2SO_4$ saturation with solid $(NH_4)_2SO_4$. Ammonium fractionation and dialysis were conducted as described elsewhere.[24]

Purification of G6PD by Affinity Chromatography

Dried 2′,5′ ADP-Sepharose 4B was resuspended in 0.1 M K-acetate/0.1 M K-phosphate buffer (pH 6.0), and then packed in a small column (1 × 10 cm) which was equilibrated with the same buffer. The dialysed homogenate was loaded on the column. The gel was then sequentially washed with 25 ml of 0.1 M K-acetate/0.1 M K-phosphate (pH 6.0), then 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 a solution of 80 mM K-phosphate + 80 mM KCl + 0.5 mM $NADP^+$ + 10 mM EDTA (pH 7.85). Protein determination at 280 nm was not performed on the elutes, since the $NADP^+$ absorbance masked the actual protein absorbance. Active fractions were collected. All procedures were performed at 4°C.[24,29]

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was conducted after purification of the enzyme. It was carried out according to Laemmli[30] in 10% and 4% acrylamide concentrations for the running and the stacking gel, respectively, containing 0.1% SDS.

In Vitro Drugs Studies

In order to determinate I_{50} values for remifentanil hydrochloride, fentanyl citrate, alfentanil hydrochloride, pethidine hydrochloride, all analgesics, as inhibiting drugs, inhibition percent values were obtained from five different inhibitor concentrations with 0.6 mM constant substrate concentration. Regression analysis graphs were drawn using inhibition percent values using a statistical packing program on a computer and the inhibitor concentrations causing up to 50% inhibition were determined from the graphs.

RESULTS AND DISCUSSION

In this study, a large group was examined by statistical methods for G6PD deficiency and the G6PD activity of normal and enzyme-deficient persons was determined. We found that of 1,183 children aged 0.5–6 year's old tested, three children (less than 1%) were G6PD-deficient. The activity value for the G6PD enzyme was determined as 9.7 ± 2.27 EU/gHb. Although the haemolysate for person-1 exhibited least measurable activity (8%), the purified enzyme had a more detectable activity. The haemolysate for person 2 and 3 had less activity than the normal one (20% and 48%, respectively).

Some kinetic properties of the G6PD enzyme were studied after the enzyme had been purified by ammonium fractionation, dialysis and 2′,5′-ADP Sepharose 4B affinity chromatography. Purification factor for the normal person and G6PD-deficient persons 1, 2 and 3 were 13,356; 4,355; 4,75; 4,104, respectively.[32] SDS-PAGE gel for the determination of purity of the enzyme from the four persons under study was carried out and showed a single band, accepted as demonstrating high purity for the obtained enzymes.

Figure 1 exhibits agarose gel electrophoresis after PCR amplification of G6PD-coding regions (exons II, III–IV, V, VI–VII, VIII, IX, X) for the healthy person and persons 1, 2 and 3. The fragment containing exons XI–XIII was then amplified too. All exons were successfully amplified. Long fragments were previously digested with restriction enzymes in order to obtain a better resolution for the SSCP procedure. Digestions with EcoRI of the 540 bp PCR fragment containing exons VI–VII and with NIaIII of the 550 bp PCR fragment containing exons XI–XIII generated two fragments (Figure 2).

SSCP analysis is quite useful for rapidly searching various known and unknown mutations in a large number of specimens with high sensitivity. The SSCP analysis results (Figure 3) showed that there was a mutation on the region of the exons 6 and 7 of one person (person-1) and the exon 5 of two G6PD-deficient persons (persons 2 and 3) examined.

This study confirms that G6PD deficiency is present in Erzurum, Turkey but that the frequency of G6PD enzyme deficiency appears to be

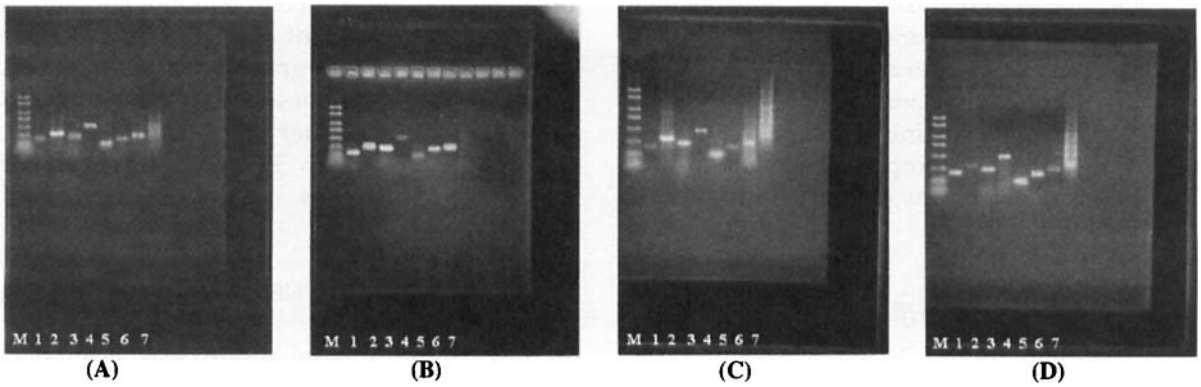

FIGURE 1 Agarose gel electrophoresis of PCR products shows the following: **A**; for healthy one, **B**; for G6PD-deficient Person-1, **C**; for G6PD-deficient Person-2, **D**; for G6PD-deficient Person-3. Lane M; marker (2000-1500-1000-750-500-300-150-50 bp), lane 1; exon 2 (240 bp), lane 2; exons 3–4 (320 bp), lane 3; exon 5 (300 bp), lane 4; exons 6–7 (540 bp); lane 5; exon 8 (164 bp), lane 6; exon 9 (253 bp), lane 7; exon 10 (310 bp).

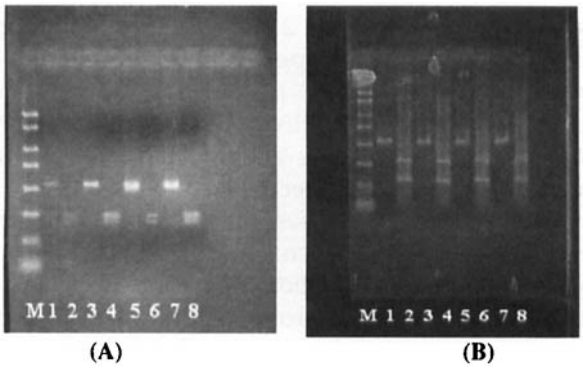

FIGURE 2 Agarose gel electrophoresis show products of exons 6–7 and exons 11–13 undigested and digested with EcoRI (**A**) and NIaIII (**B**), respectively: Lane M; marker (2000-1500-1000-750-500-300-150-50 bp), lane 1, 3, 5, 7; undigested PCR product for healthy one, Person-1, Person-2, Person-3, respectively, lane 2, 4, 6, 8; digested PCR product for healthy one, Person-1, Person-2, Person-3, respectively.

lower compared with that found in the malaria-endemic Mediterranean region of Turkey.[20,33] Biochemical studies done for G6PD deficiency don't provide sufficient data about mutation on the G6PD gene. For this reason, it is necessary to determine abnormality on the gene to understand the etymology of G6PD deficiency. Turkey is a Mediterranean country so it is not suprising that G6PD deficiency has been found in its population. Further studies will have to be carried out to sequence the exons showing abnormal mobility.

Biochemical analysis has revealed great diversity in the properties of the G6PD enzyme in deficient subjects as identified in different laboratories.[14] The kinetic properties of G6PD variants determined world-wide have been found different from each other. For example;

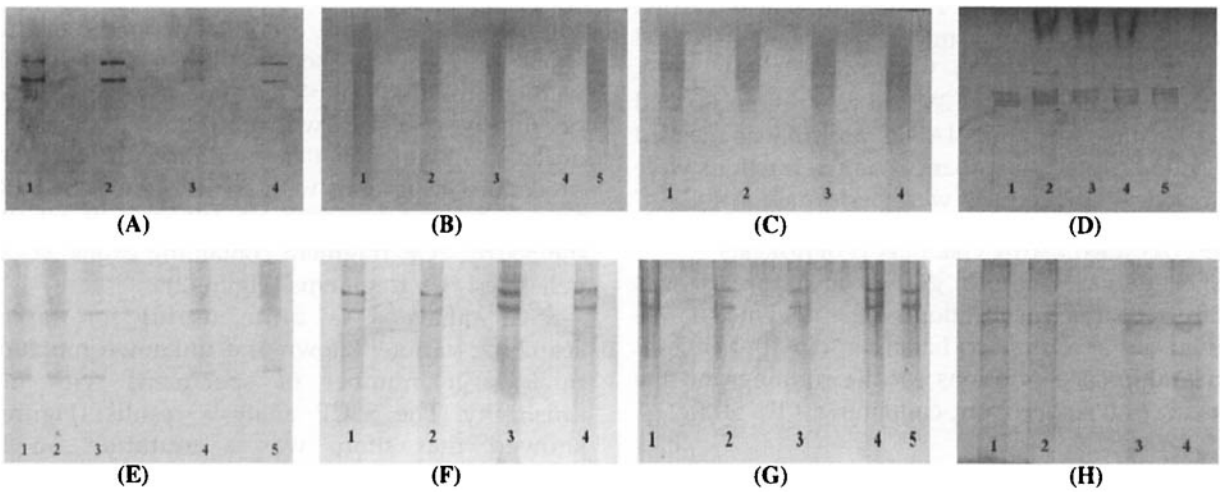

FIGURE 3 SSCP analysis for exon 2 (A), exons 3–4 (B), exon 5 (C), exons 6–7 (D), exon 8 (E), exon 9 (F), exon 10 (G), exons 11–13 (H), **A**; lane 1; healthy one, lane 2; Person-1, lane 3; Person-2, lane 4; Person-3, **B**; lane 1; healthy one, lane 2; Person 1, lane-3; Person 2, lane 4 and 5; Person-3, **C**; lane 1; healthy one, lane 2; Person-1, lane-3; Person 2, lane 4; Person-3, **D**; lane 1 and 5; healthy one, lane 2; Person-1, lane 3; Person-2, lane 4; Person-3, **E**; lane 1 and 2; healthy one, lane 3; Person-1, lane 4; Person-2, lane 5; Person-3, **F**; lane 1; healthy one, lane 2; Person-1, lane 3; Person-2, lane 4; Person-3, **G**; lane 1; Person-1, lane 2; Person-2, lane 3; Person-3, lane 4 and 5; healthy one, **H**; lane 1; healthy one, lane 2; Person-1, lane 3; Person-2, lane 4; Person-3.

TABLE I I_{50} values obtained from regression analysis graphs for G6PD in the presence of different drugs

	remifentanil hydrochloride (mM)	fentanyl citrate ($mM \times 10^{-3}$)	alfentanil hydrochloride (mM)	pethidine hydrochloride (mM)
Normal	1.05	4.8	NI*	NI
Person-1	NI	NI	NI	NI
Person-2	NI	NI	NI	NI
Person-3	1.45	6.1	NI	NI

*NI = no inhibition.

sulfamethoxazole has been shown to produce shortening of erythrocyte life span[34] in Asian subjects with G6PD deficiency, but in patients with G6PD A$^-$ significant haemolysis did not appear when this drug was used.[35]

G6PD deficiency is the cause of neonatal haemolysis and jaundice.[1] Although drug-induced haemolysis has attracted the most attention, it is probable that haemolysis induced by infection may be more common cause of clinical haemolysis. Many reports attest to the importance of infection in causing hemolytic anemia[36,37] and it is difficult to be certain in some cases whether there is relationship between ingestion of a drug and haemolysis.[5] Some studies have demonstrated a protective effect of G6PD-deficient genotypes;[38,39] 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]

We have found interesting results from the drug studies carried out on the enzyme from persons with G6PD-deficiency and normal (Table I). I_{50} values for the enzyme belonging to a healthy person were determined as 1.05 mM for remifentanil hydrochloride and 4.8×10^{-3} mM for fentanyl citrate (Figures 4 and 5). The corresponding I_{50} values for remifentanil hydrochloride and fentanyl citrate for Person 3 were 1.45 mM, 6.1×10^{-3} mM, respectively. Alfentanil hydrochloride and pethidine hydrochloride were not inhibitors effect of the enzyme from healthy and G6PD-deficient persons.

It is clear that the I_{50} values of the enzyme in deficient subjects are slightly higher than those for the normal person. Remifentanil hydrochloride, fentanyl citrate did not effect the G6PD enzyme of persons 1 and 2. Those results are advantageous for G6PD-deficient persons. We conclude that G6PD-deficient persons may show some persistence against the inhibition effect of remifentanil hydrochloride and fentanyl citrate, since the enzymes for G6PD-deficient persons have slightly higher I_{50} values in comparison to those of a healthy person. Moreover, the enzymes for person 1 and 2 were unaffected by remifentanil hydrochloride and fentanyl citrate.

Although there was a mutation on exon 5 of two G6PD-deficient persons (persons 2 and 3), remifentanil hydrochloride and fentanyl citrate did not inhibit the G6PD enzyme for person-2 but only that of person-3. We have shown that streptomycin and metamizol activated the G6PD enzyme for person-2 but inhibited that for Person-3 in our previous studies.[32] According to results of drug studies obtained from these persons, it may be said that they have a different mutation point on exon 5 for persons 2 and 3. Those results are been supported by our previous studies.[32]

Both a healthy person and G6PD-deficient persons did not have their G6PD inhibited by alfentanil hydrochloride and pethidine hydrochloride. Healthy and G6PD-deficient persons may use those drugs confidently from the point of view of the G6PD enzyme.

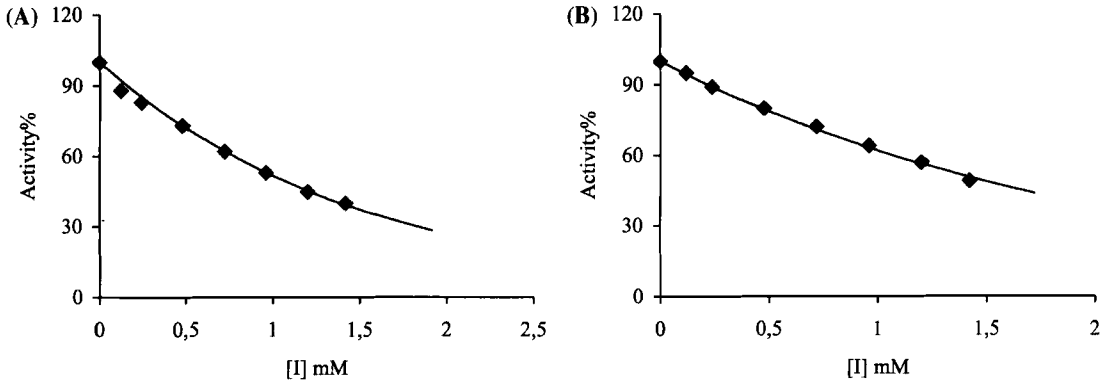

FIGURE 4 Activity%-[I] regression analysis graphs in 1 M Tris-HCl for G6PD with remifentanil for normal (**A**) and Person-3 (**B**).

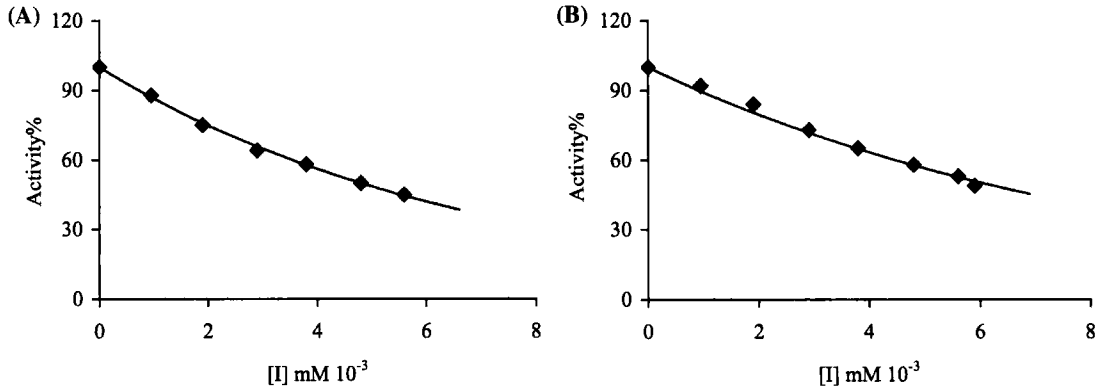

FIGURE 5 Activity%-[I] regression analysis graphs in 1 M Tris-HCl for G6PD with fentanyl citrate for normal (**A**) and Person-3 (**B**).

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