



Original Article

# *In vitro* effects of estrogen and progesterone containing drugs on human erythrocyte carbonic anhydrase I and II isozymes in women smokers and nonsmokers

Mine Islimye Taskin <sup>a,\*</sup>, Cigdem Bilen <sup>b</sup>, Adem Ergun <sup>b</sup>, Nahit Gencer <sup>b</sup>, Umit Inceboz <sup>a</sup>

<sup>a</sup> Department of Obstetrics and Gynecology, Balikesir University School of Medicine, Balikesir, Turkey

<sup>b</sup> Department of Chemistry, Faculty of Art and Sciences, Balikesir University, Balikesir, Turkey

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## Abstract

**Background:** Carbonic anhydrases (CAs), a group of metalloenzymes, are involved in numerous physiological and pathological processes such as acid–base balance, gluconeogenesis, lipogenesis, ureagenesis, electrolyte secretion in various tissues, bone resorption and calcification, and tumorigenicity. In the current study, we aimed to determine and compare possible alterations in the activity of carbonic anhydrase I (CA I) and carbonic anhydrase II (CA II) isozymes by using estrogens and progestagens in female smokers and nonsmokers.

**Methods:** Blood samples from 30 smoker and 30 nonsmoker volunteers were drawn after obtaining informed consent. The blood samples were centrifuged to separate the plasma and erythrocytes. Thereafter, hemolysate was prepared from the red cells. CA I and CA II were purified from human erythrocytes with a simple one-step procedure using Sepharose 4B-I-tyrosine-sulfonamide affinity column. CA I and CA II isozymes were treated with estrogen and progesterone-containing drugs, after which the inhibition or activation of the enzyme was determined.

**Results:** CA I and CA II enzyme activity was observed to be increased in female smokers. The results of this study show that dienogest is the most effective inhibitor for human erythrocytes CA I when compared with micronized progesterone, hydroxyprogesterone caproate, estradiol valerate, and estradiol hemihydrate in both female smokers and nonsmokers. All active ingredients have been shown to have a stronger inhibition in smokers than nonsmokers for CA I activity. Additionally, estradiol valerate and hydroxyprogesterone caproate have stronger inhibition against CA II enzyme activity in women who smoke.

**Conclusion:** The results of the current study provide important information to clinicians about how to consider the possible adverse effects of these drugs which are produced as a result of inhibition of CA I and CA II enzyme. Clinicians should take into consideration the side effects caused by CA I and CA II enzyme inhibition when prescribing these drugs in the treatment of different clinical conditions, especially in women who smoke.

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**Keywords:** carbonic anhydrases; enzyme inhibition; estrogens; progestagens

## 1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are zinc metalloenzymes which reversibly catalyze the hydration of carbon dioxide to bicarbonate and hydrogen ions, and are abundantly presented in mammalian tissues.<sup>1</sup> The CA reaction is involved in many physiological and pathological processes. These include respiration and transport of CO<sub>2</sub> and bicarbonate

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\* Corresponding author. Dr. Mine Islimye Taskin, Department of Obstetrics and Gynecology, Balikesir University School of Medicine, Bahcelievler Mah, Yay Sit, 35/2 10050, Balikesir, Turkey.

E-mail address: [minetaskin1302@yahoo.com.tr](mailto:minetaskin1302@yahoo.com.tr) (M.I. Taskin).

between tissues and lungs, pH, and CO<sub>2</sub> homeostasis, electrolyte secretion in various tissues and organs, biosynthetic reactions such as gluconeogenesis, lipogenesis and urea-genesis, bone resorption, calcification, and tumorigenicity.<sup>2,3</sup> Because of the many CA isoenzymes involved in these processes, CA enzyme inhibitors are used in the treatment of some disorders including edema, glaucoma, obesity, cancer, epilepsy, and osteoporosis.<sup>4,5</sup>

Oral contraceptives (OCs) offer safe, effective, and reversible fertility regulation, and contain either a combination of estrogen and progestin, or only progestin. However, OCs may have some adverse effects. In some cases, OCs have been shown to induce rare cardiovascular events and venous thromboembolism. The estrogen component of these drugs alters the production of liver enzymes, coagulation, and fibrinolysis factors. Progestogens have various metabolic effects apart from binding to the progesterone receptor, also interacting with the androgen, glucocorticoid, or mineralocorticoid receptors. Adverse effects of the progesterone component can also include altering of the lipid metabolism negatively, fluid retention, glucose tolerance deterioration, and an increase of peripheral insulin resistance. New progestins with high specificity have been designed to avoid interaction with other receptors and prevent androgenic, estrogenic, or glucocorticoid related side effects, and to prevent other undesirable side-effects especially on lipoproteins, clotting factors, and insulin sensitivity.<sup>6</sup>

Cigarette smoking (CS) is the primary cause of preventable morbidity and mortality, and abundant clinical evidence suggests that CS is more harmful to women than men. CS alters endothelial function, the redox state, inflammation, and global DNA methylation. Cardiovascular risk factors seem to appear earlier in young healthy female smokers than in young healthy male smokers.<sup>7</sup> In young women, the risk of cardiovascular disease is very low in OC users but can be strongly influenced by smoking and the presence of other risks factors, such as hypertension, obesity, and diabetes mellitus, more so than for women over 35 years of age.<sup>8</sup>

In the present study we aimed to determine and compare possible alterations in activity of isozymes CA I and CA II caused by the use of estrogen (estradiol valerate and estradiol hemihydrate) and progesterone-containing drugs (hidroxyprogesterone caproate, micronized progesterone, and dienogest) in female smokers and nonsmokers. CA isoenzymes present in different tissues and consist of metabolic wastes due to respiration diffuse through the blood, thereby containing venous blood. Mammalian red blood cells mainly express CA II and CA I. For this reason, we focused on plasma CA isoenzymes I and II in this article. The aim of this study was to determine whether these drugs can be a risk factor for the presence of CA I and CA II isoenzyme inhibition in smokers. Furthermore, smoking is a risk factor for the enzyme inhibition degree of these drugs, and side effects of the drugs can be increased by smoking.

## 2. Methods

The necessary materials Sepharose 4B, l-tyrosine, sulfonamide, protein assay reagents, phenol red, and chemicals for electrophoresis were obtained from Sigma-Aldrich Co, St. Louis, MO, USA. All other chemicals were of analytical grade and obtained from either Sigma or Merck (Kenilworth, NJ, USA). Those medical drugs used in this study were provided by the local pharmacy.

### 2.1. Purification of CA from human erythrocytes using affinity chromatography

The study comprised of 30 smoker and 30 nonsmoker women aged 18–30 years. The smoker group was chosen from women who smoke approximately five cigarettes per day. Informed consent was obtained from all participants, and the study was also approved by the local Ethics Committee of Balikesir University. Erythrocytes were purified from fresh heparinized human blood obtained from the healthy volunteers. The blood samples were centrifuged at 1000g for 20 minutes, after which the plasma and buffy coat were removed. The red cells were isolated and washed twice with 0.9 % NaCl, and hemolyzed with 1.5 volumes of ice-cold water. The ghost and intact cells were removed by centrifugation at 3100g for 30 minutes at 4°C. The pH of the hemolysate was adjusted to 8.7 with a solid Tris base, and applied to the prepared Sepharose 4B-L-tyrosine-sulfonamide affinity column equilibrated with 25 mM Tris-HCl/22 mM Na<sub>2</sub>SO<sub>4</sub> (pH 8.7).<sup>9</sup> The affinity gel was washed with the same buffer. The hCA I and CA II were eluted with 0.1 M NaCH<sub>3</sub>COO/0.5 M NaClO<sub>4</sub> (pH 5.6). The absorbance of the protein in the column effluents was determined spectrophotometrically at 280 nm. CO<sub>2</sub>-hydratase activity in the eluates was determined, and the active fractions were collected. The purified enzymes were then stored at 4°C, in order to maintain activity. The active contents of selected drugs are estradiol hemihydrate, dienogest, estradiol valerate, micronized progesterone, and hidroxyprogesterone caproate. The structures of active substances of the drugs are shown in Fig. 1.

### 2.2. Total protein determination

A rate of absorbance at 280 nm was used to monitor the protein in the column effluents. Quantitative protein determination was achieved by absorbance measurements at 595 nm according to the method described by Bradford<sup>10</sup> using the bovine serum albumin standard.

### 2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of enzymes

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in order to verify the purified enzyme. It was carried out in 12% and 3% acrylamide

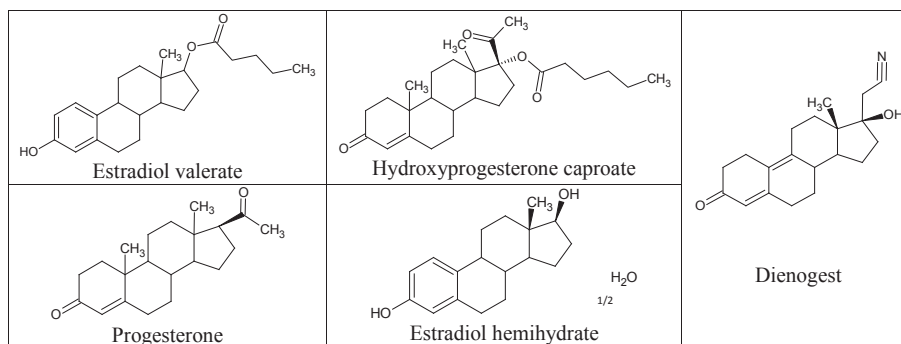


Fig. 1. Structures of the active substances.

concentrations for the running and the stacking gel, respectively, containing 0.1% SDS according to the method described by Laemmli.<sup>11</sup> A 20 mg sample was applied to the electrophoresis medium. Gel was stained for 1.5 hour in 0.1% Coomassie Brilliant Blue R-250 dye (Sigma—Aldrich, Germany) in 50% methanol and 10% acetic acid, and then destained with several changes of the same solvent without the dye.

#### 2.4. CA I and CA II enzyme activities assay

The CA I and CA II enzyme activities were assayed by following the hydration of CO<sub>2</sub> according to the method

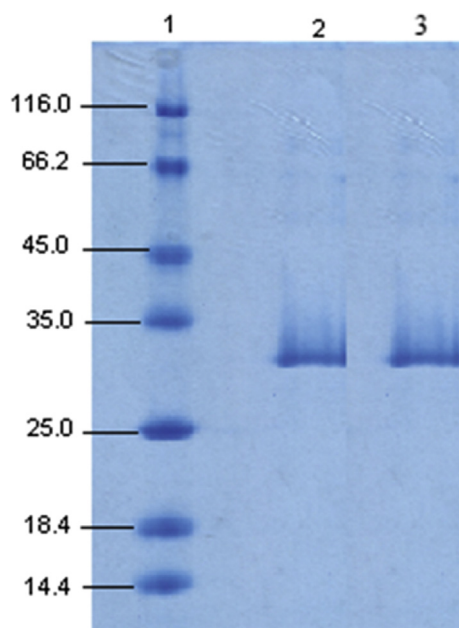


Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of human carbonic anhydrase isozymes. The pooled fractions from affinity chromatography (Sepharose 4-B, L-tyrosine, sulfanilamide) was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (12% and 3%) and revealed with Coomassie Blue staining. Experimental conditions were as described in the Methods. Lane 1 contained 5  $\mu$ g of various molecular mass standards:  $\beta$ -galactosidase (116.0), bovine serum albumin (66.2), ovalbumin (45.0), lactate dehydrogenase (35.0), restriction endonuclease (25.0),  $\beta$ -lactoglobulin (18.4), and lysozyme (14.4). One-hundred microgram of purified human carbonic anhydrase I and II (lane 2 and lane 3) migrated with a mobility corresponding to an apparent Mr 33.0 kDa.

described by Wilbur and Anderson.<sup>12</sup> CO<sub>2</sub>-hydratase activity of the enzyme was determined at room temperature in a 0.15 M Na<sub>2</sub>CO<sub>3</sub>/0.1 M NaHCO<sub>3</sub> (pH 10.0) buffer using phenol red (pH 8.6) as an indicator, and saturated carbon dioxide concentration as substrate in a final volume of 4.2 mL. The duration (in seconds) of the color change from red to yellow in solution was measured in a 10-mL glass tube with 1 cm diameter. The enzyme unit (EU) was calculated using the equation  $(t_0 - t_c/t_c)$ , where  $t_0$  and  $t_c$  are the times for pH changes of the nonenzymatic and enzymatic reactions, respectively.

#### 2.5. In vitro studies for estrogen and progesterone containing drugs

In this study, estradiol valerate, estradiol hemihydrate, micronized progesterone, dienogest, and hydroxyprogesterone caproate were used as drugs. Different concentrations of the drugs (5.13  $\mu$ M; 10.26  $\mu$ M; 15.38  $\mu$ M; 20.51  $\mu$ M; and 25.64  $\mu$ M) were added to the enzyme activity determination medium in 4.2 mL of total volume. Duration (in seconds) of the color change from red to yellow in solution was measured in a 10-mL glass tube with 1 cm diameter. Control cuvette activity in the absence of inhibitor was taken as 100%. All compounds were tested in triplicate at each concentration used. The EU was calculated using the equation  $(t_0 - t_c/t_c)$ , where  $t_0$  and  $t_c$  are the time periods for pH change of the nonenzymatic and enzymatic reactions, respectively. For each inhibitor, an activity%-[inhibitor] graph was drawn (Fig. 2).

### 3. Results

In this study, human erythrocyte CA I and CA II were purified, with a simple one-step method, by using Sepharose 4B-Ltyrosine-sulfonamide affinity gel with the elution buffer 0.1 M NaCH<sub>3</sub>COO/0.5 M NaClO<sub>4</sub> (pH 5.6). Purity of the enzyme was confirmed by SDS-PAGE (Fig. 2). Inhibition or activation effects of drugs on enzyme activity were tested under *in vitro* conditions. Inhibition graphs using the drugs are shown in Fig. 3. The overall purification gave CA in a yield of 31.74% with a specific activity of 5359.48 EU/mg proteins and the overall purification was 259.16-fold (Table 1). IC<sub>50</sub> values were calculated from Activity %-[inhibitor] graphs and are

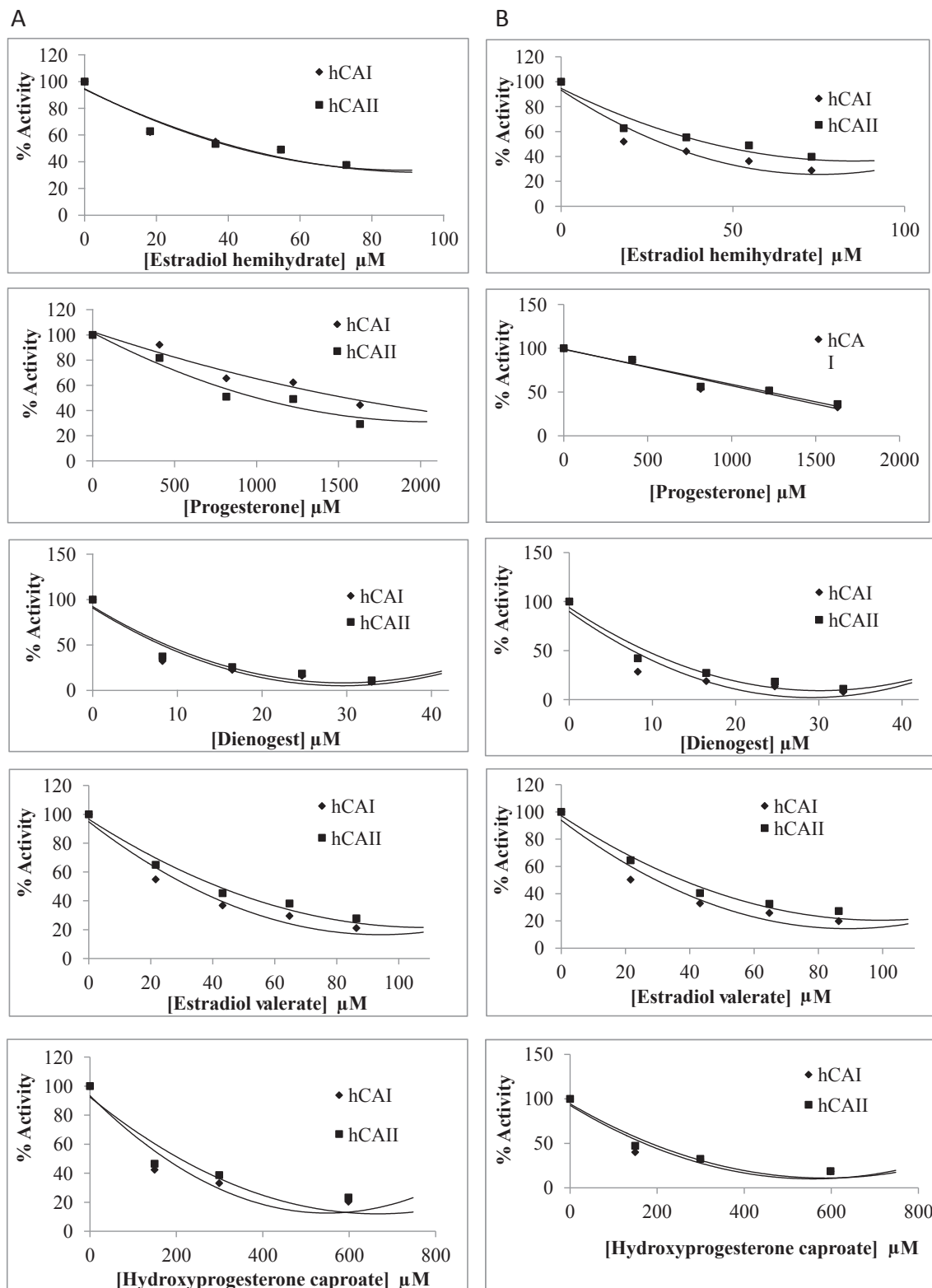


Fig. 3. (A) Nonsmokers. (B) Smokers. hCA I = human carbonic anhydrase I; hCA II = human carbonic anhydrase II.

given in Table 2. CA activity in the absence of a drug was designated as 100% activity. Dienogest has been shown to have the strongest inhibition for CA I and CA II activities in both smoker and nonsmoker women (Fig. 2). Specific

activities of human CA I and CA II for each group were provided in Table 3. It was observed that CA I and CA II enzyme activity was increased in smoker women. All active ingredients have been shown to manifest stronger inhibition in

Table 1  
Summary of the purification of human carbonic anhydrase I and II.

Step	Volume (mL)	Activity (U/mL)	Total activity (U)	Protein amount (mg/mL)	Total protein (mg)	Specific activity (U/mg)	Overall yield %	Overall purification (fold)
Hemolysate	25	41.33	1033.25	1.9980	49.9500	20.68	100.00	—
Affinity chromatography	4	82	328	0.0153	0.0612	5359.48	31.74	259.16

smokers than nonsmokers for CA I activity. It was seen that estradiol valerate and hydroxyprogesterone caproate have stronger inhibition against CA II enzyme activity in women smokers. Smoking has not deteriorated CA II enzyme activity with estradiol hemihydrate, micronized progesterone, and dienogest. Micronized progesterone has the weakest inhibitor activity against CA I and CA II (Table 2).

#### 4. Discussion

In the present study, the effects of the estrogen and progesterone-containing drugs on human erythrocyte CA enzyme activity were investigated in female smokers and nonsmokers. There are various studies in literature about CA enzyme inhibition. But according to our review of the literature, this is the first study that associated smoking and drugs with enzyme inhibition. The results of the current study revealed that human erythrocyte CA I and CA II activities have been inhibited at different levels (Fig. 2). Dienogest was the strongest inhibitor for human CA. However, the inhibitor effect of micronized progesterone was found to be lower than the other drugs.

CAs, a group of metalloenzymes, are involved in numerous physiological and pathological processes. These are gluconeogenesis, lipogenesis, ureagenesis, tumorigenicity, and the growth and virulence of various pathogens. CA enzyme inhibitors are used as antidiuretics and antiglaucoma drugs. In addition to their established roles, CA inhibitors have been

investigated for possible antiobesity, anticancer, and anti-infective roles in recent years.<sup>13</sup>

Many drugs and chemicals at low dosages affect the metabolism by changing normal enzyme activity, especially the inhibition of specific enzymes. Estrogen and progesterone-containing drugs are commonly used in obstetrics and gynecology. OCs are used for birth control, but also have many noncontraceptive benefits. These benefits include improved cycle control and relief from menstrual symptoms, improved acne and hirsutism, improved bone health, and prevention of ovarian, endometrial, and colorectal cancer.<sup>14</sup> Estradiol valerate and estradiol hemihydrate are the components of OCs or hormone replacement therapy. Estradiol valerate is a natural estrogen and recently has been used as a component in new oral OCs formulations.<sup>6</sup>

Dienogest is a new nonethyl progestin, and is also an estrane. In contrast with the ethyl estranes, dienogest is anti-androgenic and has no estrogenic and glucocorticoid activity. It has a high endometrial efficacy, so when used in combination with ethinyl estradiol or estradiol valerate can easily stabilize the menstrual cycle of women.<sup>15</sup> Endometriosis is defined as the presence of functional endometrial tissue outside the uterus, causing diverse progressive symptoms such as infertility, pelvic pain, and dysmenorrhea. Because of its strong endometrial efficacy, dienogest has been used to treat endometriosis in recent years. Dienogest has antiproliferative and anti-inflammatory effects in the treatment of endometriotic lesions.<sup>16</sup> Though it was believed that dienogest has a minimal clinically significant effect on metabolic, lipid, and hemostatic parameters because of its antiandrogenic activity, we determined that dienogest has a marked inhibitory effect on CA I and CA II.

Micronized progesterone is a natural progestagen, and is widely used in the treatment of secondary amenorrhea, premenopausal bleeding disorders, and luteal phase disorders; it is also used for the prevention of preterm labor.<sup>17</sup> Transdermal estrogen and micronized progesterone may be also a choice for hormone replacement therapy.<sup>18</sup> Hydroxyprogesterone caproate is a synthetic progestin and is used for preterm birth prevention in pregnancy.<sup>19</sup> Micronized natural progesterone has a weak inhibitory effect on CA enzymes than hydroxyprogesterone caproate. Therefore it can be a first choice for preventing preterm labor in pregnancy. Micronized progesterone can also be a component choice for hormone replacement therapy in the postmenopausal period.

Tobacco smoke contains large numbers of organic and inorganic compounds. A broad variety of these substances are absorbed from inhaled cigarette smoke. Among these numerous substances, many could be considered as a means to

Table 2  
The IC<sub>50</sub> values of drugs.

Active ingredients	Nonsmokers		Smokers	
	hCA I	hCA II	hCA I	hCA II
	IC <sub>50</sub> μM	IC <sub>50</sub> μM	IC <sub>50</sub> μM	IC <sub>50</sub> μM
Progesterone	1536	1141	1174	1221
Estradiol hemihydrate	43.71	42.82	30.01	43.81
Dienogest	8.14	8.73	7.59	9.21
Estradiol valerate	32.61	41.68	29.48	37.66
Hydroxyprogesterone caproate	180.49	213.25	175.35	178.6

hCA I = human carbonic anhydrase I; hCA II = human carbonic anhydrase II.

Table 3  
The specific activities of human carbonic anhydrase I and II for each group.

	Specific activity (EU/mg)	
	hCA I	hCA II
Nonsmokers	5.34	5.65
Smokers	6.81	6.11



explain an effect on CA activity. One of the best known constituents of cigarette smoke is nicotine. Nicotine as a respiratory stimulant forces CO<sub>2</sub> elimination.<sup>20</sup> CA catalyses the reversible hydration of CO<sub>2</sub> and provides acid–base regulation.<sup>1</sup> Although Abel et al<sup>20</sup> have reported that erythrocyte CA activity was decreased in smokers, we found that CA I and CA II activity were increased in women smokers. This increased level of CA can be attributed to increased CO<sub>2</sub> levels in smoker women for adaptation to acute influences on the acid–base balance. According to our results, CA enzymes, especially CA I enzyme, were inhibited by estrogen and progesterone-containing drugs in smoker women with lower doses than nonsmokers. Therefore, it can be said that smoking is a risk factor for the enzyme inhibition degree of these drugs, and side effects of the drugs can be increased by smoking.

The effects of various drugs on human CA enzymes were investigated in previous studies. In one particular *in vitro* study, the effect of antipsychotic drugs on the CA I and CA II isoenzymes were investigated.<sup>21</sup> Their results showed that all of the antipsychotic drugs inhibited CA enzyme activity. Among such drugs, aripiprazole and pramipexole were found to be the strongest inhibitors of CA I (IC<sub>50</sub>: 3.64 mM and 5.37 mM) and CA II (IC<sub>50</sub>: 4.16 mM and 4.81 mM) activity. In the current study, dienogest was the strongest inhibitor. IC<sub>50</sub> values of dienogest were found to be 8.14 μM and 8.73 μM in nonsmokers; 7.59 μM and 9.21 μM in smokers for CA I and CA II, respectively. Ottlecz et al<sup>22</sup> have shown that ampicillin sulfate inhibits human CA enzyme. The IC<sub>50</sub> value of this work is 385 μM for human CA I and 774 μM for human CA II. In another study, *in vitro* effects of some analgesic drugs on CA enzyme activity were evaluated. Dexketoprofen trometamol has been shown to be the strongest inhibitor against the erythrocyte human CA I and human CA II, whereas diclofenac sodium increased enzyme activities.<sup>23</sup>

Gencer et al<sup>24</sup> investigated the effects of the some anabolic compounds on CA I and CA II enzymes, and the report that 17-β estradiol is the strongest inhibitor against CA I and diethylstilbestrol is the strongest inhibitor against CA II enzyme activity. In our study, dienogest, a synthetic new progestin, was found to be the strongest inhibitor against both CA I and CA II enzymes. In another study, researchers evaluated the *in vivo* effects of OCs on CA, paraoxonase, and catalase enzyme activities in a mouse model; they revealed that OCs did not change the erythrocytes CA activity.<sup>3</sup> In the current study we showed the enzyme alteration, but our results could be obtained with additional *in vivo* studies. The previous study in mice used a microgram drug as the designated amount, but in the current study a micromolar drug was used. It was determined that there was an enzyme inhibition in both studies. The earlier study was made by our group in our laboratory. However, the drugs used in this study are different. In the present study, instead of combined estrogen and progesterone regimens we used pure estrogen or progesterone. Our results contain human erythrocyte CA enzyme alterations. This is because it is apparent that every experimental model does not reflect human physiology identically.

There are some limitations of our study. Due to the necessity of high blood volume for each patient, the IC<sub>50</sub> value could not be calculated for each patient. The blood from 30 patients was used to create a pool for purifying CA. Only one IC<sub>50</sub> value for 30 patients was calculated in each group so statistical analysis could not be performed. However, there are different articles in the literature which describe the use of this method.<sup>21–26</sup>

In conclusion, according to the authors' knowledge, this is the first study which associated smoking and estrogen and progesterone-containing drugs with enzyme inhibition. In this study, dienogest was the strongest inhibitor against human CA I and human CA II enzyme activity. All active ingredients have been shown to have a stronger inhibition against smokers than nonsmokers for human CA I activity. The results of the current study gives information about how to consider the possible adverse effects produced as a result of inhibition of CA I and CA II enzyme inhibition when prescribing these drugs, particularly to women who smoke.

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