Quantification of Salicylate in Serum by Use of Salicylate Hydroxylase

Kwan-sa You¹,³ and John A. Bittiköfer²

In this enzymatic method for salicylate in serum, *Pseudomonas* salicylate hydroxylase (EC 1.14.13.1) is used. This stable enzyme catalyzes the stoichiometric, unidirectional conversion of salicylate and NAD(P)H to catechol and NAD(P)⁺ in the presence of molecular oxygen. The concentration of salicylate in a clinical sample is determined by measuring the ΔA at 340 nm as compared with a standard. This new method is rapid, highly specific, requires 40 μL or less of sample, and we saw no interference by any of 61 commonly used drugs. Lipemic, icteric, or hemolyzed samples can be used. Furthermore, this method does not involve extraction, deproteinization, or derivatization. Results are precise and agree well with those obtained by the Trinder test.

Numerous analytical methods have been developed for quantification of salicylate in body fluids, especially serum or plasma. Of these, the Trinder test (1) is by far the most commonly used. Unfortunately, this colorimetric method is not very specific; many phenolic (e.g., tyrosine and gentisate) and aliphatic enolic (e.g., acetacetate) metabolites as well as drugs (e.g., phenothiazines) show color reactions similar to that for salicylate (2, and refs. therein). In addition, background values for serum are often high unless salicylate is extracted with an organic solvent. Hence, cautionary reports concerning results obtained by the Trinder test continue to appear (2, 3).

Figure 1 shows the reaction catalyzed by salicylate hydroxylase (salicylate 1-monoxygenase, EC 1.14.13.1) from *Pseudomonas cepacia*. This reaction proceeds irreversibly until one of the reactants is exhausted. Either NADH or NADPH can be used, because the Vₘₐₓ is the same with either of them (5).

![Reaction catalyzed by salicylate hydroxylase from *Pseudomonas cepacia*](image)

The numbers shown under salicylate, NAD(P)H, and catechol are their absorbance maxima, in nm

The concentration of salicylate in a given clinical sample can be determined by measuring the decrease in absorbance of the reaction mixture at 340 nm (ΔA₃₄₀); this value may be converted to salicylate concentration by use of a standard curve (ΔA₃₄₀ vs serum salicylate concentration).

The present method exhibits a high reaction rate and specificity for the substrate (salicylate), characteristic of enzymatic reactions, with no interference from serum proteins. A preliminary report of this work was presented previously (6).

Materials and Methods

Materials. Salicylate hydroxylase was purified from *Pseudomonas cepacia* (ATCC 29351) by affinity-chromatography (7). The reduced pyridine nucleotide coenzymes were purchased from P-L Biochemicals, Milwaukee, WI 53205, sodium salicylate from Aldrich Chemical Co., Milwaukee, WI 53201, and "Salicylate Rapid Stat" kit (the Trinder reagent and salicylate standard solutions) from Lancer Division of Sherwood Medical Industries, St. Louis, MO 63103. Various test drugs and clinical serum samples were provided by Duke University Hospital.

Procedure. The reaction mixture—consisting of NADH (0.05–0.25 mmol/L), 0.04 mL of serum, and enough potassium phosphate (20 mmol/L, pH 7.6) to make the final volume 1.00 mL—is placed in a 1-mL cuvette. Molecular oxygen is already present in the medium at a concentration of about 0.24 mmol/L (5). The initial A₃₄₀ is marked on the recorder chart, and the reaction (at room temperature) is initiated by adding 0.05–0.10 U of salicylate hydroxylase in 5 to 10 μL of the phosphate buffer. The absorbance decrease is recorded until the reaction ends when all salicylate has reacted.

Prepare a standard curve, ΔA₃₄₀ vs sample salicylate concentration (mg/dL), from data obtained by reacting serum samples supplemented with known amounts of salicylate.

The Trinder test was carried out without extraction, according to the procedure furnished with the kit.

Results

Figure 2 illustrates the spectrophotometric tracings for the decrease in A₃₄₀ of reaction mixtures containing serum with various known quantities of added salicylate. When the full recorder scale was set at 0 to 2 A, the sample having a salicylate concentration of 4.70 mg/dL caused a considerable decrease in A₃₄₀ and the absorbance change progressively increased with the increasing salicylate concentration. Generally, the reactions were complete within 2 min. Adding more enzyme shortened the reaction time.

By expanding the recorder scale by fourfold so that the full scale is 0–0.5 A (inset, Figure 2), it could be demonstrated that a sample containing salicylate in concentrations as low as 1.57 mg/dL caused a detectable decrease in A₃₄₀.

Under the conditions specified, we used NADH there was a slight blank reaction (i.e., oxidation of the coenzyme in the absence of salicylate), which resulted in an apparent salicylate concentration equivalent to about 0.2 mg/dL. This reaction is caused by the low inherent NADH
Fig. 2. The spectrophotometric tracings of the absorbance decrease at 340 nm of the reaction mixtures containing 0.25 mmol of NADH per liter (0.05 mmol/L for the reactions in the inset). Other concentrations as described in text (except 50 µM of enzyme in inset). For the experiment illustrated at right, the full recorder scale was from 0 to 2 A; for the inset, it was 0 to 0.5 A.

oxidase activity of the enzyme (5). (Note in Figure 2 that the NADH concentration was reduced to 0.05 mmol/L from the usual 0.25 mmol/L for the scale-expansion experiment.)

The data shown in Figure 2, when plotted, show an excellent linear relationship between the ΔA340 and serum salicylate concentration up to about 70 mg/dL, with a deviation at higher salicylate concentrations because of depletion of dissolved molecular oxygen in the medium. Thus, this procedure can be used to measure salicylate in clinical samples containing less than 70 mg/dL, a value well into the toxic range (8). Samples with higher concentrations must either be diluted or less than 40 µL added to the reaction mixture.

Table 1 presents our precision data. The CVs were consistently <4.5%.

Figure 3, a graphic representation of the least-squares analysis of the enzymatic method against the Trinder test, shows that the individual points are well represented by the straight line and shows the good agreement between results by the two methods. The following least-squares parameters were obtained from the plot: proportion error, −17%; constant error, 0.89 mg/dL; random error, 4.42 mg/dL; and correlation coefficient, 0.97.

Table 2 lists 61 commonly administered drugs, none of which interfered. Addition of salicylate to the mixtures containing these drugs imparted the decrease in A340 immediately. Lipemia, icterus, or hemolysis also did not interfere.

Salicylate hydroxylase is a highly stable enzyme. The purified enzyme did not lose its original activity on storing at either 4 or −20 °C for three weeks, the longest interval tested, nor did seven freeze–thaw cycles affect its activity.

### Table 1. Precision of the Present Method

<table>
<thead>
<tr>
<th>x, mg/dL</th>
<th>SD, mg/dL</th>
<th>CV, %</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within run</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3.6</td>
<td>0.10</td>
<td>3.27</td>
<td>9</td>
</tr>
<tr>
<td>11.84</td>
<td>0.43</td>
<td>3.63</td>
<td>12</td>
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<tr>
<td>21.76</td>
<td>0.85</td>
<td>3.91</td>
<td>11</td>
</tr>
<tr>
<td>32.59</td>
<td>1.07</td>
<td>3.28</td>
<td>12</td>
</tr>
<tr>
<td>50.32</td>
<td>1.29</td>
<td>2.56</td>
<td>9</td>
</tr>
<tr>
<td><strong>Between run</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>12.50</td>
<td>0.55</td>
<td>4.40</td>
<td>8</td>
</tr>
<tr>
<td>22.28</td>
<td>0.80</td>
<td>3.59</td>
<td>8</td>
</tr>
<tr>
<td>31.88</td>
<td>0.63</td>
<td>1.98</td>
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</tr>
<tr>
<td>48.78</td>
<td>0.70</td>
<td>1.44</td>
<td>8</td>
</tr>
<tr>
<td>56.47</td>
<td>0.66</td>
<td>1.11</td>
<td>8</td>
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### Table 2. Drugs Showing No Interference with the Enzymatic Method

<table>
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<tr>
<th>Drug</th>
<th>Digoxin</th>
<th>Morphine</th>
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<tbody>
<tr>
<td>Acetaminophen</td>
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<tr>
<td>Amitriptyline</td>
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<td>Amobarbital</td>
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<td>Dopamine</td>
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<tr>
<td>Aprobarbital</td>
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<tr>
<td>Barbituric</td>
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<tr>
<td>Benztropine</td>
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<tr>
<td>Bromide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butobarbital</td>
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<tr>
<td>Caffeine</td>
<td></td>
<td></td>
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<tr>
<td>Carbazepine</td>
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<td></td>
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<tr>
<td>Chloral hydrate</td>
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<tr>
<td>Chlorodiapoxide</td>
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<tr>
<td>Chlorpheniramine</td>
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<tr>
<td>Chlorpromazine</td>
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<tr>
<td>Clozapine</td>
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<tr>
<td>Cocaine</td>
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<tr>
<td>Codeine</td>
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<tr>
<td>Desipramine</td>
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<td></td>
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<tr>
<td>Desmethyl doxepin</td>
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<td>Diazepam</td>
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</table>

### Discussion

The procedure described here is novel for salicylate analysis, because it utilizes an enzyme-catalyzed reaction.

Because of the simplicity and low cost, the Trinder test is currently by far the most popular choice for salicylate analysis. However, this method is well known to be subject to many interferences, and also gives variable background values if an extraction step is not included. Thus, Mitchel et al. (3) cautioned about the interlaboratory variability of the results obtained by the Trinder test. Kang et al. (2) list 31 common metabolites that gave false-positive results with this colorimetric test; thus results tend to be underestimated in certain clinical situations in which these metabolites increase, such as Reye's syndrome.

The present procedure does not have these drawbacks. None of the 61 drugs we tested exhibited positive reaction (Table 2), nor did they inhibit the enzymatic reaction.
Bilirubin also does not interfere, because icteric serum gave
the expected results.

However, the enzyme does oxidize NAD(P)H in the presence of benzoate, p-hydroxybenzoate, p-aminosalicylate, or any of the four monohydroxy-substituted salicylates, including gentisate (5). Hence, these structural analogs of salicylate may be expected to interfere in this method.

The Trinder method tends to yield higher salicylate values, as evidenced by the noteworthy negative proportion- al error.

Either NADH or NADPH can be used. The former is less expensive, but it causes a greater background reaction than the latter. White-Stevens and Kamin (5) reported that the NADH and NADPH oxidase activities of salicylate hydroxylase were 2-4% and 0.4% of the physiological activity, respectively. Toxicologically as well as therapeutically, the blank reaction associated with NADH can be ignored—or it can be cancelled out by use of a reference mixture containing every component but salicylate, and a double-beam spectrophotometer. Or the blank reaction can be decreased by 80 to 90% by replacing NADH with NADPH.

Salicylate conversion can be directly monitored at 300 nm, the salicylate-specific wavelength (salicylate's absorbance maximum is at 296 nm (5)), but serum proteins also absorb significantly at this wavelength; at 300 nm the absorbance contribution from proteins is relatively small, but still >95% of maximum for salicylate (K. You, unpublished work). The reaction can be monitored at 340 nm with a simple spectrophotometer equipped with a tungsten lamp; monitoring at 300 nm requires use of a deuterium lamp.

The present method can be adapted to automated analyzers capable of monitoring the reactions catalyzed by NAD(P)-linked enzymes. Or salicylate can be quantified by monitoring the oxygen consumption in the reaction with a polarographic instrument such as the Beckman glucose analyzer 2. Results obtained by these procedures will be published elsewhere.

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References