

ORIGINAL ARTICLE

Effects of some drugs on human erythrocyte 6-phosphogluconate dehydrogenase: an in vitro study

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Abstract

The inhibitory effects of some drugs on 6-phosphogluconate dehydrogenase from human erythrocytes have been investigated. For this purpose, initially, erythrocyte 6-phosphogluconate dehydrogenase was purified 3364 times in a yield of 58% by using ammonium sulfate precipitation and 2',5'-ADP Sepharose 4B affinity gel. A temperature of +4°C was maintained during the purification process. Enzyme activity was determined with the Beutler method by using a spectrophotometer at 340 nm. This method was utilized for all kinetic studies. Many commonly used drugs were investigated in this study. Some drugs (ketotifen (K_i : 8.3 ± 1.7 μ M), dacarbazine (K_i : 10.1 ± 0.7 μ M), meloxicam (K_i : 50.9 ± 13.2 μ M), furosemide (K_i : 127 ± 37.8 μ M), methotrexate (K_i : 136.7 ± 25.3 μ M), metochloropramide hydrochloride (K_i : 2.1113 ± 0.6979 mM), ritodrine hydrochloride (K_i : 6.0353 ± 1.2783 mM), and **gadopentetic acid** (K_i : 73.4 ± 21.9 mM)) inhibited enzyme activity *in vitro*. K_i constants for the enzyme were found by means of Lineweaver–Burk graphs. All drugs showed non-competitive inhibition. In addition, IC_{50} values of the drugs were determined by plotting activity percent vs [I].

Keywords: 6-phosphogluconate dehydrogenase; erythrocyte; drug inhibition

Introduction

6-Phosphogluconate dehydrogenase (E.C.1.1.1.44; 6PGD), the third enzyme in the pentose phosphate metabolic pathway, catalyzes the conversion of 6-PGA (6-phosphogluconate) and NADP⁺ (nicotinamide adenine dinucleotide phosphate) to D-ribulose-5-phosphate and NADPH, which protects the cell against oxidizing agents by producing reduced glutathione (GSH)^{1,2}. NADPH is also a coenzyme participating in the synthesis of a number of biomolecules such as fatty acids, steroids, and some amino acids^{3,4}. In the case of lack of NADPH, the concentration of GSH in living cells declines, resulting in cell death. GSH is indirectly produced by 6PGD; therefore, 6PGD can be defined as an indirect antioxidant enzyme^{4,5}. Many agents are known to activate or inhibit enzymes *in vitro* and *in vivo*^{6–9}, so affecting metabolic pathways. Inhibition of 6PGD leads to decreased NADPH and GSH, which will cause cell damage especially in older erythrocytes, resulting in some problems in living cells^{6–8}.

The effects of many commonly used drugs (netilmicin sulfate, cefepime, amikacin, isepamycin, chloramphenicol, ceftazidim, teicoplanin, ampicillin, ofloxacin, levofloxacin,

cefotaxime, penicillin G, gentamicin sulfate, ciprofloxacin, ceftazidim, decefin, streptomycin, combisid, meronem, lar-noxicam, metronidazole, imipenem, ornidazole, vancomycin, clindamycin, and amoxicillin) on 6PGD enzyme activities have been investigated^{10,11}. However, no reports could be found in the literature on the effects of ketotifen, dacarbazine, meloxicam, furosemide, methotrexate, metochloropramide hydrochloride, ritodrine hydrochloride, and **gadopentetic acid** on human erythrocyte 6PGD, although vitamin C has been reported to stimulate 6PGD *in vitro* and *in vivo*¹⁰.

This study was aimed at the purification of human erythrocyte 6PGD, and determination of the effects of some commonly used drugs on human red blood cell 6PGD activity.

Materials and methods

Materials

6-PGA, NADP⁺, Tris-HCl, and the other chemicals were from Sigma-Aldrich Co. (Germany), and the drugs were purchased from Hoechst Marion Roussel (Turkey).

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(Received 23 May 2009; revised 23 July 2009; accepted 29 July 2009)

ISSN 1475-6366 print/ISSN 1475-6374 online © 2010 Informa UK Ltd
DOI: 10.3109/14756360903257900

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Activity determination

6PGD activity was measured spectrophotometrically at 25°C as described by Beutler¹². Briefly, the enzyme sample was added to a 1 mL (final volume) incubation mixture containing 0.1 M Tris-HCl + 0.5 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 10 mM MgCl₂, 0.2 mM NADP⁺, and 0.6 mM 6-PGA, and the increase in absorption at 340 nm due to the reduction of NADP⁺ was measured at 25°C. One enzyme unit represents the reduction of 1 μmol of NADP⁺ per min at 25°C, pH 8.0.

Preparation of hemolysate

Fresh human blood samples were collected in tubes containing EDTA, then centrifuged (15 min, 2500 × g), and the plasma and buffy coat (leukocytes) removed. The packed red cells were washed three times with KCl (0.16 M), hemolyzed with five times the volume of ice-cold water, and then centrifuged (4°C, 10,000 × g, for 30 min), to remove the ghosts and intact cells¹¹.

Ammonium sulfate precipitation

The hemolysate was subjected to precipitation with ammonium sulfate (gradient 35–65%). Enzyme activity was determined both in the supernatant and in the precipitate for each respective precipitation. The precipitated enzyme was dissolved in phosphate buffer (50 mM, pH 7.0), and gave a clear solution which contained partially purified enzyme¹¹.

Purification of 6PGD

The ammonium sulfate fraction (35–65%) of the hemolysate obtained as above was loaded onto a 2',5'-ADP Sepharose 4B affinity column and the flow rate was adjusted to 20 mL/h. The column was then sequentially washed with a 25 mL buffer of 0.1 M K-acetate and 0.1 M K-phosphate

(pH 6.0) and 25 mL of a buffer of 0.1 M K-acetate and 0.1 M K-phosphate (pH 7.85). Washing continued until an absorbance of 0.05 at 280 nm was obtained. Elution of the enzyme was carried out with a mixture containing 80 mM K-phosphate, 80 mM KCl, 5 mM NADP⁺, and 10 mM EDTA (pH 7.85). Enzyme activity was measured in all fractions, and the activity-containing fractions were pooled, then dialyzed in 50 mM K-acetate + 50 mM K-phosphate buffer (pH 7.0) for 2 h with two changes of buffer. All procedures were performed at 4°C¹¹.

Protein determination

The protein content in all samples was quantified spectrophotometrically at 595 nm according to Bradford's method¹³, using bovine serum albumin as standard.

SDS polyacrylamide gel electrophoresis

Enzyme purity was examined using Laemmli's procedure¹⁴ with 3% and 8% acrylamide concentrations for running and stacking gel, respectively. Bovine albumin (66,000), chicken ovalbumin (45,000), and bovine carbonic anhydrase (29,000) were used as standards (Sigma: MW-SDS-200) (see Figure 1).

In vitro effect of drugs

In order to determine the effects of some drugs on human 6PGD, concentrations of ketotifen (0.0018–0.0282 mM), dacarbazine (0.0049–0.054 mM), meloxicam (0.02–0.285 mM), furosemide (0.03–0.6 mM), methotrexate (0.036–0.55 mM), metochloropramide hydrochloride (0.83–8.335 mM), ritodrine hydrochloride (1.54–15.4 mM), and **gadopentetic acid (24.99–249.935 mM)** were added to the reaction mixture and the enzyme activity was measured. An experiment in the absence of drug was used as control (100% activity). The IC₅₀ values were obtained from activity (%) vs. drug concentration plots (for example Figure 2). In order to determine the K_i values, the substrate (6-PGA) concentrations were 0.006, 0.012, 0.024, 0.036, 0.048 mM for ketotifen, dacarbazine, meloxicam, furosemide, methotrexate, and 0.003, 0.006, 0.012, 0.024,

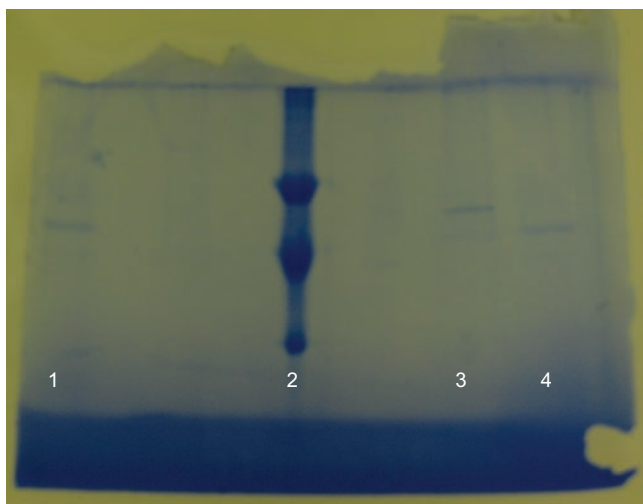


Figure 1. SDS-PAGE bands of 6PGD. Lane 1, human erythrocyte glutathione reductase; lane 2, standards: bovine erythrocyte carbonic anhydrase-I (29 kDa), chicken ovalbumin (45,000), bovine albumin (66,000); lane 3, human erythrocyte glucose 6-phosphate dehydrogenase; lane 4, human erythrocyte 6PGD.

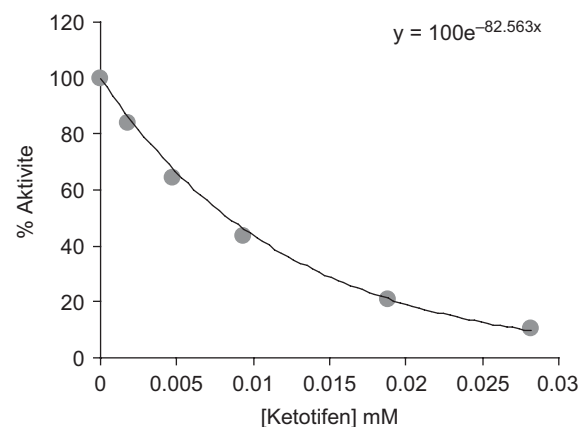


Figure 2. Activity % vs. [Ketotifen] regression analysis graph for human erythrocyte 6PGD in the presence of five different ketotifen concentrations.

0.036 mM for metochloropramide hydrochloride, ritodrine hydrochloride, **gadopentetic acid**. Inhibitor (drug) solutions were added to the reaction mixture at three different fixed concentrations. Lineweaver-Burk graphs¹⁵ were drawn using $1/V$ vs. $1/[S]$ values, and the K_i values were calculated from these graphs (see Figure 3). Regression analysis graphs were drawn using percentage inhibition values vs. drug concentration using a statistical package (SPSS for Windows; version 10.0) on a computer (Student *t*-test; $n=3$).

Results

Purification of the enzyme led to a specific activity of 13.12 EU/mg protein, a yield of 58%, and 3364-fold purification (Table 1). Sodium dodecyl sulfate (SDS)

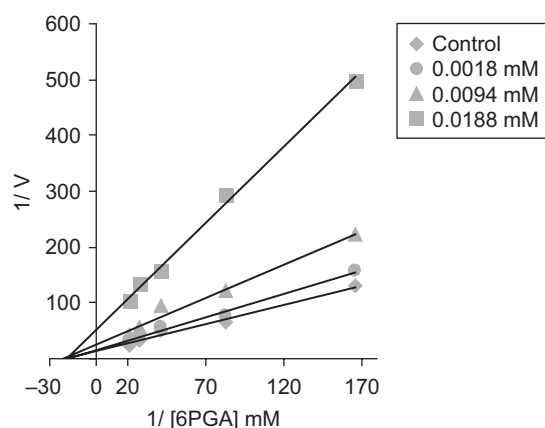


Figure 3. Lineweaver-Burk graph for five different substrate (6-PGA) concentrations and three different ketotifen concentrations for determination of K_i .

Table 1. Purification scheme of 6-phosphogluconate dehydrogenase from human erythrocytes.

Purification step	Total volume (mL)	Activity (U/mL)	Total activity (U)	Protein (mg/mL)	Specific activity (U/mg)	Yield (%)	Purification factor
Hemolysate	60	0.120	7.2	30.88	0.0039	100	1
Ammonium sulfate precipitation (35-65%)	20	0.305	6.1	32.12	0.0090	84.72	2.3
2',5'-ADP Sepharose 4B affinity chromatography	8	0.525	4.2	0.04	13.1200	58.33	3364

Table 2. K_i values obtained from Lineweaver-Burk graphs for 6PGD in the presence of three fixed inhibitors and five substrate concentrations for different drugs.

Drug	IC_{50} (mM)	K_i	Inhibition type
Ketotifen	0.0084	$8.3 \pm 1.7 \mu\text{M}$	Non-competitive
Dacarbazine	0.0120	$10.1 \pm 0.7 \mu\text{M}$	Non-competitive
Meloxicam	0.0550	$50.9 \pm 13.2 \mu\text{M}$	Non-competitive
Furosemide	0.1250	$127 \pm 37.8 \mu\text{M}$	Non-competitive
Methotrexate	0.1420	$136.7 \pm 25.3 \mu\text{M}$	Non-competitive
Metochloropramide hydrochloride	3.2700	$2.1113 \pm 0.6979 \text{ mM}$	Non-competitive
Ritodrine hydrochloride	3.6600	$6.0353 \pm 1.2783 \text{ mM}$	Non-competitive
Gadopentetic acid	209.10	$73.4 \pm 21.9 \text{ mM}$	Non-competitive

polyacrylamide gel electrophoresis (PAGE) was performed after purification of the enzyme, and the electrophoretic pattern is shown in Figure 1. IC_{50} values of ketotifen, dacarbazine, meloxicam, furosemide, methotrexate, metochloropramide hydrochloride, ritodrine hydrochloride, and **gadopentetic acid** were 0.0084, 0.0120, 0.0550, 0.1250, 0.1420, 3.2700, 3.6600, and 209.10 mM, and the K_i constants were $8.3 \pm 1.7 \mu\text{M}$ (non-competitive), $10.1 \pm 0.7 \mu\text{M}$ (non-competitive), $50.9 \pm 13.2 \mu\text{M}$ (non-competitive), $127 \pm 37.8 \mu\text{M}$ (non-competitive), $136.7 \pm 25.3 \mu\text{M}$ (non-competitive), $2.1113 \pm 0.6979 \text{ mM}$ (non-competitive), $6.0353 \pm 1.2783 \text{ mM}$ (non-competitive), and $73.4 \pm 21.9 \text{ mM}$ (non-competitive), respectively (Table 2). Representative graphs are shown for ketotifen (Figures 2 and 3).

Discussion

It is known that many drugs have adverse effects on the body when used for therapeutic or other purposes¹⁶ which may be dramatic and systematic¹⁷. A good example is that of pamaquine used for malaria treatment, which caused severe adverse effects in patients within a few days, resulting in black urination, hyperbilirubinemia, a dramatic decrease in blood Hb levels, and finally death, which occurred in cases of severe G6PD (glucose 6-phosphate dehydrogenase) deficiency¹⁸. Therefore, investigation of the effects of some drugs on the enzyme activity of human erythrocyte 6PGD is very important.

Here, human erythrocyte 6PGD enzyme was purified with a 58% yield and 3364-fold purification in 5-6 hours using 2',5'-ADP Sepharose 4B affinity gel chromatography (Table 1). SDS-PAGE showed that high purity for the enzyme had been obtained (Figure 1). The inhibitory effects of some drugs on 6PGD activity in humans have

been reported in several investigations⁹⁻¹¹. For example, it has been reported that netilmicin sulfate, cefepime, amikacin, isepamycin, chloramphenicol, ceftazidim, teicoplanin, ampicillin, ofloxacin, levofloxacin, cefotaxime, penicillin G, gentamicin sulfate, ciprofloxacin, ceftozin, decefim, streptomycin, combisid, meronem, larnoxicam, metronidazole, imipenem, ornidazole, vancomycin, clindamycin, and amoxicillin inhibit human erythrocyte 6PGD^{10,11}, and that ampicillin and amikacin inhibit rat red-cell 6-PGA⁹. However, to the best of our knowledge, the inhibitory effects of the drugs examined here on human erythrocyte 6PGD have not been studied. In order to show the inhibitory effects, while the most suitable parameter is the K_i constant, some researchers use the IC_{50} value^{9,10}. Therefore, in this study, both the K_i and IC_{50} parameters of these drugs for 6PGD were determined. IC_{50} values of ketotifen, dacarbazine, meloxicam, furosemide, methotrexate, metochloropramide hydrochloride, ritodrine hydrochloride, and **gadopentetic acid** were 0.0084, 0.0120, 0.0550, 0.1250, 0.1420, 3.2700, 3.6600, and 209.10 mM, respectively. K_i constants of ketotifen, dacarbazine, meloxicam, furosemide, methotrexate, metochloropramide hydrochloride, ritodrine hydrochloride, and **gadopentetic acid** were $8.3 \pm 1.7 \mu\text{M}$ (non-competitive), $10.1 \pm 0.7 \mu\text{M}$ (non-competitive), $50.9 \pm 13.2 \mu\text{M}$ (non-competitive), $127 \pm 37.8 \mu\text{M}$ (non-competitive), $2.1113 \pm 0.6979 \text{ mM}$ (non-competitive), $6.0353 \pm 1.2783 \text{ mM}$ (non-competitive), and $73.4 \pm 21.9 \text{ mM}$ (non-competitive), respectively (Table 2). In this study, the drugs inhibited 6PGD activity, compared with the control group. The drugs can cause non-competitive inhibition by binding to other sites affecting the three-dimensional structure of the enzyme²⁰.

The K_i constants show that ketotifen had the highest inhibitory effect, followed by dacarbazine, meloxicam, furosemide, methotrexate, metochloropramide hydrochloride, ritodrine hydrochloride, and **gadopentetic acid**. The IC_{50} values showed the same trend. From this investigation, by using the obtained K_i and IC_{50} values, undesirable side effects of these drugs on 6PGD activity and body metabolism and fatty acid synthesis can be reduced. According to these data, if it is required to administer ketotifen, dacarbazine, and meloxicam to patients, their dosage should be very well controlled to decrease hemolytic and other side effects due to possible inhibition of 6PGD.

Declaration of interest

This research was financed by grants from of the Scientific and Technological Research Council of Turkey (TÜBİTAK) (Project No: 108T213).

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