TECHNICAL METHODS

A MICRO-METHOD FOR BLOOD SALICYLATE ESTIMATIONS

BY

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In the treatment of rheumatic fever by the administration of massive doses of salicylates, the importance of maintaining an optimum blood level of the drug has been generally recognized. The therapeutic level, however, is very close to the toxic level, and, since different patients receiving the same dosage of salicylates may have widely differing plasma levels, repeated blood estimations may be of great value, particularly during the first few days of treatment.

The methods available for the estimation, though numerous, have certain inherent drawbacks in their routine use. The chief of these is the comparatively large volume of serum or plasma usually required. Also, most methods give appreciable values when applied to the blood of patients not receiving salicylates, due to the presence of interfering substances in the blood. This may entail the application of an approximate correction, by the use of a calibration curve constructed from pooled normal blood serum or plasma. Finally, many methods are not simple or rapid enough for routine use by technicians in small laboratories.

The methods which have been most commonly employed are based on the violet colours formed when ferric salts are added to salicylate solutions. Keller (1947) and Peters (1947) used direct addition of acidified ferric nitrate to serum or plasma, but Brodie, Udenfriend, and Coburn (1944) extracted the plasma with ethylene dichloride, and then returned the salicylates to an aqueous phase as a coloured ferric complex. A micro-method based on the same colour reaction has recently been published by Miller and Whitehead (1949), but a special technique is required.

The colour reaction of Jorissen, obtained when salicylate solutions are boiled with copper sulphate and nitrous acid, was utilized by Mallick and Rehmann (1945) and by Reid (1948) for the estimation of blood salicylates, while Volterra and Jacobs (1947) first nitrated the salicylates and then estimated the nitro derivatives colorimetrically in alkaline solution. Weichselbaum and Shapiro (1945) and Smith and Talbot (1950) have used the blue colour which results when a salicylate solution is mixed with Folin and Ciocalteu's phenol reagent and made alkaline with sodium carbonate.

Diazo reactions have been widely employed for the estimation of *p*-aminosalicylic acid in blood, but do not appear to have been used for the determination of salicylic acid itself. This, no doubt, is due to the comparative slowness of coupling diazo compounds with salicylates. The difficulty, however, can be overcome by using a

A MICRO-METHOD FOR BLOOD SALICYLATE ESTIMATIONS 209

large excess of the diazo reagent under carefully controlled conditions. The present method is based on coupling with diazotized *p*-nitraniline to give an orange dye, and was evolved to fill what was felt to be a need for a method which would (1) give blanks on normal blood which would be sufficiently low to be negligible by comparison with the salicylate levels commonly encountered during treatment, (2) be applicable to 0.2 ml. of whole blood collected by finger prick, and (3) be sufficiently rapid and simple for routine use in small laboratories.

In carrying out the estimation on whole blood, laking with water followed by precipitation of the proteins by trichloroacetic acid was tried initially, but this procedure was found to lead to high blank values on normal blood, due presumably to liberation of interfering substances from the red cells. Dilution of the blood with isotonic sodium sulphate and protein precipitation by zinc hydroxide, as used by King (1946), gave more satisfactory results, and this technique has been adopted. Filtration, rather than centrifugation, has been preferred, as it was found that, in the latter case, small floating fragments of protein precipitate were occasionally captured by the pipette and caused increased colour. Since only traces of salicylates are present in the red cells (Smith, Gleason, Stoll, and Ogorzalek, 1946; Lester, Lolli, and Greenberg, 1946), whole blood levels are lower than the corresponding plasma levels. The former, however, can be converted to the latter, to a close approximation, by use of the equation: $p = b/100 - h$, where p and b are the plasma and whole-blood levels in mg./100 ml. and h is the haematocrit. Table II shows a comparison of plasma levels calculated in this way and the observed levels. An exception was found in a fatal case of aspirin poisoning, in which a considerable concentration of salicylate was present in the red cells.

Method

Reagents.—The following are required: (1) Isotonic sodium sulphate (2% $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$); (2) 10% zinc sulphate; (3) 0.5N sodium hydroxide; (4) 1.5% *p*-nitraniline in 5N hydrochloric acid; (5) 10% sodium nitrite (stored in the ice-chest, this keeps for several months); (6) 4N sodium hydroxide; (7) standard salicylate solution (50 mg. sodium salicylate dissolved in 100 ml. water. Store in the ice-chest).

Procedure.—Serum, plasma, or whole blood, 0.2 ml., are mixed with 6.6 ml. of isotonic sodium sulphate. Then 0.6 ml. of zinc sulphate solution followed by 0.6 ml. of 0.5N sodium hydroxide are added to precipitate the proteins, and the mixture is filtered. A Whatman No. 1 paper is suitable. A standard and a reagent blank are carried through simultaneously with the unknown by substituting 0.2 ml. of salicylate solution and 0.2 ml. of isotonic sodium sulphate for the blood, and 4 ml. of filtrate are mixed with 0.8 ml. of *p*-nitraniline solution. The solution is chilled in ice water. Sodium nitrite solution, 0.6 ml., is added, and, after standing in the ice water for two or three minutes, the solution is made alkaline by adding 1.2 ml. of 4N sodium hydroxide. The colours develop immediately and are stable for a considerable time.

Fig. 1 shows the light absorption of the reagent blank and salicylate standard respectively. Since the diazo reagent is present in large excess, subtraction of these two curves gives the absorption spectrum of the azo dye formed in the reaction. The wavelength of maximum light absorption of the latter compound is 500 $\text{m}\mu$. If a spectrophotometer is used for the estimation, this wavelength setting should therefore be used. Alternatively an Ilford spectrum blue-green filter (No. 603) may be used. For routine use we have found a Chance blue filter (No. OB 2), used in conjunction with a Hilger "Biochem" absorptiometer, to give satisfactory results.

FIG. 1

FIG. 1.—Absorption spectra of (a) reagent blank, (b) salicylate standard, (c) salicylate azo compound, obtained by subtracting (a) from (b). Light absorption was measured on a "Unicam" spectrophotometer.

FIG. 2

FIG. 2.—Calibration curve obtained by carrying out the analysis on salicylate solutions of the concentrations shown. Extinction coefficients were measured on a Hilger "Biochem" absorptiometer.

Thirty-five blood specimens from normal persons, not receiving salicylates, were examined by the present method. The estimations on whole blood gave values ranging from 0.0 to 3.0 mg./100 ml., with an arithmetical mean of 1.5 mg./100 ml. The range for plasma was 1.5 to 3.5 mg./100 ml., with a mean of 2.0 mg./100 ml.

Fig. 2 shows a typical calibration curve. A linear relationship between colour intensity and salicylate concentration was observed up to a concentration of 70 mg./100 ml. If levels in excess of this figure are encountered, they may be found by reference to the calibration curve, or alternatively the estimation may be repeated on a smaller volume of

TABLE I
RECOVERY OF SODIUM SALICYLATE FROM WHOLE BLOOD AND PLASMA

Added (μ g.)	Whole Blood		Plasma	
	Found (μ g.)	Recovery (%)	Found (μ g.)	Recovery (%)
50	43	86	41	82
60	53	88	54	90
70	64	91	62	89
80	73	91	68	85
90	87	90	81	90
100	87	87	88	88

Mean recovery from whole blood 89%. Mean recovery from plasma 86%.

A MICRO-METHOD FOR BLOOD SALICYLATE ESTIMATIONS 211

TABLE II
BLOOD SALICYLATE LEVELS

Plasma Salicylate Level* (mg. %)	Whole Blood Salicylate Level† (mg. %)	Haematocrit	Plasma Level Calculated from Whole Blood Level (mg. %)	Plasma Level (Smith and Talbot Method mg. %)
12.5	9.0	36	14.0	15.0
13.5	9.0	42	15.5	16.5
21.0	14.0	40	23.5	19.0
22.5	15.5	31	22.5	19.5
22.5	13.5	41	23.0	22.5
23.0	16.5	37	26.0	21.5
76‡	61	48	117	86

* A blank of 2.0 mg. % has been subtracted. † A blank of 1.5 mg. % has been subtracted. ‡ A fatal case of aspirin poisoning.

filtrate, the volume being corrected to 4 ml. with distilled water. The recoveries of sodium salicylate added to plasma and whole blood are shown in Table I.

A series of estimations on blood from patients receiving sodium salicylate is given in Table II. Simultaneously with the present method, the plasma salicylate level was determined by Smith and Talbot's method. Except for one exceptional case the greatest difference found was 3 mg. %.

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