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Inhibition Effects of 5-Amino-1,3,4-thiadiazole Derivatives on Carbonic Anhydrase Enzyme

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In this study, new carbonic anhydrase inhibitors's effects on bovine carbonic anhydrase enzyme, to resembling human carbonic anhydrase-II (hCA-II), as candidates for treatment of glaucoma were investigated. Therefore, carbonic anhydrase enzyme was purificated from bovine erythrocyte cells by the affinity column. The inhibition effects of 5-amino-1,3,4-thiadiazole-2-sülfonamide (**1**), acetazolamide (**2**) and new synthesized amides (**5**, **6**, **7**) on this enzyme have been studied *in vitro*. The IC_{50} concentrations (the concentration of inhibitor producing a 50 % inhibition of bovine erythrocyte carbonic anhydrase activity) against hydratase activity values were 0.179, 0.758, 0.978 µM for bovine erythrocyte carbonic anhydrase. The IC_{50} values against esterase activity values were 0.225, 0.194 and 0.205 µM for this enzyme. The K_i values were observed 0.351, 0.208 and 0.232 µM for this enzyme. The comparison of new synthesized amides (**5**, **6**, **7**) to **1** and **2** indicated that the new synthesized compounds (**5**, **6**, **7**) inhibit carbonic anhydrase activity more effectively than parent compounds.

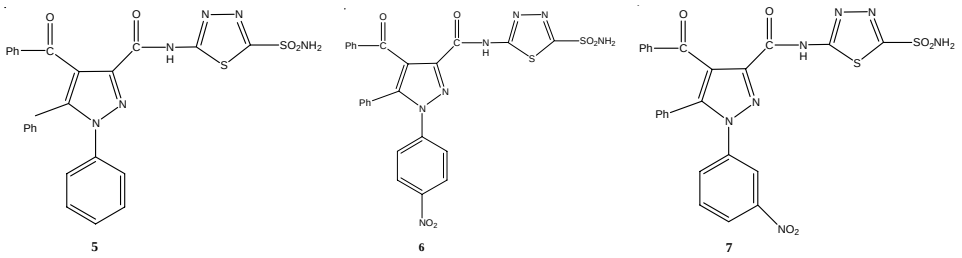
Key Words: Glaucoma, Carbonic Anhydrase, Sulfonamide.

INTRODUCTION

Carbonic anhydrase (CA, EC 4.2.1.1) catalyzes the reversible hydration of carbon dioxide, a very simple but critically important physiological reaction[1-5]. This enzyme has 16 different isoenzymes presently known in human. Some of these isoenzymes (CA-I, CA-II and CA-IV) are expressed in the human eyes[6-9]. Glaucoma is a group of diseases characterized by gradual loss of visual field due to intraocular pressure (IOP) and thus this disease is the second leading cause of blindness worldwide[10]. Carbonic anhydrase inhibitors have been used for the treatment of glaucoma for last many years[11,12]. Sulfonamides are the best known inhibitors of carbonic anhydrase enzyme in the treatment of glaucoma in clinical medicine. These inhibitors are very effective in this treatment of the disease by reducing the elevated intraocular pressure[1-13]. 5-Amino-1,3,4-thiadiazole-2-sulfonamide (**1**) has several biological activites and antibacterial properties[9].

Derivatives of this ring compound have been used in treatment of glaucoma for last several years, one of the drugs is acetazolamide **2** having a number of side effects. However, many of these drugs (*e.g.* acetazolamide) have a number of side effect on account of systemic use. Therefore, such systemic inhibitors have not been used in the treatment of glaucoma for decades. Recently dorzolamide **3** and brinzolamide **4** are available for clinical use as topically acting inhibitors[14-16]. These drugs show to reduce intraocular pressure exclusively by lowering the aqueous humour flow[2,17-19]. The common ocular side effects of dorzolamide and brinzolamide are stinging, burning, blurred vision, itching and tearing[2,16,17,20]. Due to side effects of these drugs, it is required to develop new compound and new drugs.

In present study, the synthesis of new carbonic anhydrase inhibitors with 5-substitued-1,3,4-thiadiazole and their effects on bovine carbonic anhydrase (BCA), to resembling hCA-II, purified from bovine erythrocytes are reported. The role of the substituted pyrazole carboxylic acid derivatives which have antipyretic, analgesic and antiinflammatory effects, moiety attached to a 1,3,4-thiadiazole ring on carbonic anhydrase inhibition[21,22] were also investigated. The IC_{50} and K_i values were determined to compare the inhibitory effects of 5-amino-1,3,4-thiadiazole-2-sulfonamide (**1**), acetazolamide (**2**) and newly synthesized compounds (**5**, **6**, **7**) on bovine erythrocyte carbonic anhydrase (BCA)[23].



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EXPERIMENTAL

Sepharose 4B for affinity column and electrophoresis reagents were obtained from Sigma Chem. Co. All other used chemicals were analytical grade and purchased from either Sigma or Merck.

Purification of bovine carbonic anhydrase from bovine erythrocytes: Erythrocytes were obtained from bovine blood. The blood samples were centrifuged at 1500 rpm for 20 min and plasma and buff coat were removed. Cell membranes were removed by centrifugation at 4 ºC, 20,000 rpm for 0.5 h. The homogenate was applied to affinity column having a structure of Sepharose-4B-L-tyrosine-*p*-aminobenzene sulfonamide. Bovine erythrocyte carbonic anhydrase was purified from this column[24,25].

SDS polyacrylamide gel electrophoresis: SDS polyacrylamide gel electrophoresis was performed after the purification of the enzyme. It was carried out with

10 % separating gel and 4 % stacking gel concentrations for running and the stacking gel, respectively, containing 0.1 % SDS according to Laemmli[26]. Equal amounts of each sample were applied to the electrophoresis gel. Gels were stained or 45 min in 0.1 % Coommassie Brillant Blue R-250 dissolved 50 % methanol and 10 % acetic acid and then detained with several changes of the same solvent above without the dye (Fig. 1).

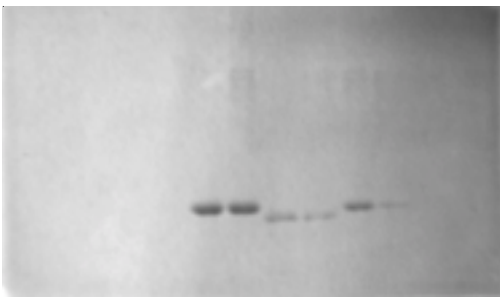

Fig. 1. SDS-PAGE analysis of carbonic anhydrase isoenzymes. Standard enzymes: Lane-1 (hCA-I), 3 (BCA), 5 (hCA-II). Enzymes used here:Lane-2 (hCA-I), 4 (BCA), 6 (hCA-II)

Measurement of carbonic anhydrase activity: CO_2-hydratase activity of the enzyme was determined at 0 ºC in a veronal buffer (pH 8.15) with pH-state method and saturated carbon dioxide solution as substrate in a final volume of 4.2 mL. The time (s) taken for the solution to change from 8.15 to 6.3 in pH was measured. The enzyme unit (EU) is the enzyme amount resulting in 50 % decrease of the non enzymatic reaction time. Activity as an enzyme unit was calculated by using the equation $(t_0\text{-}t_c/t_c)$ where t_0 and t_c are times for pH change of the non enzymatic and enzymatic reactions, respectively[27-30].

Esterase activities of carbonic anhydrase enzymes were assayed by the hydrolysis of *p*-nitro phenyl acetate. 1.2 mL of 3 mM *p*-nitro phenyl acetate was used as substrate. The substrate was added to a 1.5 mL volume of 5 different concentrations of inhibitors and water. Reaction was started by adding of 1.4 mL of 0.05 M *Tris*-SO_4 (pH 7.4) and 0.1 mL enzyme solution for a final volume of 3 mL. The absorbance was determined at 348 nm after 3 min[30].

Determination of IC_{50} and K_i values of compounds: The percentage of carbonic anhydrase activity values were assayed by the hydration of CO_2. I_{50} values for 5-amino-1,3,4-thiadiazole-2-sulfonamide (**1**), acetazolamide (**2**) and the synthesized compounds were determined on bovine erythrocyte carbonic anhydrase. In order to determine IC_{50} values, a saturated solution of CO_2 was used as substrate. To a total volume of 1.7 mL of various aliquots (10-250 mL) of a suitable concentration of inhibitors and water were added to 1 mL of *Tris*-HCl and 0.1 mL enzyme solution. The reaction was started, by adding 2.5 mL of CO_2 solution. The activity was determined by

using the equation (t_0-t_c/t_c) where t_0 and t_c are times for pH change of enzymatic reactions for each inhibitor concentration, respectively[27-30]. Regression analysis graphs were drawn on X axis of inhibitor concentrations and Y axis of % enzyme activity by using Microsoft Excel Program. The data were then fitted with non-linear regression using second degree polynomial equation. The I_{50} value was obtained by solving the equation derived from second degree polynomial as well as by simply extrapolating the X value (compound concentration) corresponding to the Y axis indicating the 50 % inhibition[1,27-30] (Fig. 2). Esterase activities of carbonic anhydrase enzymes were assayed by the hydrolysis of *p*-nitro phenyl acetate. IC_{50} and K_i values of the inhibitors (**1, 2, 5, 6** and **7**) were determined on bovine erythrocyte carbonic anhydrase (BCA). In order to determine IC_{50} values, 1.2 mL of 3 mM *p*-nitro phenyl acetate was used as substrate. The volume of substrate was added to 1.5 mL with 5 different concentrations of inhibitors (50, 100, 150, 200 and 300 mL) and water. Reaction was started by addition of 1.4 mL of 0.05 M *Tris*-SO_4 (pH 7.4) and 0.1 mL enzyme solution for total volume of 3 mL. The absorbance was determined at 348 nm after 3 min. The experiment was repeated 3 times for each inhibitor. In order to determine IC_{50} values, regression analysis graphs were drawn by using % inhibition values by a statistical packing program on a computer. The IC_{50} concentrations of the compounds (**1**, **2**, **5**, **6** and **7**) were determined from graphs (Fig. 2).

It was followed to determine K_i values. In the media with or without inhibitor, the substrate concentrations were 0.6, 0.8, 1.0 and 1.2 mM. For this purpose, the substrate was used between 0.6 and 1.2 mL. The solutions of the compounds (**1**, **2**, **5**, **6** and **7**) were added as inhibitors to the reaction medium as 0.1, 0.2 and 0.3 mL resulting in 3 different fixed concentrations of inhibitor. K_i values of these compounds (**1**, **2**, **5**, **6** and **7**) were obtained by calculating from the Lineweaver-Burk graphs[30] (Fig. 3).

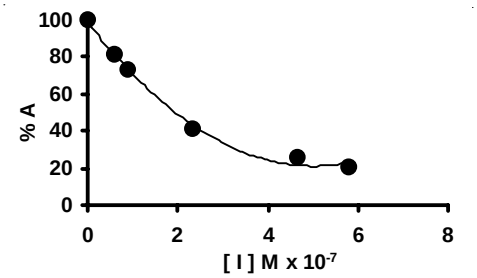

Fig. 2. I_{50} graph which were obtained from *in vitro* studies for compound **6** on hydratase activity of bovine erythrocyte carbonic anhydrase

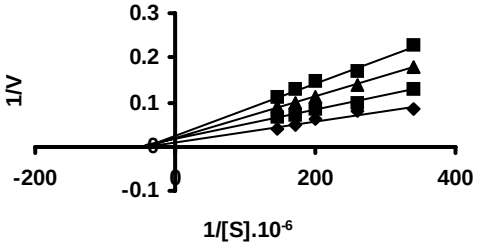

Fig. 3. K_i graph which were obtained from *in vitro* studies for compound **6** on bovine erythrocyte carbonic anhydrase

RESULTS AND DISCUSSION

The treatment of glaucoma with inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) is used in reducing elevated intraocular pressure (IOP) characteristic of this disease. In addition to the classical inhibitors used *via* the systemic route of administration [acetazolamide (**2**), methazolamide (**8**), ethoxzolamide (**9**) and dichlorophenamide (**10**)][1,2]. But, these inhibitors provoke a wide range of deleterious side effects due to inhibition of the enzyme present in other tissues (kidneys, lungs, red cells, stomach, *etc.*) than in the eye. The peripheral inhibition of carbonic anhydrase with systemic inhibitors produce a wide array of side effects which most of the patients are unable to tolerate and hence they could not continue the therapy[2,16,17,20]. Important drugs in this field have then been discovered by the Merck group, with the discovery compounds of the first clinically used, topically effective anti glaucoma sulfonamide, dorzolamide **3** and brinzolamide **4** known as the 'ring approach[17-20]. The two compounds also had side effects such as stinging, burning, blurred vision, itching and tearing[16-20]. The new amides of 5-amino-1,3,4-thiadiazole-2-sulfonamide (**1**) with some pyrazole carboxylic acids is synthesized with an alternative approach for the design of topically acting antiglaucoma sulfonamides (**5**, **6** and **7**). In newly synthesized three compounds, the inhibitor concentrations causing 50 % inhibition (IC_{50} values) were determined from regression analysis graphs (Fig. 2). IC_{50} values obtained, for bovine erythrocyte carbonic anhydrase purified by affinity chromatography are shown in Table-1.

TABLE-1
IC_{50} VALUES OBTAINED FROM *in vitro* STUDIES FOR THE COMPOUNDS (**1**, **2**, **5**, **6** AND **7**) ON HYDRATASE ACTIVITY OF BOVINE ERYTHROCYTE CARBONIC ANHYDRASE (BCA)

Hidrataz için, IC_{50} (µM)			
Inhibitor	BCA	Inhibitor	BCA
1	1.500	6	0.758
2	3.400	7	0.978
5	0.179	–	–

According to *in vitro* studies, IC_{50} values of newly synthesized compounds (**5**, **6** and **7**) are generally lower (respectively 0.179, 0.758 and 0.978 µM) than IC_{50} values of 5-amino-1,3,4-thiadiazole-2-sulfonamide **1** (1.5 µM) and acetazolamide **2** (3.4 µM) on hydratase activity of bovine erythrocyte carbonic anhydrase (Table-1). At the same time these new compounds (**5**, **6** and **7**) also have lower values of IC_{50} (respectively 0.225, 0.194 and 0.205 µM) than IC_{50} values of 5-amino-1,3,4-thiadiazole-2-sulfonamide **1** (2.4 µM) and acetazolamide **2** (5.9 µM) on esterase activity of this enzyme inhibition (Table-2).

TABLE-2
I_{50} VALUES OBTAINED FROM *in vitro* STUDIES FOR THE COMPOUNDS (**5**, **6** AND **7**) ON ESTERASE ACTIVITY OF BOVINE ERYTHROCYTE CARBONIC ANHYDRASE (BCA)

Esteraz için, I_{50} (µM)			
Inhibitor	BCA	Inhibitor	BCA
1	2.400	6	0.194
2	5.900	7	0.205
5	0.225	–	–

Nevertheless K_i values of these compounds (**5**, **6** and **7**) are lower (respectively 0.351, 0.208 and 0.232) than 5-amino-1,3,4-thiadiazole-2-sulfonamide **1** (8 µM) and acetazolamide **2** (2.3 µM) (Table-3). Therefore the whole obtained inhibition values for these compounds on bovine erythrocyte carbonic anhydrase are appropriate to use on glaucoma treatment for hCA-II and hCA-IV.

TABLE-3
K_i VALUES OBTAINED FROM *in vitro* STUDIES FOR SYNTHESIZED COMPOUNDS (**5**, **6** AND **7**) ON ESTERASE OF BOVINE ERYTHROCYTE CARBONIC ANHYDRASE (BCA)

Esteraz için, K_i (µM)			
Inhibitor	BCA	Inhibitor	BCA
1	8.000	6	0.208
2	2.300	7	0.232
5	0.351	–	–

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