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Bazı Yapay Gözyaşı Damlalarının İnsan Karbonik Anhidraz Enzimi-II (hCA-II) Üzerindeki İnhibitör Etkileri

Büşra ÇALIŞKAN[1*], Mine AKSOY[2]

Öne Çıkanlar:
- CA inhibitörleri göz içi basıncını düşürmede ve glokom tedavisinde önemlidir.
- DES ile glokom ilaçları arasında bir ilişki olduğu bilinmektedir.
- DES tedavisinde kullanılan suni gözyaşı damlalarının CAII inhibisyonu araştırıldı.

ÖZET:

Karbonik anhidrazlar (CA), canlılarda karbondioksitin hidrasyonu ve bikarbonat anyonunun dehidrasyonu reaksiyonlarını tersinir olarak katalize eden, aktif bölgelerinde çinko iyonları içeren bir metalloenzimdir. Bu çalışmada, insan eritrositlerinden karbonik anhidraz II (hCAII), afinite (sefaroz 4B/L-tirozin-sülfanilamid) kolonu ile saflaştırıldı. Yapay gözyaşı damlalarının hCAII izoenzimi üzerindeki inhibitör etkisi araştırıldı. IC_{50} değerleri sodyum hiyalüronat için 1.01 µL, polivinil alkol+povidon için 140.06 µL, polietilen glikol+propilen glikol için 49.51 µL olarak hesaplandı. Sodyum hiyalüronat için K_i değeri 0.339±0.121 mM olarak bulundu. Bu sonuçlara göre, incelenen üç ilaç arasında uygulama hacmi (mikrolitre) açısından en etkili inhibitörün sodyum hiyalüronat olduğu görülmektedir.

Anahtar Kelimeler:
- İnsan karbonik anhidraz II
- suni gözyaşı damlaları
- inhibisyon

Inhibitor Effects of Some Artificial Tears Drops on Human Carbonic Anhydrase Enzyme-II (hCA-II)

Highlights:
- CA inhibitors are essential in lowering intraocular pressure and treating glaucoma
- There is a relationship between DES and glaucoma medication.
- hCAII inhibition of artificial tear drops used in DES treatment was investigated.

ABSTRACT:

Carbonic anhydrases (CAs) are a metalloenzyme that contains zinc ions in their active sites, and they reversibly catalyze the reactions of hydration of CO_2 and dehydration of HCO_3^- in the living. In this study, human carbonic anhydrase II (hCAII) isoenzyme was purified from human erythrocytes by affinity column (Sepharose 4B-L-tyrosine-sulfanilamide). The inhibitory effect of artificial tear drops on hCAII isoenzyme was investigated. IC_{50} values were calculated as 1.01 µL for sodium hyaluronate, 140.06 µL for polyvinyl alcohol+povidone, and 49.51 for polyethylene glycol+propylene glycol. The K_i value for sodium hyaluronate was found as 0.339±0.121 mM. According to these results, sodium hyaluronate was the most effective inhibitor in terms of application volume (microliter) among the three drugs examined.

Keywords:
- Human carbonic anhydrase II,
- artificial tears drops,
- inhibition

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INTRODUCTION

Carbonic anhydrase (CA) is a critical metalloenzyme that contains zinc ions in their active site. It reversibly catalyzes the reactions of hydration of CO_2 and dehydration of HCO_3^- in the living organism. CA is widely present in all organisms and participates in varied pathological and physiological processes, such as fluid balance, pH regulation, carbohydrate oxidation and glaucoma (Taslimi et al. 2016).

Various studies have shown that CA I, CA II, and CA IV are the most common isoforms in the human eye. Only CA I and CA II are expressed in the corneal endothelium, while CA II is expressed in both retina and ciliary processes. Isoform CA IV is found in the choriocapillaris and retinal pigment epithelium. These isoforms play an essential role in aqueous humor secretion, which is the main controller of intraocular pressure in the eye (Sugrue, 2000; Mincione et al., 2007; Scozzafava and Supuran, 2014).

CA enzyme activity may play an essential role in maintaining the acid-base balance of the corneal surface fluid by enabling the transepithelial flow of carbon dioxide and bicarbonate anions. The acid-base state of the corneal surface is inevitably affected by this flow. CA I-II-IV are possible candidates for maintaining acid-base balance in the corneal epithelium (Supuran and Simone 2015).

The CA VI activity in rat tears and the CA I-II activities in the interlobular ducts were found by Ogawa et al. (1995). In the rabbit, CA was detected in the acinar cells of the lacrimal gland where lacrimal fluid is secreted. CA II-IV are possible candidates that play a role in tear secretion (Bromberg et al., 1993).

Dry eye syndrome (DES) is a common, chronic and multifactorial disease characterized by tear film instability and damage to the ocular surface, causes symptoms such as burning, pain and visual impairment. The surface of the cornea and conjunctiva, meibomian glands, lacrimal glands and ocular surface innervation form a functional unit (Stern et al., 2013).

Artificial eye drops are the primary drugs used to cure dry eye in all degrees of severity. Many preparations are available, such as sodium hyaluronate, hydroxypropyl guar, polyvinyl alcohol, povidone, and cellulose derivatives (Messmer, 2015)

Loss of tear film and homeostasis of the ocular surface causes dry eye (Craig et al., 2017). Carbonic anhydrase may be one of the critical enzymes providing ocular hemostasis. Therefore, we investigated the inhibitory effects of artificial tear drops containing sodium hyaluronate, polyvinyl alcohol+povidone and polyethylene glycol-propylene glycol, which are frequently used in the cure of dry eye, on human carbonic anhydrase II isoenzyme.

MATERIALS AND METHODS

Materials

Artificial tears

Artificial tear medicines were obtained from the pharmacy. ***Sodium hyaluronate***: A single dose drop of 0.3 mL solution contains 0.45 mg sodium hyaluronate ($C_{28}H_{44}N_2NaO_{23}$: MW: 799.6 g/mol). ***Polyvinyl alcohol + povidone***: 0.4 ml contains 5.6 mg of polyvinyl alcohol and 2.4 mg of povidone. ***Polyethylene glycol + propylene glycol***: 1 mL of the drug contains 4 mg of polyethylene glycol 400 and 3 mg of propylene glycol.

Method

Enzyme activity assay

The enzyme activity assay described by Verpoorte et al. (1967) was used to measure the esterase activity of the hCA-II isoenzyme. This method is based on the hydrolysis of p-nitrophenyl acetate (PNF) to p-nitrophenol with maximum absorption at 348 nm, catalyzed by the enzyme CA. The increase in absorbance occurring at 348 nm is recorded. The amount of enzyme that hydrolyzes one μmol of PNF to p-nitrophenol in one minute is defined as an enzyme unit.

The affinity chromatography method (Sepharose 4B/L-Tyr-sulfanilamide) was used to purify of CA-II from human erythrocyte cells. First, erythrocyte cells were hemolyzed with ice water. It was centrifuged to remove cell debris (10.000 g, 15 min.). The hemolysate was loaded onto a pre-prepared affinity column (Nalbantoğlu et al., 1996). The hCA-I was firstly eluted from the column using 25 mM pH:6.3 phosphate buffer+1 M NaCl, followed by hCA-II using 0.1 M pH:5.6 acetate buffer+0.5 M $NaClO_4$. Purified hCA-II was used in this study. It was dialyzed with tris buffer at pH 7.4.

Polyacrylamide gel electrophoresis (PAGE) in denatured conditions

SDS (sodium dodecyl sulfate)-PAGE method was used at 3% stacking-8% separation gel concentrations to control enzyme purity (Laemmlli, 1970). Protein bands were made clear by staining with Coomassie Brilliant Blue R-250. Photographs were taken after the protein bands became apparent.

Inhibition studies

To examine the effects of artificial tear drops on hCA-II activity, enzyme activities were measured at various (at least five different) drug concentrations. 100% activity is measured without artificial tear drops and is considered a control. Activity%-[Inhibitor] graphs were drawn with the calculations made due to the measurement. IC_{50} values (drug concentration causing 50% inhibition) were calculated from these graphs.

The K_i value was determined only for sodium hyaluronate because other teardrops have two active ingredients. For sodium hyaluronate, 1/V-1/S values were calculated by measuring activities at five substrate concentrations and three different inhibitor concentrations. Inhibition type and K_i were determined by Lineweaver-Burk plots.

RESULTS AND DISCUSSION

hCA-II was obtained by affinity chromatography with a specific activity of 4771 EU/mg protein. Purification results are given in Table 1.

Table 1. Purification table for hCAII

	Activity (EU/mL)	Protein (mg/mL)	Total volume (mL)	Total activity	Total protein (mg)	Specific activity (EU/mg protein)	Purification fold
Hemolysate	1521.93	65.98	49	74574.57	3233.02	23.07	1
hCA-II	143.13	0.03	3	429.39	0.09	4771	206.81

The purity of the enzyme was demonstrated using the SDS-PAGE method, and a single band visible in Figure 1 confirmed the purity.

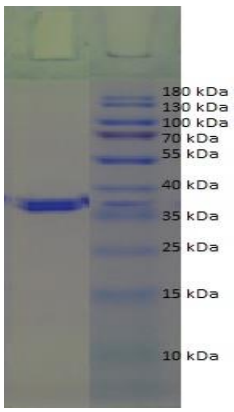

Figure 1. SDS-PAGE photo for hCA-II

Activity%-inhibitor graph and Lineweaver-Burk graph for sodium hyaluronate, %Activity-inhibitor graphs for polyvinyl alcohol+povidone and polyethylene glycol+propylene glycol are given in Figure 2.

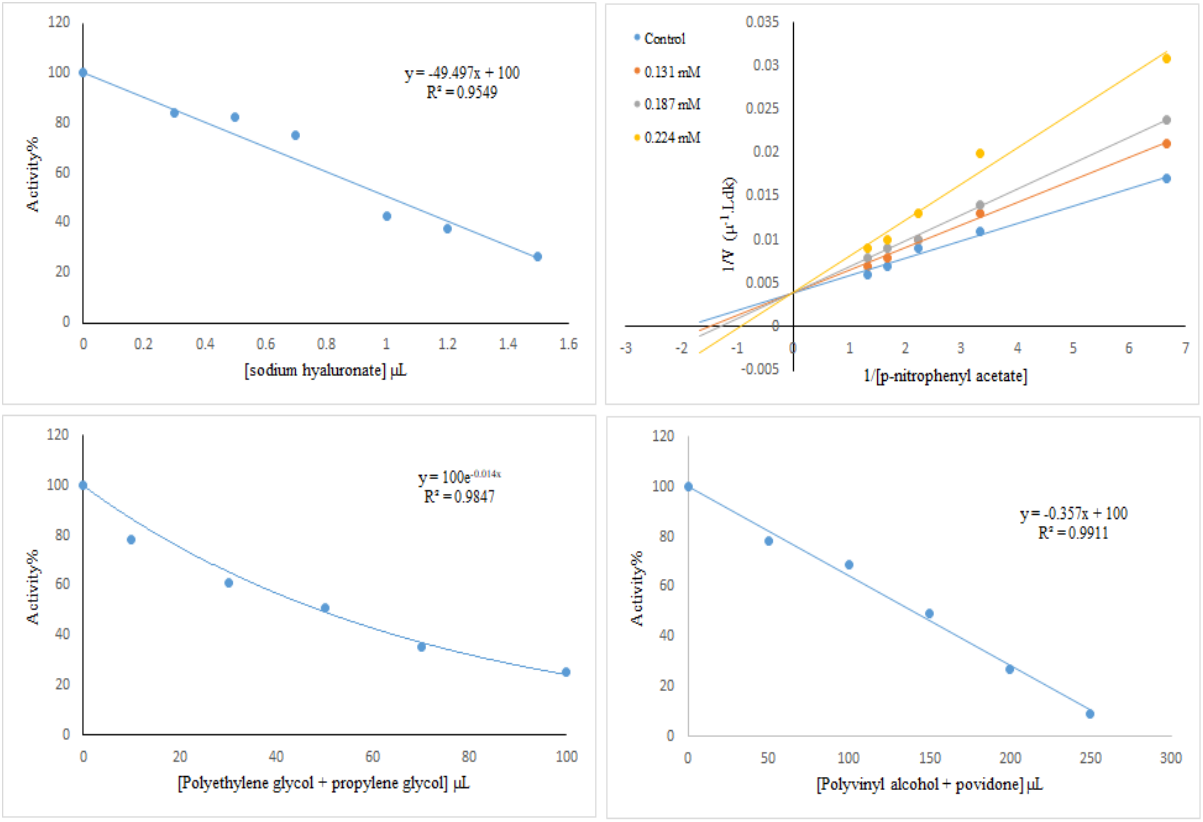

Figure 2. Activity%-inhibitor graph and Lineweaver-Burk graph for sodium hyaluronate, Activity%-inhibitor graphs for polyvinyl alcohol+povidone and polyethylene glycol+propylene glycol

All three artificial tear drops (sodium hyaluronate, polyvinyl alcohol+povidone, and polyethylene glycol+propylene glycol) were found to have an inhibitory effect, and IC_{50} values were determined as 1.01, 140.06 and 49.51 µL, respectively. While the K_i contents could not be determined since polyvinyl alcohol+povidone and polyethylene glycol+propylene glycol contain two active ingredients, the K_i value for sodium hyaluronate was determined as 0.339±0.121 mM (Table 2). In addition, this inhibitor showed a competitive inhibition type.

Table 2. The inhibition results of artificial tear drops

İnhibitör	IC_{50} (µL)	K_i mM
Sodium hyaluronate	1.01	0.339±0.121
Polyvinyl alcohol + povidone	140.06	ND*
Polyethylene glycol + propylene glycol	49.51	ND*

*Not determined

DES is an ocular disorder that causes blurred vision, deterioration of the corneal barrier, eye irritation, and irregular cornea (Bron et al., 2014; Milner et al., 2017). Strong junctions in the apical corneal epithelium have been proven to be disrupted in DES patients, an essential increase in the level and activities of matrix metalloproteinase have been observed in the ocular surface and tear (Wei and Asbell, 2014; Pflugfelder and de Paiva, 2017).

MMPs are a family of calcium and Zn^{2+} ion-dependent enzymes involved in physiological processes such as wound healing, embryogenesis, and bone growth (Cerd`a-Costa and Xavier Gomis-Rüth, 2014; Cui et al., 2017). Since MMPs are associated with changes in corneal barrier function observed in the pathophysiology of dry eye, there are many studies investigating the inhibition of MMPs in treatment (Lanza et al., 2016). Considering the structural similarity between carbonic anhydrases and MMPs, which are physiologically expressed metalloenzymes in the eye, there is also a study examining the inhibition of CA isoenzymes (Richichi et al., 2016). Also, glaucoma itself and intraocular pressure-lowering agents have been found to be associated with impaired basal tear cycle (Kuppens et al., 1995; Erb et al., 2008). It has also been reported that patients taking glaucoma medication more often showed symptoms of dry eye syndrome than participants not taking glaucoma medication (Rossi et al., 2009). Glaucoma itself and intraocular pressure lowering agents are associated with impaired basal tear cycle. The incidence of dry eye symptoms in patients with glaucoma 59%. Dry eye causes optical aberrations that may be associated with decreased the retinal image and visual function (Kuppens et al., 1995; Erb et al., 2008; Leung et al., 2008). CA inhibitors play an essential role in ophthalmology as they reduce high intraocular pressures and are used in the treatment of glaucoma (Carradori et al., 2015). CA inhibitors (CAIs) reduce ocular pressure in glaucomatous patients by lowering the rate of bicarbonate formation and, thus the secretion of the aqueous humor (Supuran et al., 2019). If CA in the lacrimal gland is involved in tear secretion, it has been reported in the literature that ionic composition and tear flow may be affected after topical medication use of CA inhibitors to the eye (Wistrand, 2000).

Although none of the current treatment options for patients with DES can successfully regenerate the regular human tear film, the topical application of artificial tear agents to patients to supplement the natural tear film is a safe approach (Labetoulle et al., 2022). In this study, we investigated whether three types of artificial tear drops used in patients with DES cause inhibition of the hCAII isoenzyme (Figure 2). The active ingredients of the artificial tear drops are sodium hyaluronate, polyvinyl alcohol+povidone and polyethylene glycol+propylene glycol.

CONCLUSION

In our study, we investigated the inhibitory effects of artificial tears containing sodium hyaluronate, polyvinyl alcohol+povidone and polyethylene glycol+propylene glycol on hCAII isoenzyme. According to these results, sodium hyaluronate was determined as the most effective inhibitor application volume (microliter) among the three drugs.

CA inhibitors are used in reducing intraocular pressure in the treatment of glaucoma. Antiglaucomatous agents are known to be associated with ocular surface problems. Considering the possible roles of CA activity on acid-base balance in corneal epithelium, tear production and

homeostasis of the ocular surface, the inhibition effect of drugs used in dry eye treatment is suggestive in terms of the effectiveness of treatment.

Also, considering that inhibition of metalloproteinases is essential in the treating of DES, it is noteworthy that CA isoenzymes (especially membrane-dependent CAIV and cytosolic CAII) show similarities with zinc-dependent metalloproteinases. It is suggest that the inhibition effect of artificial tear drops used in the treatment of DES in the study will help to consider the assumptions of a connection between DES treatment and CA inhibition in future studies.

Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

The authors declare that they have contributed equally to the article.

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