Effects of Omeprazole, Famotidine, and Ranitidine on the Enzyme Activities of Carbonic Anhydrase from Bovine Stomach in Vitro and Rat Erythrocytes in Vivo

Yaşar Demir, Hayrunnisa Nadaroğlu, and Nazan Demir

Department of Chemistry, Faculty of Education, Atatürk University; Department of Food Technology, Oltu Technical School, Atatürk University; Department of Chemistry, Faculty of Science, Atatürk University; 25240, Erzurum-Turkey.

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In this study, the effects of omeprazole, famotidine, and ranitidine on bovine stomach carbonic anhydrase (EC 4.2.1.1.) isoenzymes have been investigated in vitro. Bovine stomach carbonic anhydrase (CA) was purified from four different cell localisations of bovine stomach using affinity chromatography by Sepharose 4B–l-tyrosine sulphanilamide. The inhibition or activation effects of three different medical drugs on CA isoenzymes were determined using esterase activity and the CO2–hydratase method by plotting activity % vs. [medical drug]. The \( K_i \) values for omeprazole, famotidine, and ranitidine were determined in all localization CA, respectively. The \( I_{50} \) values of the drugs exhibiting an inhibition effect were found by means of these graphs. It was observed that omeprazole, famotidine, and ranitidine showed inhibition of bovine stomach CA activity. In addition, in vivo studies were performed for these medical drugs in Sprague-Dawley rats. It was demonstrated that CA in erythrocytes was significantly inhibited by these drugs to 3 h.

Key words carbonic anhydrase; stomach; omeprazole; famotidine; ranitidine

Carbonic anhydrase (carbonate hydrolyase, E.C. 4.2.1.1) isoenzymes are a family of zinc metalloenzymes that catalyze the interconversion of \( CO_2 \) and \( HCO_3^- \). The enzyme is abundantly present in mammalian red blood cells and to a lesser extent in different types of tissues and secretory organs. In addition, carbonic anhydrase have been obtained and characterized from plant, yeast and bacteria. The important roles of the enzyme in various cell types have been extensively reviewed. Human and most mammalian red blood cell carbonic anhydrases are known to comprise two isozymes, I and II, however, ruminants and cats have only one isozyme II.

Carbonic anhydrases facilitate the one-for-one electroneutral exchange of \( Cl^- \) for \( HCO_3^- \) and thereby contribute to pH regulation, \( CO_2 \) metabolism, volume regulation, and maintenance of \( Cl^- \) and \( HCO_3^- \) levels. In our stomach lining the play a role in secreting acid, while the same enzymes help to make pancreatic juices alkaline and our saliva neutral. Thus, carbonic anhydrase isozymes perform different functions at their specific locations, and their absence or malfunction can lead to diseased states, ranging from the loss of acid production to stomach failure.

Therefore, we examined the effects of omeprazole, famotidine, and ranitidine on stomach CA and erythrocyte CA, and compared the results for the 3 drugs with each other.

It has been reported that the activity levels of CA isoenzymes in human erythrocytes vary considerably under certain pathological and physiological conditions. Changes in CA activity have been associated with metabolic diseases such as diabetes mellitus and hypertension. It has been reported that the inhibition of CA was found to impair proton secretion into the proximal tubule lumen and thereby decreased bicarbonate reabsorption. At the same time, inhibition of CA was also found to decrease the rate of acidification of urine, producing alkaline urine and eventually metabolic acidosis.

Omeprazole is a specific inhibitor of the \( H^+ \), \( K^+ \)-ATPase or ‘proton pump’ in parietal cells. This enzyme is responsible for the final step in the process of acid secretion; omeprazole blocks acid secretion in response to all stimuli. It has also been shown to be highly effective in healing ulcers which have failed to respond to \( H_2 \)-receptor antagonists, and has been extremely valuable in treating patients with Zollinger–Ellison syndrome. Famotidine and ranitidine are \( H_2 \) blockers. They are available in prescription strength and over the counter. These drugs provide short-term relief, but over doses \( H_2 \) blockers.

In the present study, the in vitro effects of omeprazole on CA isoenzymes purified from bovine stomach and the in vivo effects on the CA enzyme from rat erythrocytes were investigated. Furthermore, in vitro and in vivo studies were performed with famotidine and ranitidine, which are used as \( H_2 \) blockers. Using the \( I_{50} \) values obtained (causing 50% inhibition of enzyme activity), clinically, undesirable side effects on CA activity and body metabolism can be diminished.

MATERIALS AND METHODS

Materials Sepharose 4B, protein assay reagents, and chemicals for electrophoresis were obtained from Sigma Chem. Co. Sulfanilamide and l-tyrosine were purchased from E. Merck. All other chemicals used were analytic grade and obtained from either Sigma or Merck. The test drugs were provided by a research hospital affiliated with Atatürk University.

Purification of Carbonic Anhydrase from Bovine Stomach by Affinity Chromatography The bovine stomach samples were obtained during the slaughter of animals at the Erzurum Municipal Slaughterhouse and were rinsed in a solution of 0.9% NaCl. Each pieces of stomach was cut into small pieces and washed with physiological saline.

Outer Peripheral CA: The washed samples were mixed with 1 M KCl for 2.5—3 h at room temperature in order that to remove the outer peripheral proteins. Mixture was then
centrifuged for 20 min at 20000 rpm at 4 °C in Heraeus Sepa-tech Suprafuge, and the precipitant was saved for purification of cytosolic CA at 4 °C.

The outer peripheral CA was purified from the supernatant. For this purpose, the pH of the supernatant was adjusted to 8.7 with solid Tris. This solution containing outer peripheral CA was purified by affinity column.(19)

Cytosolic CA: The precipitant obtained above was suspended within 0.05 m Tris buffer at pH: 7.4. The suspension was frozen and thawed using liquid nitrogen in order to break the inorganic matrix of the cell and disturbing. The suspension was homogenized to break the cell membranes in 0.05 m Tris buffer at pH: 7.4 by a Sonic Disembrator for 4 h. The resultant content was filtered and centrifuged (20000 rpm, 20 min). The precipitant was saved for purification of inner peripheral CA at 4 °C. The pH of the solution was adjusted to 8.7 with solid Tris, and it was applied to affinity column.(19)

Inner Peripheral CA: The supernatant was mixed in the presence of 1 m KCl for 2.5—3 h at room temperature, and the content was centrifuged (20000 rpm; 10 min). The precipitant was saved for purification of integral CA. The pH of the supernatant was adjusted to 8.7 with solid Tris and then was applied to the affinity column.(19)

Integral CA: The precipitant was suspended with 0.05 m Tris–SO₄, pH: 7.4, and Triton X-100 (%1) was added to the suspension to dissolve the integral proteins. The final solution was homogenized using a Sonic Disembrator for 4 h. It was then centrifuged (20000 rpm, 1 h). The supernatant was dialyzed against distilled water to remove the Triton X-100 and then against 0.05 m Tris–SO₄ (pH: 7.4). The pH of the dialysate was adjusted to 8.7 with solid Tris before application to affinity column.(19)

Outer peripheral, inner peripheral, cytosolic and integral CA from bovine stomach were purified by an affinity column containing Sepharose-4B–L-tyrosine-sulfanilamide, as previously described.(19) The column was balanced with 25 mM Tris–HCl/0.1 m Na₂SO₄ (pH: 8.7). The enzyme solution was applied and the enzyme molecules bound to the column. The column was washed with 400 ml of 25 mM Tris–HCl/22 mM Na₂SO₄ (pH: 8.7) in order to remove the other proteins and substances. The enzyme was then eluted with 0.1 m CH₃COONa/0.5 m NaClO₄ (pH: 5.6). The elution procedure was followed by 280 nm absorbance tracing and then the column was rebalanced.

Measurement of CA Activity Carbonic anhydrase activity was assayed by two different methods. First, hydration of CO₂ was measured by the method of Wilbur and Anderson as modified by Rickli et al. In this procedure, 2 ml of veronal buffer (pH: 8.2), 0.4 ml of brome thymol blue (0.004%), 0.8 ml of diluted enzyme solution, and 2 ml of a CO₂ solution (saturated at 0 °C) were mixed. The time (tc) interval was determined between the addition of CO₂ solution and the occurrence of a yellow-green colour. The same interval was recorded without enzyme solution (to). The activity was calculated using the formula:

\[ \text{activity} = \frac{(t_o - t_c)}{t_c} \]

Second, the principle of this determination is that the substrate of CA (p-nitrophenol acetate) is hydrolysed to p-nitrophenol and acetic acid. The reaction is detected at 348 nm.

For this procedure, 1.5 ml of a buffered enzyme solution (0.1 ml enzyme + 1.4 ml 0.05 m Tris–SO₄, pH: 7.4) and 1.5 ml of substrate were mixed in a measured cuvette and three minutes later the absorbance was measured (348 nm, 25 °C). A blank measurement was obtained by preparing the same cuvette except that saline was added instead of enzyme solution.(23)

Protein Determination To determined specific activity and purification rate after all subsequent steps. The protein concentration was determined according to the method of Bradford using bovine serum albumine as a standard.(23) The absorbance at 280 nm was used to monitor protein in the column effluents.

In Vitro Studies for Medical Drugs Omeprazole, famotidine, and ranitidine were selected as study drugs. To obtain the Kᵢ value at pH 7.4, the enzyme activity was measured for seven different substrate concentrations at 20 °C by measuring the absorbance at 348 nm. In the media with or without inhibitor, the substrate concentrations were 1.4, 1.2, 1, 0.8, 0.6, and 0.4 mM. Inhibitor concentrations in the reaction medium are given in Tables 2a and 2b. Kᵢ values were calculated from Lineweaver–Burk plots graphs, and the average Kᵢ values were calculated for each drug.(23)

To determine the I₅₀ values of the inhibitors, 5 different volumes (0.1, 0.2, 0.3, 0.4, 0.5 ml) of the drugs at a constant concentration were added to the enzyme activity determination medium (total volume: 4.2 ml) Carbonic anhydrase activity was assayed by following the hydration of CO₂.(21) The % activity % values for outer peripheral, inner peripheral, cytosolic, and integral CA for the five different concentrations of each drug were drawn using regression analysis graphs on a computer.

Carbonic anhydrase activity without a medical drug was accepted as 100% activity. For the drugs having an inhibitory effect, the inhibitor concentrations causing up to 50% inhibition (I₅₀ values) were determined from the graphs.

In Vivo Studies for Medical Drugs Ten units from adult (200—250 g) rats, which were raised for in vivo studies, for each medical drug were selected. The units comprised 0.5 ml blood samples from each rat which were placed into test tubes containing EDTA. Then, for the first group, 8 mg kg⁻¹ tissue omeprazole; and for the second group, 4 mg kg⁻¹ tissue famotidine and 10 mg kg⁻¹ tissue ranitidine were injected intraperitoneally into each rat. Blood sample were taken from each rat at 1, 3 and 6 h after injection. All blood sample were centrifuged at 2500×g for 15 min at 5 °C (HERMLE Z383K) and then the erythrocyte pellet was washed with 0.16 m KCl three times and the supernatant was discarded. One volume of the erythrocyte pellet was hemolyzed in five volumes ice-water. To prepare the hemolysate. Studies were carried out at 4 °C. Carbonic anhydrase activity was assayed by following the hydration of CO₂ according to the method of Wilbur and Anderson as modified by Rickli et al. and analyses of the data obtained were performed using the t-test, and are presented as X ± S.D.

RESULTS AND DISCUSSION

When administered at relatively low doses, many chemicals affect metabolism by altering normal enzyme activity, particularly through inhibition of a specific enzyme. The
As shown in Tables 2a and 2b, the general, cytosolic inner peripheral and integral CA, respectively, were determined on famotidine, and ranitidine were 3.23 and 6.17 μM, respectively.

HCO₃⁻ rapidly mobilize H⁺ ions are transported in large amounts across the membrane. In all these cases, it appears to be the function of the membrane itself is ideally suited for such a task.

Given the importance of CA in pH regulation in stomach membranes, the activity of the control, which did not contain any drug, was determined to be 48777.7 ± 5311.0 EU (g Hb)⁻¹. Drug injection was then performed on the control groups intraperitoneally. The activities of the groups after the drug injection were measured at 1, 3 and 6 h, and the corresponding activities were observed to be 29282.8 ± 1012.0 EU (p < 0.001), 13684.2 ± 646.0 EU (p < 0.05), and 33333.4 ± 613.0 EU (g Hb)⁻¹ (p > 0.05), respectively (Table 3). The greatest inhibition was found 3 h after injection. These results indicated that omeprazole entered into erythrocytes. Thus, in vivo studies related to this drug supported the results of the in vitro studies.

For famotidine, the activity of the control, which did not contain any drug, was determined to be 38333.2 ± 1213.0 EU (g Hb)⁻¹. Drug injection was then performed on the control groups intraperitoneally. The activities of the groups after the drug injection were measured at 1, 3 and 6 h, and the corresponding activities were observed to be 29282.8 ± 342.0 EU (p < 0.001), 22666.7 ± 1520.0 EU (p < 0.05), and 38181.8 ± 2460.0 EU (g Hb)⁻¹ (p < 0.05), respectively (Table 3). The greatest inhibition was found 3 h after injection. These results indicated that omeprazole entered into erythrocytes. Thus, in vivo studies related to this drug supported the results of the in vitro studies.

For ranitidine, the activity of the control, which did not contain any drug, was determined to be 33846.2 ± 1520.0 EU (g Hb)⁻¹. Drug injection was then performed on the control groups intraperitoneally. The activities of the groups after the drug injection were measured at 1, 3 and 6 h, and the corresponding activities were observed to be 25333.4 ± 671.0 EU (p < 0.001), 22000.0 ± 613.0 EU (p < 0.05), and 38000.0 ± 346.0 EU (g Hb)⁻¹ (p < 0.05), respectively (Table 3). The greatest inhibition was found 3 h after injection. These results indicated that ranitidine entered into erythrocytes. Thus, in vivo studies related to this drug supported the results of the in vitro studies.
drug cannot be recommended for this enzyme. Therefore, the use of this drug inhibits the CA activities of outer peripheral, cytosolic, inner peripheral and integral stomach carbonic anhydrase isoenzymes. According to our results [Table 2a and Table 2b], use of this drug inhibits the CA activities of outer peripheral, cytosolic, inner peripheral and integral stomach carbonic anhydrase isoenzymes. Therefore, the use of this drug cannot be recommended for this enzyme.

For normal injections, a 20 mg (5 ml)−1 dosage of famotidine (molecular weight: 337.43 g mol−1) is used intravenously. In this way, it is thought that the drug is homogeneously spread throughout the blood at a concentration of approximately 0.033 mM. According to our results [Table 2], the use of this drug inhibits the CA activities of outer peripheral, cytosolic, inner peripheral and integral stomach carbonic anhydrase isoenzymes. Therefore, the use of this drug cannot be recommended for this enzyme.

For normal injections, a 20 mg (5 ml)−1 dosage of famotidine (molecular weight: 337.43 g mol−1) is used intravenously. In this way, it is thought that the drug is homogeneously spread throughout the blood at a concentration of about 0.012 mM. According to our results [Table 2a and Table 2b], use of this drug inhibits the CA activities of outer peripheral, cytosolic, inner peripheral and integral stomach carbonic anhydrase isoenzymes. Therefore, the use of this drug cannot be recommended for this enzyme.

On the other hand, a 50 mg (5 ml)−1 dosage of ranitidine (molecular weight: 314.41 g mol−1) is used intravenously. By

### Table 2a. $K_i$ Values Obtained from Lineweaver–Burk Plots for Outer Peripheral and Cytosolic CA in the Presence of Three Inhibitors and Six Substrate Concentrations

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>[Solvent] (m)</th>
<th>$K_i$ (m)</th>
<th>Mean value</th>
<th>Inhibition</th>
<th>I₅₀ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Omeprazole</td>
<td>1 × 10⁻²</td>
<td>3.69 × 10⁻⁹</td>
<td>3.25 × 10⁻⁹</td>
<td>Uncompetitive</td>
<td>0.96</td>
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<tr>
<td></td>
<td>1 × 10⁻³</td>
<td>3.26 × 10⁻⁹</td>
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<tr>
<td></td>
<td>1 × 10⁻⁴</td>
<td>2.80 × 10⁻⁹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Famotidine</td>
<td>1 × 10⁻²</td>
<td>2.31 × 10⁻⁶</td>
<td>2.59 × 10⁻⁹</td>
<td>Uncompetitive</td>
<td>0.292</td>
</tr>
<tr>
<td></td>
<td>1 × 10⁻³</td>
<td>3.13 × 10⁻⁶</td>
<td></td>
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<td></td>
<td>1 × 10⁻⁴</td>
<td>2.34 × 10⁻⁶</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ranitidine</td>
<td>1 × 10⁻²</td>
<td>2.89 × 10⁻⁶</td>
<td>2.43 × 10⁻⁶</td>
<td>Uncompetitive</td>
<td>1.924</td>
</tr>
<tr>
<td></td>
<td>1 × 10⁻³</td>
<td>2.38 × 10⁻⁶</td>
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<tr>
<td></td>
<td>1 × 10⁻⁴</td>
<td>2.02 × 10⁻⁶</td>
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</table>

### Table 2b. $K_i$ Values Obtained from Lineweaver–Burk Plots for Inner Peripheral and Integral CA in the Presence of Three Inhibitors and Six Substrate Concentrations

<table>
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<tr>
<th>Inhibitor</th>
<th>[Solvent] (m)</th>
<th>$K_i$ (m)</th>
<th>Mean value</th>
<th>Inhibition</th>
<th>I₅₀ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Omeprazole</td>
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<td>5.79 × 10⁻⁷</td>
<td>5.25 × 10⁻⁷</td>
<td>Uncompetitive</td>
<td>0.872</td>
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<tr>
<td></td>
<td>1 × 10⁻³</td>
<td>5.17 × 10⁻⁷</td>
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<td></td>
<td>1 × 10⁻⁴</td>
<td>4.78 × 10⁻⁷</td>
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<tr>
<td>Famotidine</td>
<td>1 × 10⁻²</td>
<td>5.29 × 10⁻⁶</td>
<td>6.17 × 10⁻⁶</td>
<td>Uncompetitive</td>
<td>0.148</td>
</tr>
<tr>
<td></td>
<td>1 × 10⁻³</td>
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<td></td>
<td>1 × 10⁻⁴</td>
<td>5.06 × 10⁻⁶</td>
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<tr>
<td>Ranitidine</td>
<td>1 × 10⁻²</td>
<td>4.20 × 10⁻⁶</td>
<td>4.59 × 10⁻⁶</td>
<td>Uncompetitive</td>
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<td></td>
<td>1 × 10⁻³</td>
<td>3.38 × 10⁻⁶</td>
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<td>6.21 × 10⁻⁶</td>
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</table>

I₅₀ values obtained from regression analysis graphs for outer peripheral CA and cytosolic CA in the presence of three inhibitors and 3 mM substrate concentrations.

I₅₀ values obtained from regression analysis graphs for inner peripheral CA and integral CA in the presence of three inhibitors and 3 mM substrate concentrations.
assuming homogeneous distribution of this drug in the blood at a concentration of about 0.032 mM, the results indicate that it inhibits the activity of outer peripheral, cytosolic, inner peripheral and integral stomach carbonic anhydrase isoenzymes [Table 2a and Table 2b]. Hence, the use of this drug is undesirable for this enzyme.

The findings suggest that omeprazole, ranitidine and famotidine bind near the active site and disrupts the interactions of the water bound to the zinc ion, blocking the enzyme action. Unfortunately, prolonged use of these drugs can affect the same enzyme present in stomach and other tissues and lead to side effects such as kidney and liver damage.

These observations have been demonstrated by in vitro studies of the outer peripheral, cytosolic, inner peripheral, and integral stomach carbonic anhydrase isoenzymes activities and by in vivo studies after 3 h in Sprague–Dawley rats in which erythrocyte CA activity was shown to be significantly inhibited by these drugs.

REFERENCES