ESTERASE-LIKE ACTIVITY OF HUMAN SERUM ALBUMIN TOWARD PRODRUG ESTERS OF NICOTINIC ACID

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ABSTRACT:
The esterase-like activity of human serum albumin (HSA) toward esters of nicotinic acid was investigated under a variety of conditions such as protein concentration, temperature, pH, ionic strength, nature of buffers, and presence of organic solvents. Initial rate constants of hydrolysis of 18 nicotinates in the presence of 50 μM HSA were measured at pH 7.4 and 37°C. The substrates displayed half-lives ranging from less than 15 min (2-butoxyethyl nicotinate) to more than 95 hr (methyl nicotinate). The hydrolysis of tert-butyl nicotinate was too slow to be measurable, whereas 1-carbamoyl methyl nicotinate was stabilized against hydrolysis by the presence of HSA. The rate constants of HSA-catalyzed hydrolysis were well correlated (r² = 0.85; N = 12) with previously published data obtained in human plasma, indicating similar substrate specificities in the two biological preparations. All evidence points to serum albumin as the possible major catalyst of hydrolysis of nicotinate esters in human plasma.

HSA is a major component of blood plasma in approximately 60% of plasma proteins, MW 66,500 (1) and has been found to catalyze the hydrolysis of various compounds such as p-nitrophenyl acetate (2, 3), aspirin (4), cinchomycin imidazole (5), organophosphate insecticides (6), and long- and short-chain fatty acid esters (7). While albumin thus appears as one of the proteins exhibiting esterase activity in serum, it has been suggested that the enzymatic activity displayed by the purified protein might be a result of contamination by other soluble hydrolases (7). However, an intrinsic activity of albumin has been demonstrated under experimental conditions unfavorable to other esterases, e.g. absence of necessary ions (8), heat pretreatment (7), or presence of specific inhibitors (9). Thus, albumin seems to play important pharmacokinetic and physiological roles not only by binding and transporting drugs and endogenous compounds, but also by acting as an esterase. In other words, serum albumin contains both binding site(s) and catalytic site(s) (10).

HSA appears to have one markedly reactive site (11) and/or multiple non-specific catalytic sites (2). Its primary reactive site (the R-site), which corresponds to Sudlow’s site II, is composed of a tyrosine residue (Tyr-411) and a histidine residue (12). The secondary site of HSA (the T-site) makes a modest contribution to the overall esterase activity of the protein (13), and a lysine residue has been suggested as the catalytic center (14). Finally, the U-site appears to be located near to Trp-214.

Albumins from different origins, except ovalbumin, display an esterase activity whose importance depends on the animal species; e.g. HSA is more active than bovine serum albumin. Bound fatty acids seem to inhibit the catalytic activity of HSA (7). In contrast, HSA can stabilize some xenobiotics or endogenous compounds against degradation in blood, e.g. melphalan, prostacyclin PG12, and thromboxane A3 (15). It is also established that HSA-catalyzed hydrolysis can be stereoselective (16). Moreover, the enzymatic activity of HSA has even been proposed to measure albumin concentrations (17).

Despite its hydrolytic activity toward a variety of esters, HSA is not considered as a genuine hydrolase. First, its turnover numbers (measured by the appearance of the acid product) are too slow for a genuine hydrolase but classify HSA as having an “esterase-like activity.” Second, no proof exists for the presence in HSA of the genuine hydrolase but classify HSA as having an “esterase-like activity.”

A previous study from our laboratory has established the kinetic parameters for the hydrolysis by human plasma of a series of ester prodrugs of nicotinic acid (19). Binding studies to HSA gave indirect evidence for hydrolysis (20). Therefore, the present study was undertaken to demonstrate and characterize the esterase-like activity of HSA toward nicotinate esters. A newly developed, continuous spectrophotometric assay using a pH indicator was used to monitor the reaction (21). A correlation between hydrolyses in human plasma and in HSA solutions confirmed the contribution of this protein to the overall metabolic capacity of plasma.

Materials and Methods

Chemicals and Reagents. All chemicals were of analytical grade. The solutions were prepared with demineralized and purified water obtained with the system Seralpur Pro 90 C (Seral, E. Renggli AG, Rotkreuz, Switzerland). Most nicotinates used in this study were synthesized in our laboratory according to known methods (22). The remaining nicotinic acid esters were supplied by pharmaceutical companies or obtained from commercial sources. The phosphate buffer components, the buffer Tris, dimethylsulfoxide, and acetylcycline chloride purum were purchased from Fluka AG (Buchs, Switzerland). Acetonitrile and methanol were of HPLC grade and supplied by Romil Chemicals (Loughborough, UK). The buffer Hepes-Na was obtained from Merck (Darmstadt, Germany).

The pH indicator BCECF came from Calbiochem Co. (La Jolla, CA). An aqueous solution (10 μg/ml) was prepared and kept at 4°C in the dark.

Essentially fatty acid free human serum albumin (quality A-1887, lot 118P9311) was obtained from Sigma Chemical Co. (St. Louis, MO). A standard solution (33.5 mg/ml) was prepared in a phosphate buffer (pH 7.4 ± 0.1; 6.1 mM; ionic strength 0.173 adjusted with KCl), divided into portions of
0.9 ml and kept at −80°C, so that the same protein solution was used in all experiments. This HSA solution was checked at regular intervals under analysis conditions for which the possible problem of polymerization owing to freezing is negligible and no activity loss was observed during the whole series of experiments.

**Assay Procedures.** The initial rate constants of hydrolysis were determined at 37 ± 0.2°C by a continuous spectrophotometric method using the pH indicator BCECF (21) and a Perkin-Elmer Luminescence Spectrometer LS 50B (Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, UK), according to a previously described method (21). The initial substrate concentration was chosen to obtain a pseudo-first-order reaction and was 1 mM for all substrates in all experiments.

Initial rate constants were determined by linear regression from the semilogarithmic plots of the decay of C_i vs. time, where C_i was the substrate concentration at time t. The standard error on the regression slope in a single hydrolysis experiment was between 0.3 and 1.6%.

The velocities (mmol/min per mg protein) were obtained from the initial linear period (20% of total reaction) by linear regression of the decay of C_i vs. time.

Chemical hydrolyses were performed under the same experimental conditions, replacing the HSA solution with a phosphate buffer. The rate constants of HSA-catalyzed hydrolysis were obtained as total minus chemical hydrolysis.

**Results**

**Esterase-Like Activity of HSA as a Function of Protein Quality and Concentration.** Preliminary studies showed that human serum albumin had significantly greater esterase-like activity than bovine serum albumin. De-fatted albumin was more active in hydrolyzing acetylcholine than untreated albumin. Furthermore, the hydrolytic activity of HSA was found to vary non-negligibly from one batch to the other, HSA being guaranteed by manufacturers for its purity but never for a catalytic activity. Up to 20-fold differences were found between different batches of HSA in their hydrolytic activity toward acetylcholine and 2-butoxyethyl nicotinate. As a consequence, a single batch of HSA was used throughout to obtain comparable results.

Fig. 1 shows initial rate constants of HSA-catalyzed hydrolysis (corrected for chemical hydrolysis) as a function of HSA concentration for two substrates. The rate constants for 2-butoxyethyl nicotinate and phenyl nicotinate showed an apparent linear relation with protein concentrations up to 50 μM (r^2 = 0.986 and 0.998, respectively, N = 4). The curvature observed at higher protein concentrations could be a result of albumin polymerization.

**Esterase-Like Activity of HSA as a Function of pH, Ionic Strength, and Temperature.** Using 2-butoxyethyl nicotinate as a substrate, the influence of pH on the catalytic activity of HSA was investigated. The explorable pH range was limited by the constraints of the method, such that the hydrolysis of substrate produces stoichiometric amounts of protons only at pH values higher than the pKa of the liberated acid (pK_a of nicotinic acid 4.85) (21). As shown in fig. 2, the initial rate constants of hydrolysis catalyzed by HSA were markedly pH-dependent and increased about 5-fold from pH 6.0 to 8.2. The rates of chemical hydrolysis were obviously also affected by pH, a 10-acceleration being observed from pH 6.0 to 8.2.

Increasing ionic strength from 0.06 to 0.173 (by addition of KCl) slightly decreased the rates of HSA-catalyzed hydrolysis (data not shown). All experiments presented here were performed at an ionic strength of 0.173. Temperature also had a marked influence on rates of HSA-catalyzed hydrolysis, as investigated using 2-butoxyethyl nicotinate as a substrate (detailed results not shown). The highest activity was observed between 37° and 42°C, whereas 59 ± 3% and 17 ± 2% of maximal activity were found at 25° and 51°C, respectively.

**Influence of Organic Co-solvents and Chemical Structure of Buffers on the Esterase-Like Activity of HSA.** A number of nicotinates in this study were not sufficiently water-soluble to be examined without using a co-solvent. To assess the influence of co-solvents on enzymatic activity, three organic solvents (MeCN, MeOH, and DMSO) were tested at three concentrations (1%, 2%, and 5%) using three substrates (2-butoxyethyl nicotinate, phenyl nicotinate, and acetylcholine). The general observations (detailed results not shown) were that the effects of these co-solvents were comparable (initial rate constants of hydrolysis of acetylcholine decreased to 85 ± 3, 81 ± 1, and 73 ± 2% with MeOH, MeCN, and DMSO, respectively) at the lowest concentration of 1%, but the difference became more pronounced at the higher concentrations. DMSO and MeOH had the highest and lowest effects, respectively. MeCN had a different influence on each substrate, whereas MeOH shortened the time interval during which hydrolysis followed pseudo-first-order kinetics. DMSO was used as a co-solvent owing to its better solubilizing properties, and its concentration was fixed at 1% (v/v) in all assays.

The nature of the buffer used (phosphate, Tris, or Heps) had no detectable influence on the rates of reaction, excluding an inhibitory effect on the esterase-like activity of HSA (data not shown).

**Hydrolysis of Nicotinate Esters by Human Serum Albumin.** The time course of HSA-catalyzed hydrolysis of some representative nicotinate esters is shown in fig. 3, demonstrating that the reaction followed pseudo-first-order kinetics during an initial period. This allowed initial rate constants to be calculated (table 1). Chemical rate constants were subtracted from enzymatic rate constants when they were larger than the standard deviation of the total reaction (enzymatic plus chemical hydrolysis). The ratios of the rate constants of...
Assays performed at 37°C in phosphate buffer (pH 7.40 ± 0.1, μ = 0.173 adjusted with KCl, DMSO 1%) in the presence of HSA (50 μM). The numbers correspond to the compounds in table 1.

### TABLE 1

<table>
<thead>
<tr>
<th>No.</th>
<th>Nicotinate</th>
<th>k_HSA ± SD (10^{-6} · min^{-1})</th>
<th>Velocities ± SD (10^{-8} · mmol/min per mg HSA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Methyl</td>
<td>1.2 ± 0.17</td>
<td>0.188 ± 0.0082</td>
</tr>
<tr>
<td>2</td>
<td>Ethyl</td>
<td>1.5 ± 0.25</td>
<td>0.16 ± 0.018</td>
</tr>
<tr>
<td>3</td>
<td>n-Propyl</td>
<td>14.8 ± 0.61</td>
<td>1.32 ± 0.046</td>
</tr>
<tr>
<td>4</td>
<td>n-Butyl</td>
<td>56 ± 2.0</td>
<td>5.3 ± 0.27</td>
</tr>
<tr>
<td>5</td>
<td>Isobutyl</td>
<td>29 ± 2.2</td>
<td>2.9 ± 0.17</td>
</tr>
<tr>
<td>6</td>
<td>tert-Butyl</td>
<td>—</td>
<td>0.14 ± 0.033</td>
</tr>
<tr>
<td>7</td>
<td>n-Hexyl</td>
<td>32 ± 1.5</td>
<td>2.9 ± 0.15</td>
</tr>
<tr>
<td>8</td>
<td>2-Methoxyethyl</td>
<td>22.8 ± 0.89</td>
<td>2.19 ± 0.049</td>
</tr>
<tr>
<td>9</td>
<td>2-Butoxyethyl</td>
<td>504 ± 7.1</td>
<td>45 ± 1.0</td>
</tr>
<tr>
<td>10</td>
<td>Carbamoylmethyl</td>
<td>1.8 ± 0.76</td>
<td>0.54 ± 0.043</td>
</tr>
<tr>
<td>11</td>
<td>1-Carbamoyl methyl</td>
<td>—</td>
<td>0.154 ± 0.0021</td>
</tr>
<tr>
<td>12</td>
<td>2-(N,N-diethylamino)ethyl</td>
<td>410 ± 6.2</td>
<td>41.3 ± 0.45</td>
</tr>
<tr>
<td>13</td>
<td>2-Tetrahydrofurfuryl</td>
<td>231 ± 8.8</td>
<td>21.2 ± 0.38</td>
</tr>
<tr>
<td>14</td>
<td>Benzyl</td>
<td>66 ± 2.3</td>
<td>6.7 ± 0.38</td>
</tr>
<tr>
<td>15</td>
<td>2-Phenoxyethyl</td>
<td>39 ± 2.3</td>
<td>3.68 ± 0.021</td>
</tr>
<tr>
<td>16</td>
<td>Phenyl</td>
<td>224 ± 2.0</td>
<td>21.3 ± 0.50</td>
</tr>
<tr>
<td>17</td>
<td>p-Fluorophenyl</td>
<td>51 ± 5.4</td>
<td>4.7 ± 0.44</td>
</tr>
<tr>
<td>18</td>
<td>2-Methoxyphenyl</td>
<td>8 ± 1.0</td>
<td>1.29 ± 0.035</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD (N = 3 or 4).
Velocity data are calculated for total hydrolysis (chemical + enzymatic).

\(^{a}\) Reaction rate too slow to determine k_HSA.

\(^{b}\) Chemical hydrolysis faster than hydrolysis in the presence of HSA.

### Discussion

As reported by a number of authors, the hydrolytic activity of HSA depends not only on the source of the protein, but also on its content in fatty acids (17). To express the activity of HSA quantitatively, it must be ascertained that the initial velocity of the reaction it catalyzes is proportional to its concentration. Here, linearity was proved up to an upper limit of 50 μM, beyond which proportionality was lost (fig. 1).

The HSA solution displayed great differences in catalytic rates among the nicotinic acid esters investigated under fixed conditions of pH, ionic strength, and temperature. However, no structure-metabolism relationships were uncovered using steric, lipophilic, and electronic parameters. This could be an indication for the involvement of multiple catalytic sites on HSA, as concluded by Kurono et al. (30) using an excess of substrate (p-nitrophenyl14-guanidinobenzoate) over HSA. Alternatively, this could indicate a reactive site allowing a variety of binding modes. The ratios of the hydrolytic rates obtained with purified hog liver carboxylesterase (21) vs. those achieved in HSA solution (calculated in mmol/min per mmol protein) vary from 4,000 for 2-butoxyethyl nicotinate up to 900,000 for ethyl nicotinate. These findings clearly differentiate a genuine hydrolase and a protein, such as HSA, having an “esterase-like activity.”

An important finding to emerge from this work is a correlation between the rates of hydrolysis (mmol/min per mg protein) catalyzed by HSA (table 1) and by human plasma for the same substrates (19) (eq. 1 and fig. 4).

\[
\log \text{HSA velocity} = 0.9(±0.13) \times \log \text{Plasma velocity} - 1.5(±0.30)
\]

\[N = 12; \ R^2 = 0.85; \ s = 0.298; \ F = 56 \ (1)\]

A direct comparison of the two sets of data shows that the velocity of hydrolysis in human plasma is about 20 times greater than in HSA solution. This might indicate the contamination of HSA with plasma hydrolases of similar specificity towards nicotinate esters (see below). However, this difference is comparable with that seen between different batches of HSA. More significantly, freeze-drying during the preparation of HSA decreases its proportion of monomers and, hence, its catalytic activity (25,29).
The correlation in eq. 1 is of good statistical quality and accounts for 85% of the variance, indicating similar substrate specificities of HSA and human plasma toward nicotinic acid esters. This suggests either that serum albumin is mainly responsible for the hydrolysis of nicotinic esters in human plasma or that the reaction is a result of both HSA and a contaminant hydrolase with remarkably similar substrate specificity toward nicotinic esters.

The purity of the commercial albumin employed here was greater than 96%, the remainder being mostly globulins (Sigma, quality A-1887). Whereas a contamination by a genuine hydrolase (mainly pseudo-cholinesterase) cannot be excluded, it is compatible neither with the low activity of cholinesterases at pH 7.4 nor with the loss of pseudo-cholinesterase) cannot be excluded, it is compatible neither with the low activity of cholinesterases at pH 7.4 nor with the loss of activity vs. HSA concentration (fig. 1). Thus, all evidence points to HSA as the possible major catalyst of hydrolysis of nicotinic esters in human plasma.

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References