Determination of Salicylate in Blood Serum by Flow Injection with Immobilized Salicylate Hydroxylase

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A flow injection (FI) enzymatic system, based on the use of immobilized salicylate hydroxylase in glass beads, was developed for the determination of salicylate. Salicylate hydroxylase and nicotinamide adenine dinucleotide (NADH) are used to convert salicylate to catechol. The reaction of catechol with 4-aminophenol at high pH yields a colored product which is detected spectrophotometrically at 565 nm. Ten samples of human serum containing from $5.0 \times 10^{-4}$ to $5.0 \times 10^{-3}$ mol/L added salicylate were analyzed and the recovery was determined. Eight additional serum samples containing salicylate were analyzed by the Trinder test and the proposed method. The results obtained with the 2 methods showed good agreement by the statistical Student’s $t$-test. The relative precision of the method is about 3.4% (RSD of the mean recovery). Considering the lowest concentration analyzed, the quantitative limit of detection is about $0.2 \times 10^{-5}$ mol/L ($3 \times SD$). The volume of the sample used was 150 µL. The proposed method was also used to analyze medicines containing acetylsalicylic acid. The results were statistically compared with those obtained through the U.S. Pharmacopoeia procedure and showed excellent agreement.

Acetylsalicylic acid (aspirin), introduced in medicine in 1899, is still one of the most commonly used analgesic, antipyretic, and anti-inflammatory drugs. In a new therapeutic use, based on its antiplatelet aggregation property, aspirin is used to treat cardiovascular complications (1). Most of the absorbed aspirin reaches the systemic circulation as salicylate anion, reaching its maximal level in the blood serum 2 h after ingestion (2). Therefore, plasma levels of salicylate are measured in patients to assess the effectiveness of therapy and to avoid side or toxic effects during long-term treatment.

Short-term analgesic/antipyretic doses of aspirin (25–50 mg/kg per day) produce only relatively low serum salicylate concentrations (30–100 mg/L) and almost never require therapeutic monitoring. In contrast, long-term anti-inflammatory doses of aspirin, which are given primarily to patients with various forms of arthritis, generally require laboratory monitoring to maintain serum salicylate within the therapeutic range, 150–300 mg/L serum. Because the difference between therapeutic and toxic dosages is relatively small, a rapid and specific method is needed to determine salicylate levels in the blood. Severe symptoms can be observed when salicylate levels in serum are >300 mg/L (2, 3).

Various methods for determining salicylate, which have been reported in the literature, make use of a large variety of analytical techniques. High-pressure liquid chromatography and gas–liquid chromatography (4–10), fluorescence (11, 12), and ultraviolet spectrophotometry (13) have been used. The methods are highly sensitive, but many of these techniques require time-consuming sample pretreatment and are not suitable for use in emergency situations where a rapid test is required. Potentiometry analysis using ion-seleitive electrodes (ISEs) has been used to determine salicylate in pharmaceutical samples (14, 15) and blood serum (16). Common problems with the ISEs are interferences from anions normally present in biological fluids. In the clinical laboratory, colorimetric techniques are most popular and, of these, the Trinder test (17) is the most commonly used. The method is based on the formation of a colored complex when salicylate and iron(III) ions are combined. However the purple color in the weakly acid solution is nonspecific. A color reaction is obtained with a wide range of compounds found in body fluids, particularly phenols and aliphatic enols. For a more specific analysis, an enzymatic method is used (18). The hydroxylolation of the salicylate is catalyzed by salicylate hydroxylase (E.C. 1.14.13.1.), and this reaction can be used for analytical purposes. Although the enzymes are expensive and may increase the cost of the analysis, immobilization of the enzyme for repeated use significantly reduces the cost. The immobilization can be done on solid supports which are then packed in reactors (immobilized enzyme reactor; 19) or in close proximity to an electrode surface (20–22).

This study describes a flow injection (FI) system with colorimetric detection for determination of salicylate in serum samples and in medicines. It is based on the generation of catechol by the passage of samples with salicylate and nicotinamide adenine dinucleotide (NADH) through a reactor.
containing salicylate hydroxylase immobilized in controlled porosity glass beads. The enzyme reaction of salicylate with NADH and oxygen, in the presence of salicylate hydroxylase, hydrolyzes salicylate, producing catechol, NAD\(^+\), carbon dioxide, and water (Figure 1). In the enzymatic method described by Chubb et al. (23), where enzyme is not immobilized, salicylate is converted to catechol, which is detected by formation of an indophenol dye with 4-aminophenol under alkaline conditions. The salicylate does not react with the dye. The light absorption of the complex formed is measured spectrophotometrically at 565 nm. Other aspirin metabolites analogous to salicylate, such as gentisic acid and salicyluric acid which are present in the blood at low levels, do not interfere in the assay (23–25). According to Chubb et al. (23), 54 common drugs were tested and only N-acetylcysteine and cysteamine interfered to about 10%.

In June 1998, Brazilians were surprised by an astonishing stream of falsification of medicines detected in contraceptives, prostate cancer medicines, and antibiotics. It was ascertained that the falsification included almost all kinds of pharmaceutical preparations, even very simple formulas such as analgesics and antipyretics. The situation still remains and there is strong evidence that such anomalies also occur in other countries (26, 27). The specificity of the developed method was therefore tested in several pharmaceutical drugs containing acetylsalicylic acid.

**Experimental**

**Reagents**

(a) Salicylate hydroxylase (E.C.1.14.13.1), NADH (reduced form), and sodium salicylate.—Sigma Chemical Co., St. Louis, MO. All other reagents were of analytical grade.

(b) Water.—Distilled in a glass distiller and deionized with a Nanopure System.

(c) Sodium hydroxide solution.—10 g NaOH was dissolved in 1 L deionized water.

(d) Aminophenol solution.—100 mg 4-aminophenol was dissolved in 1 L 0.1 M hydrochloric acid.

(e) NADH solution.—Dissolved in phosphate buffer (0.1 M, pH 7.6) at various levels in relation to salicylate (1:1, 2:1, 3:1). The NADH solution used was always freshly prepared.

(f) Serum samples.—Kindly provided by the UNICAMP University Hospital (Campinas, São Paulo, Brazil).
Enzyme Immobilization

The silanization and subsequent immobilization of the enzyme on the glass beads were performed as described by Zaitsev et al. (28) with some modifications. Glass beads (CPG 240; 0.2 g) were boiled in 10 mL 5% nitric acid for 30 min. The beads were filtered on a glass filter, washed several times with deionized water, and dried in an oven at 95°C. About 19 mL xylene (bp 137°–144°C), 1 mL 3-aminopropyltriethoxysilane, and 0.2 g glass beads were mixed in an Erlenmeyer flask. The mixture was heated for 3 h at 100°C. The glass beads were then washed with xylene and ethanol and dried at room temperature for 12 h. Then a treatment with the cross-linking agent glutaraldehyde (2.5% solution) was performed. The solution was prepared by diluting 25% glutaraldehyde solution in phosphate buffer (0.1M, pH 7.0). About 1 mL of the glutaraldehyde–buffer solution, enough to cover the beads, was added to the prepared alkylamino-glass beads. The reaction was allowed to proceed for 1 h at room temperature. Ten units of the enzyme salicylate hydroxylase were then weighed (0.93 units/mg solid) and dissolved in 3.0 mL cold phosphate buffer (0.1M, pH 7.6) and added to the alkylamino-glass. The mixture was allowed to stand at 4°C for 12 h. The immobilized
enzyme was washed with phosphate buffer and then with cold water to remove any unlinked enzyme. The resulting immobilized enzyme was packed into a polyethylene tube 35 mm long and 3 mm in diameter.

Apparatus and Manifold

(a) Peristaltic pump.—Ismatec mp13 GJ4 (Ismatec S.A., Glattbrugg, Switzerland).

(b) Spectrophotometer.—Zeiss PM2D, equipped with a flow-through cell and connected to a chart recorder (Carl Zeiss, Goettingen, Germany).

(c) Sampling valve.—Described previously (29).
A schematic flow diagram for the colorimetric determination of salicylate is shown in Figure 2.

Blood Serum Procedures

(a) Proposed enzymatic method.—The injected sample, not previously treated, containing salicylate and coenzyme, is combined with the carrier stream, a 0.1M phosphate buffer at pH 7.6 pumped at a flow rate of 0.6 mL/min. The whole mixture is then passed through the enzyme reactor. In the next path the mixture joins with the aminophenol solution and then with the sodium hydroxide solution.

(b) Trinder procedure.—A 10 g portion of Fe(NO₃)₃·9H₂O was dissolved in 1.0 L 0.1M HNO₃. To 0.2 mL sample were added 0.8 mL water and 1.0 mL iron nitrate solution. The mixture was agitated and allowed to stand for 5 min. The light absorbance was measured in the spectrophotometer at 540 nm and compared with a calibration curve prepared in the same way.

Medicines Procedures

(a) Titrimetric determination (30).—A 1.5 g amount of acetylsalicylic acid was exactly weighed, dissolved, and diluted to 50.0 mL with 0.482M NaOH solution. The solution was gently boiled for 10 min, cooled to room temperature, and then titrated with 0.250M H₂SO₄ solution using phenolphthalein as indicator.

(b) Proposed enzymatic method.—A commercial tablet was exactly weighed, i.e., 0.5 g acetylsalicylic acid for the adult dosage and 0.1 g for the infant dosage. The tablet was...
dissolved in ca 50 mL 0.5M NaOH solution, and gently boiled for 10 min. After the solution cooled to room temperature, the pH was adjusted to 7.0–7.8 by dropwise adding a solution of 0.5M H2SO4 and the solution was diluted with phosphate buffer (pH 7.6) according to the indications below. For tablets containing 0.5 g acetylsalicylic acid, the solution was diluted to 250 mL in a volumetric flask with the same buffer solution; 25 mL of this solution was diluted to 100.0 mL with buffer, and this solution was introduced in the FI system. For the infant tablet that contained ca 0.1 g acetylsalicylic acid, after dissolution with 0.5M NaOH, the solution was diluted to 100.0 mL with phosphate buffer. From this solution 10.0 mL was diluted to 50.0 mL with the buffer. This last solution was introduced in the FI system.

The light absorption of the colored species formed was measured at 565 nm and registered on a chart recorder.

### Results and Discussion

To optimize the system, we investigated various parameters. Figure 3 shows that the signal height decreased almost linearly with the increase of the flow rate. As flow rate increases the time of contact of the salicylate with the enzyme decreases; thus less catechol is produced, leading to smaller signals. Slower rates, on the other hand, result in a poor sample throughput. Therefore, as a compromise between signal height and analytical frequency, a flow rate of 0.6 mL/min was chosen for the measurements.

The effect of pH was optimized for best activity of the enzyme. The optimum activity for free salicylate hydroxylase was at pH 7.6 and 30°C (20). However, as the optimum pH could be changed by the immobilization process, the influence of pH on the immobilized enzyme was studied in the range of pH 7.0–8.0 (Figure 4). The best results were found at pH 7.4–7.8. Therefore, pH 7.6 was chosen for the measurements.

The amount of the salicylate oxidized by the enzyme depends on the NADH concentration. The studied molar ratios between the concentrations of NADH and salicylate were 1:1, 2:1, 3:1, and 4:1. To obtain a good analytical signal, a molar ratio of NADH to salicylate equal to 3:1 is needed. Higher molar ratios do not improve the analytical response; therefore, the molar ratio 3:1 is a good compromise between the signal intensity and the cost of the analysis. Figure 5 shows the influence of the NADH/salicylate ratio on the intensity of the analytical signal.

Figure 6 shows the effect of temperature on the reaction rate. The optimum activity of the immobilized enzyme was

| Table 1. Recovery of salicylate from blood seruma |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Samples | Signal height, cm | Salicylate added, 10⁻³ mol/L | Salicylate found, 10⁻³ mol/L | Recovery, % | Salicylate found, 10⁻³ mol/L | Recovery, % |
| 1 | 11.47 ± 0.25 | 5.00 | 4.98 ± 0.14 | 99.5 ± 2.8 | 4.79 ± 0.14 | 95.8 ± 2.8 |
| 2 | 10.76 ± 0.45 | 4.50 | 4.59 ± 0.25 | 101.9 ± 5.6 | 4.49 ± 0.20 | 100.4 ± 4.6 |
| 3 | 9.86 ± 0.21 | 4.00 | 4.08 ± 0.14 | 102.1 ± 3.5 | 4.08 ± 0.10 | 102.1 ± 2.4 |
| 4 | 8.73 ± 0.17 | 3.50 | 3.50 ± 0.14 | 99.9 ± 4.0 | 3.57 ± 0.09 | 102.1 ± 2.7 |
| 5 | 7.63 ± 0.12 | 3.00 | 2.97 ± 0.07 | 99.0 ± 2.5 | 3.08 ± 0.07 | 102.6 ± 2.2 |
| 6 | 6.80 ± 0.22 | 2.50 | 2.58 ± 0.12 | 103.2 ± 5.0 | 2.70 ± 0.12 | 108.0 ± 4.8 |
| 7 | 5.66 ± 0.25 | 2.00 | 2.07 ± 0.13 | 103.3 ± 6.6 | 2.19 ± 0.14 | 109.5 ± 6.9 |
| 8 | 4.06 ± 0.06 | 1.50 | 1.39 ± 0.02 | 92.4 ± 1.5 | 1.47 ± 0.02 | 98.0 ± 1.5 |
| 9 | 3.10 ± 0.10 | 1.00 | 1.00 ± 0.04 | 99.7 ± 3.8 | 1.03 ± 0.05 | 103.0 ± 4.5 |
| 10 | 1.70 ± 0.17 | 0.50 | 0.48 ± 0.07 | 96 ± 14 | 0.40 ± 0.08 | 80 ± 17 |

Mean recovery ± SD of the mean = 99.7 ± 3.4 100.1 ± 8.2

a Flow rate, 0.6 mL/min; pH, 7.6; molar ratio NADH/salicylate, 3:1; sample loop, 150 μL; temperature, 25°C.

b Quadratic calibration curve.

c Straight line calibration curve.

| Table 2. Comparison between values obtained by Trinder and the enzymatic methods, using statistical Student's t-testa |
|-----------------------------|-----------------------------|-----------------------------|
| Samples | Trinder, 10⁻³ mol/L | Enzymatic, 10⁻³ mol/L | Student's t, calculated |
| 1 | 0.47 ± 0.05 | 0.44 ± 0.05 | 0.600 |
| 2 | 0.29 ± 0.06 | 0.24 ± 0.10 | 0.607 |
| 3 | 0.52 ± 0.05 | 0.49 ± 0.04 | 0.664 |
| 4 | 0.47 ± 0.04 | 0.43 ± 0.06 | 0.784 |
| 5 | 0.89 ± 0.05 | 0.81 ± 0.05 | 1.600 |
| 6 | 1.48 ± 0.05 | 1.34 ± 0.06 | 2.679 |
| 7 | 0.34 ± 0.06 | 0.23 ± 0.10 | 1.334 |
| 8 | 1.55 ± 0.04 | 1.49 ± 0.05 | 1.325 |

a Tabulated t value for the degree of freedom, v = 4, is 2.776 (α = 0.05).
Table 3. Comparison between recovery obtained with the U.S. Pharmacopoeial procedure and enzymatic FI proposed method, using the statistical Student’s t-test.

<table>
<thead>
<tr>
<th>Medicine (laboratory)</th>
<th>Nominal content, mg</th>
<th>Recovery, % Pharmacopoeia</th>
<th>Recovery, % FI enzymatic</th>
<th>Student’s t, calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirina (Bayer S.A.)</td>
<td>500.0</td>
<td>101.2 ± 0.8</td>
<td>103.1 ± 1.4</td>
<td>1.666</td>
</tr>
<tr>
<td>Melhoral (Sanofi Winthrop)</td>
<td>500.0</td>
<td>99.9 ± 0.5</td>
<td>99.0 ± 1.3</td>
<td>0.914</td>
</tr>
<tr>
<td>AAS (Sanofi Winthrop)</td>
<td>500.0</td>
<td>101.0 ± 0.8</td>
<td>102.3 ± 1.4</td>
<td>1.140</td>
</tr>
<tr>
<td>AAS Infantil (Sanofi Winthrop)</td>
<td>100.0</td>
<td>102.6 ± 0.9</td>
<td>103.0 ± 2.7</td>
<td>0.199</td>
</tr>
<tr>
<td>Mean recovery ± SD of the mean</td>
<td></td>
<td>101.2 ± 1.4</td>
<td>101.9 ± 2.4</td>
<td></td>
</tr>
</tbody>
</table>

a) Tabulated t value for the degree of freedom, v = 4, is 2.776 (α = 0.05).

b) pH 7.6; temperature, 25°C; flow rate, 0.6 mL/min; salicylate/NADH ratio, 3:1; sample volume, 150 µL.

Table 3 shows the analytical results of medicines containing acetylsalicylic acid. The values obtained with the proposed FI method were compared with those obtained with the U.S. Pharmacopoeia procedure (30). The results obtained with the 2 methods were in agreement as shown by the statistical Student’s t-test (v = 4 and α = 0.05), which corroborates the analytical results of the procedure proposed in this work.

Conclusions

Because of its relative simplicity and low cost, the Trinder test currently is by far the most popular choice for salicylate analysis. However, enzymes are used in a range of analytical methods because of 2 characteristics: the unique specificity of the active site, and catalytic properties (32). The method proposed in this work exhibits a relatively high reaction rate and specificity for the substrate (salicylate), characteristic of enzymatic reactions, with no interference from serum proteins. Furthermore, this method does not involve extraction, deproteinization, or derivatization.

The development and widespread use of immobilized enzymes are motivated by the need to reduce operative and cost constraints. Flow injection with integrated immobilized enzymes combines the advantages of enzymatic analysis and of FI, namely, reagent economy, operational simplicity, good analytical frequency, and specificity. Therefore it is recommended for use in the analysis of clinical samples and pharmaceutical products.

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