A Tableted Enzymic Reagent for Salicylate, for Use in a Discrete Multiwavelength Analytical System (Paramax®)

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A fully enzymic reagent for determination of salicylate in serum has been developed for use in the Paramax® analytical system. The assay, run as an equilibrium determination, is based on the reaction of salicylate with NADPH and oxygen in the presence of salicylate hydroxylase (EC 1.14.13.1) to form catechol, NADP⁺, carbon dioxide, and water. The reaction is complete within 7 min, after which time the resulting absorbance change at 340/405 nm is measured. The sample:reagent ratio is 1:60 (5 μL of sample in a 300 μL final reaction volume). A single 30-mg tablet contains all of the reactants with tableting excipients. The use of NADPH eliminates interferences from reactions involving NADH. The large sample:reagent ratio, high sensitivity, and choice of bichromatic wavelengths minimize sample error. Results are linearly related to salicylate concentration to 1500 mg/L. Precision (CV) is 1.7% at 540 mg/L and 2.4% at 280 mg/L.

Additional Keyphrases: bichromatic analysis • drug assay

Rapid, accurate determination of salicylate is important in accidental or suicidal overdose, particularly in children and in patients being medicated with or who are taking combinations of prescription and nonprescription dosages that may increase salicylate concentrations above the therapeutic range, i.e., up to 200 mg/L. Above 300 mg/L, symptoms of toxicity appear; concentrations in serum exceeding 600 mg/L are usually lethal (1-3).

All of the several methods currently in use (4, 5) have one or more of the following disadvantages: requirement for precipitation and separation of the specimen, interference from other compounds in the sample or high "blank" values for specimens containing no salicylate. A rapid method offering the specificity of an enzymic procedure, which could be performed directly without pretreatment of the specimen, would be an improvement.

In 1962 Katagiri et al. (6) reported an enzyme, salicylate hydroxylase (EC 1.14.1.a), which catalyzed the reaction:

\[
\text{Salicylate + NADH + O}_2 \rightarrow \text{catechol + NAD}^+ + \text{H}_2\text{O} + \text{CO}_2
\]

Later, White-Stevens and Kamin (7-9) described a similar enzyme (EC 1.14.13.1), differing in molecular size, with which either NADP or NADH could be used. You and Roe (10) recently described the purification of this enzyme in quantities sufficient for a salicylate assay, with the advantage of eliminating interferences by other reactions involving NADH.

Materials and Methods

Materials

We used a recording spectrophotometer (Model 250; Gilford Instrument Laboratories Inc., Oberlin, OH 44074) equipped with cuvet temperature control and 1-cm "Thermo-cuvet" cuvets accommodating a 300-μL reaction volume for enzymic assays and combined this with an ABA-100 discrete analyzer (Abbott Laboratories, South Pasadena, CA 91030) for the manual studies. A Gilford 30N microsample spectrophotometer with flow-through cell was used for the manual chemical method. Correlation and precision studies were performed with a Du Pont acu (Du Pont Clinical Systems Division, Wilmington, DE 19898) and a pre-production "breadboard" model of the Paramax analyzer (American Dade, Costa Mesa, CA 92626).

Salicylate hydroxylase was obtained from Mr. Carl Wooten, Duke University, Durham, NC 27706, and β-NADPH (tetrasodium tetrabrate, 99.8%, formula weight 905.41) from U.S. Biochemical Corp., Cleveland, OH. Buffer salts were Mallinckrodt AR grade, obtained commercially. All other reagents were of the highest purity available. Tablet ed salicylate reagent was obtained from American Dade's production material. The same lot was used throughout the study.

Enzymic Assay

Prepare the following reagents: potassium phosphate buffer, 100 mmol/L, pH 7.6, at ambient temperature; sodium salicylate (100 mmol/L), 16.0 mg of 99+% sodium salicylate per milliliter of phosphate buffer (final concentration in assay mixture, 0.99 mmol/L); NADPH (1.5 mmol/L), 1.36 mg of β-NADPH per milliliter of phosphate buffer (final concentration in assay mixture, 0.25 mmol/L).

Monitor the reaction in 1-cm cuvet at 340 nm and 30 °C.

To perform the assay, place 240 μL of buffer in the cuvet. Add 50 μL of NADPH solution, mix, and allow to equilibrate at 30 °C. Add 10 μL of enzyme solution (reaction volume = 300 μL), mix, and record the absorbance change for 5 min. This represents the blank rate. Initiate the reaction by adding 3.0 μL of salicylate solution (final reaction volume = 303 μL) and record the reaction rate for 3 to 5 min. If it exceeds 0.10 A/min, repeat, using a smaller sample volume or a further dilution of enzyme. Make all enzyme dilutions with buffer.

Calculate enzyme activity as follows:

\[
\text{Activity, U/L} = \frac{A_{\text{min}} \cdot 1000 \cdot \text{total reaction vol}}{6.22 \cdot \text{light path (cm)} \cdot \text{sample vol}}
\]

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Repeat the calculation for the blank. The enzyme activity is then: \( \frac{U/L_{\text{sample}} - U/L_{\text{blank}}}{U/L_{\text{enzyme activity}}} \).

**Results**

**Optimization of the Reagent**

Because the reagent is intended to be used in an equilibrium reaction, we therefore optimized the individual constituents accordingly. Limitations included maximum initial reagent absorbance and a desired reaction time of 10 min or less. A 1:100 sample:reagent ratio was maintained throughout the study. Salicylate concentration, where not otherwise specified, refers to the concentration in the sample. All reactions were at 30 °C.

**pH.** Potassium phosphate buffers, 20 mmol/L, of pH 7.0, 7.2, 7.4, 7.6, 7.8, and 8.0 were prepared, and to each was added NADH, to a final concentration of 0.25 mmol/L, and sodium salicylate, to 10 mg/L, representative of the contribution of a sample containing 1000 mg of salicylate per liter. After allowing the temperature to equilibrate, the reaction was initiated by adding enzyme, diluted in the same buffer, pH 7.6, to give an activity in the reaction mixture of 4 U/L. The rate of absorbance change at 340 nm for 5 min, except at pH 7.0, where activity was slightly less, showed that the enzyme is relatively insensitive to pH differences in this range. We chose to use pH 7.6 in the assay.

**Buffer concentration.** Varying the potassium phosphate buffer concentration to 20, 40, 60, 80, and 100 mmol/L, and keeping the pH at 7.6, produced no significant difference in reaction rate. We chose to use 100 mmol/L buffer concentration for its higher buffering capacity.

**NADH and NADPH concentration.** The enzyme can utilize either NADH or NADPH as coenzyme. A contaminating NADH oxidase activity in the enzyme preparation produced a high reagent blank rate with NADH, which was substantially decreased by substituting NADPH. When we compared the two coenzymes in an experiment in which buffer, pH, and salicylate and enzyme concentrations were identical but the concentrations of the NADH or NADPH were varied, the NADPH reaction mixtures had greater initial absorbances at 340 nm and required >30 min to reach equilibrium in the assays, as compared with 10–12 min for NADH. However, the NADPH reaction time could be shortened to 10 min by increasing the concentration of enzyme. We therefore selected an NADPH concentration of 0.25 mmol/L for subsequent testing; higher concentrations produced no further decrease in completion time.

**Enzyme.** We repeated the above experiment, using potassium phosphate buffer at 100 mmol/L, pH 7.6, 0.25 mmol/L NADPH, and increasing the sodium salicylate to 16 mg/L in the reaction mixture (equivalent to 1600 mg of salicylate per liter in a sample). We initiated the reaction by adding enzyme diluted in buffer. No further decrease of completion time was obtained for enzyme activity concentrations exceeding 120 U/L. This excess enzyme concentration also assures completion of the reaction at the temperature used in the instrument, 37 °C.

The complete reagent consists, per liter, of 100 mmol of potassium phosphate buffer, pH 7.6, 0.26 mmol of NADPH, and 120 U of salicylate hydroxylase. In its tabulated form non-interfering excipients are included. In the Paramax® analyzer the 30-mg tableted reagent is reconstituted with 200 μL of water, solubilized by sonication, and the 5-μL sample is introduced together with 73 μL of washout water. The reaction endpoint is read 7 min later. The stated volumes take into account the expansion volume of the tablet, as previously described (11).

**Analytical Variables**

**Precision.** Within-run: A lyophilized serum-based control (Moni-Trol ES; American Dade) was reconstituted to yield three salicylate concentrations, which we tested in 10 four-sample runs in the Paramax analyzer. Precision (CV) was 7.6% at 84 mg/L, 2.4% at 284 mg/L, and 1.7% at 541 mg/L (n = 40).

Between-run: Samples prepared as above were also run in the Paramax analyzer on 10 separate days. The CVs were 5.5% at 84 mg/L, 2.3% at 284 mg/L, and 1.6% at 541 mg/L (n = 40: 10 days, mean of four replicates each per day).

**Linearity.** Sodium salicylate was added quantitatively to aliquots of pooled normal human serum and tested on the Paramax analyzer. Results varied linearly with concentration to at least 1500 mg/L.

**Method comparison.** We analyzed by the Du Pont aca procedure (x) and by the present method (v) 39 individual sera from normal individuals and patients. Because the patients’ samples did not represent values throughout the test range, we also analyzed normal sera to which sodium salicylate had been added. Results showed a slope of 1.159, intercept of −1.78, and correlation of 0.9987.

**Analytical recovery.** Pooled human serum, supplemented with increasing amounts of pure sodium salicylate, was assayed in the Paramax analyzer and the aca. Results for recovery were as follows: 150 mg/L—present method, 96.5%, aca 87.3%; 500 mg/L—present method 97.2%, aca 87.4%; 850 mg/L—present method 97.2%, aca 86.0%; 1200 mg/L—present method 97.4%, aca 85.7%.

**Effect of Medium**

**Salicylate in serum and aqueous solution.** For manual assays we dissolved the tableted reagent in 273 μL of distilled water per tablet and dispensed 295 μL of the resulting solution into the cuvet, followed by 5 μL of sample.

In the ABA-100, tablets are each dissolved in 272 μL of distilled water with sonication, followed by 5 μL of sample, and the absorbance change is measured at 340/380 nm and 37 °C, with use of a 5-min carousel revolution in the down/rate mode.

**Standardization throughout was by use of freshly prepared aqueous solutions of pure (99%+) sodium salicylate.**

**Interferences.** A series of solutions of substrate analogs prepared in potassium phosphate buffer, 100 mmol/L, pH 7.6, were each run as a sample, manually. These included materials not likely to be encountered clinically as well as those found more frequently. The purpose was to examine substrate specificity of the enzyme as well as its clinical usefulness, and to compare our findings with previously published results (12).

We also prepared a 1000 mg/L solution of sodium salicylate in pooled normal serum, and added various potentially interfering substances to aliquots of the solution to give the final concentrations shown in Table 1, which we then assayed manually.

**Salicylate in urine.** Trinder (4) states that, before analysis for salicylate in urine, the sample must be diluted to contain about 100–400 mg/L. To investigate this, we prepared a series of nine concentrations of salicylate, from 0 to 2000 mg/L, in 250 mg/L increments, in each of three matrices: distilled water, pooled normal human serum, and urine freshly collected from a normal donor who had not ingested aspirin or other medication for at least 48 h before. Tests were run in the Paramax instrument as previously described. Analytical recoveries were the same for serum and water, but markedly less for urine. The urine-based samples were then diluted fivefold with distilled water, and reas-
butyric acid or 2,5-dihydroxyphenylacetic acid (homogentisic acid), which may appear in abnormal states and which interfere in the chemical test for salicylate, interfere in the enzymatic method. The short reaction time required and absence of requirement for sample pretreatment also mean the test is adaptable to use on an emergency basis.

Serum or plasma is the sample of choice. The data obtained for both the method comparison and recovery experiments show a bias of approximately 10% in the values obtained by the enzymatic vs the chemical method. Differences in calibration may be responsible, because the enzymatic determinations were calibrated against aqueous standards, whereas the published assay value for the comparison method calibrator was used to standardize that method. Although we have addressed the issue of the use of aqueous standard for assigning the calibrator value, we have not verified the value for the chemical method calibrator by this method.

The inhibition of the reaction in urine, observed in both the chemical and enzymatic methods, suggests a reaction of a urinary component with the salicylate salt to produce an unreactive species. The behavior of urine-based samples on dilution follows that observed when an inhibitor is diluted or a formed complex is dissociated. Interestingly, the amount by which the corrected results for 10-fold dilutions of 1000, 1500, and 2000 mg/L samples each fall short of the absorbance change produced by their corresponding undiluted aqueous counterparts appears to be proportional to the concentration of salicylate known to be present. At the lowest salicylate concentration (500 mg/L), the most likely source of the additional absorbance of the 10-fold diluted urine-based material is the imprecision of measurement at the very low concentration attained (50 mg/L).

Salicylate salts are known to occur in urine, but addition of neither urea or uric acid under the conditions tested reproduced this matrix-associated effect, as was also the case with the other urinary components tested. The phenomenon is to be investigated further.

References