Journal of Animal and Veterinary Advances 2 (3): 143-146, 2003

The *In vitro* Effects of Some Medical Drugs on Rat Carbonic Anhydrase

Sükrü Beydemir*, İlhami Gülcin
[1]Department of Chemistry, Faculty of Arts and Sciences,
Atatürk University,25240-Erzurum-Turkey

Abstract: The effects of streptomycin sulfate, gentamicin sulfate and sodium dipyrone have been investigated on rat erythrocyte carbonic anhyrase enzyme (CA) (EC 4.2.1.1) *in vitro*. For this purpose, rat erythrocyte carbonic anhyrase (CA) was purified. Rat erythrocyte carbonic anhyrase (CA) was purified. The purification steps comprised high-speed centrifugation, Sepharose-4B-L tyrosine-sulfanilamide affinity gel chromatography and dialyze. The yield was 44.4 % and the enzyme was found to be specific activity of 13873.2 EU/mg proteins. The overall purification was about 629.74-fold. Temperature of +4°C was maintained during the purification process. Inhibition or activation effects of three different medical drugs on rat CA were determined using the Esterase method by using spectrophotometer at 348 nm. Streptomycin sulfate showed highly inhibition effect the enzyme activity in *in vitro* conditions. I_{50} value of the streptomycin sulfate was determined as 32.8 mM by means of these graphs Activity %-[Drug] graphs. However, gentamicin sulfate showed *in vitro* inhibition effect on CA esterase activity up to 2.8 mM concentration, and then it was observed activation effect on the enzyme activitiy. Beside, sodium dipyrone has not any effect on rat erythrocyte CA.

Key words: Carbonic anhyrase, rat, erythrocyte and medical drugs

Introduction

Carbonic anhydrase (EC 4.2.1.1., CA) has been a well characterized pH regulatory enzyme, and the enzyme is abundantly present in mammalian red blood cells and to a lesser extent in different types of tissues and secretory organs. (Beydemir, 2000; Krungkrai, 2001). It is a zinc metalloenzyme with a high turnover that catalyses the interconversion of CO_2 and HCO_3^-. Also metalloenzymes are an important class of enzymes used to catalyze the conversion of fatty acids to amides, promote dioxygen and electron transport, and have roles in cellular structure composition (Lippard and Berg, 1994). Although this is the principal role of CA enzyme, it also has esterase activity (Verpoorte, 1967). In mammals at least four-teen isoenzymes of CA have been identified to date (Supuran, 2002). These isoenzymes can be differentiated based on specifik activity, subcellular and tissue distribution, and their sensitivites to certain inhibitors (Dogson, 1991; Maren and Sanyal, 1983; Sanyal, 1984). Soh as, CA-I is found together with CA-II in erythrocytes; CA-III is the most abundant soluble protein in skeletal muscle; CA-IV, a membran-bound form, has been isolated from the brush-border and basolateral membranes and microsomes of tubular cells of human kidneys and lungs; CA-V is found in mitochondria of certain tissues; CA-VI is secreted into the saliva, and CA-VII is a cytosolic isozymes (Beydemir, 2000).

Many drugs are being used in therapies. Several studies could be found related to changes of enzyme activities. Such as effects of metamizol, amikacin sulfate, ampicillin, netilmicine sulfate as drugs were investigated in rat erythrocyte *in vitro* and *in vivo* 6-phosphogluconate dehydrogenase enzyme activity (Çiftçi, 2002). In addition, *in vitro* effects of gentamicin sulfate on human erythrocyte carbonic anhydrase, and on rat erythrocytes *in vivo* effects of this drug were investigated by Beydemir et al In the present study, the *in vitro* effects of streptomycin sulfate, gentamicin sulfate and sodium dipyrone on CA enzyme purified from rat erythrocytes were investigated.

This study was aimed to determine any possible effect of some commonly used drugs on red blood cell CA activity.

Materials and Methods

Materials: In all experiments, Spraque-Dawley rats were used. Sepharose 4B, protein assay reagents, and chemicals for electrophoresis were purchased from Sigma-Aldrich Co. (Sigma-Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany). Para-aminobenzene sulfonamide and L-tyrosine were from E.Merck (Merck KGaA Frankfurter strasse 250, D-64293 Darmstadt Germany). All other chemicals used were analytical grade and

obtained from either Sigma-Aldrich or Merck. Drugs was provided by the University Hospital Pharmacy (Atatürk University, Erzurum, Turkey).

Purification of trout erythrocyte CA enzyme by affinity chromatography: Fresh blood samples from the rats, collected to EDTA-containing tubes, were centrifuged (15 min, 2,500xg) and plasma and buffy coat (leucocytes) were removed. The pack of red cells was washed twice with 0.9 % w/v NaCl, and hemolyzed with 1.5 volumes of ice-cold water. Ghost and intact cells were then removed by high-speed centrifugation (48745xg for 30 mins.) (Heraeus Sepatech, Suprafuge 22) at 4 °C and the pH of the haemolysate adjusted to 8.7 with solid Tris. The pH-adjusted haemolysate was then subjected to affinity chromatography [*Chromatography cystem:* chromatography column: 1.36x30 cm (Sigma Chemical Company), bed volume: 25 ml; peristaltic pump (Pharmacia Chemical Company), and fraction collector (AO Instrument Company, U.S.A.)] at 4 °C for the purification of carbonic anhydrase enzyme (Arslan, 1996)
25 ml of pH-adjusted rat erythrocyte haemolysate was applied to the Sepharose 4B-L-tyrosine-sulfanylamide affinity column pre-equilibrated with 25 mM Tris-HCl/ 0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris-HCl/ 22 mM Na_2SO_4 (pH 8.7). The rat carbonic anhydrase enzyme was eluted with 0.1 M $NaCH_3COO$ / 0.5 M $NaClO_4$ (pH 5.6) (flow rate: 20 ml h^{-1}, fraction volume: 4 ml). All procedures were performed at 4 °C (Beydemir, 2002).

Measurement of CA activity: Carbonic anhydrase activity was assayed by following the change in absorbance at 348 nm of p-nitrophenyl acetate to p-nitrophenylate ion over a period of 3 min at 25 °C using a spectrophotometer according to the method described by Verpoorte et al (Verpoorte, 1967).

Protein determination: During the purification steps, protein levels were determined spectrophotometrically (595 nm) according to the Bradford method, using bovine serum albumin as the standart (Bradford, 1976). Protein amounts in column fractions were observed via absorbance variations at 280 nm.

SDS-polyacrylamide gel electrophoresis: The enzyme purity was controlled by SDS-polyacrylamide gel electrophoresis. This technique was performed according to the Laemmli method using a verticalslab gel apparatus (Laemmli 1970). It was carried out in 10 % and 4% acrylamid concentrations for running and stacking gel, respectively, containing 10 % SDS. Gel eas stained with Coomassie brillant blue R-250 dye reagent overnight. The electrophoretic pattern was photographed (Fig. 1).

***In vitro* drug studies**: In order to determine the effects of some drugs on CA, some concentrations of streptomycin sulfate (6.0, 11.0, 17.0, 28.0, 40.0, and 51.0 mM), gentamicin sulfate (0.7, 2.8, 4.2, 7.0, 14.0, and 21.0 mM), and sodium dipyrone (4.0, 12.0, 16.0, 24.0, 30.0, and 36.0 mM) were added to separate tubes containing purified enzyme. The enzyme activity was measured in these tubes taking the tubes containing no drug as control (100 % activity). For the drug having an inhibition effect, the inhibitor concentration causing up to 50 % inhibition (I_{50}) value was determined from the graph.

Results and Discussion

Many chemicals when administered at relatively low doses affect metabolism by altering normal enzyme activity, particularly through inhibition of a specific enzyme (Hochster, 1972). The effects can be dramatic and systemic (Christensen, 1982). For example, Beydemir et. al. have reported the effects of some antibiotics in human erythrocyte carbonic anhydrase *in vitro* and rat erythrocyte carbonic anhydrase *in vivo* enzyme activity (Beydemir, 2000). Additionally, metamizol and magnesium sulfate have inhibitory effect on the G6PD enzyme activity (Çiftçi, 2001). In another study, vitamin C has been reported to stimulate 6PGD (Puskas, 2000). There has been a few study concerned with carbonic anhydrase activity for streptomycin sulfate, gentamicin sulfate and sodium dipyrone used in our study. Whereas, these drugs are used commonly in therapies. Due to the pH regulation of CA isozymes in most tissues, these isozymes have important role for body metabolism. Therefore, the effects of increasing concentration of streptomycin sulfate, gentamicin sulfate and sodium dipyrone administration on rat CA enzyme were undertaken in this study.
Thus, rat CA was purified with a specific activity of 13873.2 EU/mg proteins, a 44.4% yield and 629.74-fold by using Sepharose 4B-L-tyrosine-sulfanylamide affinity chromatography after the haemolysate step (Table 1) and purity confirmed by SDS-PAGE (Fig. 1). A high purity for the enzyme has been obtained. Streptomycin sulfate, gentamicin sulfate and sodium dipyrone

Table 1: Purification scheme for carbonic anhydrase from rat erythrocytes

Purificiation	Activity	Total	Protein	Total	Total	Specific	Yield	Purification
Haemolysate	710	25	32.218	805	17750	22.03	100	1
Sepharose-4B-L Tyrosine-sulfanilamid	1970	4	0.142	0.568	7880	13873.2	44.4	629.74

Table 2: Results which were obtained from *in vitro* study

Drug	I50 value (mM)
Streptomycin sulfate	32.8

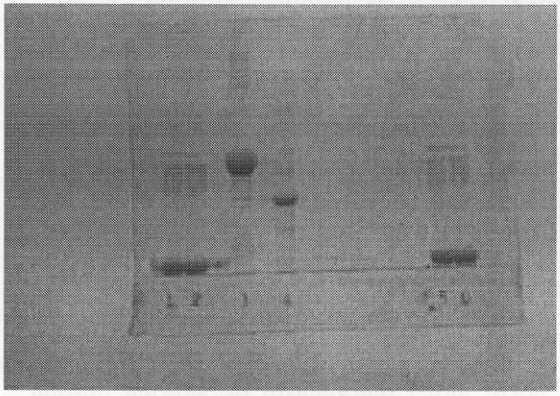

Fig. 1: SDS –polyacrylamide gel electrophoresis of CA purified by affinity gel. (Lane 1,2,5 and 6. are rat erythrocyte CA; Lane 3 is yeast hexokinase; Lane 4 is rabbit heart creatine phosphokinase)

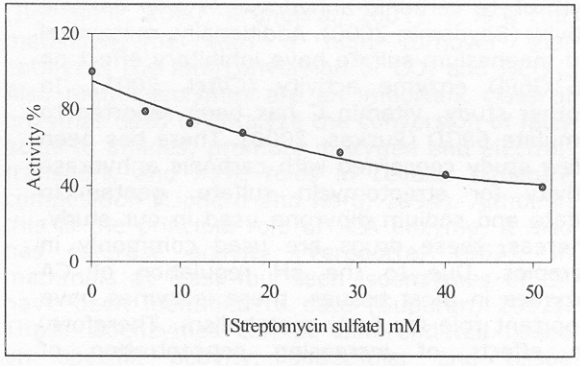

Fig. 2: Activity % - [Drug] graph for G6PD in presence of streptomycin sulfate.

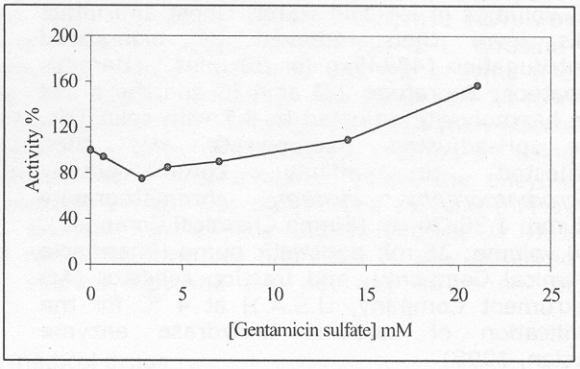

Fig. 3: Activity % - [Drug] graph for G6PD in presence of gentamicin sulfate.

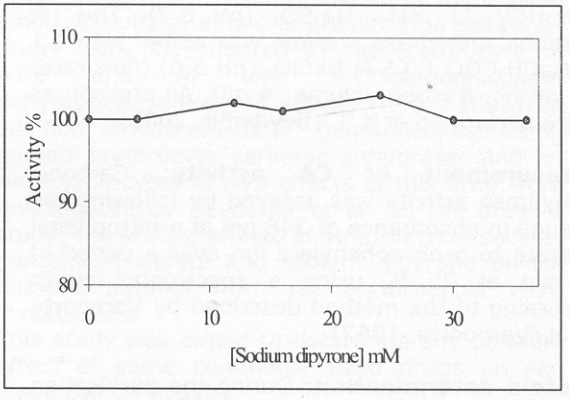

Fig. 4: Activity % - [Drug] graph for G6PD in presence of sodium dipyrone.

as medical drugs were chosen for the investigation of inhibition or activation effects. To show inhibition or activation effects, activity% values of rat CA for six different concentrations of each medical drug were determined. Carbonic anhydrase activity without a medical drug was accepted as 100 % activity (Fig.2, 3, 4). For the drug exhibiting an inhibition effect, the inhibitor concentration causing up to 50 % inhibition (I_{50} value) was determined from the regression analysis graph.

It was seen from *in vitro* studies that streptomycin sulfate has strongly inhibition effect on erythrocyte CA activity. I_{50} value (I_{50}= 32.8

mM) obtained for rat CA are shown in Table 2. According to the results of the study, gentamicin sulfate showed *in vitro* inhibition effect on CA esterase activity up to 2.8 mM concentration. 4.2 mM and higher concentrations of gentamicin sulfate excessively activated the *in vitro* rat CA activity. Beydemir et al. have reported gentamicin sulfate showed *in vitro* (= 2 mM) inhibition effect on erythrocyte CA activity. However, 4 mM and higher concentrations of gentamicin sulfate excessively activated the *in vitro* Human CA-I and CA-II activities (Beydemir, 2002). Conversely, has also been seen in sodium dipyrone activation effect on human erythrocyte CA-I and CA-II (Beydemir, 2000). However, in our study, sodium dipyrone has not any effect on this enzyme.

As a result, the use of streptomycin sulfate in a patient can cause serious side-effects and worsen healt of this patient. For this reason, streptomycin sulfate must be carefully used and their dosages should be very well ordered to decrease the side-effects. The inhibitory effect of gentamicin sulfate on CA activity may be it's one cause of side-effects. Additionally, increase of side-effect due to use of gentamicin sulfate at high dose is possible. Sodium dipyrone is not any effect on this enzyme, but effects of this drug are necessary to investigate on the other enzymes in body metabolism.

References

Arslan O., B. Nalbantoğlu, N. Demir, H. Özdemir, Ö. İ. Küfrevioğlu, 1996. Trop. J. Med. Sci., 26; 163-66.

Beydemir, Ş.O., M. Çiftçi, Ö.İ. Küfrevioğlu, M. E. Büyükokuroglu, 2002. Effects of gentamicin sulfate on enzyme activities of carbonic anhydrase from human erythrocytes *in vitro* and from rat erythrocytes *in vivo*. Biol Pharm. Bull, 25: 966-69.

Beydemir, S., M. Çiftçi, I. Özmen, M. E. Büyükokuroglu, H. Özdemir, Ö. I. Küfrevioglu, 2000. Effects of some medical drugs on enzyme activities of carbonic anhydrase from human erythrocytes *in vitro* and from rat erythrocytes *in vivo*. Pharmacol. Res., 42: 187-91.

Bradford M.M. 1976. A rapid and sensitive method for the quntitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-51.

Christensen, G.M., D. Olson, B. Riedel,1982. Chemical effects on the activity of eight enzymes: A review and discussion relevant to environmental monitoring. Environ. Res., 29: 247-55.

Çiftçi, M., Ş. Beydemir, H. Yilmaz, E. Bakan, 2002. Effects of some drugs on rat erythrocyte 6-phosphogluconate dehydrogenase: an *in vitro* and *in vivo* study. Pol. J. Pharmacol., 54: 275-80.

Çiftçi M, İ. Özmen, M. E. Büyükokuroğlu, Sadrettin Pençe, Ö. İ. Küfrevioğlu, 2001. Effects of metamizol and magnesium sulfate on enzyme activity of glucose 6-phosphate dehydrogenase from human erythrocyte *in vitro* and rat erythrocyte *in vivo*. Clinical Biochemistry, 34: 297-302.

Dogson, S. J., 1991. The carbonic anhydrases: Overview of their importance in cellular physiology and in molecular genetics. In: Dodgson S.J, Tashian R.E, Gros G, Carter N.D. editors. The carbonic anhydrases. New York: Plenum press, p: 3-14.

Hochster, R.M., M. Kates, J. H. Quastel, 1972. *Metabolic Inhibitors*. New York: Academic Press 197; 3 and 4: 71-89 and 66-82.

Krungkrai, S.R., N. Suraveratum, S. Rochanakij, J. Krungkrai, 2001. Characterisation of carbonic anhydrase in *Plasmodium falciparium* Int. J. Parasitol., 31: 661-668.

Laemmli D.K., 1970. Cleavage of structural proteins during assembly of the head of bacteriophage Nature, 227: 680-83.

Lippard, S.J., J. M. Berg, 1994. Principles of bioorganic chemistry. University Science Books, Mill Vally, CA, p: 411.

Maren, T.H., G. Sanyal, 1983. The activity of sulfonamides and anions against the carbonic anhydrases of animals, plants, and bacteria. Ann. Rev. Pharmacol. Toxicol., 23: 439-59.

Puskas, F., P. J. Gergely, K. Banki, A. Perl, 2000. Stimulation of the pentose phosphate patway and glutathione levels by dehydroascorbate, the oxidized form of vitamin c. FASEB J., 14: 1352-1361. Sanyal, G., 1984. Comparative carbon dioxide hydration kinetiks and inhibition of carbonic anhydrase isozymes in vertebrates. Ann. NY. Acad. Sci., 429: 165-78.

Supuran, C., A. TScozzafava, 2002. Applications of carbonic anhydrase inhibitors and activators in the rapy, Exp. Opin. Ther. Pat., 12: 217-242.

Verpoorte, J.A., 1967. Esterase Activities of Human Carbonic Anhydrases B and C Mehta S, Edsall J.T. J. Biol. Chem., 242: 4221-4229.