100. A Note on the Estimation of the Sulphonamide Drugs by the Method of Werner

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The usual methods for estimation of sulphonamide drugs in biological fluids depend upon diazotization and coupling with an amine (cf. Bratton & Marshall [1939] and Hynes [1940]). Although they are very accurate these methods call for reagents not commonly available in clinical laboratories, the solutions of which deteriorate rather rapidly. A method described by Werner [1939], using the yellow colour produced by adding *p*-dimethylaminobenzaldehyde to a solution containing the drug, avoids the inconveniences inherent in the diazotization procedure. Morris [1941] has modified Werner's method by the use of toluenesulphonic acid for protein precipitation and has introduced a citrate buffer which considerably increases the relative density of the colour and at the same time eliminates random errors which occur with the original method. However, the aldehyde reagent in Morris's method is dissolved in alcohol and it has been found that when a microcolorimeter is used fine bubbles form under the plunger which give rise to a fictitiously high result, especially if a neutral grey screen is used as a standard.

By the use of salicylsulphonic acid for protein precipitation a blood filtrate having pH 1.26 can be obtained which, if the aldehyde reagent is made up in aqueous solution containing sulphuric acid, gives a colour of similar relative density to that obtained in Morris's method. In the absence of alcohol no bubbles are formed.

EXPERIMENTAL

Reagents. (1) 30 g. 'Analar' salicylsulphonic acid made up to 100 ml. with water.

(2) 7 % aldehyde reagent. Wash 7 g. 'Analar' *p*-dimethylaminobenzaldehyde into a 100 ml. flask with 7 % *v/v* H₂SO₄ and make up to 100 ml. with 7 % H₂SO₄. Shake until dissolved, add about 0.3 g. 'Decolorizing Charcoal B.D.H.' and shake again for 2 min. Filter.

Method

Determination of sulphonamides in blood. Add 0.5 ml. blood to 6.5 ml. water, mix and leave 3 min. Add 0.5 ml. 30 % salicylsulphonic acid, shake vigorously and leave for 10 min. Filter through two filter papers folded together (Whatman No. 2, 3 or 40). If the filtrate is cloudy, return the first few ml. through the filter papers.

To 2 ml. clear filtrate add 0.5 ml. 7 % aldehyde reagent. Compare the colour produced with a standard, using a violet or blue Ilford 'Spectrum' filter on the eyepiece of the colorimeter.

Standards. The best standard to use is one of the neutral grey screens supplied by Messrs Ilford. The 0.25 screen is suitable for blanks and usually the 0.50 or 0.75 is appropriate for test solutions. If a permanent standard of this type is used a calibration curve may be constructed for each drug by addition of varying amounts to blood known to be free from sulphonamides. This may conveniently be done by replacing the 6.5 ml. water in the method by standard aqueous solutions of the drug. Owing to the low solubility of the substituted sulphonamides 10 or 20 mg. of the substance should be dissolved in the minimum of warm alcohol and transferred to 800 ml. water in a litre flask,

which is then filled to the mark with water. Using a violet 'Spectrum' filter on the eyepiece of a Klett microcolorimeter it has been found that if

$$D_v = \frac{\text{density of neutral grey screen}}{\text{cm. depth of solution}}$$

then mg. sulphapyridine/100 ml. blood = $14.4 \times D_v - 2.7$. The appropriate conversion factors for other sulphonamides may be derived by direct calibration or by making the assumption (cf. Morris [1941]) that an equal amount of colour is produced by one molecule of each of the drugs.

The yellow colour of the aldehyde reagent gives rise to a considerable blank which introduces difficulties if direct comparison between coloured solutions is preferred to the use of a neutral grey standard. Satisfactory results can, however, be obtained with a sulphanilic acid solution as the standard if the calculation is carried out by means of the equation of Delory & Jacklin [1942]. If C_S and C_T are the concentrations of the substance under investigation in the standard and test solutions and R_S , R_T and R_B are the colorimeter readings of standard, test and blank respectively, the usual colorimetric calculation is given by

$$C_T = \frac{R_S}{R_T} \times C_S. \quad \text{.....(1)}$$

The improved equation of Delory & Jacklin states that

$$C_T = \frac{R_B - R_T}{R_B - R_S} \times \frac{R_S}{R_T} \times C_S. \quad \text{.....(2)}$$

The relative density of the colour produced by adding 0.5 ml. aldehyde reagent to 2 ml. of a solution of sulphanilic acid in 2% salicylsulphonic acid has been compared with that produced by blood filtrates containing known amounts of sulphapyridine and it has been found that, for equal colour densities, mg. sulphapyridine/100 ml. blood = $15.4 \times$ mg. sulphanilic acid/100 ml. standard - 0.85. Substituting this in equation (2)

$$C_T = 15.4 \times \frac{R_B - R_T}{R_B - R_S} \times \frac{R_S}{R_T} \times C_S - 0.85, \quad \text{.....(3)}$$

where R_B is the reading of a blank obtained by addition of aldehyde reagent to 2% salicylsulphonic acid and C_S is the concentration of the sulphanilic acid standard.

Table 1 shows that the results obtained by direct comparison with sulphanilic acid standards using equation (1) are unsatisfactory, especially when concentrations of sulphapyridine are small.

Table 1. *Results obtained by direct comparison using formulae based on equations (1) and (2)*

	mg. sulphapyridine/100 ml. blood	
	By direct comparison	
	mg./100 ml.	mg./100 ml.
	$= 15.4 \times \frac{R_S}{R_T} \times C_S - 0.85$ (from equation 1)	$= 15.4 \times \frac{R_B - R_T}{R_B - R_S} \times \frac{R_S}{R_T} \times C_S - 0.85$ (from equation 2)
From reference curve		
13.7	13.4	14.0
10.4	10.8	10.2
7.2	7.6	7.2
5.0	5.5	4.9
3.1	2.8	3.2
1.6	1.9	1.4
1.2	0.3	1.1
0.8	0.2	0.9
0.1	-0.04	0.1

R_S = reading of standard. R_T = reading of test. R_B = reading of sulphanilic acid blank (4 ml. 2% salicylsulphonic acid + 1.0 ml. aldehyde reagent).

C_S = mg./100 ml. sulphanilic acid in standard. SAA = sulphanilic acid.

The use of the equation of Delory & Jacklin, however, gives good correspondence throughout the range even when the depth of colour of the standard differs very considerably from that of the test solution.

Stock standard solution. 20 mg. 'Analar' sulphanilic acid dissolved in 100 ml. 2% salicylsulphonic acid.

'Blood standard' solutions. 1 ml. stock standard diluted to 20 ml. with 2% salicylsulphonic acid.

If equation (3) is used for the calculation of results this standard is suitable for most purposes, but higher dilutions with 2% salicylsulphonic acid may be used if desired. The sulphanilic acid solutions are not subject to rapid deterioration—the 'blood standard' may be heated in a boiling water bath for 6 hr. without alteration of the density of the final yellow colour.

Estimation of total sulphonamides

If it is desired to estimate total sulphonamide the acetyl derivative may be hydrolysed with acid and the colour developed as for free sulphonamide. Reference curves must be constructed with addition to the reagents of the amount of acid used for hydrolysis.

Method. To 2 ml. blood filtrate in a graduated tube add 0.5 ml. 8% *v/v* (about 3 *N*) H_2SO_4 . Shake and stopper with a cork in the side of which a small V-shaped notch has been cut. Heat in a gently boiling water-bath for 40 min., cool, add water to 2.5 ml. and then 0.5 ml. 7% aldehyde reagent.

In the particular case of the estimation of acetylsulphapyridine a complication arises owing to hydrolysis of the sulphapyridine itself during the heating. Furthermore, the rate of hydrolysis of free sulphapyridine differs significantly from that of sulphapyridine as liberated from the acetyl compound. Since the original free sulphapyridine is partially destroyed during hydrolysis of the acetyl compound the amount of colour it forms is less than that obtained by adding aldehyde reagent to unheated blood filtrate plus acid. The proportion destroyed under the conditions given in the method has been found to be 13%. Hence in order to calculate what proportion of the colour obtained after hydrolysis is due to the original free sulphapyridine it is necessary to multiply the density of colour obtained with unheated blood filtrate plus acid by 0.87. The remaining colour is due to hydrolysed acetylsulphapyridine, the amount of which may be derived from a reference curve constructed by means of addition of aqueous solutions of monoacetylsulphapyridine to normal blood.

The monoacetyl derivatives of the sulphonamides are easily prepared by warming with a slight excess of acetic anhydride in presence of sodium carbonate, followed by recrystallization from hot alcohol.

It should be noted that the various constants quoted above apply only to the instruments and conditions in the author's laboratory.

SUMMARY

A modification of Werner's method for the estimation of the sulphonamide drugs has been described.

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