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Effects of Morphine and Hashish (*Cannabis sativa*) on Activities of Human Red Blood Cell Carbonic Anhydrase Isoenzymes

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Abstract

Carbonic anhydrase (CA) exists in all living organisms and converts CO_2 (waste product) to H_2CO_3. Two different forms of CA are present in human erythrocytes. These forms are known as CA-I and CA-II. Whereas CA-I has a low activity, CA-II is highly active. In the current study, CA-I and CA-II were purified by affinity chromatography from human blood. Then the effects of hashish (*Cannabis sativa* L.) and morphine on the CA-I and CA-II activities were measured by following esterase activity, and the results are presented.

Keywords: Affinity chromatography, carbonic anhydrase, hashish (*Cannabis sativa*), morphine.

Introduction

Carbonic anhydrase (CA; carbonate hydrolase: E.C. 4.2.1.1) was first isolated from mammalian red cells. It is a zinc-containing metalloenzyme (Sly & Hu, 1995). CA catalyzes the reaction given below:

$$H_2O + CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

The enzyme plays an important role both in CO_2 removal and in acid-base balance by producing H^+ and CO_2. CA-I and CA-II have been known for several decades to occur in mammalian red blood cells. CA-I has six times lower activity then CA-II (Keha & Küfrevioğlu, 2000). The human red cell contains trace amounts of CA-III with minor activity (Carther, 1972). The deficiency of CA-I and CA-II results in pathological disorders (Rickli et al., 1964; Demir et al., 2001).

Morphine is the principal alkaloid that is extracted from opium. Morphine is a prototype opiate, and many opiates, for example heroin (dactyl morphine), oxymorphine, and codeine (methyl morphine) are morphine derivatives. It was first synthesized in the early 1800s and has widely been used all over the world since the nineteenth century, as both a prescription and nonprescription drug. With the invention of the hypodermic needle in 1856, morphine could be injected subcutaneously, intramuscularly, or intravenously (Tejador-Real et al., 1995; Hamilton & Baskett 2000).

The types of hashish (*Cannabis sativa* L. Cannabaceae) called "marijuana," "bag," and "press" are known as commonly abused substances causing dependence (Fairbairn, 1972). "Grass" (marijuana) is made by cutting and drying the flowers and leaves of *Cannabis sativa*. Marijuana is made from any part of the plant *Cannabis sativa*. In addition to marijuana, the following forms of *Cannabis* are also abused: hashish, a resin of the plant, hashish oil, and a liquid concentrate derived from *Cannabis sativa*. "Press" and "bag" are made of resins of *Cannabis sativa* (De Zeeuw et al., 1962; Pate, 1994). The isoenzymes, CA-I and CA-II, could be affected by substances of abuse such as morphine and three commercial forms of hashish, "grass," "press," and "bag." This study was designed to investigate the effect of morphine and hashish (forming substances) on CA-I and CA-II activities. THC is the cannabinoid that is responsible for the main psychoactive effects of most of the *Cannabis* drugs (Bradford, 1972; Coney & Xue-Min, 1989).

Materials and Methods

All chemical and morphine were obtained from Sigma Chemical Co. Hashish kinds, said to have high purity, were

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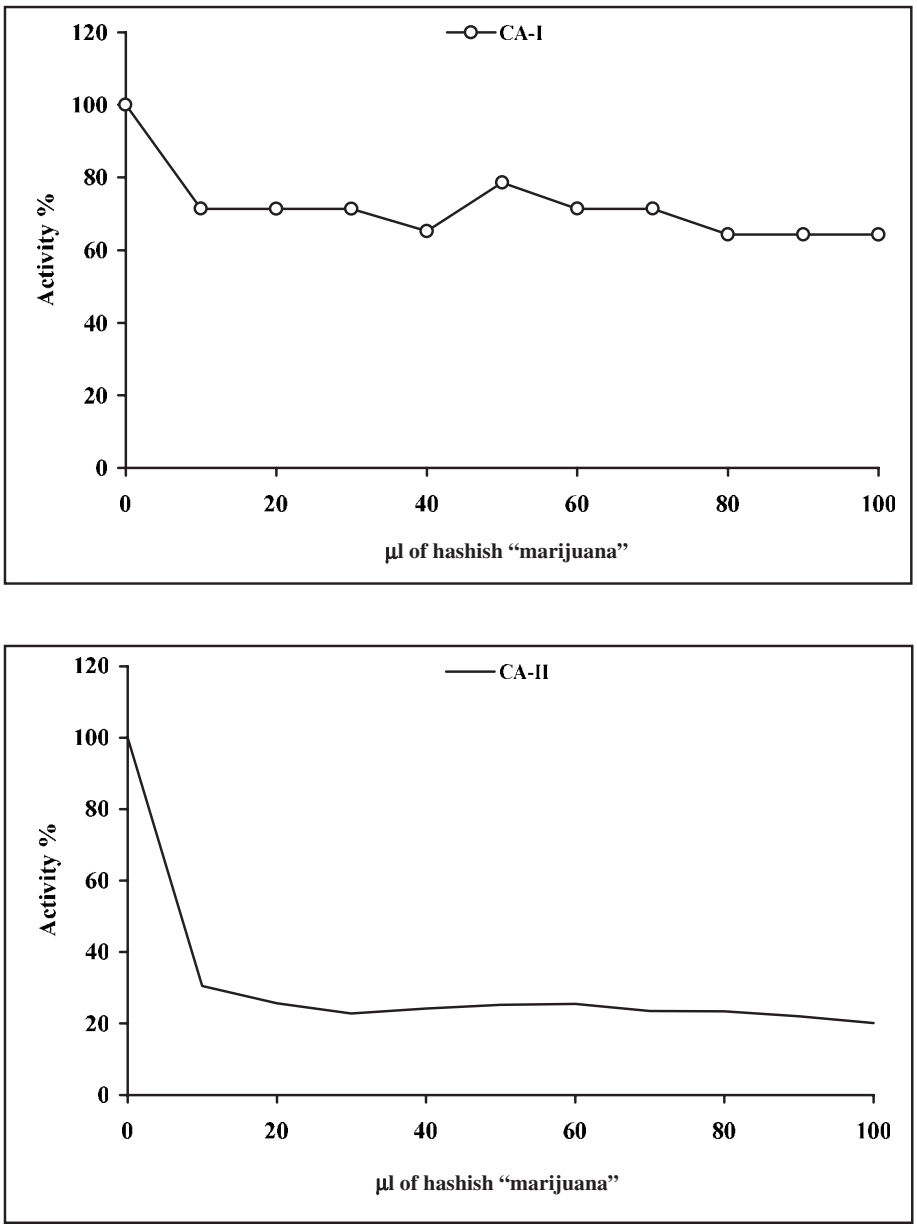

Figure 1. The effect of marijuana, "grass", on CA-I and CA-II activities.

Table 1. The purification of CA-I and CA-II from human erythrocytes.

Steps	Volume (ml)	Activity (EU/ml)	Total activity		Protein (mg/ml)	Specific activity (EU/mg)	Purification (Times)
			EU	%			
Hemolysate	400	2.80	1120	100	158	0.0177	–
Pure enzyme CA-I	35	5.65	197.7	17.65	0.273	20.695	1169
Pure enzyme CA-II	30	6.35	190.5	17.00	0.210	30.238	1708

supplied from the narcotic department of military security of Turkey.

Purification of human erythrocyte CA isoenzymes (CA-I and CA-II)

Hemolysate preparation

Blood samples obtained from the blood bank of Atatürk University Hospital were centrifuged (2500 rpm, 15 min, 4 °C). The plasma and buffy coat were removed carefully. The precipiates were washed with saline three-times. Forty to 50 ml of red blood cell pack was obtained from 100 ml of whole blood with this procedure. The resultant red blood cell pack was mixed with chilled distilled water at a ratio of 1 : 15 and left for 30 min for completion of hemolysis. For isolation of membranous structure, the hemolysate was centrifuged at 20,000 rpm for 30 min at 4 °C. The supernatant was taken and its pH was adjusted to 8.7 with solid Tris. It was then ready for application to the column (Poker & Sarkonen, 1979).

Affinity chromatography

Sepharose-4B activated by CNBr and having covalently bound L-tyrosine was used for separation of CA; 100 ml of the hemolysate was applied to the column. Then, it was washed with 400 ml of 25 mM Tris-HCl/22 mM Na_2SO_4

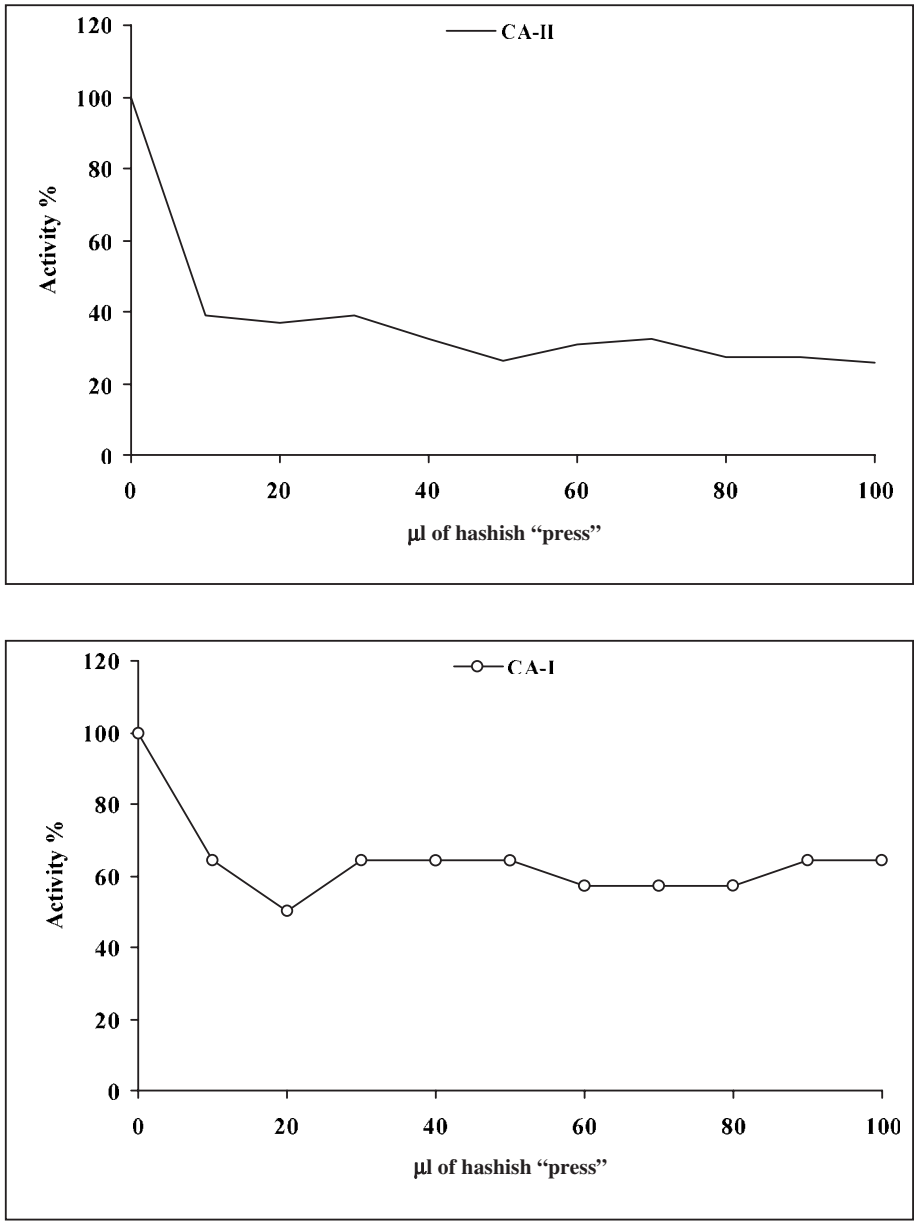

Figure 2. The effect of hashish, "press", solution on CA-I and CA-II activities.

(pH 8.7), allowing to rise CA attachment and remove undesirable substances. CA-I was eluted with 25 mM $Na_2HPO_4 \cdot 12\ H_2O$/1 M NaCl (pH 6.3) until the eluate did not show adsorbance at 280 nm. Then, CA-II was eluted with 0.1 M NaCHCOO/$NaClO_4$ (pH 5.6). The eluates were collected as 5-ml fractions with a fraction collector. The flow rate was adjusted to 20 ml/h and the process was carried out at 10–15 °C according to O'Carra et al. (1996), Arslan et al. (1996), and Cuatrecases (1970).

Determination of protein content

The tubes showing high absorbance at 280 nm were pooled and protein content of the solution was determined by the Coomassie brilliant blue G-250 method of Bradford (1976).

Determination of enzyme activity (esterase activity)

This method is based on hydrolysis of *p*-nitrophenylacetate (which is a substrate of CA) to *p*-nitrophenol and acetic acid. The reaction is followed at 348 nm. For this procedure, 1.5 ml of a buffered enzyme solution (0.1 ml of enzyme +1.4 ml of 0.005 M Tris-SO_4, pH 7.4) and 1.5 ml of substrate were mixed in a measurement cuvette, and 3 min later, the absorbance was measured at 348 nm and 25 °C. The blank contained the substrate and buffer without enzyme (Rickli et al., 1964).

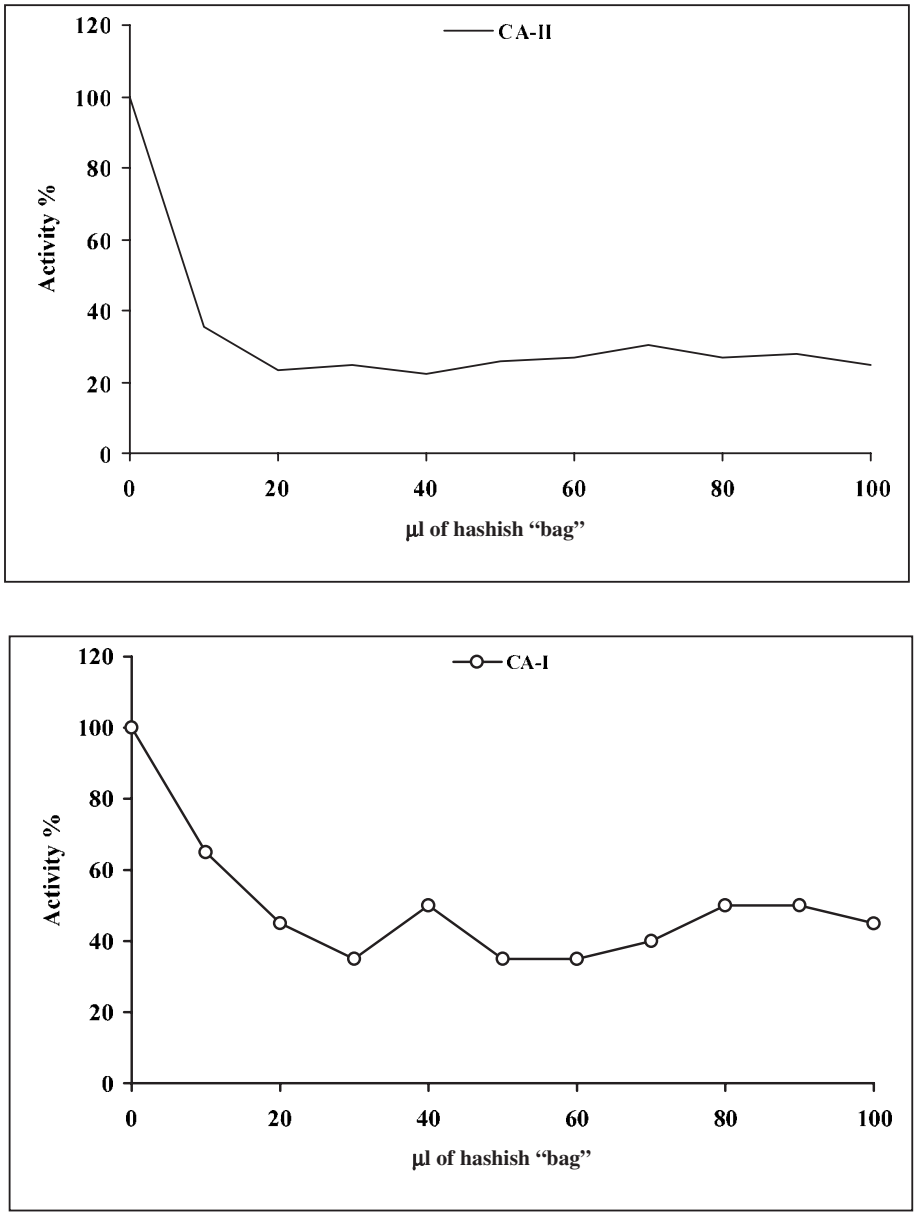

Figure 3. The effect of hashish, "bag", solution on CA-I and CA-II activities.

SDS-polyacrylamide gel electrophoresis

The purity of enzyme was controlled by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) as reported by Laemmli (1970).

Results and Discussion

Table 1 shows high purity of CA-I and CA-II, which were obtained by means of affinity chromatography. The current study demonstrated that marijuana "grass," hashish "press" and "bag" (20 mg/ml) decreased the esterase activity (Figs. 1–3) of both CA isoenzymes. CA-II isoenzyme was affected more than CA-I. I_{50} values of CA-I and CA-II are shown in Table 2. I_{50} values of CA-I and CA-II for grass solution were calculated and found to be 159.6 and 3.56 µl, respectively. I_{50} values of CA-I and CA-II for press solution were 137 and 21.60 µl, respectively. I_{50} values of CA-I and CA-II for bag

Table 2. I_{50} values of CA-I and CA-II.

I_{50} values	CA-I	CA-II
20 mg/ml press solution	137 µl	21.60 µl
20 mg/ml grass solution	159.6 µl	3.56 µl
20 mg/ml bag solution	50.68 µl	2.3 µl
1×10^{-3} M morphine solution	16.41 µl	37.24 µl

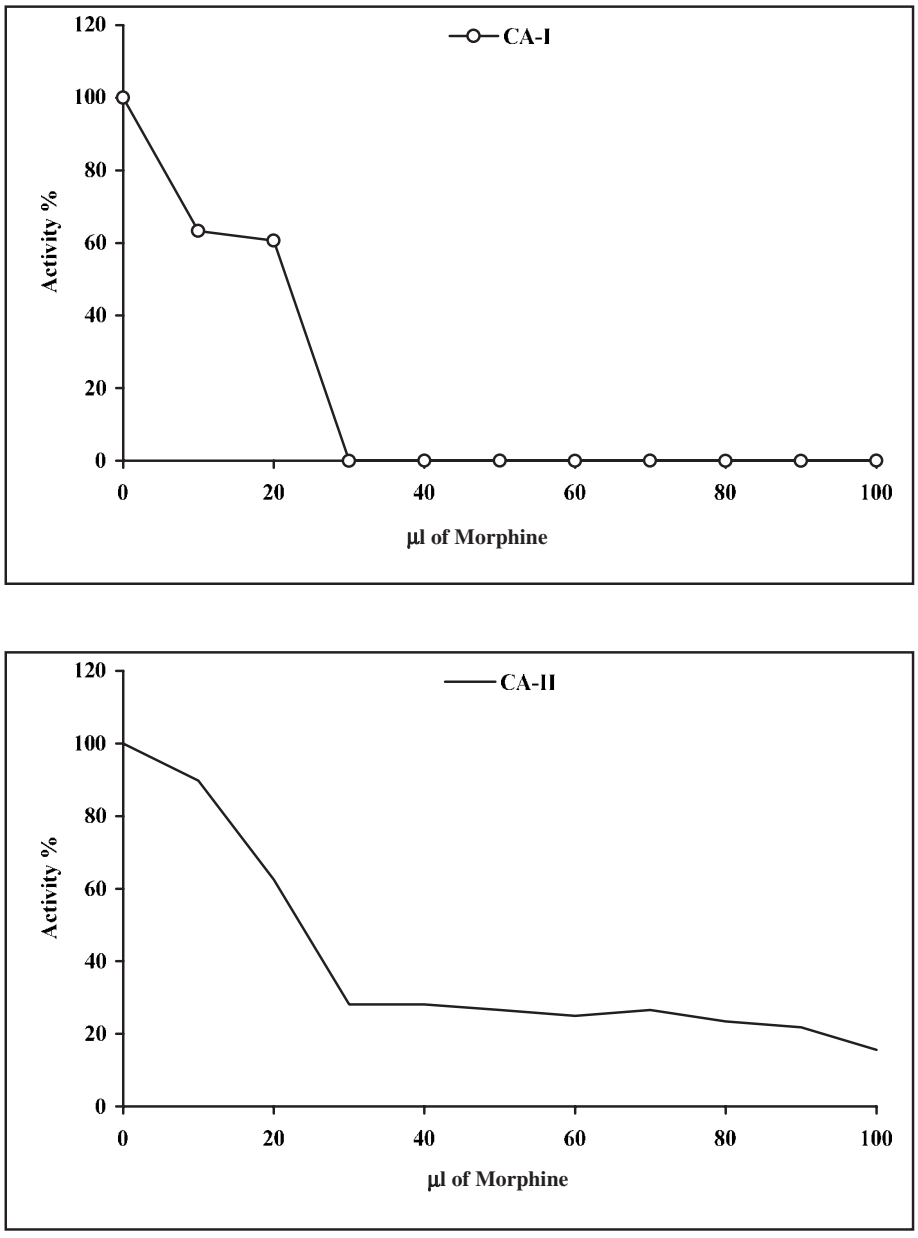

Figure 4. The effect of morphine solution on CA-I and CA-II activities.

solution were 50.68 and 2.3 µl, respectively. All of the studied forms of hashish were able to inhibit the enzymes when used at the tested concentrations.

Morphine exerted a similar effect on CA activity as marijuana and hashish. The effect of morphine solution (1×10^{-3} M) on CA-I and CA-II is shown in Fig. 4. I_{50} values of CA-I and CA-II for morphine solution were 16.41 and 37.24 µl, respectively. As can be seen from the figure, this compound inhibited the activities of both isoenzymes. Morphine and hashish samples inhibited CA-I much stronger than CA-II.

It is difficult to explain the changes in the activity of CA isoenzymes induced by morphine and different forms of hashish (*Cannabis sativa*). *In vivo* experiments are needed to determinate the physiological importance of the activity change. Our future studies will aim to clarify whether morphine and hashish components are used as substrates by human CA isoenzymes.

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