



Some drugs inhibit *in vitro* hydratase and esterase activities of human carbonic anhydrase-I and II

Deniz Ekinci, Şükrü Beydemir, Zuhâl Alim

Atatürk University, Faculty of Science and Arts, Department of Chemistry, 25240 Erzurum, Turkey

Correspondence: Şükrü Beydemir, e-mail: beydemir@atauni.edu.tr

Abstract:

In this study, we determined the *in vitro* inhibitory effects of ceftriaxone sodium, imipenem and ornidazole on hydratase and esterase activities of human erythrocyte carbonic anhydrase-I and II isozymes (CA I and II). Human erythrocyte CA I and II isozymes were purified by Sepharose-4B L-tyrosine affinity chromatography column with a yield of 30% and 40%, a specific activity of 920 and 8,000 EU/mg protein, respectively. In the overall purification procedure, human carbonic anhydrase (hCA)-I and (hCA)-II were purified 104 and 900-fold, respectively. In order to determine the purity of the enzymes, SDS-PAGE was performed. Inhibitory effects of the drugs on hCA-I and hCA-II were determined by using colorimetric method for CO₂-hydratase activity assay and spectrophotometric method for esterase activity assay. P-Nitrophenyl acetate was used as a substrate in the spectrophotometric esterase activity assay. The obtained *IC*₅₀ values (inhibitor concentrations which cause 50% inhibition of *in vitro* enzyme activity) for esterase activity were 1.900, 0.008, 0.318 mM for hCA-I and 2.542, 0.0258, 0.343 mM for hCA-II for ceftriaxone sodium, imipenem and ornidazole, respectively. *IC*₅₀ values for CO₂-hydratase activity were 0.864, 0.00354, 0.131 mM for hCA-I and 1.118, 0.0214, 0.263 mM for hCA-II for ceftriaxone sodium, imipenem and ornidazole, respectively. In conclusion, ceftriaxone sodium, imipenem and ornidazole showed inhibitory effects on human erythrocyte carbonic anhydrase-I and II isozyme activities under *in vitro* conditions.

Key words:

human carbonic anhydrase, erythrocyte, drug

Abbreviation: hCA – human carbonic anhydrase

Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are widespread metalloenzyme family that catalyzes the hydration of carbon dioxide to form bicarbonate and a proton: $\text{CO}_2 + \text{H}_2\text{O} \xrightleftharpoons{\text{CA}} \text{H}^+ + \text{HCO}_3^-$ [18, 32]. The mammalian carbonic anhydrases have generally monomeric structure [13]. Beside physiological functions, the carbonic anhydrase enzymes also catalyze

some non-physiological reactions under *in vitro* conditions [34], for instance, it was observed that the purified enzyme has esterase activity under *in vitro* conditions [38].



Sixteen isozymes of the zinc-binding enzyme have been described that differ in their subcellular localization, catalytic activity and susceptibility to different classes of inhibitors. Some of these isozymes are cytosolic (CA I, CA II, CA III, CA VII and CA XIII), others are membrane bound (CA IV, CA IX, CA XII and CA XIV), two are mitochondrial (CA VA and CA

VB), and one is secreted in saliva (CA VI). Hilvo et al. [17] has reported that CA XV isoform is not expressed in humans or in other primates but it is abundant in rodents and other higher vertebrates. There are also three acatalytic forms called CA-related proteins (CARPs): CARP VIII, CARP X and CARP XI [35]. The CA isozymes play an important role in different tissues [8, 34]. These isozymes differ in terms of tissue-sensitive expression, subcellular localization, kinetic features, and affinity for various inhibitors [32].

Carbonic anhydrases have been purified, characterized and their kinetic properties from a wide range of sources including: human erythrocytes [5], fish gills [6] and fish erythrocytes [1], rat saliva and rat erythrocytes [14], *Plasmodium falciparum* [22], insect [9], bovine bone [31] and bovine leukocytes [15] have been established. In addition, it has been reported that CA was partially characterized from plant, yeast and bacteria [22]. The CAs are present in so many tissues and different isoforms have been attractive enough for scientist to design the inhibitors with biomedical applications. The inhibitors of the enzyme are often used in clinical practice [33]. So, inhibitory and activating effects of many chemicals and drugs on the enzyme were determined in our laboratory and by other scientist. For example, the *in vitro* and *in vivo* inhibitory effects of some sulfonamide derivatives on rainbow trout erythrocyte carbonic anhydrase activity were investigated in a previous study. In addition, Beydemir et al. [4] reported that sodium ampicillin inhibited both human carbonic anhydrase (hCA)-I and hCA-II isozymes, also, magnesium sulfate showed an inhibitory effect. Moreover, many synthesized inhibitory compounds have been tested on CA isozymes [39]. However, we have not found any studies in the literature regarding the *in vitro* inhibitory effects of ceftriaxone sodium, imipenem and ornidazole on human erythrocyte carbonic anhydrase-I and II isozymes.

Ceftriaxone is a third-generation cephalosporin antibiotic. It has excellent antimicrobial activity against Gram-positive and Gram-negative bacteria. This drug is often used for the treatment of serious bacterial infections. For example, it is an important drug for treatment of bacterial meningitis [2, 21]. Imipenem is an intravenous beta-lactam antibiotic. The drug has a broad spectrum of activity against bacteria by inhibiting cell wall synthesis of various Gram-positive and Gram-negative bacteria [26]. Ornidazole, a nitroimi-

dazole derivative, is used for the treatment of susceptible protozoan infections and prophylaxis of anaerobic bacterial infections [37].

In conclusion, we have purified the carbonic anhydrase I and II from human erythrocytes in one-step and examined *in vitro* inhibitory effects of ceftriaxone sodium, imipenem and ornidazole on these important enzymes.

Materials and Methods

Materials

CNBr-activated Sepharose 4B, protein assay reagents, and chemicals for electrophoresis were obtained from Sigma-Aldrich Co. (Sigma-Aldrich Chemie GmbH, Germany). Para-aminobenzene sulfonamide and L-tyrosine were from E. Merck (Merck KGaA, Germany). All other chemicals used were of analytical grade and were obtained from either Sigma-Aldrich or Merck. Ceftriaxone sodium, imipenem and ornidazole were provided from the University Hospital Pharmacy (Atatürk University, Erzurum, Turkey).

Hemolysate preparation

Erythrocytes were purified from fresh human blood, which was obtained from the University Hospital Blood Center, and from fresh rat blood (10 ml). Following low-speed centrifugation (1,500 rpm for 15 min) (MSE, MISTRAL 2000) and removal of plasma and buffy coat, the red blood cells were isolated, washed twice with 0.9% NaCl, and hemolyzed with 1.5 volumes of ice-cold water. Ghost and intact cells were then removed by high-speed centrifugation (20,000 rpm for 30 min.) (Heraeus Sepatech, Suprafuge 22) at 4°C and the pH of the hemolysate adjusted to 8.7 with solid Tris.

Affinity chromatography

Sepharose-4B L-tyrosine affinity chromatography column was prepared according to our previous studies [4]. The pH-adjusted human erythrocyte hemolysate (100 ml) was applied to the Sepharose 4B-L-tyrosine-sulfanylamide affinity column pre-equilibrated with 25 mM Tris-HCl/0.1 M Na₂SO₄ (pH 8.7). The

affinity gel was washed with 25 mM Tris-HCl/22 mM Na₂SO₄ (pH 8.7). The human carbonic anhydrase isozymes (hCA-I and hCA-II) were eluted with 1.0 M NaCl/25 mM Na₂HPO₄ (pH 6.3) and 0.1 M NaCH₃COO/0.5 M NaClO₄ (pH 5.6), respectively (flow rate: 20 ml/h, fraction volume: 4 ml).

During purification procedures of hCA-I and hCA-II, the absorbance at 280 nm was measured to monitor protein elution by affinity chromatography. CO₂-hydratase activity was determined in eluted fractions and the active fractions were collected [25, 38].

Hydratase activity assay

Carbonic anhydrase activity was assayed by following the hydration of CO₂ according to the method described by Wilbur and Anderson [38]. CO₂-Hydratase activity was calculated by using the equation

$$\left(\frac{t_0 - t_c}{t_c}\right)$$

where t_0 and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively, and this was used to express enzyme unit (EU).

Esterase activity assay

Esterase activity of human erythrocyte carbonic anhydrase was assayed by following the change in absorbance at 348 nm of 4-nitrophenyl acetate to 4-nitrophenolate ion over a period of 3 min at 25°C using a spectrophotometer (CHEBIOS UV-VIS) according to the method described by Verpoorte et al. [36]. The enzymatic reaction, in a total volume of 3.0 ml, containing 1.4 ml of 0.05 M Tris-SO₄ buffer (pH 7.4), 1 ml of 3 mM 4-nitrophenyl acetate, 0.5 ml of H₂O and 0.1 ml of enzyme solution. A reference measurement was obtained by preparing the same reagents in cuvette without enzyme solution.

Quantitative protein determination

Quantitative protein determination was done by means of measuring the absorbance at 595 nm according to Bradford, using bovine serum albumin as a standard [7].

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was conducted according to our previous studies [18]. The hCA-I and hCA-II isozyme samples were loaded into each slot of the stacking gel (slab gel dimensions: 16 × 18 cm). Initially, a voltage of 80 V was applied until the bromphenol blue reached the running gel. Then it was increased to 200 V for 3–4 h (Hoefer Scientific Instruments SE 600). Gels were stained for 1.5 h in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, and destained with methanol/acetic acid. The electrophoretic patterns were photographed (Fig. 2).

In vitro inhibition studies

The effects of increasing concentrations of ceftriaxone sodium, imipenem and ornidazole on hCA-I and II isozyme activities were determined colorimetrically using CO₂-hydratase assay [3].

The drugs were also tested in the esterase activity assay in triplicate at each concentration used. Different concentrations of drugs were examined in preliminary assays. Enzyme activities were measured at five different cuvette concentrations of ceftriaxone sodium, imipenem and ornidazole. Control enzyme activity in the absence of a drug was taken as 100%.

For each drug an activity % vs. inhibitor concentration curve was drawn using conventional polynomial regression software (Microsoft Office 2000, Excel). Drug concentrations that produced 50% inhibition (IC_{50}) were calculated from graphs.

Results

Human erythrocyte carbonic anhydrase I and II were purified from hemolysate by affinity chromatography on CNBr-activated Sepharose 4B (Fig. 1). SDS-PAGE gels (Fig. 2) revealed that both hCA-I and II isozymes migrated as a single band.

The overall purification yield of hCA-I and II was 30 and 40%, specific activity was 920 and 8,000 EU/mg protein, and these enzymes were purified 104 and 900-fold, respectively, (Tab. 1). Figure 3 and 4 show the *in vitro* effects of ceftriaxone sodium, imipenem and ornidazole on hydratase and esterase activi-

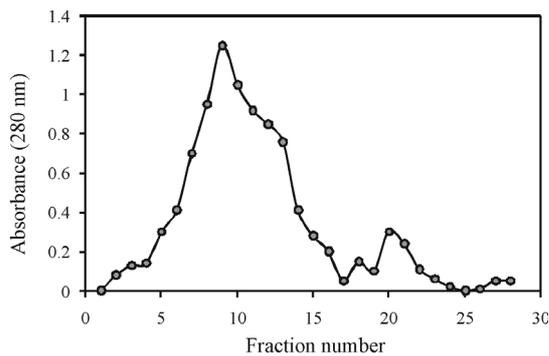


Fig. 1. Elution of hCA-I and hCA-II isozymes from human erythrocytes with 1.0 M NaCl/25 mM Na₂HPO₄ (pH 6.3) and 0.1 M CH₃COONa/0.5 M NaClO₄ (pH 5.6), respectively. Flow rate: 20 ml/h, fraction volume: 3 ml

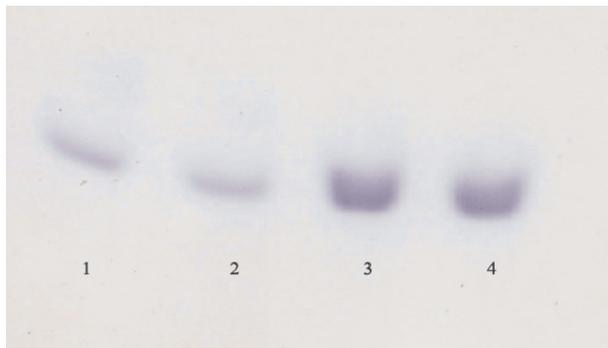


Fig. 2. SDS-PAGE bands of hCA-I and II [Number 1 and 2: hCA-I, Number 3 and 4: hCA-II in SDS-PAGE photograph]

ties of hCA-I. Figure 5 and 6 show the *in vitro* effects of ceftriaxone sodium, imipenem and ornidazole on hydratase and esterase activities of hCA-II. *IC*₅₀ values were determined from activity% vs. drug concentration curves and are given in Table 2.

Discussion

The zinc-binding enzyme family, CAs have important physiological roles in a number of tissues. For example, pH and CO₂ homeostasis, respiration, electrolyte secretion in different tissues/organs and biosynthetic reactions (such as gluconeogenesis, lipogenesis and ureagenesis). In addition, the enzyme has an important role in red blood cells. In all cells, metabolism produces carbon dioxide, which must be removed from the body. For its transport, the red blood cells convert most of carbon dioxide into bicarbonate by means of carbonic anhydrase. After transport, it is converted back into carbon dioxide in order to be exhaled from the lungs [23]. The two major CA isozymes (CA-I and CA-II) are present at high concentrations in the cytosol in erythrocytes, and CA-II has the highest turnover rate of all CAs [24]. Besides, the carbonic anhydrase enzymes also catalyze some non-physiological reactions under *in vitro* conditions [30], for instance, it was observed that the purified enzyme

Tab. 1. Affinity chromatography purification scheme for human erythrocyte carbonic anhydrase-I and II isozymes

Purification step	Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Hemolysate	135	90	15.7	1368	12150	8.88	100	1
hCA-I	368	10	0.4	4	3680	920	30	104
hCA-II	800	6	0.1	0.6	4800	8000	40	900

Tab. 2. *IC*₅₀ values of three drugs for the esterase and hydratase activities of human erythrocytes CA-I and II

Drugs	Hydratase activity of hCA-I	Esterase activity of hCA-I	Hydratase activity of hCA-II	Esterase activity of hCA-II
	<i>IC</i> ₅₀ (mM)	<i>IC</i> ₅₀ (mM)	<i>IC</i> ₅₀ (mM)	<i>IC</i> ₅₀ (mM)
Ceftriaxone sodium	0.864	1.9	1.118	2.542
Imipenem	0.00354	0.0081	0.0214	0.0258
Ornidazole	0.131	0.318	0.263	0.343

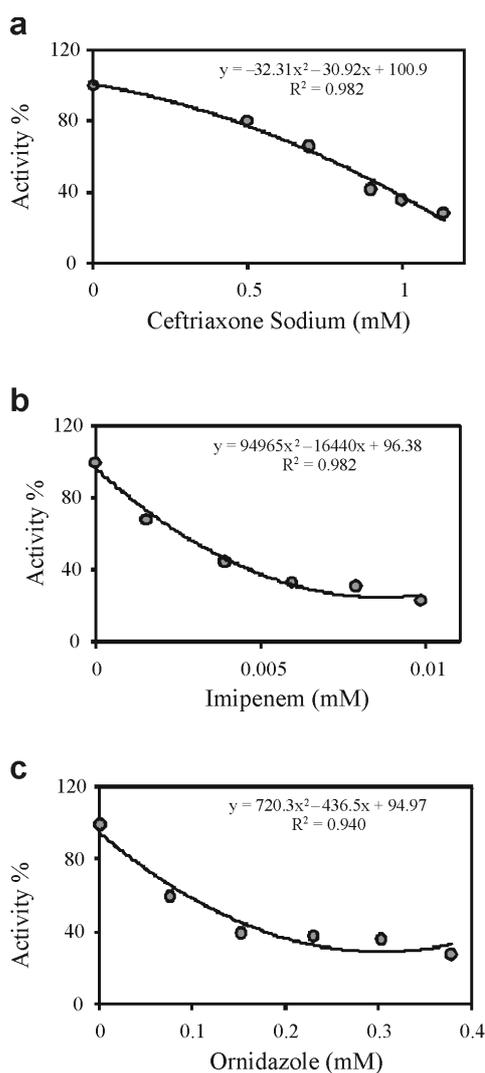


Fig. 3. Effect of the drugs – (a) Ceftriaxone sodium, (b) Imipenem, (c) Ornidazole at different concentrations on CO_2 -hydratase activity of hCA-I

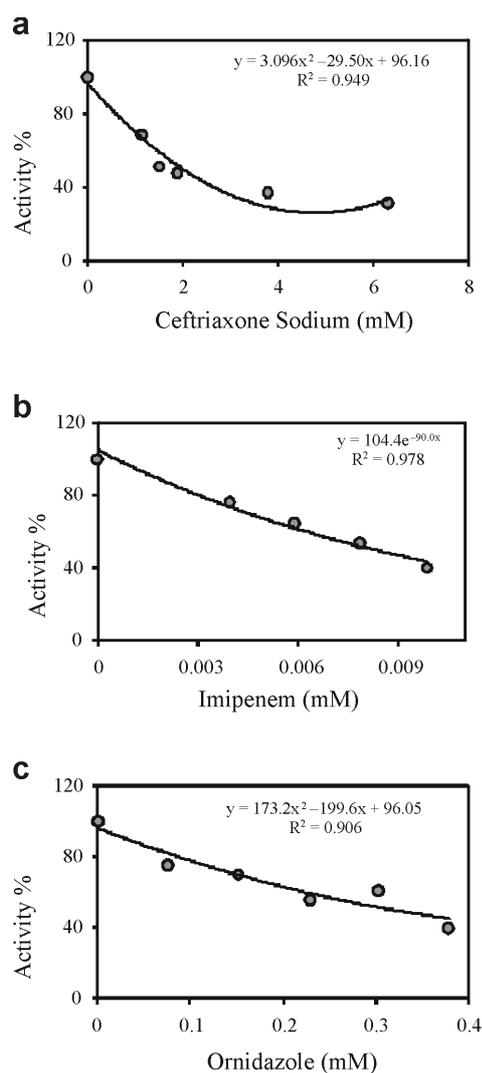


Fig. 4. Effect of the drugs (a) Ceftriaxone sodium, (b) Imipenem, (c) Ornidazole at different concentrations on esterase activity of hCA-I

had esterase activity under *in vitro* conditions [36]. This activity provides an advantage for monitoring.

Carbonic anhydrases have been studied by a lot of scientist up to now. The first studies on the biochemical properties of CAs were published in the 1940s [27–29]. The enzyme has been purified many times from different organisms and investigated for effects of various chemicals, pesticides and drugs on its activity [5, 12, 20]. Inhibitors of CA isozymes have been utilized in the treatment of many diseases particularly in humans. Because of this, the enzyme is the target for many drugs. For example, some sulfonamides, such as acetazolamide, dorzolamide and brinzolamide are also strong inhibitors of CA isozymes

[8]. Those CA inhibitors which reduce intraocular pressure are widely used pharmacological agents for the treatment of glaucoma [10, 11, 16].

Ceftriaxone sodium, imipenem and ornidazole are drugs that are often used for medicinal applications. Ceftriaxone sodium belongs to a group of cephalosporins, imipenem belongs to the subgroup of carbapenems are used as therapeutic antibiotics [21]. Ornidazole is a derivative of nitroimidazole that cures some protozoan (usually microscopic, eukaryotic organisms) infections [21]. Their impacts on hCA activity have not previously been reported. As explained above, given the importance of CA in pH regulation in most tissues, the effects of increasing concentra-

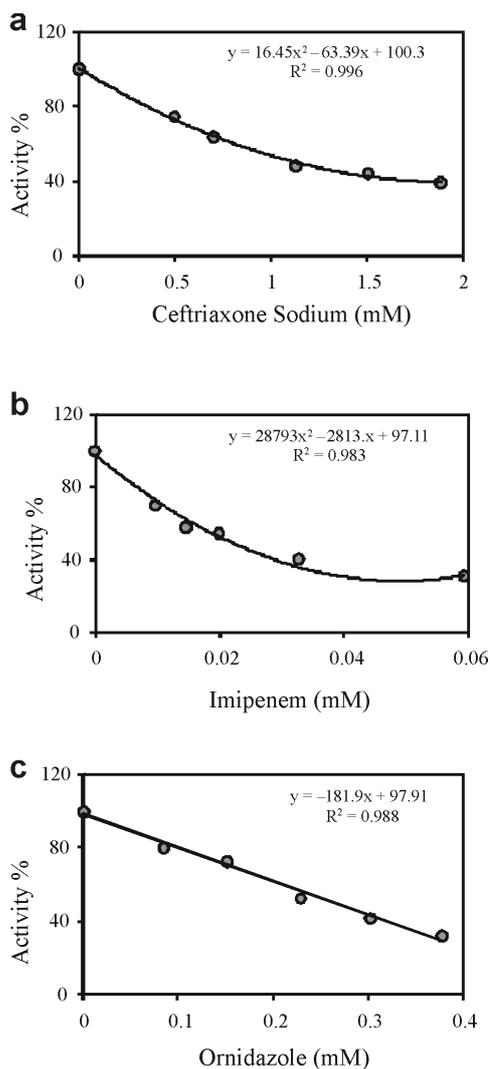


Fig. 5. Effect of the drugs (a) Ceftriaxone sodium, (b) Imipenem, (c) Ornidazole) at different concentrations on CO_2 -hydratase activity of hCA-II

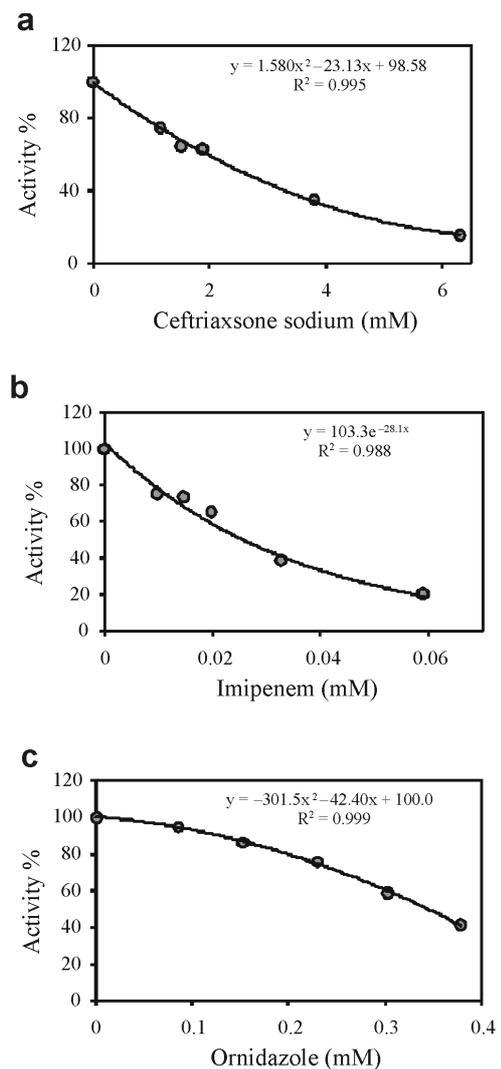


Fig. 6. Effect of the drugs (a) Ceftriaxone sodium, (b) Imipenem, (c) Ornidazole) at different concentrations on esterase activity of hCA-II

tions of drugs on human erythrocyte hCA-I and hCA-II isozymes were investigated in this study.

For this purpose, hCA-I and hCA-II were purified by Sepharose 4B-L-tyrosine-sulfanylamide affinity chromatography (Fig. 1 and Tab. 1) and the purity was confirmed by SDS-PAGE (Fig. 2). hCA-I and hCA-II isozymes were obtained with a yield of 30% and 40%, and a specific activity of 920 and 8,000 EU/mg protein, and these enzymes were purified 104 and 900-fold, respectively (Tab. 1). Activities of hCA-I and II isozymes were measured by CO_2 -hydratase activity assay, physiological function of CA in living organisms, and esterase activity assay, a non-physiological function of CA under *in vitro* conditions. IC_{50}

values of the drugs were determined by means of these methods. If the IC_{50} values of the drugs for CO_2 -hydratase activity of hCA-I were compared, it could be seen that imipenem showed inhibitory effect approximately 100-fold higher than other drugs. For esterase activity of hCA-I, imipenem showed inhibitory effect approximately 1,000-fold higher than ceftriaxone sodium and 100-fold higher than ornidazole. When the IC_{50} values of the drugs for hCA-II CO_2 -hydratase activity were compared, imipenem showed inhibitory effect approximately 100-fold higher than ceftriaxone sodium and 10-fold higher than ornidazole. For esterase activity of hCA-I, imipenem showed inhibitory effect approximately 100-fold higher than

ceftriaxone sodium and 10-fold higher than ornidazole. As can be seen from the above data, the results obtained from esterase and CO₂-hydratase methods support each other.

In conclusion, human erythrocyte 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 ceftriaxone sodium, imipenem and ornidazole were demonstrated. Ceftriaxone sodium, imipenem and ornidazole at low concentrations showed *in vitro* inhibitory effects on hCA-I and hCA-II activity. Imipenem had the strongest inhibitory effects on hCA-I and II, when compared to the ceftriaxone sodium and ornidazole. However, ceftriaxone sodium and ornidazole inhibited hCA-I and II at low concentrations. Because of this, uncontrolled usage of these drugs can cause serious side effects and can be deleterious to health. For this reason, these drugs must be used carefully and the dosage should be closely monitored to decrease side effects.

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Received:

March 2, 2007; in revised form: September 24, 2007.