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Investigation of the effects of some sulfonamide derivatives on the activities of glucose-6-phosphate dehydrogenase, 6-phospho gluconate dehydrogenase and glutathione reductase from human erythrocytes

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Abstract

In this study, the *in vitro* effects of some sulfonamide derivatives, which are carbonic anhydrase inhibitors, on the enzymes activities of glucose-6-phosphate dehydrogenase, 6-phospho gluconate dehydrogenase and glutathione reductase were investigated. For this purpose, these three enzymes were purified from human erythrocytes. Purification procedure composed of four steps; preparation of the hemolysate, ammonium sulfate precipitation, 2′,5′-ADP Sepharose 4B affinity chromatography, and gel filtration chromatography on Sephadex G-200. 5-(3α-Hydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**1**), 5-(3α,12α-Dihydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**2**), 5-(3α,7α,12α-Trihydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**3**), 5-(3α,Acetoxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**4**), 5-(3α,7α,12α-Triacetoxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**5**), 5-(3,7,12-Trioxo-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**6**), acetazolamide, and dorzolamide were tested in this experiment. Compounds **3**, **5**, and dorzolamide showed inhibitory effects on the activity of 6-phosphogluconate dehydrogenase, and I_{50} values and K_i constants were calculated as 0.0601 mM, 0.00253 mM, and 1.41 mM and 0.0878 ± 0.0274 mM, 0.0042 ± 0.0009 mM, and 3.1446 ± 0.2081 mM, respectively. Glutathione reductase was also inhibited by **1** and **2**. I_{50} values and K_i constants were 0.0471 mM and 0.0723 ± 0.0388 mM for **1** and 0.0045 mM and 0.0061 ± 0.0014 mM, for **2**. If these sulfonamide derivatives are proposed as drugs, some of which are being used in glaucoma treatment such as acetazolamide and dorzolamide, these results should be taken into consideration concerning via these enzymes.

Keywords: *Glucose-6-phosphate dehydrogenase, 6-Phosphogluconate dehydrogenase, Glutathione reductase, Sulfonamide derivatives, Human erythrocytes*

Introduction

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ oxidoreductase EC 1.1.1.49; G6PD) and 6-phospho gluconate dehydrogenase (6-phospho-D-gluconate-$NADP^+$ oxidoreductase, decarboxylating, EC 1.1.1.44; 6PGD) are the crucial enzymes of the pentose phosphate metabolic pathway, which produce NADPH in the metabolism. NADPH is a coenzyme participating in the synthesis of a number of biomolecules such as fatty acids, steroids, and some amino acids [1]. Glutathione reductase (Glutathione: $NADP^+$ oxidoreductase, E.C.1.8.1.7; GR) is also an ubiquitous NADPH-dependent enzyme which catalyzes the reduction of oxidized glutathione (GSSG) to its reduced form (GSH) with NADPH as the reducing cofactor. GSH plays an important role in many cellular functions, including protection against oxidative stress [1,2]. GSH is also a reaction partner for the detoxification of endobiotics and xenobiotics, and a storage and transport form of cysteine [2]. Its function is important for maintaining the thiol redox potential in cells keeping sulfhydryl groups of intracellular proteins in the reduced form

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and in the production of deoxyribonucleotides [2]. Increasing the intracellular concentration of GSSG by oxidative stress is positively correlated with an increase in protein-GSH mixed disulfides [3]. Formation of mixed disulfides of protein thiols and GSH may affect protein structure and serve a regulatory function. For example, S-glutathiolation of Cys residues by this process has been shown to modulate the phosphatase activity of carbonic anhydrase (CA) III [4].

CA inhibitors, which reduce aqueous production with a corresponding decrease in intraocular pressure (IOP), are ocular hypotensive agents for the treatment of glaucoma [5]. Acetazolamide was the first of this potent CA-II to be developped pharmaceutically. At that time it was suggested that reducing aqueous humor secretion might provide an effective means of lowering IOP to treat glaucoma [6]. Thereafter some systemic sulfonamide drugs have been used clinically mainly as antiglaucoma agents, for a long time [7]. The orally administered drugs affected various CA isozymes present in other tissues, and leaded to an entire range of side effects. To decrease systemic side effects of oral carbonic anhydrase inhibitors, dorzolamide hydrochloride and brinzolamide ophthalmic suspension have been used as the topical carbonic anhydrase inhibitors approved for the treatment of glaucoma [5, 8].

Each drug is an effective antiglaucoma agent, but these drugs tend to pose tolerability problems in many patients because of local side effects [9]. Thus, both the search for novel types of topically acting antiglaucoma sulfonamides and the search for adverse effects for these drugs have continued by some time [10].

A greater understanding of the safety and side effects on some enzymes associated with the use of these antiglaucoma medications can significantly affect treatment-oriented decisions, particularly in the chronic clinical management of this disease. The objective of this paper is to review the safety and side effects on the activity of G6PD, 6PGD, and GR associated with the use of the topical agents most commonly used which may be utilized for new compounds in the treatment of glaucoma (Figure 1).

Materials and methods

Materials

2′,5′-ADP Sepharose 4B was obtained from Pharmacia. Sephadex G-200, $NADP^+$, NADPH, glucose-6-phosphate, 6-phosphogluconate, GSSG, protein assay reagent, and chemicals for electrophoresis were obtained from Sigma Chem. Co. All other used chemicals were analytical grade and obtained from either Sigma or Merck. Medical drugs were from the Hospital of Atatürk University, other sulfonamide derivatives were synthesized in our Laboratory of Organic Chemistry.

Preparation of the hemolysate

Fresh human blood was obtained from the Hospital of Atatürk University and was centrifuged at 2500 rpm for 15 min to separate the plasma and buffy coat. The plasma was isolated and washed three times with 0.9% NaCl solution. The erythrocytes were hemolysed with four volumes of ice-cold water, and then was separated the ghost and intact cells by centrifugation at 20.000 rpm for 30 min at 4°C. The hemolysate was used for purification of the enzyme [11].

Purification of the enzymes

G6PD and GR were purified in a single chromatographic step as in our previously published procedure [11]. Purification of 6PGD was also performed according to Akyüz et al's procedure [12]. Ammonium sulphate fraction (30–70%) of the hemolisate was dissolved in and dialyzed against the buffer, 50 mM K-phosphate buffer including 1 mM EDTA, pH 7.5, (Buffer A). Dialysate was applied to a column of 2′,5′-ADP Sepharose 4B (1 × 10 cm) and the column was washed with buffer A. The G6PD bounded on the gel was eluted with of 80 mM K-phosphate + 80 mM KCl + 0.4 mM $NADP^+$ + 1 mM EDTA, pH 7.85. After the activity of G6PD had ended, the GR was eluted with the buffer of 50 mM K-phosphate + 1 mM EDTA, pH 7.5, including 0.4 mM NADPH and 0.4 mM GSH. For the purification of 6PGD, the other dialyzed sample was loaded on the same washed affinity column. The column was washed with 0.1 M K-acetate and 0.1 M K-phosphate, pH 6.0, and 0.1 M K-acetate and 0.1 M K-phosphate, pH 7.85. Elution of the 6PGD bounded on the gel was performed with 80 mM K-phosphate 80 mM KCl + 5 mM $NADP^+$ 10 mM EDTA (pH 7.85). For each enzyme, active fractions were collected. The enzyme samples one by one loaded onto a column of Sephadex G-200 (1.6 × 50 cm) equilibrated with 50 mM Tris-HCl, 50 mM KCl, pH 7.5. After then, the enzymes were eluted by the same buffer and active fractions were collected in a separate tube for the use. During all the procedure, the temperature was kept at 4°C.

Confirmation of the enzyme purity

In order to confirm the enzyme purity, SDS-PAGE was performed according to the method used by Laemmli [13], with bovine carbonic anhydtase (29 kDa), chicken ovalbumin (45 kDa), bovine albumine (66 kDa), rabbit phosphorylase B (97.4 kDa), *E.coli* β-galactosidase (116 kDa), and rabbit myosin (205 kDa) used as standard proteins (Sigma: MW-SDS-200).

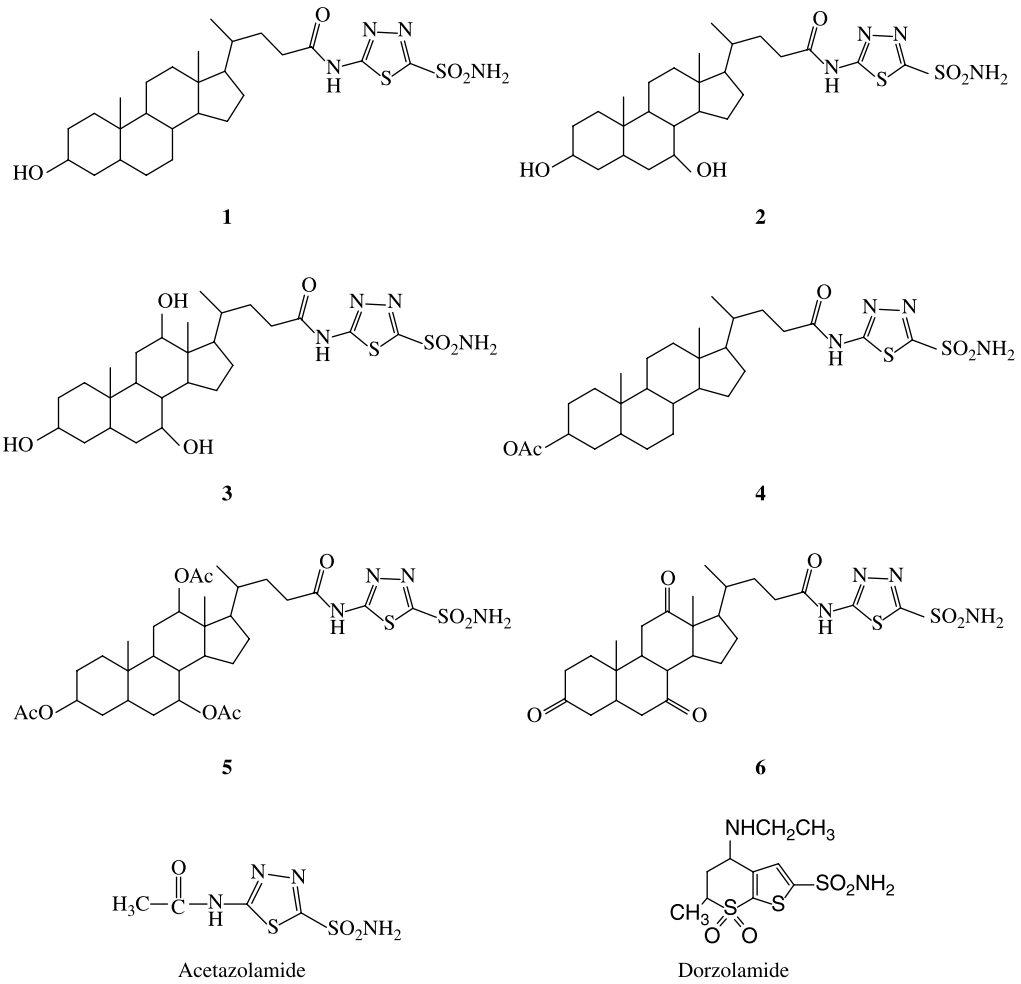

Figure 1. Structures of some sulfonamide derivatives: 5-(3α-Hydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**1**), 5-(3α,12α-Dihydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**2**), 5-(3α,7α,12α-Trihydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**3**), 5-(3α,Acetoxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**4**), 5-(3α,7α,12α-Triacetoxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**5**), 5-(3,7,12-Trioxo-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**6**), N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-acetazolamide (acetazolamide), and (4S)-trans-4-ethylammonio-6-methyl-5,6-dihydro-4H-thieno[2,3-b]thiopyran-2-sulfonamide 7,7-dioxide (dorzolamide).

Measurement of enzyme activities

G6PD and 6PGD activities were measured by Beutler's method [14], and GR activity was determined to the Carlberg and Mannervik's methods [15] with a Shimadzu Spectrophotometer UV-(1208). One unit of enzyme activity (EU) was defined as the reduction of 1 μmol $NADP^+$ for G6PD and 6PGD, and the oxidation of 1 μmol NADPH for GR per min under the assay conditions (extinction coefficient at 340 nm = 6.2 mM^{-1} cm^{-1} for NADPH).

Determination of the in vitro effects of the sulfonamide derivatives on enzymes activity

Sulfonamide derivatives, some of which had been synthesized previously by us as CA inhibitors in our laboratory [16], were tested to determine their effects on human erythrocytes G6PD, 6PGD, and GR activities. G6PD and 6PGD activities were performed in the cuvette at concentrations of **1** (0.0093–0.0462 mM), **2** (0.0009–0.0045 mM), **3** (0.0085–0.0655 mM), **4** (0.0095–0.0475 mM), **5** (0.0007–0.0036 mM), **6** (0.0088–0.044 mM), and **7** and **8** (0.22–1.1 mM). GR activities were also measured in the cuvette at concentrations of **1** (0.0056–0.0333 mM), **2** (0.0005–0.0032 mM), **3** (0.005–0.0816 mM), **4** (0.0038–0.0228 mM), **5** (0.0003–0.0014 mM), **6** (0.0053–0.0211 mM), and **7** and **8** (0.132–0.792 mM). Three measurements were performed and mean values were used for each data point. The enzyme activities in the absence of drugs or chemicals were taken as 100%. For each drug or chemical, an Activity%-[Drug] graph was drawn and drug concentrations producing 50% inhibition (I_{50}) were calculated for the inhibitors.

Table I In order to determine K_i constants, three fixed inhibitor concentrations (0.05, 0.06, and 0.07 mM for **3**, 0.002, 0.0025, and 0.003 mM for **5**, 1.3, 1.45, and 1.6 mM for dorzolamide, 0.04, 0.05,

Table I. The effects of some sulfonamide derivatives on the enzyme activity of G6PD, 6PGD, and GR (I: inhibition; WI: weak inhibition; A: activation; WA: weak activation; IN: ineffective).

Sulfonamide derivatives	G6PD	6PGD	GR
5-(3α-Hydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**1**)	IN	A	I
5-(3α,12α-Dihydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**2**)	WA	IN	I
5-(3α,7α,12α-Trihydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**3**)	WI	I	A
5-(3α,Acetoxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**4**)	A	A	IN
5-(3α,7α,12α-Triacetoxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**5**)	WI	I	WI
5-(3,7,12-Trioxo-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**6**)	IN	WI	A
N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-acetazolamide (acetazolamide)	A	IN	IN
(4S)-trans-4-ethylammonio-6-methyl-5,6-dihydro-4H-thieno[2,3-b]thiopyran-2-sulfonamide 7,7-dioxide (dorzolamide)	IN	I	WA

and 0.06 mM for **1**, and 0.003, 0.004, and 0.005 mM for **2**) were tested with five different substrate concentrations. Concentrations of 6PGA, substrate of 6PGD, were 0.0186, 0.0375, 0.075, 0.15, and 0.3 mM. Concentrations of GSSG, substrate of GR, were also 0.0625, 0.125, 0.25, 0.5, and 1 mM. Lineweaver-Burk graphs were drawn for each inhibitor by using 1/V and 1/[S] values [17]. K_i constants and inhibition types were estimated from these graphs and the values were given as $\bar{X} \pm SD$ (Tables II and III).

Results

The enzymes G6PD, 6PGD, and GR were purified from human erythrocytes 8500, 1220, and 4845 times with specific activities 25.00, 18.00, and 47.00 EU/mg protein, respectively. Purity of the enzymes was controlled by means of SDS-PAGE analysis as in our previously published papers [11,12]. Pure G6PD was inhibited weakly by **3** and **5**; however, the enzyme also activated by **4** and acetazolamide. Compounds **1**, **2**, **6**, and dorzolamide did not show important inhibitory or activatory effects on G6PD activity. 6PGD was inhibited effectively by **3**, **5**, and dorzolamide, and I_{50} values were calculated as 0.0601 mM, 0.00253 mM, and 1.41 mM; K_i constants were determined as 0.0878 ± 0.0274 mM, 0.0042 ± 0.0009 mM, and 3.1446 ± 0.2081 mM respectively (Table II). Compounds **1** and **4** were activators but **2** and acetazolamide did not exhibit any effects on 6PGD activity. As for GR, **1** and **2** were potent inhibitors for the enzyme, and I_{50} values were calculated as 0.0471 mM and 0.0045 mM; K_i constants were 0.0723 ± 0.0388 mM and 0.0061 ± 0.0014 mM, respectively (Table III). Inhibition type of all inhibitors was competitive compounds **5** and **7** were weak inhibitors; but **3**, **6**, and dorzolamide were found to be activators of the enzyme. The results obtained are shown in Table I.

Discussion

Many chemicals at relatively low dosages affect the metabolism of the body by altering normal enzyme activity, particularly inhibition of specific enzymes [18]. It has been reported that some drugs used for the treatment of the bacterial infectious inhibited the enzyme activities of G6PD, 6PGD, and GR. For example, ampicillin inhibits G6PD from human red cells [19] and sheep liver [20]. Amikacin also inhibits human red cell G6PD [19]. Rat erythrocyte 6PGD has been inhibited by amikacin, ampicillin, and netilmicin [21]. Ofloxacin and cefepime have also inhibited chicken liver 6PGD [22]. Ofloxacin, levofloxacin, cefepime, and cefazolin have inhibited GR from sheep liver [23] and chicken liver [24].

Table II. I_{50} and K_i values of some sulfonamide derivatives for human erythrocytes 6PGD.

Inhibitors	I_{50} values (mM)	K_i constants (mM)	Mean K_i constants (mM)	Inhibition types
5-(3α,7α,12α-Trihydroxy-5-β-cholanamido) -1,3,4-thiadiazole-2-sulfonamide (**3**)	0.0601	0.1121 0.0933 0.0581	0.0878 ± 0.0274	Competitive
5-(3α,7α,12α-Triacetoxy-5-β-cholanamido) -1,3,4-thiadiazole-2-sulfonamide (**5**)	0.00253	0.00488 0.00450 0.00322	0.0042 ± 0.0009	Competitive
(4S)-trans-4-ethylammonio-6-methyl-5,6-dihydro -4H-thieno[2,3-b]thiopyran-2-sulfonamide 7,7-dioxide (dorzolamide)	1.41	3.364 3.120 2.950	3.1446 ± 0.2081	Competitive

Table III. I_{50} values of some sulfonamide derivatives for human erythrocytes GR.

Inhibitors	I_{50} values (mM)	K_i constants (mM)	Mean K_i constants (mM)	Inhibition types
5-(3α-Hydroxy-5-β-cholanamido) -1,3,4-thiadiazole-2-sulfonamide (**1**)	0.0471	0.0389	0.0723 ± 0.0388	Competitive
		0.0632		
		0.1152		
5-(3α,12α-Dihydroxy-5-β- cholanamido) -1,3,4-thiadiazole-2-sulfonamide (**2**)	0.0045	0.0046	0.0061 ± 0.0014	Competitive
		0.0063		
		0.0074		

Some sulfonamide derivatives perform several biological activities and are effective chemotherapeutic agents used in the treatment of bacterial infectious by means of their 1, 3, 4-thiadiazole rings [25]. Some of them, such as dorzolamide, acetazolamide, brinzolamide, **1**, **2**, **3**, **4**, **5**, and **6** are also effective inhibitors of CA isozymes [8,26]. They have a diuretic effect by inhibiting CA isozymes in human kidney tubule cells, and are pharmacological agents used for the treatment of glaucoma because of reducing intraocular pressure [10,27,28]. In addition, some aromatic sulfonamide derivatives have been reported to be very powerful inhibitors of growth for many types of tumor cells [29].

To our knowledge, although some of these sulfonamide derivatives are used as antibacterial, antiglaucomal, and antitumoral agents, no study has yet been encountered about their effects them on the enzyme activity of G6PD, 6PGD, and GR. Therefore, in the present study, investigation of effects of some sulfonamide derivatives on the activity of enzymes from human erythrocytes was undertaken. For the purposes of this study, these enzymes were purified from human erythrocytes by ammonium sulphate precipitation, 2′,5′-ADP Sepharose 4B affinity chromatography, and gel filtration chromatography on Sephadex G-200, respectively. Purification of the enzymes was 8500-, 1220-, and 4845-fold with yields of 50%, 23%, and 30%, respectively. Given the SDS-PAGE analysis, it was seen that the enzymes had high purities.

5-(3α-Hydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide(**1**), 5-(3α,12α-Dihydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**2**), 5-(3α,7α,12α-Trihydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**3**), 5-(3α,Acetoxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**4**), 5-(3α,7α,12α-Triacetoxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**5**), 5-(3,7,12-Trioxo-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**6**), N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-acetazolamide (acetazolamide), and (4S)-trans-4-ethylammonio-6-methyl-5,6-dihydro-4H-thieno[2,3-b]thiopyran-2-sulfonamide 7,7-dioxide (dorzolamide) were chosen for investigation of their effects.

All sulfonamide derivatives used in the experiment were not effective inhibitors for human erythrocyte G6PD. However, (**4**) and acetazolamide were effective activators. Although inhibition or activation of a non-target enzyme by a drug used in therapy is an undesired result, inhibition of these enzymes from human erythrocytes, especially G6PD, are crucial for the persons having haemolytic anemia. Thus, the effectiveness of these sulfonamide derivatives as inhibitors on G6PD may be a desirable result. 6PGD was inhibited by **3**, **5**, and dorzolamide, effectively, and I_{50} values were determined as 0.0601 mM, 0.00253 mM, and 1.41 mM for the compounds, respectively; K_i constants were 0.0878 ± 0.6274 mM for **3**, 0.0042 ± 0.0009 mM for **5**, and 3.1446 ± 0.2081 mM for dorzolamide. GR was also inhibited by **1** and **2**, and I_{50} values were calculated as 0.0471 mM and 0.0045 mM: K_i constants were 0.0723 ± 0.0388 mM and 0.0061 ± 0.0014 mM, respectively.

From the findings of the present study, **3**, **5**, and dorzolamide were inhibitors for 6PGD, but **5** had the greatest potency with its low I_{50} and K_i values. For GR enzyme, both **1** and **2** were also potent inhibitors with very low I_{50} and K_i values.

Because 6PGD and GR are important antioxidant agents in metabolism, inhibition of these enzymes is important in the antioxidant defense system. Thus, if these sulfonamides (**1**, **2**, **3**, **5**, and dorzolamide) are used in therapy, the results obtained from this study oncoming 6PGD and GR enzymes should be taken into consideration.

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