In vitro inhibitory effects of ofloxacin hydrochloride, ampicillin sodium, cefotaxime sodium, and ceftizoxime sodium on purified paraoxonase-1 (hPON1) from human serum

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In this study, hPON1 enzyme was purified from human serum using ammonium sulphate precipitation, DEAE-Sephadex A-50 ion-exchange chromatography and Sephadex G-200 gel-filtration chromatography. It was purified approximately 295.1 fold with 53.9% yield. For purity determination of the enzyme, SDS polyacrylamide gel electrophoresis was used and molecular mass was approximately determined 43 kDa by this method. In addition, we investigated the effects of antibacterial drugs (ofloxacin hydrochloride, ampicillin sodium, cefotaxime sodium, and ceftizoxime sodium) on hPON1 enzyme activity from human serum in vitro conditions. \textit{IC}_{50} values for ofloxacin hydrochloride, ampicillin sodium, cefotaxime sodium, and ceftizoxime sodium were determined to be 2.358, 41.254, 112.108 and 172.414 mM, respectively.

Key words: Paraoxonase, inhibition, ofloxacin hydrochloride, ampicillin sodium, cefotaxime sodium, ceftizoxime sodium.

INTRODUCTION

Ofloxacin (9-fluoro-2, 3-dihydro-3-methyl-10 (4-methyl-1-piperazinyl)-7-oxo-7H pyrido [1, 2, 3-de]-1, 4-benzoxazine-6-carboxylicacid) (Figure 3) is a synthetic fluoroquinolone antibacterial agent. It is one of the most frequently used fluorinated quinolones antibiotics in the world. It inhibits the topoisomerase enzyme which is essential in the reproduction of the bacterial DNA (Tripathy, 2003). Ofloxacin has a broad spectrum of activity against gram-positive, gram-negative aerobic, facultatively anaerobic bacteria, chlamydiae, and some related organisms, such as mycoplasmas or mycobacteria (Xu et al., 2011). It is approved for use in the treatment of gastrointestinal infections, respiratory tract infections and urinary tract infections (Zivanovic et al., 2006).

Ampicillin (Figure 3) is an active \beta-lactam broad-spectrum oral antibiotic that is indicated for respiratory, urinary, and gastrointestinal tract infections and meningitis. It has been used in the treatment of pneumonia, intestinal infections, urinary tract infections, post-operative infection in the soft tissue, otitis media, Haemophilus influenzae, and other infections caused by susceptible microorganisms (Wu et al., 2010).

Cefotaxime sodium (Figure 3) is a drug of the third generation cephalosporin family widely used for the treatment of gram-negative bacteria. It is a broad-spectrum beta-lactam antibiotics and treats several kinds of infections, including those of the skin, bone, stomach, brain, blood, respiratory tract, sinuses, ears, urinary tract, gonorrhea, meningitis, pneumococcal, staphylococcal and streptococcal infections (Chena et al., 2011; Sharma et al., 2012).

Ceftizoxime sodium is chemically designated as mono sodium \(\{6R,7R\}-7-\{(\text{Z})-2-(2-aminothiazol-4-y1)-2-methoxyiminoacetamido\}-3-\text{cephem}-4-\text{carboxylate}\) (Figure 3). Its molecular formula is \(\text{C}_{13}\text{H}_{12}\text{N}_{5}\text{NaO}_{5}\text{S}_{2}\) and molecular weight is 405.39 (Bharath et al., 2007). Ceftizoxime is a third generation cephalosporin antibiotic and is used to reduce the infection caused by both gram-negative and gram-positive bacteria. Ceftizoxime exhibits a broad
Figure 1. SDS-PAGE analysis of purified PON1; lane (A) is standard proteins (kD): Bovine serum albumin (66.000), aldolase (47.500), triosephosphate isomerase (32.000), and soy bean trypsin inhibitor (24.000) and lane B contains a human serum sample.

Figure 2. In vitro effect of antibacterial drugs: (a) ofloxacin hydrochloride, (b) ampicillin sodium, (c) cefotaxime sodium, and (d) ceftizoxime sodium at five different concentrations on paraoxonase-1 activity.
spectrum of activity against bacteria isolated from patients with respiratory and urinary tract infections. It also has more potent activity against gram-negative rods than other oral cephalosporins, when administered intravenously or intramuscularly (Saito et al., 1980).

hPON1 catalyses the hydrolysis of organophosphates, ary1 esters, and lactones (Billecke et al., 2000; Draganov, 2010; Tavori et al., 2008). hPON1 serves as an antioxidant enzyme by protecting Low-Density Lipoproteins (LDL) and High-Density Lipoprotein (HDL) from oxidative stress, which is known to be associated with many vascular diseases, including atherosclerosis (Aviram, 1999). Actually, higher hPON1 activity plays a significant role in the prevention of atherosclerosis (Watson et al., 1995). Epidemiological studies indicate that low hPON1 activity is correlated with increased risk of cardiovascular events and cardiovascular disease (Jarvik et al., 2000).

Due to the epidemics affected by the change in hPON1 activity, inhibition of drugs is vital. If any medication causes a reduction in hPON1 enzyme activity, many vascular diseases including atherosclerosis can occur due to the increased oxidative stress. Indeed, further studies on the inhibitory effects of drugs should be performed, because of the physiological role of hPON1. However, there are many studies regarding the effects of medications on the activity of hPON1 (Alici et al., 2008; Ekinci and Beydemir, 2009a, b; Ekinci and Beydemir, 2010; Isgor and Beydemir, 2010; Senturk et al., 2011).

As can be seen from the aforementioned statements, these drugs are widely used in the treatment of serious bacterial infections. However, it is extremely important regarding the adjustment of patient drug doses. In this study, we used a simple and rapid procedure for purification hPON1 from human serum and investigated the in vitro effects of antibacterial drugs on enzyme activity.

MATERIALS AND METHODS

The materials used in this study, including DEAE-Sepharose A50, Sepharose 4B, 1-naphthylamine, paraoxon, protein assay reagents, and chemicals for electrophoresis, were obtained from Sigma Chemical Company. All of the other chemicals used were of analytical grade and were obtained from either Sigma-Aldrich or Merck. Ofloxacin hydrochloride, ampicillin sodium, cefotaxime sodium, and ceftizoxime sodium were obtained from local pharmaceutical manufacturing companies. For the enzyme activity assays, we used a Chebios UV-VIS spectrophotometer. The peristaltic pump used for enzyme purification was obtained from Ismatec (ISM833), the centrifuge machine was purchased from Herrme Labotechnic, and the electrophoresis system was a BioRad Mini Protean system.

Paraoxonase activity assay

Human serum samples were supplied from the Research Hospital at Ataturk University. hPON1 activity was
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Determined at 25°C with paraoxon (diethyl p-nitrophenyl phosphate) (1 mM) in 50 mM glycine/NaOH (pH 10.5) containing 1 mM CaCl₂. hPON1 assay was based on the estimation of p-nitrophenol at 412 nm. The molar extinction coefficient of p-nitrophenol (ε=18.290 M⁻¹cm⁻¹ at pH 10.5) was used to calculate hPON1 activity (Renault et al., 2006). One enzyme unit was defined as the amount of enzyme that catalyses the hydrolysis of 1 µmol of substrate at 25°C (Mackness and Durrington, 1995). Assays were performed using a spectrophotometer (CHEBIOS UV-VIS).

Ammonium sulphate precipitation

Human serum precipitated with 60 to 80% ammonium sulphate was carried out in our previous studies. The precipitate was obtained after centrifugation at 15,000 × g for 20 min and redissolved in a 100 mM Na-phosphate buffer (pH 7.0).

DEAE-sephadex A50 anion exchange chromatography

At first, the anion exchange column was equilibrated with a 100 mM Na-phosphate buffer (pH 7.0). Then, the enzyme solution, which had been dialyzed in the presence of 1 mM Na-phosphate buffer (pH 7.0) for two hours, was loaded onto the DEAE-Sephadex A50 anion exchange column (3 cm × 30 cm). Later, the chromatography column was washed with a 100 mM Na-phosphate buffer (pH 7.0), and then, elution carried out by an increasing linear gradient of 0 to 1.5 M NaCl. The elution fractions which were collected were checked for enzyme activity at 412 nm. Tubes which displayed the same enzyme activity were combined. All these procedures were performed at 4°C.

Sephadex G-200 gel filtration chromatography

In the first process, the sephadex G-200 column (60 cm × 2 cm) was equilibrated with a 100 mM Na-phosphate buffer (pH 7.0). The fractions obtained from the DEAE-Sephadex A50 anion exchange column were the mixed with glycerol and loaded onto the gel filtration column with the same buffer. Finally, the enzyme solutions were eluted from the sephadex G-200 column. The protein amount (280 nm) and enzyme activity (412 nm) for all tubes was recorded. The tubes which are showed enzyme activity were combined for other kinetic studies.

Protein determination

In the previous studies that were also performed in our laboratory, Protein determination was found spectrophotometrically at 595 nm according to the Bradford method to quantitative protein assay during the purification steps.

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was applied to check that the enzyme was purified according to the Laemmil’s procedure as in previous studies which were conducted in our laboratory. The obtained single band was photographed after electrophoresis.

In vitro studies for the drugs

We examined the inhibitory effects of three antibacterial drugs: Ofloxacin hydrochloride, ampicillin sodium, cefotaxime sodium, and ceftizoxime sodium. All compounds were tested in triplicate for each concentration used. PON activities were measured in the presence of different drug concentrations. Control activity was assumed to be 100% in the absence of an inhibitor. For each drug, a percentage of activity versus drug concentration graph was drawn.

RESULTS

We purified PON1 from human serum using only three procedures, which are ammonium sulphate fractionation (60 to 80%), DEAE-Sephadex anion exchange chromatography and Sephadex G-200 gel filtration chromatography. The enzyme was obtained with a specific activity of 4060 EUxmg⁻¹ proteins and approximately, 295-fold with a yield of 53.9% (Table 1).

Figure 1 shows the SDS-PAGE to determine the purity and molecular Weight (Mw) of PON1. The molecular weight of the purified human serum paraoxonase was found to be 43 kDa, which is in agreement with other studies (Alici et al., 2008; Ekinci and Beydemir, 2009a, b; Ekinci and Beydemir, 2010; Isgor and Beydemir, 2010; Senturk et al., 2011; Rodrigo et al., 2003; Golmanesh et al., 2008).

Antibacterial drugs showed inhibition effects on paraoxonase activity. IC₅₀ values for (a) ofloxacin hydrochloride, (b) ampicillin sodium, (c) cefotaxime sodium, and (d) ceftizoxime sodium were determined to be 2.358, 41.254, 112.108 and 172.414 mM, respectively through activity% versus drug plots (Figure 2 and Table 2).

DISCUSSION

PON1 is a calcium-dependent esterase that hydrolyses...
Table 1. Summary of the PON1 purification procedure.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Activity (EU/ml)</th>
<th>Total volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (EU)</th>
<th>Specific activity (EU/mg)</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>110.7</td>
<td>10</td>
<td>8</td>
<td>80</td>
<td>1100.7</td>
<td>13.76</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation (60 to 80%)</td>
<td>90.5</td>
<td>8</td>
<td>4</td>
<td>32</td>
<td>724</td>
<td>22.6</td>
<td>65.77</td>
<td>1.64</td>
</tr>
<tr>
<td>DEAE-Sephadex A50 anion exchange chromatography</td>
<td>50.2</td>
<td>6</td>
<td>0.1</td>
<td>0.6</td>
<td>301.2</td>
<td>502</td>
<td>41.6</td>
<td>36.48</td>
</tr>
<tr>
<td>Sephadex G-200 gel filtration chromatography</td>
<td>40.6</td>
<td>4</td>
<td>0.01</td>
<td>0.04</td>
<td>162.4</td>
<td>4060</td>
<td>53.9</td>
<td>295.1</td>
</tr>
</tbody>
</table>

Table 2. $IC_{50}$ values.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>$IC_{50}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ofloxacin hydrochloride</td>
<td>2.358</td>
</tr>
<tr>
<td>Ampicillin sodium</td>
<td>41.254</td>
</tr>
<tr>
<td>Cefotaxime sodium</td>
<td>112.108</td>
</tr>
<tr>
<td>Ceftizoxime sodium</td>
<td>172.414</td>
</tr>
</tbody>
</table>

In an in vitro study, gentamycin sulphate and cefazolin sodium decreased PON1 activity (Sinan et al., 2006). In our laboratory studies, various enzyme-drug interaction studies were conducted and have contributed a great deal to the literature (Gulcin et al., 2008a, b). In our laboratory studies, some cardiovascular drugs such as digoxin, metoprolol tartrate, verapamil, diltiazem, amiodarone, dobutamine and methylprednisolone were examined for their in vitro effects on PON1. They had a negative impact on PON1 activity (Isgor and Beydemir, 2010). Pharmacology, including enzyme-drug interaction studies is of vital importance and is becoming increasingly important with each passing day (Ekinci and Beydemir, 2010; Senturk et al., 2011; Gulcin et al., 2008a, b; Robertson, 2007; Coban et al., 2009, 2007; Ciftci et al., 2008).

In this study, we investigated the in vitro effects of antibacterial drugs, such as (a) ofloxacin hydrochloride, (b) ampicillin sodium, (c) cefotaxime sodium, and (d) ceftizoxime sodium on PON1 activity. It is of crucial importance that the antibacterial drugs are potent inhibitors for human serum PON1 (Table 2). The
compounds (a) ofloxacin hydrochloride, (b) ampicillin sodium, (c) cefotaxime sodium, and (d) ceftizoxime sodium have inhibitory effects (Table 2 and Figure 2).

It is known that an adult human has approximately 5 L of the blood in total. Accordingly, blood concentrations of (b) ampicillin sodium, (c) cefotaxime sodium, and (d) ceftizoxime sodium were calculated as 0.135, 0.418 and 0.493 mM, respectively. These values are observed under IC50 values. However, the blood concentration of (a) ofloxacin hydrochloride, was determined to be 1.106 mM, respectively, which is similar this IC50 value. This mechanism can be clarified by in vivo studies. These drugs are widely used for the treatment of serious bacterial infections. As a result, we purified PON1 using three simple purification steps and investigated the in vitro effects of antibacterial drugs on PON1.

REFERENCES


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