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Spectrophotometric Assessment of Vitamin B2 (Riboflavin) by Diazotization and Coupling Reaction with 2-Amino-4-(3-Pyrimidyl) Pyrimidine Reagent

Rahma Abdulhameed Hasan
Chemistry Department, College of Education for Pure Science, Tikrit University, Iraq

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ABSTRACT

Objective: *The diazotization and coupling method were employed as a simple and sensitive spectrophotometric analysis technique for the determination of riboflavin.* ***Method:*** *The method is based on the reaction between diazotization of the reagent using sodium nitrite in acidic medium and the maximum absorption wavelength of the resulting compound is 505 nm, since the compound is colorless and gets orange red when it couples with the medicine in basic medium. Stoichiometry of the produced compound was determined using the Job's method and mole-ratio method; the drug-to-reagent binding ratio was 1:1 by both methods.* ***Results:*** *Based on Beer's law, linearity in the concentration range of 1.25-29 µg/mL was observed. The molar absorptivity value is 8317.777 L .mol-1. cm-1, the coefficient of determination (R2) is 0.997, the relative standard deviation (RSD) is less than 0.12%, the limit of quantification (LOQ) is 0.263 µ g/mL, the limit of detection (LOD) is 0.087 µg/mL, and the Sandell's sensitivity value is 0.0452 µg/cm2.* ***Novelty:*** *The presented method was successfully applied for determination of riboflavin in pharmaceutical preparations.*

INTRODUCTION

Riboflavin, a key water-soluble vitamin in the B complex group of vitamins, is necessary for the functioning of the metabolic processes and energy production in cells [1]. The formation of coenzymes such as FMN and FAD, which take active part in the mitochondrial electron transport chain, is dependent on this vitamin as the basic precursor [2]. It initiates oxidation-reduction reactions to control the metabolism of energy from carbohydrates, fats, and proteins [3]. Besides its metabolic properties, this vitamin possesses strong antioxidant properties, which aid in increasing the endogenous antioxidant defense and protect cells from oxidative damage and lipid peroxidation [4]. With the help of its action in the absorption and metabolism of iron, it also performs a key role in the maintenance of the health of blood, skin, and mucosa tissues [5]. Recent medical studies have proved that riboflavin dosages have high efficacy in prevention and therapy of chronic migraine attacks and brain responses [6]. Moreover, it protects neuronal cell activities and ensures the health of the central and peripheral nervous systems [7]. Concerning the gastrointestinal tract, various scientific researches proved the efficiency of riboflavin supplementation in improving the morphology, epithelial differentiation, and redox state in experimental model [8]. Moreover, riboflavin is clinically significant for the improvement of the condition of the blood vessels, kidneys and decreasing the risk of metabolic disorders related to the presence of diabetes mellitus [9]. Dryness of skin, angular cheilitis, cheilosis/glossitis and anemia caused by improper hemoglobin production are symptoms of severe deficiency [10]. There are plenty of sources of riboflavin in food products, such as milk and other dairy products, fish, meat, greens, and fortified cereals [11]. Due to the water-solubility of excess amounts of the

substance, riboflavin is safely excreted through urine; regular daily intake does not cause any toxicity [12]. Clinical research continues the study of the advanced use of riboflavin in the treatment of metabolic diseases and hereditary mitochondrial disorders [13]. Modern pharmaceutical research is focused on the investigation of the use of the derivatives of the vitamin for the development of novel drug targets and cell defenses [14]. "The combination of these properties makes vitamin B2 an essential element for the maintenance of public health and prevention of chronic diseases in human body [15]." Besides, there are various methods for the identification of the substance using spectrophotometric [16], chromatographic [17], and electrochemical [18] techniques.

RESEARCH METHOD

Devices used: T90UV-VIS Spectrophotometer "double bean with 1cm quartz cells from PG Instruments Ltd." Balance Kern 770 GS/GJ from Sartorius BL 210 S .

Reagents and chemicals used: High-purity liquid and solid chemicals were used in this study.

Chemical solutions and reagents:

A. Standard riboflavin vitamin solution 1000 µg/mL

Distilled water was added to a 100 mL volumetric flask after 0.1000 g of the riboflavin medication had been dissolved in 20 mL of water.

B. Standard riboflavin vitamin solution 250 µg/mL

This solution was made by diluting 25 mL of the 1000 µg/mL standard solution with distilled water in a 100 mL volumetric flask.

C. Solution of a molar reagent 1×10^{-2}M

In a 100 mL volumetric flask, 0.0172 g of the reagent powder was dissolved in 10 mL of ethanol, and the volume was then adjusted with distilled water.

D. Sodium nitrite (NaNO2) reagent solution

In order to produce the solution, 0.0345 g of it was dissolved in a certain volume of distilled water. The volume was then adjusted using distilled water in a 100 mL volumetric flask.

E. A solution of around 1 M hydrochloric acid

In a 100 mL volumetric flask, 8.28 mL of a 12.77 M hydrochloric acid solution was diluted and the volume was adjusted with distilled water.

F. A sodium hydroxide solution of around 1 M

In a 100 mL volumetric flask, 4.0 g of sodium hydroxide was dissolved to create a solution.

G. Solution of sulfamic acid 0.5%

In 100 ml of distilled water were used to dissolve 0.5 grams of it.

H. Pharmaceutical preparation solutions 250 µg/mL

The mass of ten tablets of the medicine B-plexin, which has 5 mg of riboflavin vitamin per tablet, is 3.5511 g when the tablets have been thoroughly mixed and weighed. The resulting solution was dissolved in water and filtered to give a stock solution with a concentration of 250 µg/mL in 100 mL volumetric flask.

RESULTS AND DISCUSSION

General principle of the method

The solution of vitamin B2 or riboflavin is then added to the diazotized product in a basic medium and a colorless intermediate is produced. When some quantity of acid

and diazotizing agent solution is added to the reagent solution, then the above process takes place rapidly.

Preliminary study

The mixing of 1 mL of reagent solution and 1 mL of diazotizing agent solution (sodium nitrite) in the presence of HCl solution resulted in the formation of a colorless intermediate compound. One milliliter of the riboflavin drug solution was mixed into the compound solution after it was mixed and allowed to cool for five minutes in an ice bath. Five more minutes later, the addition of sulfamic acid in the solution took place to remove any leftover nitrous acid until the reaction reached completion. The product of the reaction turned out to be reddish-orange. The absorbance of the colored product was determined against its blank solution in a 25 mL volumetric flask after diluting the volume with distilled water. The product showed maximum absorbance at 505 nm wavelength, whereas the blank solution showed no absorbance at that wavelength.

Studying the optimal conditions for producing the product

A solution containing riboflavin at a concentration of 250 µg/mL was diluted by adding one milliliter of the solution to a final volume of 25 milliliters. The absorbance of the solution was measured using 505 nm as the wavelength.

Effect of volume of hydrochloric acid

Through gradual increase in volume of 1 M hydrochloric acid, the volume of maximum absorbance was attained. The best volume of acid to use was found to be 1.5 mL based on the results shown in Table 1.

Table 1. Effect of acid volume.

Volume	Abs
0.3	0.17
0.5	0.21
0.8	0.22
1	0.37
1.5	0.411
2	0.38

Effect of base size

The amount of 1.0 M NaOH solution that gave the maximum absorbance value was found gradually. These results are shown in Table (2), and the ideal amount is found to be 1.5 mL.

Table 2. Effect of base volume.

Volume of NaOH	Abs
0.3	0.205
0.5	0.285
0.8	0.314
1	0.39
1.5	0.411

Effect of fuel size

Different volumes of diazotizing reagent (1 x10-2 M NaNO2) in increasing amounts were added to determine the effect on absorbance of the product, as shown in Table (3). It was found that the optimum volume of addition is 1 mL.

Table 3. Effect of diazotizing agent volume.

Volume of $NaNO_2$	Abs
0.2	0.23
0.5	0.311
0.8	0.383
1	0.412
1.5	0.35

Effect of detector size

The effect of increased reagent amounts using the concentration of 1×10-2 M on the absorbance of the product is presented in Table (4). The best reagent amount for adding was found to be 2 mL.

Table 4. Effect of reagent volume.

Volume of Regent	Abs
0.5	0.168
1	0.243
1.5	0.346
2	0.411
2.5	0.361

Effect of sequence of additions

A lot of experiments were carried out in 25 mL volumetric flasks with different orders of addition; more experiments were carried out based on ideal values and quantities, as shown in Table (5). Order of addition No. 5 gave the highest absorbance and was selected for future experiments.

Table 5. Effect of addition sequence on absorbance values.

No.	Sequence of addition	Abs
1	D + R + A + N + B	0.327
2	R + N + D + A + B	0.355
3	D + A + N + R + B	0.385
4	D + N + A + R + B	0.39
5	R + N + A + D + B	0.413

(D) riboflavin medication; (R) reagent; (N) diazotizing agent (sodium nitrite); (A) hydrochloric acid; and (B) sodium hydroxide base.

Study of the time of nitrogen

The set of 25 mL volumetric flasks was prepared in order to study the effect of time required for completion of the diazotization and coupling reactions. Two milliliters of the reagent, one milliliter of sodium nitrite, and one and a half milliliters of hydrochloric acid were placed into each flask. After placing them into an ice bath for different periods of time (0-35 minutes), 1 mL of riboflavin solution and 1.5 mL of sodium

hydroxide were added to each combination. Afterwards, the solutions were diluted to the total volume of 25 mL with distilled water. The absorption of each solution was recorded at the wavelength of 505 nm by comparison with its own blank solution. The obtained results are given in Table (6). It was established that the reaction time of five minutes was enough for completing the diazotization reaction.

Table 6. Study of diazotization time.

Time (min)	0	5	10	15	20	25	30	35
Abs	0.35	0.411	0.411	0.41	0.409	0.409	0.408	0.407

The effect of time on stability

It becomes very important to determine the stability of the final product for understanding the stability time period. The results obtained from monitoring the reaction in optimal conditions are presented in Table (7) .

Table 7. Effect of time on the stability of the colored product.

Time min	direct	5	10	15	20	30	40	50	60
Abs	0.411	0.411	0.41	0.41	0.41	0.409	0.408	0.407	0.405

The absorbance of the product was constant up to 60 minutes, which is sufficient time for carrying out the experiment, and the values of absorbance in the table were uniform at the wavelength.

Final absorption spectrum

After the establishment of the optimal conditions, 2 mL of 1 ×10-2M reagent, 1 mL of 1 ×10-2M diazotizing agent (NaNO), and 1.5 mL of 1.0 M HCl were added. The solution was left in an ice bath for five minutes and 0.5 mL of 0.5% sulfamic acid was added to the solution. 1.5 mL of NaOH was added following 1 mL of Riboflavin 250 mu g/mL. After the reaction was completed for five minutes, the solution was diluted with distilled water in a 25 mL volumetric flask to give an absorption spectrum of the reddish-orange product. The absorption spectra of the blank solution was then measured in comparison with that of the product. As observed from Figure (1), maximum absorption of the product was attained at 505 nm wavelength.

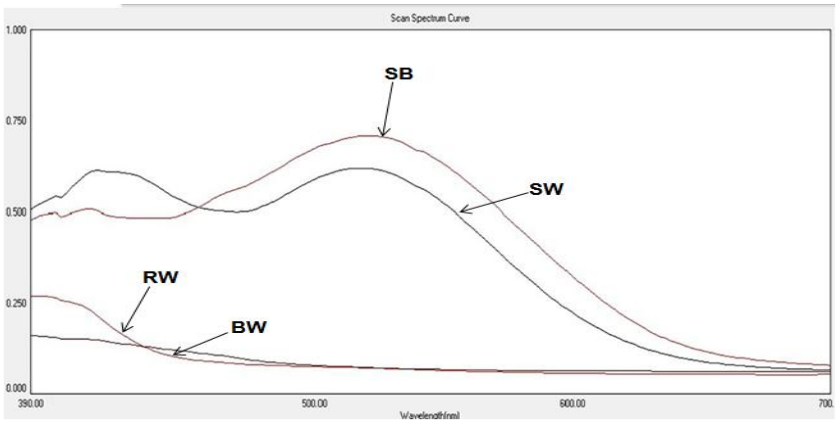


Figure 1. Final absorption spectrum for the determination of riboflavin

Where:
SW: Absorption spectrum of the complex against distilled water
SB: Absorption spectrum of the complex against the blank solution
BW: Absorption spectrum of the blank solution against distilled water
RW: Absorption spectrum of the reagent solution against distilled water

Calibration curve

Each of the 25 mL volumetric flasks was charged with 2 mL of 1 × 10-2 M reagent solution, 1 mL of 1 ×10-2 M diazotizing agent ($NaNO_2$), and 1.5 mL of 1.0 M HCl. Five minutes after being placed in an ice bath, 0.5 mL of 0.5% sulfamic acid was added to the solutions. Next, 1.5 mL of 1.0 M NaOH solution was added after supplying the riboflavin solution to have the concentration range of 1.25–29 mug/mL (or quantities of 0.125–2.9 mL from the 250 mug/mL stock solution). After diluting the solutions to their corresponding levels in 25 mL volumetric flasks with distilled water, the absorbance of each solution was read against the blank solution at 505 nm wavelength. Calibration curve (Figure (2)) has the R2 value of 0.997 and follows the Beer's law over the concentration range of 1.25-29 mug/mL of riboflavin. Sandell's sensitivity was 0.0452 mug/cm2, and the molar absorptivity was 8317.777 $L.mol^{-1}.cm^{-1}$.

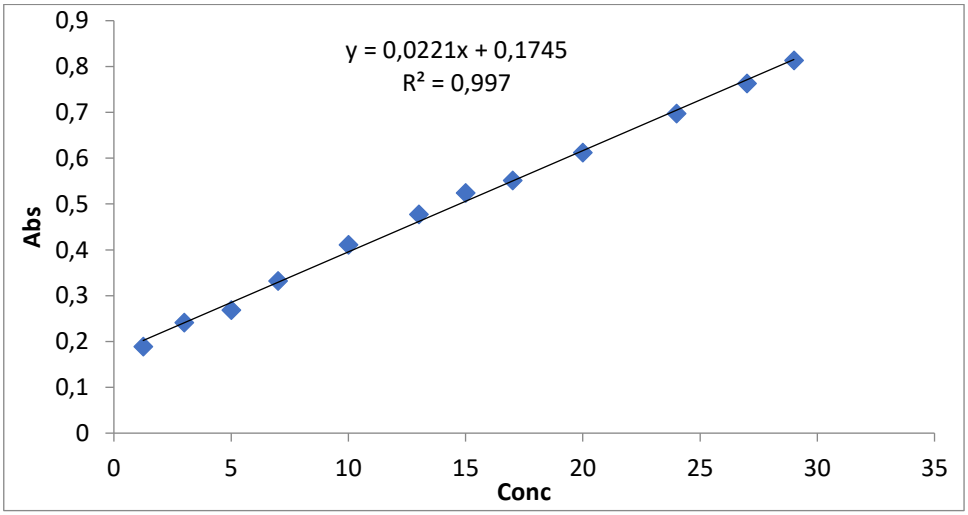


Figure 2. Calibration curve for the determination of riboflavin

Accuracy and compatibility

The precision and accuracy of the proposed method of riboflavin estimation have been confirmed through measuring of three drug concentrations from the calibration curve in the optimal working conditions. The obtained data prove that the method has high accuracy and precision, which can be seen from Table (8).

Table 8. Accuracy and precision of the proposed method
*(Each value represents the average of three readings)

Studied conc mu g/mL	Observed conc mu g/mL	RE%	%RSD*
7	7.126	1.77	0.174
17	17.03	0.213	0.105
24	23.64	-1.51	0.082

Moreover, the limit of quantification (LOQ) and limit of detection (LOD) were calculated; the results indicate that LOQ is 0.263 µg/mL, while the LOD is 0.087 µg/mL.

Equivalence of the resulting product

For equimolar concentrations, 1 x10-3M was used for both drug and reagent solutions to determine the stoichiometry of the product and the binding ratio of drug and reagent using Job's continuous variation method.

Volumetric flasks of 25 mL volume containing varying amounts of the reagent between 4.5and 0.5 mL volumes were made. After this, 1 mL of sodium nitrite (a diazotizing agent) and 1.5mL of HCL were added to it. Riboflavin solution of 0.5-4.5mL was used along with 1.5 mL of sodium hydroxide. The measurement was taken at 505 nm after diluting it to the proper amount with distilled water. As shown in Figure (3).

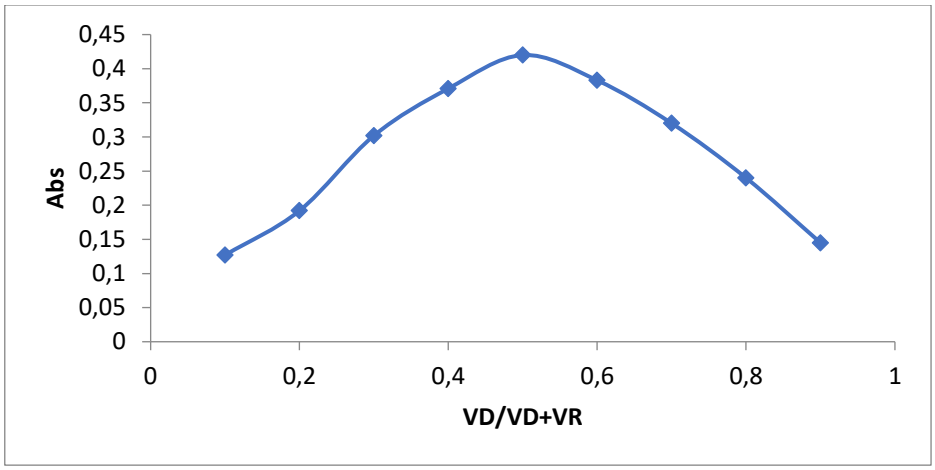


Figure 3. Job's method.

It was proven that the reaction between the riboflavin solution and the reagent was 1:1.

There were several 25 mL volumetric flasks containing varying amounts (0.1 to 0.9 mL) of the reagent, then followed by 1.5 mL of acid, 1 mL of diazotizing agent, 1 mL of riboflavin, and 1.5 mL of base in performing the mole-ratio method. Upon dilution of the solution in distilled water, the absorbance was measured at 505 nm compared to the blank solution. The results showed that the mole-ratio method agreed with Job's method of continuous variation. As in figure 4.

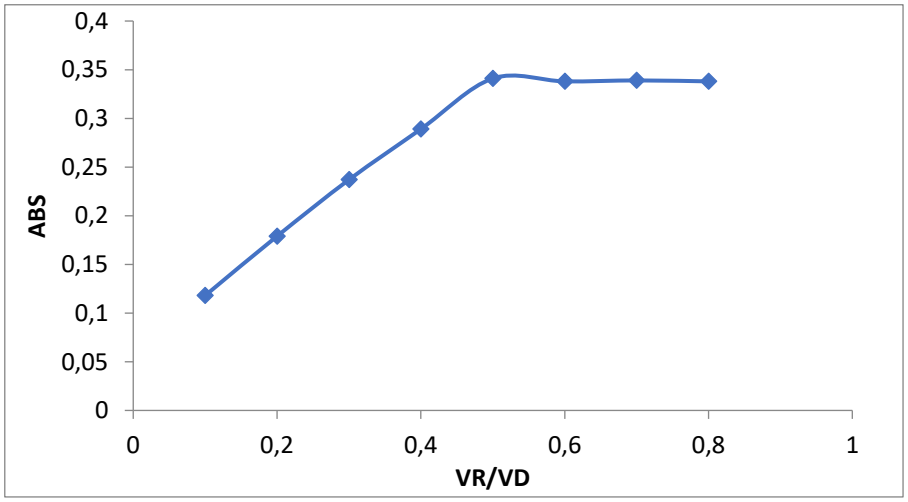
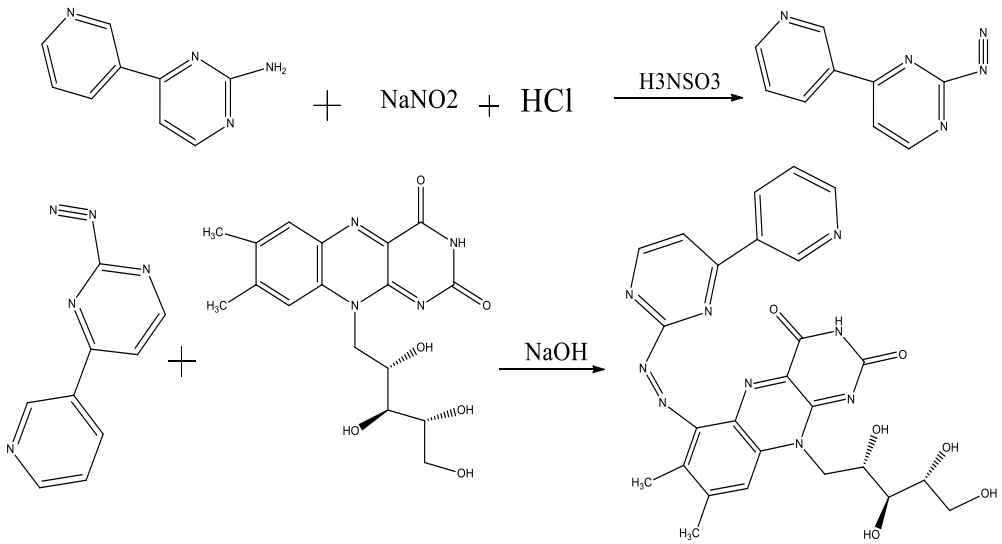


Figure 4. Mole-ratio method.

The proposed reaction can be represented according to the following equation [19]:



Applications

Concentrations of the solution of the pharmaceutical formulation (250 mug/mL) were taken three times through this method. The solutions were then placed in 25 mL volumetric flasks, having been prepared in the same way as the calibration curve. Absorbance for each concentration was determined at 505 nm (by averaging three readings) with respect to the blank solution. The validity and applicability of the proposed method for pharmaceutical formulations have been determined, as illustrated in Table (9).

Table (1-9) Application of the proposed method for the determination of the drug in pharmaceutical preparations.

Table 9. Application of the proposed method.

Studied conc g/mlμ	Observed conc g/mlμ	%RE	RSD%
3	3.01	0.31	0.24
6	6.08	1.43	0.19
12	12.10	0.86	0.13

CONCLUSION

Fundamental Finding : The maximum wavelength for the formation of colored compound was 505 nm. From Beer's law, linearity was attained between the concentration of 1.25-29 mu g/mL. With the detection limit of 0.087 mu g/mL and quantitation limit of 0.263 mug/mL, the molar absorptivity was 8317.777 L. mol-1.cm-1. Sandell sensitivity was 0.0452 mu g/cm2, the coefficient of determination (R2) was 0.997 while relative standard deviation (RSD%) did not exceed 0.12%, which showed that the method is accurate and precise. **Implication :** The proposed method was effectively applied in estimating the content of riboflavin in pharmaceutical preparations. **Limitation :** The study was limited to the spectrophotometric determination of riboflavin using the diazotization-coupling reaction and its application to pharmaceutical preparations. **Future Research :** Future research could investigate the applicability of the

proposed method to a wider range of pharmaceutical formulations and complex sample matrices.

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***Rahma Abdulhameed Hasan (Corresonding Author)**
Chemistry Department, College of Education for Pure Science, Tikrit University, Iraq
Email: Rahma.abdulhameed@tu.edu.iq