SPECTROPHOTOMETRIC ESTIMATION OF FOLIC ACID (VITAMIN B9) USING AN OXIDATIVE COUPLING METHOD WITH (E)-N'-(1-(2-NITROPHENYL)ETHYLIDENE)QUINOLINE-6-CARBOHYDRAZIDE(M4)

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Abstract: *A simple, cost-effective, and highly sensitive spectrophotometric method was developed for the determination of folic acid (vitamin B9). The method is based on an oxidative coupling reaction with a synthetic reagent, (E)-N'-(1-(2-nitrophenyl)ethylidene)quinoline-6-carbohydrazide (M4), in the presence of potassium dichromate as the oxidizing agent and sodium hydroxide in a basic medium. This reaction produces a yellow, water-soluble compound with an absorbance at 380 nm. The Beer-Lambert law was applied over the concentration range of 0.5 to 7.5 µg/mL. The recovery was 98.71%, and the correlation coefficient was 0.9955. It was determined that the Sandell sensitivity was 0.0085 µg/cm² and the molar absorptivity was 51599.66 L mol cm. The limits of detection (LOD) and quantification (LOQ) were 0.059 µg/mL and 0.196 µg/mL, respectively. Using the suggested technique, the amount of folic acid found in pharmaceutical formulations has been successfully determined.*
Keywords*: Folic acid, oxidative coupling, Spectrophotometric, estimation, Vitamin B9*

Introduction

Folic acid is a water-soluble, naturally occurring form of vitamin B9. It is found naturally in plants and vegetables such as dark leafy greens, citrus fruits, and meat products. Folic acid is sold as a dietary supplement when added to foods in its synthetic form. This synthetic form is absorbed more efficiently than folic acid from many dietary sources, with an absorption rate of approximately 85% compared to about 50%, respectively [1]. Folic acid is one of the essential vitamins that provide significant health benefits to the human body. Its structure, molecular formula, and molecular weight are important parameters for understanding its chemical and biological properties [2] (Fig.1).

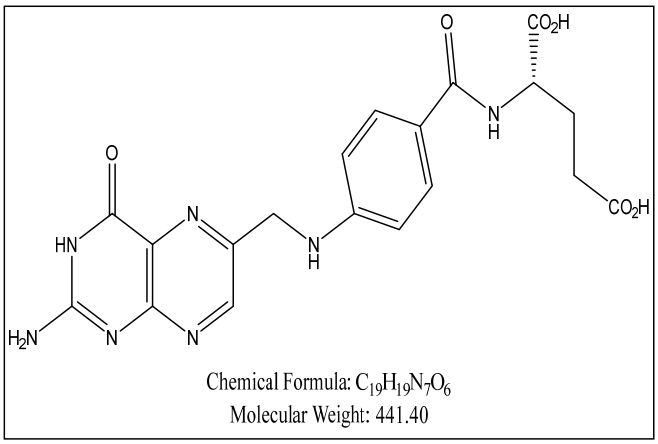

Fig. 1. Structural formula of Folic Acid (Vit. B9) [3]

Folic acid is considered one of the essential vitamins that provide significant benefits to the human body in general [4]. One of the main factors leading to folic acid deficiency is poor nutrition. Among the indications of this deficiency are softness, shortness of breath, and anemia [5]. Vitamin B9 plays a crucial role in one-carbon metabolism, especially as a methyl group donor during DNA synthesis [6]. Additionally, pregnant women are at risk of folic acid deficiency, as pregnancy significantly increases the need for folate [7]. When getting folic acid from foods or supplements, it

is absorbed in the small intestine and then gradually converted to dihydrofolate (DHF) and tetrahydrofolate (THF) by the enzyme dihydrofolate reductase (DHF R) inside living cells [8]. Consumption of this vitamin has been associated with a lower risk of cardiovascular disease, neurological disease, and colorectal cancer [9]. This vitamin has been estimated using different analytical methods such as spectrophotometry [10], high-performance liquid chromatography [11], electrochemical methods [12], nuclear magnetic resonance techniques [13], and molecular and biological spectroscopic methods [14], as well as oxidation and reduction reaction methods [15] and using flow injection technology [16].

In this study, vitamin B9 was estimated using an oxidative coupling method with the prepared reagent (M4). The method was characterized by its simplicity, fast, and applicability to pharmaceutical formulations.

Experimental part

Instrumentation Used. UV-Vis Spectrophotometer T80 UV-VIS Spectrophotometer, "double beam" model, 1 cm quartz cell, Thermosi water bath, England, manufactured by PG Instruments Ltd. Balance BL210 Kern 770GS/GJ, Hot Plate MS300HS, Harry Gestigkeit GmbH.

Materials and Solutions. The materials used in this study were obtained in pure form from FLUKA (USA) and Avonchem (UK). Distilled water and ethanol were used as solvents for the solutions.

Standard Solution (VitB9) 250 µg·mL^{-1}. Dissolving 0.025 g of VitB9 (molecular weight: 441.4 g/mol) in 10 mL of one molar NaOH solution, completing to 100 mL volumetric flask with D.W. Then, 10 mL of this solution was diluted to 25 mL with distilled water to obtain a solution with a concentration of 100 µg/mL.

Reagent solution (E)-N'-(1-(2-nitrophenyl)ethylidene)quinoline-6-carbohydrazide (M4)250 µg.mL^{-1}. Dissolving 0.025 g of the reagent powder, synthesized as described in [17], with a molecular weight of 334 g/mol, in a small amount of ethanol. The solution was heated to boiling and completed to 100 mL with the same solvent.

Potassium dichromate solution at a concentration of 10^{-3} M. 0.014 g of potassium dichromate powder (molecular weight: 294.18 g/mol) was diluted to the mark with distilled water in a 50 mL volumetric flask. Solutions of KI, KIO_3, KIO_4, $KMnO_4$, and K_2CrO_4 were prepared at 10^{-3} M, and solutions of 0.1 M NaOH and HCl acid (36%, density 1.19 g/mL) were prepared.

Pharmaceutical Preparation Solution (Folic Acid). Ten capsules of the pharmaceutical preparation Folic Acid, manufactured by Accord and containing 5 mg of vitamin B9 per capsule, were weighed and thoroughly mixed to ensure homogeneity. The total weight of the capsules was 0.62 g. The mixture was dissolved in 10 mL of 1 M NaOH solution and filtered by Whatman No. 42. In a 100 ml measuring flask, a solution containing 50 µg/ml of folic acid was obtained.

Results and discussion

Preliminary Study. It was observed that when 1 mL of Vit.B9 100 µg/mL was added, followed by 0.5 mL of potassium dichromate 10^{-3} M, and then 0.5 mL of the reagent (E)-N'-(1-(2-nitrophenyl)ethylidene)quinoline-6-carbohydrazide (M4) 250 µg/mL in a 20 mL volumetric flask, a yellow solution was formed. This solution exhibited an absorption spectrum at 380 nm.

Effect of Oxidizing Agent. The study was conducted by adding 0.5 mL of various oxidizing agents at 0.001 M, such as KI, KIO_3, KIO_4, $KMnO_4$, K_2CrO, and $K_2Cr_2O_7$, to 1 mL of Vit.B9 (100 µg/mL), followed by 0.5 mL of the reagent (M4) (250 µg/mL). The results showed that potassium dichromate solution ($K_2Cr_2O_7$) provided the highest absorption for the colored product at 380 nm. Therefore, potassium dichromate was chosen for subsequent experiments.

Effect of oxidizing agent amount. The optimal amount of potassium dichromate (0.001 mol) as an oxidizing agent was investigated. Different volumes of the oxidizing agent (ranging from 0.1 to 1.5 ml) were added to a 20 ml volumetric flask containing 0.5 ml of reagent (M4) (at a concentration

of 250 µg/ml) and 1 ml of vitamin B9 (at a concentration of 100 µg/ml). The total volume was then adjusted to 20 ml using distilled water. The optimum volume of the oxidizing agent was found to be 0.8 ml, where maximum absorbance was achieved, and this amount was chosen for the subsequent experiments.

Reagent Volume Effect. The effect of varying volumes of (M4) reagent solution (at a concentration of 250 µg/mL) ranging from 0.1 to 1 mL was studied. The results showed that a volume of 0.7 mL of the reagent solution produced the highest absorbance of the colored product at a wavelength of 380 nm compared to the other volumes tested. Therefore, 0.7 mL was selected as the optimal volume for subsequent experiments.

Effect of Oxidation Time for Folic Acid. The procedure involved transferring 0.8 mL of the oxidizing agent solution into a 20 mL volumetric flask, followed by the addition of 1 mL of folic acid solution. To determine the optimum oxidation time, the duration was varied from 5 to 30 minutes. After this, 0.7 mL of the (M4) reagent solution was added, and the flask was filled to the mark with distilled water. It was observed that the oxidation process occurred optimally at 15 minutes, which was identified as the ideal time for the oxidation of folic acid.

Effect of Addition Sequence. The effect of the addition sequence of the solutions used was studied to determine its influence on the intensity of the resulting color. To this end, several experiments were conducted with different addition sequences using 20 mL volumetric flasks, adhering to the same optimal conditions established in the previous experiments, as shown in the following Table 1.

Table 1. Effect of Addition Sequence

Addition Sequence	Absorbance
A + B + C	**0.672**
B + A + C	0.611
C + B + A	0.532
B + C + A	0.554

Where, A = Folic acid; B = Potassium dichromate solution; C = M4 reagent. Table 1 indicates that the best addition sequence is the first one (A + B + C). Therefore, this sequence was employed in the following experiments.

Temperature Effect. The effect of temperature on the absorbance of the complex was analyzed over a range from 5°C to 55°C. The experiment utilized 1 mL of vitamin B9 (at a concentration of 100 µg/mL), 0.8 mL of an oxidizing agent at 1×10^{-3} M, and 0.7 mL of the (M4) reagent (at 250 µg/mL). After combining these components, the mixture was transferred into multiple 20 mL volumetric flasks and diluted to the appropriate concentration with distilled water. The results indicated that room temperature, ideally not exceeding 25°C, was the best condition for absorbance.

Effect of pH. The effect of pH on the prepared solution was investigated by adding hydrochloric acid and sodium hydroxide. When hydrochloric acid was introduced, a decrease in absorption and a noticeable loss of color were observed. Conversely, when sodium hydroxide was added, an increase in absorption was recorded, indicating that the product remained stable in a basic medium. Subsequently, varying volumes (0.2 to 1 ml) of sodium hydroxide solution, with a concentration of 1×10^{-1} M, were added. The results, summarized in the table below, show a significant increase in absorption with the addition of these basic solution volumes, as the pH values ranged from 9 to 14. Therefore, a volume of 0.4 ml of the basic solution was selected for further experiments, as it yielded the best absorption results.

Stability Time. A study was conducted to evaluate how stability affected the absorbance of a generated complex over a duration ranging from five to sixty minutes. The experimental procedure involved using 1 milliliter of vitamin B9 (100 µg/mL), 0.4 milliliters of a 10^{-3} M sodium hydroxide solution, 0.7 milliliters of a 4M reagent (250 µg/mL), and 0.8 milliliters of a 10^{-3} M oxidant. Distilled water was then added to the mixture to achieve the desired volume in a series of 20 mL volumetric

flasks. The results indicated that the solution did not exceed 25°C and remained stable at room temperature for at least 30 minutes.

Final Absorption Spectrum. The final absorption spectrum (Fig. 2) was measured after achieving the optimal conditions, which involved using 1 mL of VitB9 solution, 0.8 mL of potassium dichromate, 0.7 mL of (M4) reagent, and 0.4 mL of sodium hydroxide. The volume was completed to the mark in a 20 mL volumetric flask using distilled water. The resulting yellow-colored absorption was measured against its blank solution, which exhibited determined absorption at 380 nm, but the blank solution showed no absorption in the region.

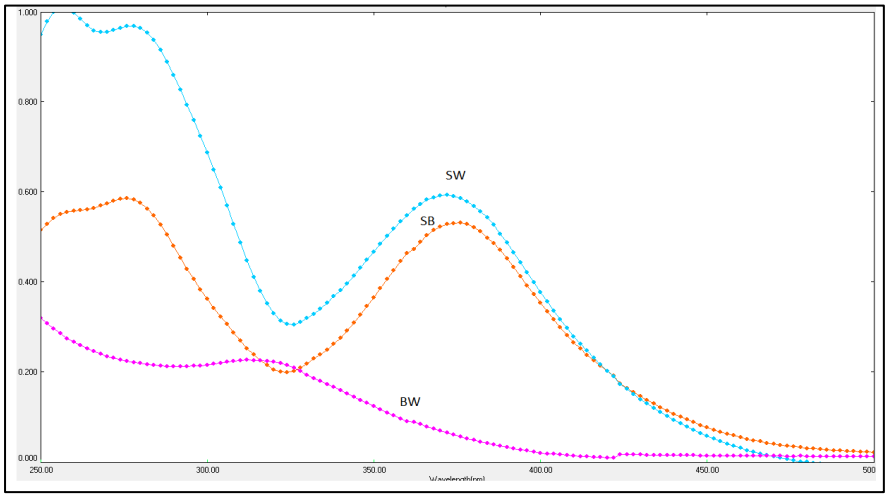

Fig. 2. Final Absorption Spectrum. **SW:** spectrum of B9 solution sample VS water; **SB:** spectrum of B9 solution sample VS blank; **BW:** Spectrum of the blank solution measured VS distilled water.

The Calibration Curve. Calibration curve solutions were prepared by adding increasing concentrations of VitB9 solution ranging from 0.5 to 7.5 µg/mL, which means volumes of 0.1–1.4 mL. 100 µg/mL were taken and added to a chain of 20 mL volumetric flasks, each containing 0.8 mL of potassium dichromate (10^{-3} M), 0.7 mL of M4 reagent (250 µg/mL), and 0.4 mL of sodium hydroxide. After 15 minutes, the solution was completed to the mark with D.W. The absorption of solutions was measured against a blank at 380 nm, as shown in Fig. 3, which represents the calibration curve for VitB9 determination. The molar absorptivity was found to be 51599.60 L/mol·cm, and Sandell's sensitivity 0.0085 µg/cm², and the correlation coefficient 0.9955, indicating the high sensitivity and accuracy of the method.

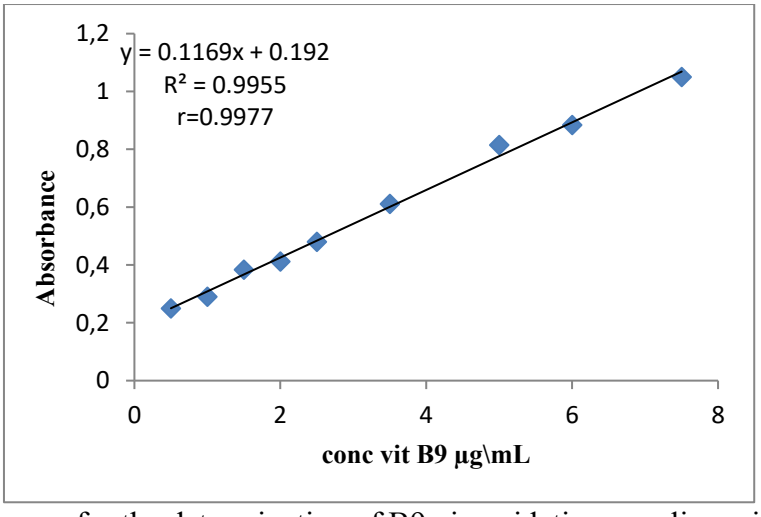

Fig. 3. Calibration curve for the determination of B9 via oxidative coupling with the (M4) reagent

Accuracy and Precision. The optimal conditions from the calibration curve were used to test the accuracy and precision of the method. Three dissimilar concentrations of vitamin B9 within the limits of Beer's law on the calibration curve were analyzed. The average recovery was 98.7%, and the relative standard deviation (RSD) was 0.4%, 0.416%, and 0.226%, indicating that the method is highly precise and accurate (Table 2).

Table 2. Accuracy and Precision

Conc. of B9 (µg/mL) Taken	Found	ER%	Recovery %	RSD %	Average Recovery %
0.5	0.496	-0.8	99.2	0.4	98.7
2.5	2.46	-1.6	98.4	0.416	
6	5.91	-1.5	98.5	0.226	

Calculation of LOD and LOQ (Table 3)

Limit of detection (LOD) planned using the following equation:

LOD=3.3(SD/Slope)

Limit of quantification (LOQ) planned using the following equation:

LOQ=10(SD/Slope)

Where **SD** is standard deviation and **Slope -** calibration curve slope.

Table 3. Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD (µg/ml) [18, 19]	LOQ (µg/ml) [20, 21]	SD (Standard Deviation)	Slope
0.059	0.196	0.0023	0.1169

Stoichiometry of Reaction. The chemical reaction between the mixture and vitamin B9 was studied using the Job method and the molar ratio method. The results obtained by both methods indicate that the reaction ratio of the water-soluble conjugate between vitamin B9 and the reagent (M4) is 1:1, as shown in Fig.s 4 and 5.

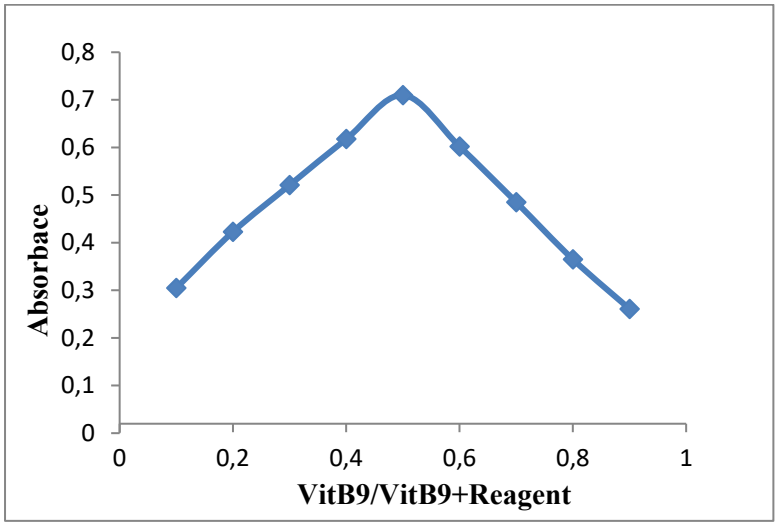

Fig. 4. Job's Method

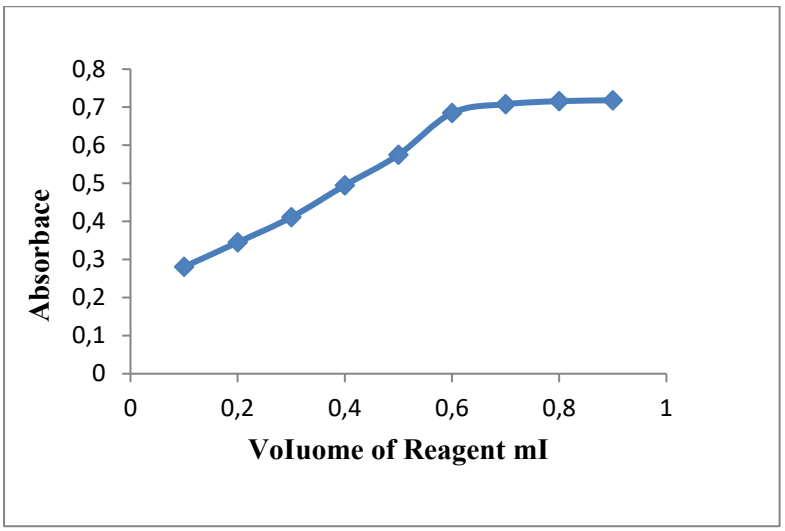

Fig. 5. Molar Ratio Method

Applications. The method can be practical on pharmaceutical preparations having vitamin B9.

Direct Method. 3 changed concentrations of the pharmaceutical formulation solution (50 µg/mL) were used: 2 µg/mL, 2.5 µg/mL, and 3.5 µg/mL. The solutions were processed following the same steps as for the calibration curve and transferred to 20 ml volumetric flasks, and the absorbance of these concentrations was measured at 380 nm, with three readings for each concentration, against their corresponding blank solutions. The efficiency of the method and its success in the determination of vitamin B9 in pharmaceutical formulation were evaluated (Table 4).

Table 4. Direct method for estimation of vitamin B9

Conc. of B9 (µg/mL)		RE%	Recovery %	Average Recovery %
Taken	Found			
2	1.98	-1	99	100.9
2.5	2.54	1.6	101.6	
3.5	3.58	2.2	102.2	

Conclusions

In this study, a spectrophotometric technique for determining the amount of vitamin B9 in pharmaceutical formulations and in its pure form was created. The method is built on the oxidation coupling reaction between the reagent (E)-N'-(1-(2-nitrophenyl) ethylidene)quinoline-6-carbohydrazide (M4) and folic acid (vitamin B9) in the presence of potassium dichromate and sodium hydroxide to obtain a yellow, water-soluble, and stable complex. The complex exhibited maximum absorption at 380 nm. Because this method does not call for heating, hydrolysis, or extraction with organic solvents, it is easy to use and effective for evaluating vitamin B9 in both its pure form and in pharmaceutical preparations.

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