Improved Measurement of Acetylsalicylic Acid Esterase in Serum

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A fixed-time incubation method for measuring acetylsalicylic acid esterase was improved by using a higher concentration of the substrate, acetylsalicylic acid, and an activator, Ca$^{2+}$. The enzymatic activity in serum was 25-fold that measured by earlier methods. Inhibition studies showed a pattern similar to that reported for cholinesterase. Use of cholinesterase inhibitor in sample tubes for determination of acetylsalicylic acid and salicylic acid is recommended.

Additional Keyphrases: aspirin • carboxylic ester hydrolases • cholinesterase inhibitors • enzyme tests • reference interval

Owing to its analgesic, anti-inflammatory, and anti-thrombotic effects, acetylsalicylic acid (ASA, aspirin) is one of the most commonly used drugs. After oral administration, ASA is hydrolyzed to salicylate (SA), either in intestinal fluids, during passage across the gastrointestinal wall, or during the first passage of the drug through the liver (1). The absorption, distribution, and elimination pharmacokinetics of ASA and SA have recently been examined (2).

Acetylsalicylic acid esterase (ASA esterase), a species of carboxylic ester hydrolase (EC 3.1.1) catalyzes hydrolysis of ASA to SA in blood, but methods for measuring ASA esterase (3–6) are poorly optimized.

I have improved this assay by using a higher substrate concentration and adding Ca$^{2+}$ as an activator. As a result the ASA esterase activity measured in normal sera was 25-fold that earlier reported. I also carried out inhibition tests for comparison with similar published studies of other esterases in human serum.

Materials and Methods

Reagents. Acetazolamide, acetylsalicylic acid, echithio- phate, neostigmine bromide, and salicylic acid were of pharmacopoeial quality; the other reagents were of analytical grade.

Buffer. Tris HCl buffer, 300 mmol/L, pH 7.6–7.7 (20 °C), containing CaCl$_2$, 200 mmol/L.

Standard. Salicylic acid, 9.05 mmol/L (1.25 g of salicylic

Received Aug. 8, 1982; accepted Nov. 9, 1982.
Table 1. Protocol for Measurement of Acetylsalicylic Acid Esterase with a Fixed-Time Incubation Method

<table>
<thead>
<tr>
<th>Pipette:</th>
<th>Vol, mL</th>
<th>Final substance concn in reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I) Buffer</td>
<td>1.275</td>
<td>Tris, 225 mmol/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CaCl₂, 170 mmol/L</td>
</tr>
<tr>
<td>(II) Serum (sample)</td>
<td>0.2</td>
<td>vol fraction = 0.13</td>
</tr>
<tr>
<td>Mix and incubate at</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37 °C for 5 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(III) Substrate</td>
<td>0.025</td>
<td>9.25 mmol/L</td>
</tr>
<tr>
<td>Incubate (37 °C, 30 min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(IV) Precipitation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>and color reagent</td>
<td>1.500</td>
<td></td>
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</tbody>
</table>

After mixing, centrifuge (2000 × g, 5 min) and measure absorbance of the supernate at 531 nm vs reagent blank.

Correct for nonenzymatic hydrolysis of ASA during incubation and for the presence of pre-existing salicylate in the samples tested.

Calculation of catalytic concentration:

\[
b = \frac{\epsilon \cdot \ell \cdot \nu \cdot \Delta A}{\Delta t} \text{kat/L} = \Delta A \times 5.24 \mu\text{kat/L}
\]

where:

- \(b\) = enzymatic activity
- \(V\) = reaction volume (L)
- \(\Delta A\) = absorbance change (corrected for spontaneous hydrolysis of the substrate and sample blank)
- \(\Delta t\) = incubation time (1800 s)
- \(\ell\) = cuvette pathlength (m)
- \(\nu\) = sample volume (L)
- \(\epsilon\) = molar absorptivity for SA: 79 500 L × mol⁻¹ × m⁻¹

Acid and 90.5 mL of 0.1 mol/L NaOH, diluted to 1000 mL with distilled water.

Substrate. Acetylsalicylic acid, 555 mmol/L of ethanol (1 g of acetylsalicylic acid in 10 mL of ethanol).

Serum. Samples from healthy subjects and patients were used either immediately after collection or after storing for various intervals to test the stability of the enzyme.

Hemolysates. From five EDTA-stabilized blood samples, erythrocytes were washed three times with isotonic NaCl (centrifugation: 650 × g, 5 min), and hemolyzed at −18 °C for 18–20 h.

Analytical System. The determination of ASA esterase is based on measurement of SA formed in an incubation mixture of buffer, ASA, and sample. The protocol for this fixed-time incubation method is shown in Table 1. Incubation was at 37 °C for 30 min, with a starting ASA concentration of 9.25 mmol/L. Corrections were made for nonenzymatic hydrolysis of ASA during incubation and for the presence of pre-existing salicylate in the samples tested. ASA esterase was activated or inhibited by incubating 200-μL samples with various substances dissolved in the Tris HCl buffer; all such experiments were performed twice. SA was determined according to Trinder (7), by use of an acid mercuric chloride–ferric nitrate solution, which both precipitates the proteins and forms colored ferric salicylate in the supernate in direct proportion to the salicylate released by the enzymatic activity. The color produced was measured at 531 nm with an LKB 2074 calculating absorbimeter. Concentration of SA and absorbance were linearly related up to at least 18 mmol/L (corresponding to an absorbance of 1.9). Maximum velocity (V) and the Michaelis constant (Kₘ) were determined with seven starting concentrations of ASA, ranging from 0.74 to 21.3 mmol/L, by plotting 1/v vs 1/s.

Results

Choice of solvent for the substrate. Ethanol was chosen as the solution medium, because ASA is 60-fold more soluble in it than in aqueous solution and does not spontaneously hydrolyze in ethanol. The small proportion of ethanol (17 mL/L) in the incubation mixture decreased the ASA esterase activity by about 15%, as determined by replacing buffer with increasing amounts of ethanol and extrapolating backwards.

Choice of buffer, pH, and temperature. Tris HCl buffer was preferred because it has no effect on Ca²⁺; a concentration of 300 mmol/L was chosen to keep the pH constant at about 7.4 at 37 °C after addition of the substrate and to allow a pH decrease of <0.10 on formation of salicylate and acetate. If the concentration of Tris HCl buffer was increased to 500 mmol/L to minimize the change in pH, the reagent blank increased threefold.

ASA esterase has a pH maximum at 8.0 at 37 °C (Figure 1). Spontaneous hydrolysis of substrate increases with increasing pH. At pH 8.0 and 8.3 the color of the reagent blank changed from light yellow to orange, sharply increasing the reagent blank. For this reason I preferred the physiological pH value of 7.4.

The temperature of 37 °C was used as standard temperature.

Choice of ASA and CaCl₂ concentrations. Starting concentrations of substrate (ASA) and activator, CaCl₂, in the incubation medium were 9.25 mmol/L and 170 mmol/L (corresponding to 200 mmol/L in the Tris HCl buffer), respectively (Figure 2). With a higher substrate concentration in the incubation medium, spontaneous hydrolysis would exceed the catalytic activity if CaCl₂ was omitted. In addition, a higher concentration of Tris HCl buffer would be needed to keep the pH constant—but then the reagent blank would increase.

With a fixed substrate concentration of 9.25 mmol/L and increasing CaCl₂ concentration in the incubation volume, the activity was greatly increased (Figure 2, insert), almost threefold compared with that without CaCl₂ in the buffer. The Kₘ was calculated to be 6.25 mmol/L and V to be 10.0 μkat/L (Figure 3). The substrate concentration used in the assay gave about 60% of V. Because the aqueous solubility of ASA is only 10 g/L (55.5 mmol/L) at 37 °C, even 10 × Kₘ used as the substrate concentration would be insoluble. During the 30-min incubation the original substrate concentration of ASA decreased by 4–5% owing to spontaneous hydrolysis and by 10–11% owing to enzymatic hydrolysis.
Fig. 2. ASA esterase activity at variable substrate and CaCl₂ concentration in the incubation medium: CaCl₂, 170(●), 85(■), 42.5(▲), 21.2(●), 10.6(△), and 0.3(●) mmol/L; (▼) spontaneous hydrolysis of ASA

Insert: activity of ASA esterase at a substrate concentration of 9.25 mmol/L and different CaCl₂ concentrations

when a serum pool from patients was used. A slight spontaneous hydrolysis continues (about 0.5% per 30 min) after the precipitation and color reagent are added.

Inhibitors. I used various inhibitors of esterase, to compare the inhibition pattern with other known esterases. Cholinesterase inhibitors such as neostigmine and echothiophate totally inhibited ASA esterase in serum at 10⁻⁴ mol/L and 10⁻⁶ mol/L, respectively. LaCl₃ had only a mild depressive effect (10% at 5 mmol/L), whereas acetazolamide had no effect at 1 mmol/L.

Stability. About 0.62 (range 0.54–0.70, n = 10) of the ASA activity was retained after 30 min at 56 °C, and only a slight decrease (1–2%) after two weeks at 4 °C.

Reference interval. ASA esterase activity was determined in serum from 15 ostensibly healthy laboratory workers: in women (n = 9), x̄ = 5.95 μkat/L (range 4.50–6.98 μkat/L); in men (n = 6), x̄ = 6.80 μkat/L (range 4.96–9.65 μkat/L). No sex-related difference was found with the Mann–Whitney rank sum test (p > 0.10). Further ASA esterase activity was measured in hemolyses from five subjects. The mean value was 0.080 μkat/mmol of hemoglobin (range 0.067–0.091 μkat/mmol Hb), corresponding to about 1.1 μkat per liter of hemolysate.

Precision. The same sample assayed on 10 consecutive days gave a CV of 3.2% (x̄ = 4.01 μkat/L, SD = 0.13 μkat/L).

Discussion

The limited aqueous solubility of ASA and the increasing reagent blank with higher concentrations of Tris HCl buffer make it difficult to establish an optimal assay system for ASA esterase, but here these difficulties have been partly overcome. As high absorbances have been obtained for normal sera, the reaction interval can be changed from 30 to 10 min, thus decreasing the percentage of the original ASA that is enzymatically and non-enzymatically hydrolyzed.

In earlier studies concerning ASA esterase determinations (3–5), the substrate concentration in the incubation mixture was considerably lower, 2.0–2.3 mmol/L, and no activator was used. Both 25 °C (3, 4) and 37 °C (5) have been used. Therefore it is not surprising that the present method gave values 25-fold greater for normal sera than those reported by Menguy et al. (4), when their results in micrograms of SA per milliliter of serum per minute are converted into microkatal per liter.

In the present study I found the enzymatic hydrolysis of ASA at 37 °C in serum from patients to be about 20% of the original substrate concentration of ASA per hour in the presence of CaCl₂, 170 mmol/L. It has previously been estimated to be 15–20% per hour at room temperature (2). This hydrolysis in plasma could be stopped by adding a cholinesterase inhibitor, as also reported by other investigators (2). But in whole blood only slight inhibition of ASA esterase activity was observed (2), probably ascribable to an esterase in erythrocytes that is resistant to the inhibitor. The nonenzymatic hydrolysis of ASA at 37 °C in this study was about 10% per hour, but only 2–3% at room temperature (8). But even in the frozen state ASA slowly hydrolyzes to SA because of an acid–base effect (2). Thus, reliable ASA and SA measurements can only be obtained if a cholinesterase inhibitor is present in the sample tubes and the sample is rapidly centrifuged and analyzed.

In several aspects the inhibition pattern for ASA esterase was similar to that reported for cholinesterase (EC 3.1.1.8) (8), but differed from that observed for arylesserases (EC 3.1.1.2) (9). Both ASA esterase and cholinesterase are affected by cholinesterase inhibitors, unlike arylesserases. ASA esterase is inhibited only 10% at 5 mmol of LaCl₃ per liter, but arylesserases is totally inactivated at the same concentration (9). ASA esterase and arylesserases also differ in regard to heat stability. Sixty percent of the activity of ASA esterase remained after 56 °C for 30 min in this study, but only 33% of arylesserase activity was left after 56 °C for 5 min (9).

There are few published methods for measuring ASA esterase in patients, although such determinations may be of clinical value because the biological half-life of SA is conditioned by the half-life of ASA, which in turn largely depends on the activity of ASA esterase (10). In cirrhotic patients the ASA esterase activity was more than halved (4). ASA esterase is present in gastric mucosa, but does not seem to be a significant factor per se in the etiology of chronic peptic ulcer (11); ASA esterase in the serum of such patients was not measured.

I am indebted to Ayerst Laboratories Inc. for kindly supplying echothiophate and to Kirsten Mikkelsen for typing the manuscript.
Radioimmunoassay of Bile Acids in Tissue, Bile, and Urine

H. Jörg Wildgrube, Hannelore Stockhausen, Peter Metz, Gerhard Mauritz, and Raza Mahdawi

Two commercially available (Abbott Labs.) radioimmunoassays for determination of conjugated cholic acid and sulfoglycolithocholic acid in serum have been modified for bile acid measurements in alcoholic tissue extracts, bile, and urine. The specificity of both radioimmunoassays has been determined with regard to 27 free and conjugated bile acids. After filtration, bile acids can be measured in urine and bile without prior extraction. Tissue is homogenized and the bile acids are extracted into methanol. Urinary excretion by 64 healthy humans was 2.09 (SD 1.09) μmol of conjugated cholic acid and 8.44 (SD 8.03) μmol of sulfoglycolithocholic acid per 24 h. In liver from 10 patients with various noncholestatic liver disease, the mean concentration of conjugated bile acids was 32.4 (SD 15.9) nmol/g wet weight. In the liver of 27 male Wistar rats, the concentration of conjugated cholic acid was 41.3 (SD 11.7) nmol/g of tissue, of sulfoglycolithocholic acid 5.1 (SD 2.3) nmol/g of tissue.

**Additional Keyphrases:** sulfoglycolithocholic acid • conjugated cholic acid • rats • “kit” methods

Investigations of bile acid metabolism require a sensitive method, because only small tissue samples are available. Gas-chromatographic and mass-spectroscopic techniques restrict the number of samples that can be assayed because of the time-consuming preparation and the need for highly qualified personnel. Therefore, we have modified two commercially available radioimmunoassay test kits so that they can be used to determine bile acids in alcoholic extracts of various tissues. Under these conditions, bile acid concentrations in biopsies of liver and other sources are measurable. Furthermore, renal excretion of conjugated and sulfated bile acids may be determined in several pathological conditions.

In healthy humans, small amounts of bile acids appear in the urine, but the nature of these bile acids is largely unknown (1, 2). Renal elimination of bile acid becomes quantitatively important in cholestasis and cirrhosis of the liver (3–6).

**Materials and Methods**

**Materials**

The following materials were from commercial sources: sodium salts or free acids of cholyglycine (CG), glycodeoxycholic acid, taurodeoxycholic acid, lithocholic acid, sulfoglycolithocholic acid (SGLC), sulfolithocholic acid, sulfodeoxycholic acid (all from P. L. hydroxyacidic acid, 17-ketosteroid mixture (all from Supelco Inc., Crans, Switzerland), glycolithocholic acid, glycoursoxyacidic acid, tauroursodeoxyacidic acid (all from Calbiochem-Behring Corp., Giessen, F.R.G.), taurochenodeoxyacidic acid, taurocholic acid, lithocholic acid, sulfoglycolithocholic acid (SGLC), sulfolithocholic acid, sulfodeoxycholic acid, sulfodeoxycholic acid (all from P. L. Biochemicals Inc., Milwaukee, WI 53205), hydroxyacidic acid (3α,6α-dihydroxycholanic acid), 3,12-diketocholanic acid, 7,12-diketolithocholic acid, coprosterol (all from Applied Science Europe B.V., Oud-Beijerland, The Netherlands), cholic acid, cholesteryl (both from Serva, Heidelberg, F.R.G.), dehydrocholic acid, 3α-hydroxy-7,12-diketocholanic acid, and 3,7-dihydroxy-12-ketocholanic acid (a gift of Casella AG, Frankfurt/M., F.R.G.).

We also used human albumin solution, 200 g/L (Blutpendienst, Frankfurt/M., F.R.G.), bovine gamma globulin (Serva, Heidelberg, F.R.G.), thimerosal, pulverized charcoal, ethanol, methanol, chloroform, 2-propanol, acetic acid, silica gel H (Merck, Darmstadt, F.R.G.), Millex filter (0.22 μm (Millipore GmbH, Neu Isenburg, F.R.G.), Sterognost 3α-Pho (Nyegaard Co., Oslo, Norway).

**Procedures**

We used the CG and SLCG radioimmunoassays (Abbott Laboratories, Diagnostics Division, North Chicago, IL)