A PHOTOELECTRIC COLORIMETRIC METHOD FOR THE ESTIMATION OF RIBOFLAVINE

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The aqueous solution of riboflavine is greenish-yellow and displays a strong yellowish-green fluorescence, which is directly proportional to the vitamin content. The fluorescence has been used for the quantitative determination of riboflavine[1,2,3,4,5], but this method has the disadvantage of the vitamin being sensitive to light. The method becomes inaccurate in cases where other fluorescent substances are present, such as in urine and yeast extracts.

The most satisfactory and generally accepted methods for the determination of riboflavine are the microbiological and animal assays[6,7,8,9,10,11]. The chief advantage of the animal assays is that they are based on biological response, which is important from the nutrition standpoint. Such methods, however, take much time and are expensive. A comparison of the rat growth, microbiological and fluorimetric methods for the determination of riboflavine in pharmaceutical products, shows the results to be similar and to give reproducible results on samples of high potency, but great differences were observed in samples of low potency[12,13].

The Photoelectric Colorimetric Method.

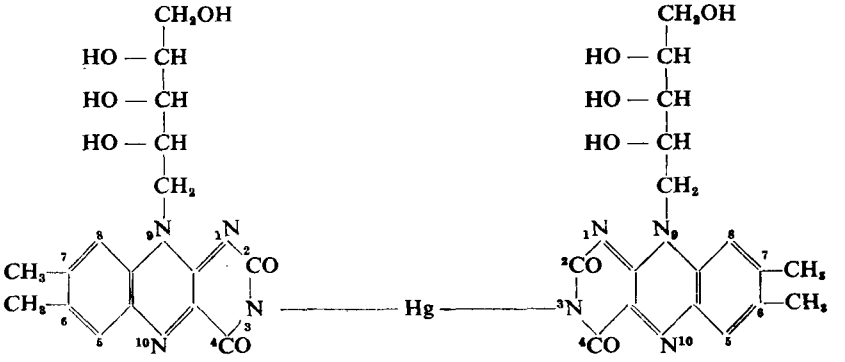
A new, simple and rapid method for the determination of riboflavine in pure solutions and in pharmaceutical products, which overcomes the disadvantages previously mentioned is advanced. This method is based on the colour reaction of Denigè's reagent (orange-red) or 10 per cent. aqueous silver nitrate solution (brick-red) with riboflavine.

The colour is immediately obtained at room temperature; the intensity is in good agreement with Beer's law and is applicable up to 40 μg. of riboflavine.

Nature of the Colour Test. Riboflavine is an imide and such compounds react readily with mercuric sulphate or silver nitrate with the formation of mercury riboflavine or silver riboflavine. The formation of these derivatives is analogous to saccharin and carbostyril[14].

The colour is stable for more than 24 hours and is not affected by the action of heat. In the case of Denigè's reagent, an orange-red colour is formed in concentrated solutions and a yellow colour in dilute solutions. The sensitivity of the test is 1 per 400,000. This colour test may be applied in biological fluids, since the following substances which might interfere have no influence on the colour: amino-acids such as glycine,

alanine and *iso*-leucine; ascorbic acid; carbohydrates such as glucose, lactose, sucrose and starch; urea and uric acid; acetone, ethyl acetate and acetoacetic ester.



Silver nitrate can only be used in a neutral medium and the presence of dibasic acids such as tartaric, oxalic or even monobasic acids, such as acetic acid, prevents the development of the colour.

Colour Tests for Riboflavine. Riboflavine gives a red-violet colour with concentrated sulphuric acid which changes to yellow on dilution. When heated with concentrated sodium hydroxide solution (50 per cent.) riboflavine gives a green colour which changes to red on dilution.

Experimental

Preparation of the Metallic Compounds. (a) A saturated solution of mercuric sulphate (1 mol.) containing concentrated sulphuric acid (98 per cent.), as in Denigè's reagent, is treated with a concentrated solution of riboflavine (2 mol.) in hot distilled water. An immediate orange-red colour is obtained followed by precipitation of a microcrystalline orange-red powder. This is filtered off and dried; m.pt. 200°C. with decomposition, leaving a residue of mercury. It is hydrolysed by weak alkalis, even by aqueous ammonia, with the production of riboflavine.

(b) A saturated solution of silver nitrate (1 mol.) in distilled water is added to a saturated aqueous solution of riboflavine (1 mol.); a brilliant red colour appears. The solution is concentrated, filtered while hot and allowed to cool; a micro-crystalline brick-red solid deposits; m.pt. 190°C. with decomposition; dark brown melt at 250°C.

Equipment and Reagents. (1) Lumetron photoelectric colorimeter using a yellow-green filter, 530 against water as the blank, set at 100 per cent. transmission. (2) Two micro-pipettes (5 ml.), one of which is graduated. (3) Denigè's reagent, prepared by dissolving 5 g. of yellow mercuric oxide in the hot solution obtained when 20 ml. of concentrated sulphuric acid is added to 100 ml. of distilled water. The solution is filtered if necessary. (4) Standard stock solution of riboflavine prepared by dissolving 0·1 g. of pure riboflavine (previously dried over concen-

trated sulphuric acid in a dessicator to constant weight) in ethanol (50 per cent.) 500 ml. Vigorous shaking or slight warming may be necessary to effect solution.

Procedure. The following general procedure has been adopted. Into a volumetric flask of 25 ml. capacity, χ ml. of the standard stock solution of riboflavine (1 ml. represents 200 μg. of riboflavine) is introduced.

The volume is then completed to 25 ml. with distilled water, well mixed, and left to stand for about 5 minutes. Then 5 ml. of the solution are transferred to a colorimeter tube and 3 ml. of Denigè's reagent added. The mixture is well mixed and its percentage transmission is read after 3 minutes in a Lumetron photoelectric colorimeter, Model 400-A using a yellow-green filter, 530 against water as the blank, set at 100 per cent. transmission. χ = 25 ml. of the standard stock solution of riboflavine (0·1 g. per 500 ml. w/v), representing a concentration of 1000 μg. of riboflavine per 5 ml. of solution or 22·5, 20, 17·5, 15, 12·5, 10, 7·5, 5 and 2·5 ml. of the standard stock solution of riboflavine representing concentrations of 900, 800, 700, 600, 500, 400, 300, 200, and 100 μg. of riboflavine per 5 ml. w/v respectively.

To obtain lower concentrations, 1, 2, 4, and 8 ml. of the standard stock riboflavine solution are diluted with distilled water to 100 ml. in a standard flask representing concentrations of 10, 20, 40 and 80 μg. of riboflavine per 5 ml. of solution respectively.

From the results obtained it was found that:—

(a) Transmission readings should be spread out sufficiently to allow a determination to be made within concentrations ranging from 40 μg. to 1000 μg. per 5 ml. w/v of riboflavine.

(b) Since the graph shows that within these concentrations there is a slight deviation from Beer's Law, a calibration table can replace the graph and give more accurate results, providing that the estimations are carried out at the same (room) temperature and under the same conditions.

TABLE I

CALIBRATION TABLE

Amount of riboflavine μg.	Percentage transmission	Amount of riboflavine μg.	Percentage transmission
1000	36	400	61
900	38	300	70
800	43	200	78
700	46	100	89
600	51	80	92
500	56	40	96
		20	99

Calibration Table. From the standard stock solution of riboflavine are prepared standard solutions so that 5 ml. of each dilution contains an amount ranging from 100 μg. to 1000 μg., increasing in the order of 100 μg.; 5 ml. of each dilution are accurately measured in a dry, colorimeter tube; 3 ml. of Denigè's reagent is added, well mixed and left to stand for about 3 minutes. The percentage transmission of the solution is then read.

The results obtained at room temperature (25°C.) are shown in Table I.

Method of Assay

(1) *Test Solutions*

A dilution of the test solution is made so that 5 ml. contains between 100 μg. and 1000 μg. of riboflavine. 5 ml. of this dilution is accurately measured into a dry colorimeter tube, 3 ml. of Denigè's reagent is added, mixed and left to stand for about 3 minutes. The percentage transmission of the solution is measured and the amount of riboflavine read from the calibration table.

(2) *Injections*

A known volume of the injection is diluted with distilled water in a standard flask so that 5 ml. contains between 400 and 600 μg. of riboflavine 5 ml. of this dilution is accurately measured and introduced into a dry colorimeter tube; 3 ml. of Denigè's reagent is added and mixed well. The percentage transmission of the solution is measured and the concentration read from the calibration table. The figure obtained is the amount in μg. contained in 5 ml. of the diluted solution.

This method has been applied to two kinds of injections obtainable in Egypt.

(a)	Riboflavine	5 mg.
	Nicotinamide	200 mg.
	in	1 ml.

1 ml. is diluted to 50 ml. with distilled water in a standard flask. 5 ml. of this solution and 3 ml. Denigè's reagent are introduced into a dry colorimeter tube and mixed well. The percentage transmission is measured and from the calibration table the amount of riboflavine corresponding to this is obtained. According to the label 500 μg. is present in 5 ml. of the diluted solution.

(b)	Lactoflavine	5 mg.
	Sodium 2-oxy-4-methoxybenzoate	80 mg.
	Sodium monophosphate	5 mg.
	Distilled water to	1 ml.

1 ml. is diluted with distilled water to 20 ml. and 5 ml. of dilute sulphuric acid (20 per cent.) added. The precipitated 2-oxy-4-methoxybenzoic acid is filtered, washed several times with distilled water and the filtrate made up to 50 ml. with distilled water in a standard flask.

According to the formula of the preparation, 5 ml. of this diluted solution contains 500 μg. of riboflavine.

(3) *Tablets.* 15 tablets are weighed and powdered and an accurately weighed quantity of the powder, equivalent to 2 mg. of riboflavine is introduced into a 25 ml. flask. Successive small quantities of an ethanol-water mixture (50 per cent.) are added with continuous and vigorous shaking and the volume made up with the same solvent mixture. The mixture is shaken at intervals of 15 minutes and filtered. The assay is carried out using 5 ml. of the filtrate, as described for injections. The result obtained, multiplied by 5, gives the amount of riboflavine in the original weight taken.

SUMMARY

(1) A new colour test for riboflavine is described. The nature of the colour test is discussed and shown to be due to the coloured mercury or silver derivative.

(2) A photoelectric, colorimetric method for the assay of riboflavine is described. This method is recommended for the assay of pharmaceutical preparations such as injections and tablets. The assay is carried out within limits of 0·04 to 1 mg. of riboflavine.

(3) New colour tests for riboflavine with concentrated sulphuric acid and sodium hydroxide are mentioned.

Work is proceeding on the application of this method for biological fluids.

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