

EFFECT OF SOME DRUGS ON SHEEP LIVER CARBONIC ANHYDRASE I AND II

BAZI İLAÇLARIN KOYUN KARACİĞERİ KARBONİK ANHİDRAZ I VE II ÜZERİNDEKİ ETKİSİ

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ABSTRACT

Effects of clindamycin, dexketoprofen trometamol, benzylpenicillin (penicillin G) and phenoxymethylpenicillin (penicillin V) were examined on purified sheep carbonic anhydrase I and II (SCAI, SCA-II). In our study, we were chosen sheep liver tissue; because the liver is responsible for detoxification of xenobiotics. The CA isozyme have a very important duties on organisms, the effect of xenobiotics on the CA enzyme was reported. It was observed these drugs exhibited the inhibition effects against liver carbonic anhydrase isozymes I and II in our study. SCA I and SCA II isozymes were purified with about a specific activity of 1108.51 EU/mg and 5706.67 EU/mg, 120.62 and 620.96 purification fold by using Sepharose-4B-L tyrosine-sulfanilamide affinity gel chromatography. Purity of the SCA I and SCA II isozyme was checked by SDS-PAGE technique and subunit molecular masses of the SCA I was found as 29 kDa and SCA II found as 31 kDa. The usage of clindamycin, dexketoprofen trometamol, benzylpenicillin (penicillin G) and phenoxymethylpenicillin (penicillin V) in sheep liver especially during necrotic hepatitis disease is important for scientific researches. Many antibiotic and antihistamine may have dangerous effect to sheep liver. Also, it is important to study the inhibition effects of these drugs on SCA I and II isozymes.

Key words: Liver, Antibiotics, Inhibition.

ÖZET

Koyun karaciğerinden saflaştırılmış karbonik anhidraz I ve II (SCAI, SCA-II) üzerine; klindamisin, deksketoprofen trometamol, benzilpenisilin (penisilin G) ve fenoksümetilpenisilin (penisilin V)'in etkileri incelenmiştir. Çalışmamızda koyun karaciğer dokusunu seçtik, çünkü karaciğer ksenobiyotiklerin detoksifikasyonundan sorumludur. Karbonik anhidraz izoenzimlerinin organizmalarda çok önemli görevleri vardır, ksenobiyotiklerin karbonik anhidraz üzerindeki etkileri rapor edilmiştir.

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Çalışmamızda bu ilaçların, koyun karaciğeri karbonik anhidraz I ve II izoenzimlerine karşı inhibisyon etkisi gösterdiği gözlenmiştir. SCA I ve SCA II izoenzimleri Sepharose-4B-L tyrosine-sulfanilamid afinite jel kromatografisi kullanılarak, yaklaşık olarak 1108.51 EU/mg ve 5706.67 EU/mg spesifik aktivite ile 120.62 ve 620.96 kat saflaştırıldı. SCA I ve SCA II izoenzimlerinin saflığı SDS-PAGE tekniği ile tespit edildi ve alt birimlerinin ağırlığı SCA I'de 29 kDa ve SCA II'de 31 kDa olarak belirlendi. Koyun karaciğerinde, özellikle nekrotik hepatitis hastalığı sırasında kullanılan; klindamisin, deksketoprofen trometamol, benzilpenisilin (penisilin G) ve fenoksümetilpenisilin (penisilin V) hakkında önemli araştırmalar yapılmaktadır. Birçok antibiyotik ve antihistamin koyun karaciğeri için tehlikeli etkilere sahip olabilir. Ayrıca, bu ilaçların SCA I ve II izoenzimleri üzerinde inhibisyon etkilerini incelemek oldukça önemlidir.

Anahtar Kelimeler: Karaciğer, Antibiyotik, İnhibisyon.

1. INTRODUCTION

The most hard-working organ of the human body is liver. Therefore function and activity of liver are vital to life. Most of investigations focus on the property of liver. This organ is a crucial organ present in vertebrates and some other animals. Functions of this organ have a wide range, such as including protein synthesis, production of biochemical necessary for detoxification and digestion. Liver plays a vital role in metabolism and has so many different functions in the body, detoxification, and decomposition of red blood cells, including plasma protein synthesis, hormone production and glycogen storage. It is the storage site for many important minerals and vitamins, such as all of fat-soluble vitamins, iron, and copper. Numerous significant metabolic processes occur in the liver such as detoxification, urea Cycle. Many different chemical reactions in the liver detoxify harmful substances. (Demirdag, 2012; Evenepoel, 2006; Kmiec, 2001).

On the other hand, enzymes are very important for the life attendance. Carbonic anhydrases is a crucial family in enzymes because they catalyze the reversible reactions of CO₂ and water (Brinkman, 1932; Meldrum, 1933; Stadie, 1933).

CA isozymes are tried a lot of because the turnover number or k_{cat} of some members of this family exceed $1 \times 10^6 \text{ s}^{-1}$ (Chegwidden, 2000). In addition, the reaction of CA isozymes are essential to several of physiological processes such as photosynthesis, calcification, respiration, acid-base and fluid balance, cell growth, ionic and

metabolism (Tashian, 2000). Therefore, most of the analysis exhibited expression of CA isoforms almost ubiquitously in living organisms. CAs consists of five different genetic families (α , β , γ , δ and ξ) (Tashian, 2000; Hewett-Emmett, 1996).

Antibiotics are widely used to treat many different disease and so many investigations reported their effects on enzyme activities. Some of these drugs were either increases or decreases in mammalian enzyme activities, and the activator or inhibitor effects of some antibiotics have been investigated. A lot of researches are reported, different type of drugs and chemicals have inhibition effects on CAs. (Britta, 1997; Cankaya, 2012; Chiche, 2009; Supuran, 2004).

Clindamycin is an antimicrobial agent used in the world for more than thirty years and clindamycin used as an effective treatment to infections of formed by many facultative and obligatory anaerobic bacteria (Brook, 2005).

Dexketoprofen is a non-steroidal drug which is water-soluble salt of an enantiomer of ketoprofen, used treatment to pain reliever, anti-inflammatory and antipyretic since 1973 (Veys, 1991). Dexketoprofen can inhibit prostaglandin synthesis *in vitro* conditions even at low doses (Barbanoj, 2001).

Benzylpenicillin (penicillin G) which is one of the natural penicillin group members is acid-labile and better absorbed by injection, in that reasons it is used in the form of parenteral and intramuscular injection. Phenoxymethylpenicillin (penicillin V), which is the oral administration of single natural penicillin. Activity against Gram-positive bacteria, penicillin G is similar. (Holten, 2000; Neu, 1982; Prince, 1983).

The effects of many of the known xenobiotics have not been checked on sheep liver CA isozymes yet. Usage of these drugs may worsen SCA 1 and II activity in liver. So, they may be used in sheep necrotic hepatitis disease; it is important to explore the effect of these on CA I and II activity during necrotic hepatitis disease. The present study was realized on the *in vitro* effect of clindamycin, dexketoprofen trometamol, benzylpenicillin (penicillin G) and phenoxymethylpenicillin (penicillin V) on SCA I and II isozymes purified from sheep liver by Sepharose-4B-L tyrosine-sulfanilamide affinity gel chromatography.

2. MATERIALS AND METHODS

Chemicals

Sepharose 4B, protein assay reagents and 4-nitrophenylacetate were obtained from Sigma-Aldrich Co. (Sigma-Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany). All other chemicals were analytical grade and obtained from Merck (Merck KGaA Frankfurter strasse 250, D 64293 Darmstadt Germany).

Purification of carbonic anhydrase isozymes from sheep liver by affinity chromatography

The purification of the two SCA isozymes from sheep liver was performed in a simple single-step method by means of Sepharose-4B-L tyrosine-sulfanilamide affinity gel chromatography. Sheep liver was obtained from Erzincan Municipal Slaughterhouse and stored at $-80\text{ }^{\circ}\text{C}$ until usage. Fifty grams of thawed liver was used in this investigation. All of the experiments were made according to Çoban and friends (Ayvaz, 2013; Çoban, 2007; Çoban, 2008).

Hydratase activity assay

CA activity was assayed by following the hydration of CO_2 according to the method described by Öztürk Sarıkaya, Gülçin and Supuran (2010). CO_2 -hydratase activity as an enzyme unit (EU) was calculated by using the equation $(t_0 - t_c/t_c)$ where t_0 and t_c are the times for pH change of the non-enzymatic and the enzymatic reactions, respectively (Öztürk, 2010).

Esterase activity assay

CA activity was assayed according to method of Verpoorte et al. (Verpoorte, 1967) described previously by Innocenti et al. (Innocenti, 2010a,b). CA activity was determined by following the change in absorbance at 348 nm of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ion over a period of 3 min at 25°C using a spectrophotometer (Perkin Elmer Lambda 35) according to the method described by Verpoorte et al. (Verpoorte, 1967). The inhibitory effects of clindamycin, dexketoprofen trometamol, Benzylpenicillin (penicillin G), Phenoxymethylpenicillin (penicillin V) were examined. All compounds were tested in triplicate at each concentration used. Different inhibitor concentrations were used. SCA I enzyme activities were measured for Phenoxymethylpenicillin

(0.123–0.247 mM), Benzylpenicillin (0.21-0.35 mM), clindamycin (11.76–23.52 mM) and dexketoprofen trometamol (4.8–8 mM) at cuvette concentrations and SCA II enzyme activities were measured for Phenoxyethylpenicillin (0,185-0,309 mM), Benzylpenicillin (0.21-0.35 mM), clindamycin (17.64-29.4 mM) and dexketoprofen trometamol (0.042–0.053 mM) at cuvette concentrations. Control cuvette activity in the absence of inhibitor was taken as 100%. For each inhibitor an Activity (%)–[Inhibitor] graphs were drawn. To determine K_i values, three different inhibitor concentrations were tested. In these experiments, 4-nitrophenylacetate was used as substrate at five different concentrations (0.15-0.75 mM). The Lineweaver–Burk curves were drawn (Lineweaver, 1934).

Protein determination

Protein during the purification steps was determined spectrophotometrically at 595 nm according to the Bradford method (Bradford, 1979), using bovine serum albumin as the standard (Hisar, 2005).

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed after purification of the sheep liver isozymes. It was carried out according to Laemmli procedure (Laemmli, 1970). The electrophoretic pattern was photographed (Figure 1).

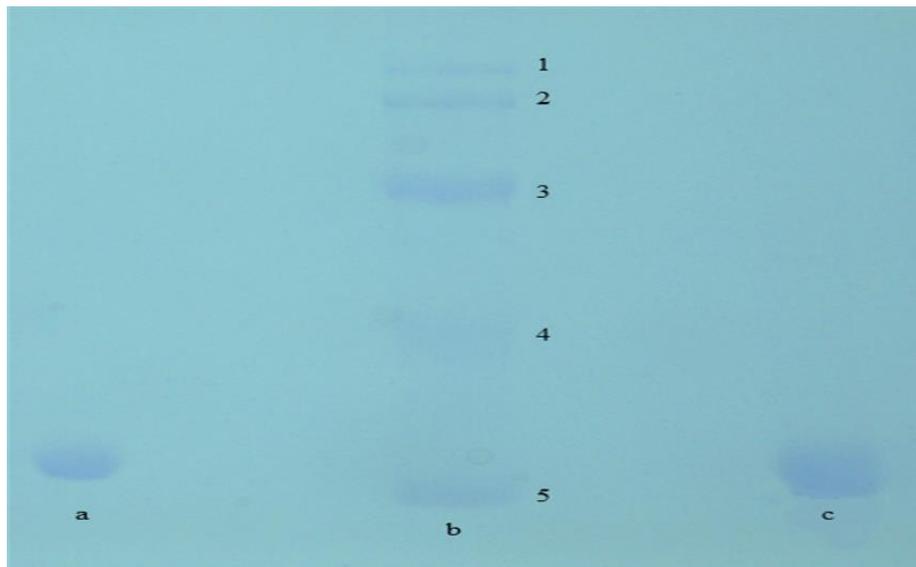
3. RESULTS AND DISCUSSION

In this study, CA I and II were purified from sheep liver by a simple one step procedure using Sepharose 6B-aniline-sulfanilamide affinity column. The activity of the effluents was determined by the hydratase method, with CO_2 as substrate and further kinetic studies were performed using the esterase activity method, with 4-nitrophenyl acetate (NPA) as substrate. SCA I was purified 120.62 fold with specific activity (1108.51 EU mg/ μL) and yield (64%) (Table 1).

Similarly, SCA II was purified 620.96 fold with specific activity (5706.67 EU mg/ μL) and yield (53%). SDS-PAGE of the enzymes showed a single polypeptide band (Figure. 1).

Table 1. Purification steps of CA I and II from sheep liver by Sepharose-4B-L tyrosine sulfanilamide affinity gel chromatography.

	Acti vity (EU/ mL)	Tot al Vol ume (mL)	Prot ein (mg/ mL)	Tota l Prot ein (mg)	Tota l Acti vity (EU)	Spec ific Acti vity (EU/ mg)	Reco very (%)	Purific ation (Fold)
Homo genate	155	42	16.86	708. 12	6510	9.19	100	1.0
CA-I	521	8	0.47	3.76	4168	1108. 51	64	120.62
CA-II	856	4	0.15	0.6	3424	5706. 67	53	620.96

**Figure 1.** PAGE of the purified CA isozymes. Lane b: Standards: 1. *E.coli* β -galactosidase (116 kDa), 2. Rabbit muscle phosphorylase-b (97 kDa), 3. Bovine serum albumine (66 kDa); 4. ovalbumine (45 kDa), 5. bovine erythrocyte CA (29 kDa),, lane a: SCA I, lane c: SCA II.

Four inhibitors were prepared and evaluated for the inhibitory effects on SCA I and II. The inhibitory effects of drugs were

tested in range of 0.001- 1000 mM. Among the four inhibitor all of them (clindamycin, dexketoprofen trometamol, benzylpenicillin and phenoxymethylpenicillin) were showed inhibition effects on SCA I and SCA II (Table 2). The results obtained from esterase and CO₂-hydratase methods support each other.

Table 2. IC values of four drugs for the esterase and hydratase activities of sheep erythrocytes CA-I and II

Compound Name	Hydratase activity of		K _i SCA I (μM)	Inhibition type	Esterase activity of		K _i SCA II (μM)	Inhibition type
	IC ₅₀ SCA-I (μM)	IC ₅₀ SCA-II (μM)			IC ₅₀ SCA-I (μM)	IC ₅₀ SCA-II (μM)		
Phenoxy methylpenicillin	0,462	0,5826	0.0686	Non-competitive	0.155	0.1938	0.0833	Non-competitive
Benzylpenicillin	0,869	0,731	0.1	Non-competitive	0.283	0.235	0.1346	Non-competitive
Clindamycin	47,23	69,58	9.157	Non-competitive	15.76	22.74	9.752	Non-competitive
Dexketoprofen trometamol	13,02	9,17	2.203	Non-competitive	4.26	2.909	2.717	Non-competitive

The liver's highly specialized tissues regulate a wide variety of high-volume biochemical reactions, including the synthesis and breakdown of small and complex molecules, many of which are necessary for normal vital functions. All enzymes involved in detoxification of xenobiotics in the liver tissue, the working pH should be stable. Carbonic anhydrase has important role in stabilizing the pH. Many antibiotic and antihistamine may have dangerous effect to sheep liver and its detoxification enzymes. In the current study, we examined the effects of some drugs on liver CA I and II enzymes activity of sheep. And as we know, sheep liver is an important source of nutrient for people. The aim of this study is purifying SCAI and II from unstudied tissue of sheep liver and examining the effects of clindamycin, dexketoprofen trometamol,

Benzylpenicillin, Phenoxymethylpenicillin on enzyme activity (Ciftci, 2000; Esbaugh, 2006; Senturk, 2008).

So far, CA consists of 16 isozymes which have been identified in mammals and several novel isozymes have also been identified in non-mammalian vertebrates. Much of the research on the evolution of the structure and function of CA has been focused on the widely distributed CA I and II isozymes (Senturk, 2008). The purification of the two CA isozymes used here was performed with a simple step method by a Sepharose-6B-aniline- sulfanilamide affinity column (Table 1). Inhibition effects of drugs derivatives on enzyme activities were tested under *in vitro* conditions; IC_{50} values were calculated Activity (%) - [Inhibitor] graphs and are given in Table 2 and K_i values were calculated from Lineweaver-Burk graphs and are given in Table 2.

CA catalyzes the conversion of CO_2 to H_2CO_3 at cells and intracellular fluid. Inhibitor of carbonic anhydrase are very important for different diseases such as antiglaucoma, diuretics, antiepileptics, in the management of mountain sickness, gastric and duodenal ulcers, neurological disorders or osteoporosis (Supuran, 2000).

CA I and II have been purified many times from different organisms and the effects of various chemicals, pesticides and drugs on its activity have been investigated (Beydemir, 2000; Demir, 2004; Otlecz, 1999; Ekinci, 2007).

As a result of our study, sheep liver CA-I and II isozymes were purified in one-step with high specific activity by the purification method used in this study. Inhibitory effects of clindamycin, dexketoprofen trometamol, benzylpenicillin and phenoxymethylpenicillin were demonstrated. Benzylpenicillin and phenoxymethylpenicillin at low concentrations showed *in vitro* inhibitory effects on SCA-I and SCA-II activity. Dexketoprofen trometamol showed less *in vitro* inhibitory effects on SCA-I and SCA-II activity than benzylpenicillin and phenoxymethylpenicillin. Clindamycin showed lowest *in vitro* inhibitory effects on SCA-I and SCA-II activity.

pH is an important chemical parameters in enzymatic activity. For this reason, the enzymes responsible for the detoxification of the liver, is in a relationship with carbonic anhydrases. If carbonic

anhydrase activity be inhibit, the activity of detoxification enzymes are not able to fully. For this reasons, uncontrolled usage of these drugs can cause serious side effects and can be deleterious to health. In conclusion, these drugs must be used carefully and the dosage should be closely monitored to decrease side effects.

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