FLUOROPHOTOMETRIC METHOD FOR THE ESTIMATION OF SALICYLATE IN BLOOD*

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While salicylates are among the most frequently prescribed drugs, methods for their determination in blood have been neither sufficiently sensitive nor precise. Brodie, Udenfriend, and Coburn (1) have devised a procedure suitable for the clinical measurement of blood salicylate levels if large doses are given. In their method the salicylate is extracted from acidified plasma with ethylene dichloride. A portion of the ethylene dichloride is removed and shaken with an aqueous ferric nitrate solution to produce a colored iron complex. Coburn (2) advises the use of quantities of plasma larger than 1 ml. if the salicyl "radical" is less than 100 γ per ml. Peters (3, 4) has drawn attention to the low sensitivity of the method. Volterra and Jacobs (5) applied the xanthoproteic reaction to trichloroacetic acid filtrates for the determination of salicylates. However, their serum blanks in normal subjects were relatively large, corresponding to 3 mg. per cent.

In the method to be presented a simple physical phenomenon, the bluish violet fluorescence of the salicylate ion on exposure to ultraviolet light, will be used for its measurement. Under suitable conditions a high degree of specificity and sensitivity can be attained.

**Principle**—Salicylates are quantitatively separated from proteins by precipitation of the latter with a dilute tungstic acid reagent. Strong alkali is added to increase the fluorescence of the salicylate ion about 9-fold. The blank fluorescence of plasma without salicylate is negligible. The fluorescence is measured directly in a fluorophotometer with the same filters as in the vitamin B₁ determination.

**Reagents**—

1. Standard salicylate solution (equivalent to 100 mg. of salicylic acid per 100 ml.). 116 mg. of sodium salicylate are dissolved in exactly 100 ml. of water. Working standards are prepared by dilution with water. Store in the refrigerator.
2. 19 per cent HCl.
3. Ethylene dichloride. Do not pipette.
4. 40 per cent NaOH.
5. Dilute tungstic acid reagent. 10 per cent sodium tungstate, 1 volume,

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mixed with 8 volumes of $\frac{N}{12}$ sulfuric acid. This solution must be prepared fresh every 2 weeks.

 Procedure 1; Clinical Method

To 1 ml. of oxalated or citrated plasma (not hemolytic) in a test-tube, add slowly, with shaking, 9 ml. of tungstic acid reagent. After 10 minutes filter. Pipette 5 ml. of filtrate into another test-tube and add 7 ml. of 40 per cent NaOH. Mix. The reagent blank reference solution contains 5 ml. of tungstic acid and 7 ml. of NaOH. Place the solutions in a fluorophotometer for measurement within 30 minutes after adding the alkali.

Fluorophotometric measurement was performed with an instrument con-
containing a balance photocell and bridge circuit (Lumetron\(^1\)). The same filters as in the vitamin \(B\) determination are used (maximum transmission of primary 3700 A, of secondary 4600 A). The sensitivity of the galvanometer (Rubicon Company) was 0.0025 milliampere per millimeter. The standard (1 ml. of the standard salicylate solution plus 11 ml. of water) is set at a slide wire reading of 30. The reagent blank is set at 0 with the zero suppressor control. The entire 12 ml. are added to the sample holder (15 ml. capacity) for measurement. Slide wire readings are taken after a 2 minute exposure to the ultraviolet light. The values are then read off a standard curve and multiplied by 2 to correct for dilution.

The standard reference curve for the clinical method (Fig. 1) is prepared by adding varying amounts of standard salicylate solution to the reagent blank mixture. With this procedure the blank reading of plasma without salicylate is of the order of 1 mg. per cent of salicylic acid.

Procedure 2; Modification of Ethylene Method (1)

To 1 ml. of plasma in a 50 ml. separatory funnel add 0.2 ml. of 19 per cent HCl and mix. After 10 minutes, 10 ml. of ethylene dichloride are added from a graduated cylinder and the separatory funnels are shaken for 3 minutes. The lower layer is transferred to a glass-stoppered test-tube in which 5 ml. of 40 per cent NaOH had previously been placed. Shake for 1 minute. Separate by centrifuging for 5 minutes at moderate speed. Plunge the pipette through the upper layer and draw off 4 ml. of the aqueous phase into a test-tube. Add 8 ml. of water, mix, and measure the fluorescence as above. A reagent blank is run simultaneously. The standard curve is made by subjecting varying amounts of standard salicylate solution to the same procedure. The blank on plasma not containing salicylate is zero.

EXPERIMENTAL.

The fluorescence of salicylate varies with the pH of the medium. Salicylic acid in distilled water does not fluoresce, whereas sodium or lithium salicylate in distilled water exhibits a bright bluish violet fluorescence on exposure to ultraviolet light. When the salicylates were investigated as fluorescent pH indicators, it was recorded that the fluorescence appears at a pH of 2.5 (6) or 3.0 (7). A sensitive galvanometer can detect fluorescence at even lower pH values. In ultraviolet light a neutralized aqueous solution of sulfosalicylic acid has the identical bluish violet fluorescence.

\(^1\) Photovolt Corporation, New York.
cyluric acid\(^2\) in distilled water does not fluoresce. If alkali is added to a salicyluric acid solution, the fluorescence appears at a pH of about 6.3.

The following experiment shows the intensification of fluorescence by alkali. The same concentration of salicylate was used (1.16 mg. of sodium salicylate in 12 ml. of water). As the concentration of alkali was increased to 10 \(\text{N}\), the fluorescence rose to twice the reading in distilled water with \(\text{NH}_4\text{OH}\) and nine times when \(\text{NaOH}\) was used. In Fig. 2 the increase in fluorescence was plotted against concentration of \(\text{NaOH}\) on a semilog graph.

With the increase in alkali concentration the fluorescence increased markedly, forming an S-shaped curve.

In strong alkali the fluorescence of salicylate deteriorates slightly during continuous exposure to ultraviolet light. No decay of fluorescence was noted at high pH in the absence of ultraviolet light. Highly reproducible readings can be made if one exposes the solution to be examined to ultraviolet light for a definite period of time. 2 minutes of irradiation with ultraviolet light were found sufficient to allow for the decrease in fluorescence of the plasma blank to a negligible quantity.

The data in Table I indicate that varying quantities of sodium salicylate

\(^2\) Kindly supplied by Dr. Bernard B. Brodie, New York University College of Medicine, New York.
added to 1 ml. of plasma are recoverable with satisfactory precision. The values given are expressed as salicylic acid in accord with routine usage. By this method the drug content of the plasma was recovered with accuracy down to 2 mg. per cent of salicylic acid.

Analyses run on plasma samples of salicylates over a period of a week gave highly reproducible results. It may be concluded, therefore, that the salicylate fluorescence is unchanged if the plasma is stored in the refrigerator.

**Table I**

**Recovery of Sodium Salicylate Added to Plasma***

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* Values expressed as salicylic acid.

**Discussion**

The fluorometric method is more sensitive than the colorimetric, quantities of salicylate as low as 1 to 2 mg. per cent being detectable. Such measurements are without significance if the plasma blank is not zero. In the ethylene dichloride procedure a zero plasma blank is obtained. The direct method on protein-free filtrates is applicable to measurement of low concentrations if the plasma blank determined prior to the administration of salicylate is known. In ordinary clinical usage with the latter procedure the plasma blank was found to be 1 mg. per cent or less. The latter method has the advantage of reference standards made up directly, whereas with the ethylene dichloride the extraction of standards is necessary.

In urine the "salicyl" is present either as free salicylic acid or as some con-
jugated product (salicyluric acid or glucuronide (8)). By use of both ethylene dichloride and carbon tetrachloride methods, Brodie et al. (1) demonstrated the absence of appreciable amounts of salicyluric acid in a series of plasma samples from patients on sodium salicylate therapy. By the direct method presented in this communication total salicylate (both free and conjugated) would be measured, whereas free salicylic acid alone is estimated by the ethylene methods.

SUMMARY

Solutions of salicylate fluoresce a bright bluish violet under ultraviolet light. The fluorescence is intensified by addition of alkali. A simple procedure for direct determination of salicylates in protein-free blood filtrates is described. The fluorescent method offers rapidity and greater sensitivity than previous methods.

BIBLIOGRAPHY

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