# Determination of Salicylic Acid and Related Substances in Serum by Ultraviolet Spectrophotometry.* (19517)

G. UNGAR, E. DAMGAARD, AND W. K. WONG.

*From the Rheumatic Fever Research Institute, Northwestern University Medical School, Chicago, Ill.*

A variety of methods are available to the clinician and the research worker for estimating salicylic acid and its derivatives in biological fluids(1-6). Any new technic, therefore, has to present some definite advantage over the existing ones. The present paper describes a simple and specific method based on ultraviolet absorption of salicylic acid and suggests similar technics for the estimation of related substances.

*Methods.* Absorption measurements were made on the Beckman Quartz Spectrophotometer, model DU, with ultraviolet attachment, using a hydrogen discharge tube as the source of radiation. The solutions were placed in silica cuvettes with a light path of 10 mm ± 0.05. Measurements were made against blanks containing all the reagents except the substance to be tested. Guinea pig blood was used for salicylate determinations. The animals were injected intraperitoneally with varying doses of sodium salicylate, and cardiac blood samples were taken prior to and one hour after injection. The blood was allowed to clot and the serum was treated as described below.

*Salicylate determination.* The ultraviolet spectrum of a $10^{-4}$M solution of sodium salicylate in water (pH = 5.1) has an absorption peak at 295 mμ. The molar extinction coefficient at this wavelength,† $E_M^{295} = 3840$. However, when sodium salicylate is dissolved in serum and the protein precipitated with trichloracetic acid, the filtrate shows a different peak (λ = 303 mμ) and a slightly different extinction coefficient ($E_M^{303} = 3680$). This difference is caused by the change of pH and not by the presence of serum. Addition of the same amount of trichloracetic acid as in the previous experiment, to sodium salicylate results in a change in pH to 1.2 and also in a shift of the peak to 303 mμ. Fig. 1 shows the two spectra of sodium salicylate, with wavelength plotted against molar extinction coefficient. For determination of salicylate the following procedure was used: The serum (or plasma) is pipetted into a centrifuge tube. Using 0.5 ml is satisfactory in most cases except when high salicylate levels are expected in which case smaller quantities should be used. The volume is made up to 5 ml with a 4% solution of trichloracetic acid. After an interval of at least 30 min the mixture is filtered or centrifuged. The clear supernatant is poured into a silica cuvette and the absorption measured at 303 mμ in the ultraviolet spectrophotometer. The test solution should be read against a blank consisting of the tri-

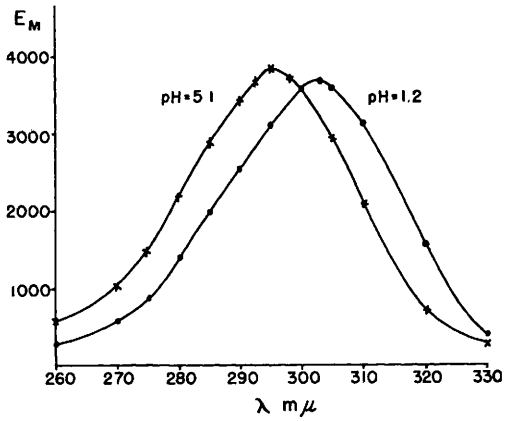


FIG. 1. Ultraviolet absorption spectrum of sodium salicylate dissolved in water (pH = 5.1) and mixed with trichloracetic acid (pH = 1.2). λ = wavelength in mμ; $E_M$ = molar extinction coefficient.

* This work was supported by a grant from the Walter P. Murphy Foundation.

† The molar extinction coefficient, $E_M = \frac{\lambda \log \left[\frac{Io}{I}\right]}{cl}$ for a particular wavelength (λ). c = the concentration expressed as moles/liter; l = length of the light path in the cell in cm; Io = intensity of the incident light and I = intensity of the transmitted light; $\log \left[\frac{Io}{I}\right]$ = (O.D.), optical density.

TABLE I. Comparison Between Blood Salicylate Levels Obtained by the Ultraviolet Method and a Colorimetric Technic(3).

| Na salicylate administered, mg/kg | Serum salicylate, mg/100 ml — Ultraviolet method | Colorimetric method |
|---|---|---|
| 150 | 19.15 | 15.9 |
| | 22.55 | 25.9 |
| | 24.15 | 24.7 |
| | | 25.2 |
| | | 25.4 |
| | Mean=21.95±1.5 | Mean=23.3±1.9 |
| 200 | 26.05 | 25.7 |
| | 28.7 | 28.6 |
| | 29.1 | 30.8 |
| | 29.55 | |
| | 29.55 | |
| | 30.7 | |
| | Mean=28.9 ± .6 | Mean=28.4±1.5 |
| 250 | 36.5 | 30.5 |
| | 41.2 | 36.5 |
| | 38.7 | 36.9 |
| | | 42.1 |
| | Mean=38.8 ±1.4 | Mean=36.5±2.4 |

chloracetic filtrate of the serum of the same subject before administration of salicylate. When this is not practicable a blank containing equivalent quantities of saline and trichloracetic acid can be used. In this case, the mean absorption of serum should be subtracted from the optical density reading. The mean absorption can be determined on a series of sera from normal untreated subjects.

The results can be calculated from the following formula: Sodium salicylate mg/100 ml serum $= \frac{(O.D.)}{\frac{E_M}{(M.W. \times 10)}} \times \frac{V}{v}$ where (O.D.) = optical density (against serum blank or after subtraction of serum absorption); (M.W.) = molecular weight of sodium salicylate; $E_M$ = molar extinction coefficient; V = total volume of the solution after adding trichloracetic acid; v = volume of serum used. In the usual case of 0.5 ml of serum diluted to 5 ml with trichloracetic acid the formula is as follows: Sodium salicylate mg/100 ml serum $= \frac{(O.D.) \times 10}{0.23}$.

This procedure was tested in guinea pigs which had received varying amounts of sodium salicylate. Table I shows the results of these determinations compared with the results obtained with a colorimetric method using the Folin-Ciocalteu reagent(3). The comparison is not based on duplicate determinations but on blood samples from different animals. The agreement, however, is satisfactory.

*Ultraviolet absorption by salicyl derivatives.* To test the specificity of the method described a number of substances related to salicylic acid were examined in the ultraviolet spectrophotometer. Acetylsalicylic acid has no absorption peak between 220 and 350 mμ. This substance cannot therefore be detected by the present method until it is hydrolyzed to salicylic acid(7). A metabolite of salicylic acid, salicyluric acid, has an absorption peak at 298 mμ and $E_M^{298} = 3498$. No change was observed in the spectrum by shifting the pH from 6 to 1. Gentisic (2, 5, dihydroxybenzoic) acid has an absorption peak at 320 mμ when dissolved in alkaline solution. ($E_M^{320} = 4784$). When, however, gentisic acid is mixed with serum, the peak shifts to 330 mμ and the extinction coefficient increases considerably ($E_M^{330} = 9152$). This shift is due to the action of serum and the development of the secondary peak depends on the amount of serum added to the substance. It has been assumed that gentisic acid is converted into another substance as soon as it is injected(8). Another dihydroxybenzoic acid, γ-resorcylic (2, 6-dihydroxybenzoic) acid, recently tested therapeutically(9), behaves in a similar manner. Its alkaline solution has an absorption peak at 302.5 mμ ($E_M^{302.5} = 3550$) but when it is mixed with serum a secondary peak develops between 290 and 300 mμ which increases with time and with increasing concentrations of serum. At the same time the original peak decreases.

*Discussion.* For routine clinical use the present method is simpler and faster than any of the technics described in the literature and requires only the use of an ultraviolet spectrophotometer. For research work, particularly the study of the metabolism of salicylate and its derivatives, the ultraviolet method has the advantage of a higher specificity than the reactions used by other methods. These are

non-specific reactions of the phenolic group with ferric ion(1), the Folin-Ciocalteu reagent(3,6) and nitrates(4) or development of fluorescence in alkaline solution(5). Separation of certain derivatives has been achieved by extraction with organic solvents(1,2,5). All of these methods require several reagents and involve a number of steps. The ultraviolet method either by itself or in combination with a colorimetric method achieves a simple, direct separate determination of the various salicyl compounds that are likely to be present simultaneously in biological fluids.

*Summary.* A method is described for determination of salicylate blood levels, based on the absorption spectrum of salicylic acid in the ultraviolet range. The method is suitable both for routine clinical use and for research work.

1. Brodie, B. B., Udenfriend, S., and Coburn, A. F., *J. Pharmacol.*, 1944, v80, 114.
2. Reid, J., *Quart. J. Med.*, 1948, v66, 139.
3. Weichselbaum, T. E., and Shapiro, I. M., *Am. J. Clin. Path.*, 1945, v9, 42.
4. Volterra, M., and Jacobs, M. D., *J. Lab. and Clin. Med.*, 1947, v3, 1282.
5. Saltzmann, A., *J. Biol. Chem.*, 1948, v174, 399.
6. Smith, M. J. H., and Talbot, J. M., *Brit. J. Exp. Path.*, 1950, v31, 65.
7. Hofstee, B. H. J., *Science*, 1951, v114, 128.
8. Meyer, K., and Ragan, C., *Science*, 1948, v108, 281.
9. Reid, J., Watson, R. D., Cochran, J. B., and Sproull, D. H., *Lancet*, 1951, v2, 321.

Received April 7, 1952. P.S.E.B.M., 1952, v80.

## Effect of Epinephrine, Glucose and Certain Steroids on Fatal Convulsive Seizures in Mice.* (19518)

E. M. VICARI, A. TRACY,† AND A. JONGBLOED.† (Introduced by C. C. Little.)
*From Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.*

This is one of a series of experiments in an attempt to define the cause of immediate death following convulsive seizures. In previous studies one of us reported that the incidence of susceptibility to sound-induced convulsive seizures was associated with age(1-3). This was established from a survey of various inbred mouse strains; the age level of maximum susceptibility varied with the strain. The experiments reported here represent attempts to locate, by preventive measures, the cause of lethal convulsion and its onset in the dilute brown, DBA, mouse strain. This closely inbred strain is characterized by its high seizure susceptibility (97% incidence) and high lethal seizures (80% incidence) at the age level of 28-34 days. The pattern of the seizure is similar in appearance to the grand mal epileptic seizure of children.

Since the blood sugar level is low in convulsive human subjects, and glucose is often administered for treatment, this substance was given to a group of lethal-seizure susceptible mice. Behavior disorder may well be dependent upon abnormalities of blood composition and the sympathoadrenal system may be associated with the blood composition, on this assumption injections of glucose and of epinephrine were tested.

*Material and procedures.* A group of highly "lethal-seizure-susceptible" mice of the dilute brown, inbred strain, DBA, was employed. Both sexes in equal numbers were used and the ages at the first injection was 28-32 days. A description of the physical characters of the strain and the method used for inducing seizures by the sound of an electric bell were given in a former paper(3). The intensity of the sound was approximately 91 decibels with a variation of about $\pm 2$ decibels in different parts of the apparatus. The animals were individually tested in a galvanized iron tub with the bell hanging inside its wall. In each group there were from 40-50 individuals. Controls genetically related to the experimental

* Supported in part by a grant from the U.S.P.H.S.
† Graduate Students, participants in the Summer Studies Program.