

CENTRAL ASIAN JOURNAL OF MEDICAL AND NATURAL SCIENCES
https://cajmns.casjournal.org/index.php/CAJMNS
Volume: 08 Issue: 01 | January 2027 ISSN: 2660-4159

Article

Spectrophotometric Determination of Folic Acid With Metheylen Blue Dye by Oxidative Reaction

Zeena Tariq Khattab[1]

1. Department of Chemistry, College of Education for Pure Sciences, Tikrit University, Iraq
* Correspondence: zeena.tariq@tu.edu.iq

Abstract: A sensitive, easy-to-perform and precise spectrophotometric technique has been developed for quantitative estimation of folic acid in its pure as well as pharmaceutical formulations in aqueous solutions. This procedure involves oxidative coupling reaction in which the oxidation of the drug (vitamin B9) is done with oxidant (ammonium metavanadate), followed by the formation of greenish-yellow colored compound due to its oxidative coupling with methylene blue dye in acidic medium. It absorbs maximum light at 650 nm wavelength and follows the law of Beer within the concentration range of 6.25-75 µg/mL. Molar absorptivity of the compound has been determined to be 3354.64 $L \bullet mol^{-1} \bullet cm^{-1}$, Sandell's sensitivity 0.1315 $\mu g \bullet cm^{-2}$, the correlation coefficient (or estimation factor) 0.9968, relative standard deviation less than 0.64% and the limit of detection 2.5 µg/mL. The method is known for its simplicity as it requires only 10 minutes for completion and stabilization, which lasts further for more than 40 minutes. The procedure has been successfully utilized for the estimation of folic acid in its pharmaceutical forms.

Graphical Abstract

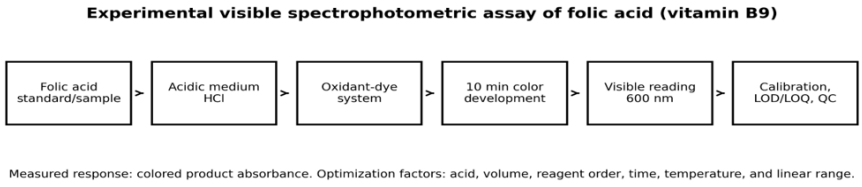

Figure 1. Graphical workflow of the experimental visible spectrophotometric assay for folic acid.

.

Keywords: Folic acid, oxidative coupling, methylene blue dye, ammonium metavanadate oxidizing agent.

Citation: Khattab, Z. T. Spectrophotometric Determination of Folic Acid With Metheylen Blue Dye by Oxidative Reaction. Central Asian Journal of Medical and Natural Science 2027, 8(1), 162-171

Received: 13th Aug 2026
Revised: 28th Sep 2026
Accepted: 15th Oct 2026
Published: 22th Nov 2026

Copyright: © 2027 by the authors. Submitted for open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/)

1. Introduction

A simple, highly sensitive, and accurate spectrophotometric method was developed for the determination of folic acid in its pure form and in pharmaceutical preparations in aqueous media [1]. The method relies on an oxidative coupling reaction, carried out by oxidizing the drug (vitamin B9) with the oxidizing agent ammonium metavanadate, yielding a greenish-yellow product upon coupling with methylene blue dye in an acidic medium. The product exhibits maximum absorbance at a wavelength of 650 nm and obeys Beer's law over the concentration range of 6.25–75 µg/mL [2]. The molar absorptivity was

found to be 3354.64 L·mol^{-1}·cm^{-1}, Sandell's sensitivity was 0.1315 μg·cm^{-2}, the correlation coefficient (or estimation factor) was 0.9968, the average relative standard deviation did not exceed 0.64%, and the limit of detection was 2.5 μg/mL[3]. The method is characterized by its simplicity; the reaction is complete and stabilizes after 10 minutes, remaining stable for more than 40 minutes. The proposed method was successfully applied to the determination of folic acid in its pharmaceutical formulations [4,5].

The IUPAC systematic name of folic acid is (2*S*)-2-[[4-[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]amino]benzoyl]aminopentanedioic acid, while its common English name is folic acid, and it is also known as vitamin B9.

- **Molecular weight:** 441.40 g/mol
- **Chemical formula:** $C_{19}H_{19}N_7O_6$
- **Chemical structure:** [6]

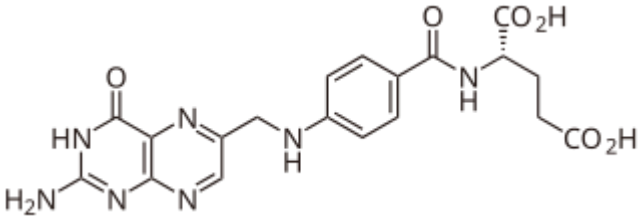

Figure 2. Chemical structure of folic acid

Folic acid occurs in yellow crystals and is stable in air, but decomposes when exposed to ultraviolet (UV) light. Folic acid is also thermally unstable in acidic medium, whereas it is highly stable in neutral and alkaline environments and stable below 100 °C temperature for 1 hour heating period. This vitamin (vitamin B9) is very important, and it has been analyzed using different methods, such as UV-visible spectrophotometric determination [7-11], electroanalytical determination [12], fluorescence method [13], HPLC [14], HPLC-DAD [15] and LC-MS/MS [16]. Because of the biological importance of vitamin B and its contribution to human health, the aim of this paper was to develop a sensitive spectrophotometric method based on the formation of an ion-pair complex with methyl violet dye [17].

Aim of the research:

The purpose of this work is to carry out an experimental investigation for the optimization and validation of a visible spectrophotometric method of the determination of folic acid. The following tasks will be fulfilled in the course of the research: (1) establishing the optimal acidic medium and experimental conditions, (2) assessing the color stability and its dependence on temperature, (3) plotting the calibration graph, and (4) calculating the preliminary LOD and LOQ values.

2. Materials and Methods

2.1 Study Design

This was a method development study using an experimental laboratory approach. Absorbance of the colored compound formed was used as the analytical signal. Independent variables studied included acid used, volume of hydrochloric acid used and its acidity, sample volume of folic acid, sequence of addition of reagents, development time of color, and temperature. Once the optimized variables were determined, they were used to develop a calibration model for quantification of folic acid.

2.2 Chemicals and Reagents

2.2.1 Standard Solution of Vitamin B9

The standard stock solution of vitamin B9 (folic acid) was prepared in a 100 mL volumetric flask by weighing 0.1 g of folic acid precisely and adding 5 mL of 1 M sodium hydroxide (NaOH) solution for ease of dissolution. The volume was made up to the mark using distilled water and mixed properly. From the stock solution, a standard working solution was made at a concentration of 250 μg/mL.

2.2.2 Dye Solution

The stock solution of methylene blue dye (100 μg/mL) was prepared by dissolving 0.01 grams of the dye in distilled water to make up the volume to 100 mL. The diluted solution (10 μg/mL) of methylene blue dye was then prepared from the stock solution.

2.2.3 Oxidizing Agent Solution (Ammonium Metavanadate, NH_4VO_3)

This was done by adding 0.116 g of ammonium metavanadate into distilled water and then filling it to the calibration mark in a 100 mL volumetric flask to form a 10^{-2} M solution.

2.2.4 Acid and Base Solutions Several 1 M solutions including sodium hydroxide, hydrochloric acid, nitric acid, acetic acid, and sulfuric acid were prepared for use during the entire experiment.

2.3 Instrumentation and Wavelength Selection

The absorbance readings were obtained from a UV-Visible spectrophotometer having optical cells that match each other with respect to a reagent blank. The wavelength chosen for analysis was 650 nm.

2.4 Preliminary Study

In a 20 mL volumetric flask, 0.5 mL of the vitamin B9 solution (concentration: 250 μg/mL) was taken and to it, 1.5 mL of the oxidant (concentration: 10^{-2} M) along with 1 mL of hydrochloric acid (concentration: 1 M) was added. After keeping it for 10 minutes, 1 mL of the dye was added and its volume was made up to the mark using distilled water. It was found that yellowish green color was formed and the solution exhibited maximum absorbance at 650 nm while reagent blank showed no absorbance.

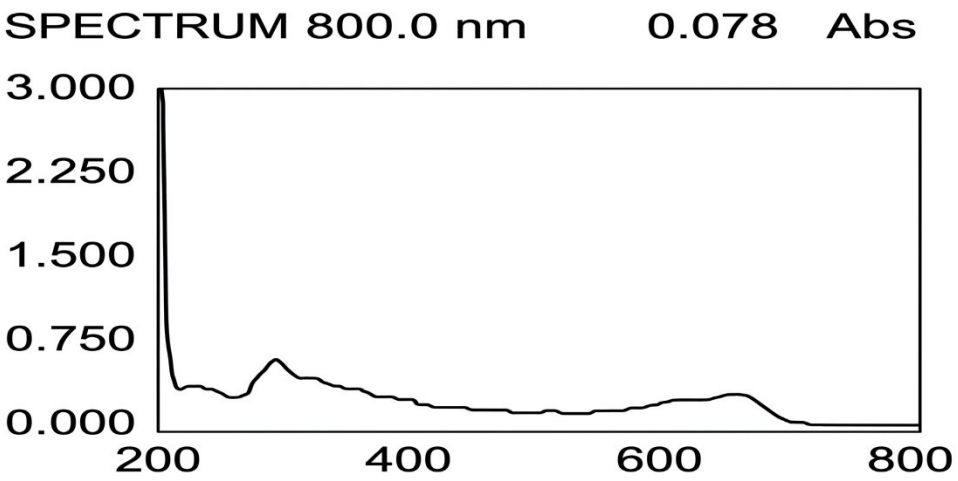

Figure 3. Absorption spectrum of the formed product

3. Results

Various optimal factors that could influence the absorbance of the resulting product were examined and how they affect the reaction was observed:

3.1 Effect of Acid Type Four acids: acetic acid, nitric acid, hydrochloric acid, and sulfuric acid, were assessed under similar conditions using 1.0 M concentration with an added volume of 1 ml to identify the acid that gives the highest absorbance value when the product forms.

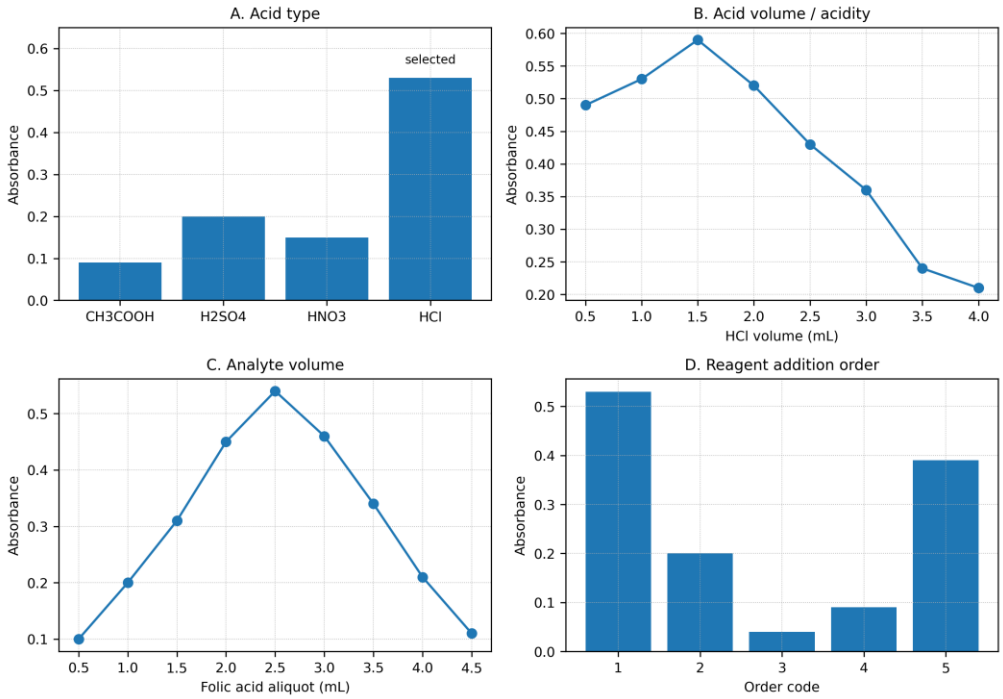

Figure 4. Effect of acid type

Table 1. Effect of acid type on absorbance response.

Acid	Abs
CH_3COOH	0.09
H_2SO_4	0.20
HNO_3	0.15
HCl	0.53

From the table above, it is evident that hydrochloric acid (HCl) was the most effective acid employed.

3.2 Effect of Dye Amount

Figure 5 and Table 2 show how the addition of different amounts of the dye solution (10 μg/mL) affects the absorbance readings, where the highest absorbance is achieved when the added amount is 1 mL.

Table 2. Effect of dye amount on absorbance values.

Volume of Aye	Abs
0.1	0.09
0.2	0.2
0.5	0.37
1	0.53
1.5	0.50
2	0.48
2.5	0.47
3	0.43

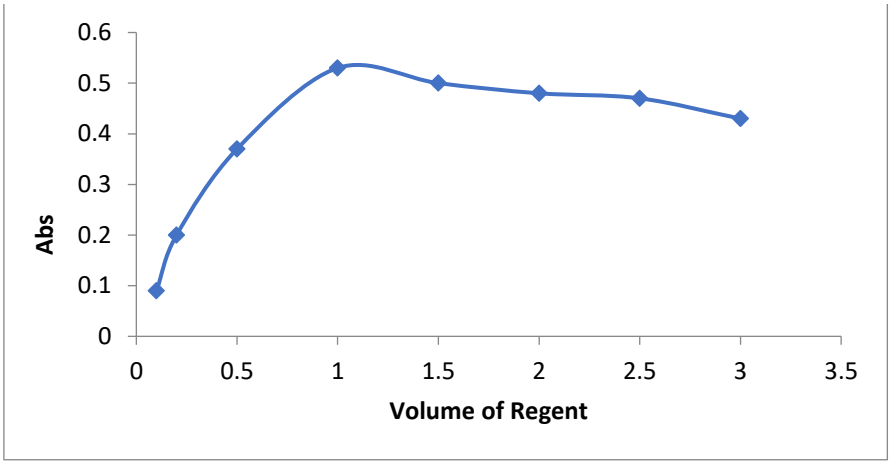

Figure 5. Effect of dye amount on absorbance.

3.3 Effect of Oxidizing Agent Volume More amounts of the oxidant (1 x 10^{-2} M) were used to determine its effect on the products of the reaction, which is shown in Table 3 and Figure 6. The amount that gave rise to the highest absorbance was found to be 1.5 mL.

Table 3. Effect of oxidizing agent volume on absorbance values.

Volume of Oxidizing	Abs
0.2	**0.17**
0.5	**0.25**
0.8	**0.38**
1	**0.48**
1.5	**0.53**
2	**0.46**
2.5	**0.47**
3	**0.44**
3.5	**0.41**

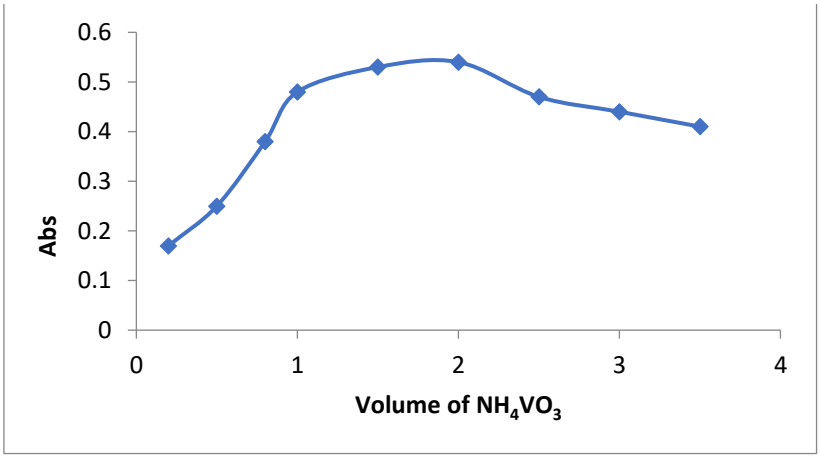

Figure 6. Effect of oxidizing agent volume.

3.4 Effect of Oxidation Time

The reaction was tested for time stability to establish the appropriate time needed to fully oxidize and produce the colored compound as presented in Table 4 and Figure 7 below.

Table 4. Effect of oxidation time.

Time of Oxidizing	Abs
0.0	0.2
5	0.34
10	0.53
15	0.48
20	0.42
25	0.37
30	0.29

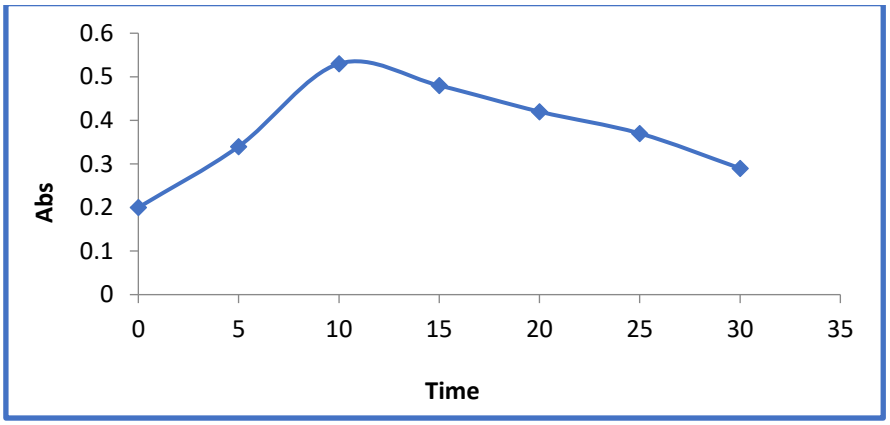

Figure 7. Effect of oxidation time.

3.5 Time Stability of the Formed Product

Stability with time of the solution formed was determined to find out how long the absorbance remained constant, as is depicted in Table 5 and Figure 8 below.

Table 5. Time stability of the formed product solution.

Time (mine)	Absorbance
0	0.53
5	0.537
10	0.538
15	0.537
20	0.536
25	0.535
30	0.532
35	0.53
40	0.49
45	0.45

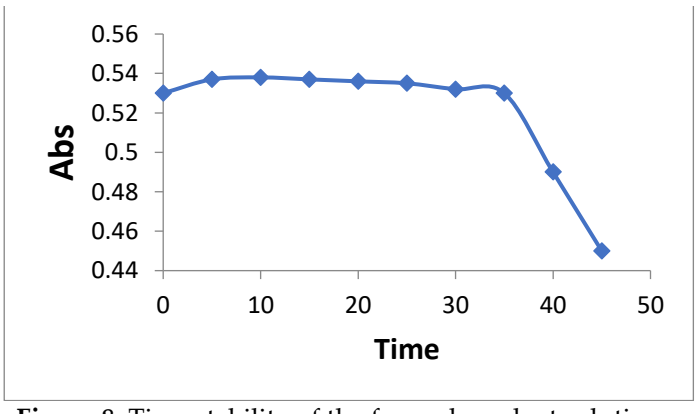

Figure 8. Time stability of the formed product solution.

3.6 Order of Addition

The following addition sequences have been studied, as shown in Table 6 below.

Table 6. Order of reagent addition.

No.	Order of Addition	Abs
1	Vitamin + Oxidizing agent + Hydrochloric acid + Dye	0.53
2	Vitamin + Hydrochloric acid + Oxidizing agent + Dye	0.2
3	Dye + Oxidizing agent + Hydrochloric acid + Vitamin	0.04
4	Dye + Vitamin + Oxidizing agent + Hydrochloric acid	0.09
5	Oxidizing agent + Dye + Vitamin + Hydrochloric acid	0.39

3.7 Calibration Curve

Into a series of 20 mL volumetric flasks, different volumes of the vitamin B9 standard solution varying from 0.5 to 6.0 mL (equivalent to 6.25–75 μg/mL) were placed, followed by adding 1.5 mL of the oxidizing agent (10^{-2} M) and 1 mL of hydrochloric acid (1 M). After 10 minutes of standing, 1 mL of the dye solution was added, and the solution was diluted to the mark with distilled water. It was noticed that yellowish-green colored solution is formed, giving maximum absorption at 650 nm, while the reagent blank does not show any absorbance at this wavelength. Fig (9) represents the standard calibration curve, obeying Beer's law at 6.25 to 75 μg/mL concentrations of vitamin B9 with a correlation coefficient (R2) of 0.9968. The value of molar absorptivity obtained was 3354.64 $L \bullet mol^{-1} \bullet cm^{-1}$, and the value of Sandell's sensitivity was 0.1315 $\mu g/cm^2$. The LOD was found to be 2.5 μg/mL, and LOQ was found to be 7.59 μg/mL.

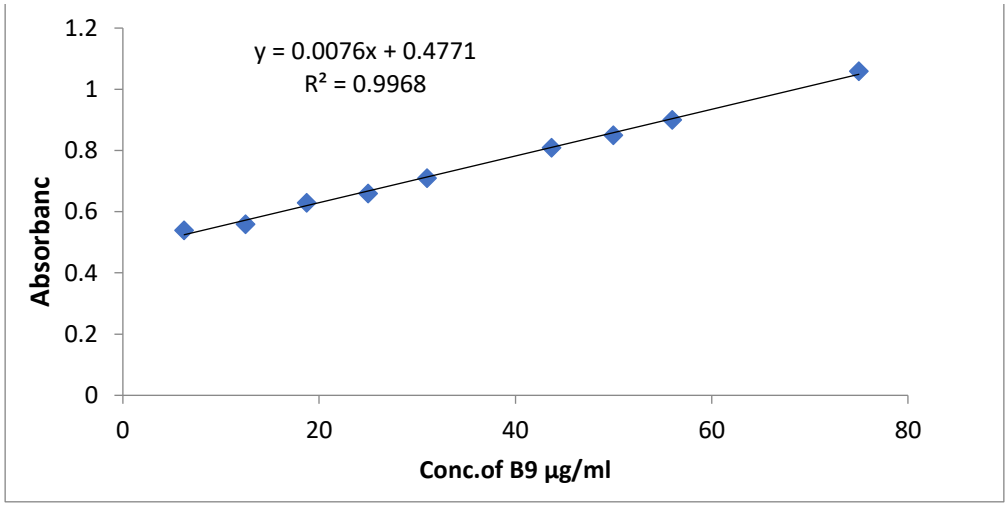

Figure 9. Calibration curve.

Accuracy and Precision of the Proposed Method

The drug concentrations in three points within the range of the calibration curve were determined under the optimal working conditions that were identified through the analytical process, and the values thus obtained were used to determine the accuracy and precision of the suggested method for determining vitamin B9. As can be seen from the table (7), the method is quite accurate and precise.

Table 7. Accuracy and precision of the proposed method

Studied conc mu g/mL	Observed conc mu g/mL	%RE	*RSD%	%Recovery
31	30.64	-1.14	0.816	98.85
43.7	43.8	0.234	0.715	100.23
56	55.64	-0.634	0.643	99.36

* Average of three determinations for each concentration.

Effect of Excipients (Additives)

The effect of the most used excipients on the measurement of vitamin B9 was tested in a 25 mL volumetric flask. After the addition of two different concentrations of the excipients, the results shown in Table (8) show that the additives don't affect the vitamin B9 or the reagent; hence, no interference was detected from the tested additives.

Table 8. Effect of excipients

Excipients (Additives)	Concentration Added (µg/mL)	%RE	Concentration Added (µg/mL)	%RE
Starch	50	-1.23	100	-1.46
Lactose	50	0.247	100	0.286
Glucose	50	-0.65	100	0.673

Applications

A preparation for a pharmaceutical formulation having a concentration of 250 µg/mL (ppm) of folic acid (vitamin B9) was made by powdering ten commercial tablets, each containing 5 mg, and mixing them. An amount equal to 25 mg of the active substance was dissolved in a dilute alkaline solution of sodium hydroxide with stirring (or sonication) while keeping the solution away from light. The solution was then filtered and the filtrate was rinsed with distilled water. The filtrate was put in a 100 mL volumetric flask and distilled water was added to make it up to 250 ppm concentration. The analysis data are presented in Table (9).

Table 9. Application of the proposed method for the determination of folic acid in pharmaceutical formulations.

Pharmaceutical Formulation	Taken (µg/mL)	Taken (µg/mL)	% Rec.	%RSD	%RE
Folic Acid	15	15.04	100.26	1.00	0.26
	30	29.93	99.78	0.50	0.22
	50	50.12	100.24	0.42	0.24

4. Discussion

The visible spectrophotometric method produced a yellowish-green analytical signal with an absorbance maximum at 650 nm. Among the acids tested, hydrochloric acid yielded the highest absorbance (0.53), compared with sulfuric acid (0.20), nitric acid (0.15), and acetic acid (0.09). The maximum response was obtained when 1.5 mL of 0.01 M ammonium metavanadate and 1.0 mL of 1.0 M hydrochloric acid were added in sequence to the folic acid solution, followed by 1.0 mL of methylene blue after a 10 min oxidation period. Changing the reagent addition order reduced absorbance, indicating that the sequence should be standardized during sample analysis. After color development, absorbance remained between 0.530 and 0.538 for the first 35 min and fell to 0.490 at 40

min. These results support measuring standards and samples at a consistent interval within the first 35 min.

The calibration showed a linear response over 6.25–75 μg/mL, with a coefficient of determination (R^2) of 0.9968 and a molar absorptivity of 3354.64 $L \cdot mol^{-1} \cdot cm^{-1}$. The estimated limits of detection and quantification were 2.50 and 7.59 μg/mL, respectively. Because the lowest calibration standard (6.25 μg/mL) was below the estimated limit of quantification, the lower end of the working range should be confirmed experimentally through accuracy and precision measurements near 7.59 μg/mL [4]. At the three standard concentrations evaluated, recoveries ranged from 98.85% to 100.23%, with relative standard deviations of 0.643%–0.816%. These results indicate good recovery and repeatability at the concentrations tested.

The tablet assay yielded recoveries of 99.78%–100.26% and relative standard deviations of 0.42%–1.00%. Starch, lactose, and glucose produced absolute relative errors of no more than 1.46% at the tested concentrations of 50 and 100 μg/mL, indicating limited interference from these excipients under the study conditions. The use of a single visible-wavelength measurement offers a straightforward approach to folic acid quantification in the tablet formulation evaluated and complements previously reported UV-spectrophotometric methods [3]. Comparison with a validated chromatographic method and testing across additional formulations would help establish agreement and assess selectivity in samples with different excipients or degradation products [2].

5. Conclusion

Oxidative coupling reaction was used as a precise spectrophotometric method for the determination of folic acid based on its sensitivity. In view of the fact that oxidation of the drug produces an intermediate which becomes yellowish-green during the coupling process, the method involves the oxidization of the drug, followed by the addition of the acid and then the dye with maximum absorption at 650 nm. Linearity was observed within the concentration range of 6.25 to 75 μg/mL according to Beer's law. Molar absorptivity is 3354.64 $L \bullet mol^{-1} \bullet cm^{-1}$, the limit of detection (LOD) is 2.5 μg/mL, limit of quantification (LOQ) is 7.59 μg/mL, coefficient of determination (R2) is 0.9968, relative standard deviation (RSD) did not exceed 0.64%, and Sandell's sensitivity is 0.1315 $\mu g/cm^2$.

REFERENCES

[1] K. S. Aziz and H. A. Qader, "Derivative spectrophotometric method for simultaneous determination of ascorbic acid and folic acid in the pharmaceutical formulation: Comparative greenness assessment," *Green Analytical Chemistry*, vol. 11, Art. no. 100157, 2024.

[2] E. Deconinck, S. Crevits, P. Baten, P. Courselle, and J. De Beer, "A validated ultra-high-pressure liquid chromatographic method for qualification and quantification of folic acid in pharmaceutical preparations," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 54, no. 5, pp. 995–1000, 2011.

[3] T. A. Elkhazein, T. A. Abdeljabar, A. N. Abdelrahman, M. E. Adam, and S. W. Shantier, "Development and validation of UV-spectrophotometric method for the determination of folic acid in bulk and tablet dosage forms," *Journal of Applied Pharmaceutical Research*, vol. 10, no. 2, pp. 19–23, 2022.

[4] International Council for Harmonisation (ICH), *ICH Q2(R2): Guideline on Validation of Analytical Procedures*, 2023.

[5] International Council for Harmonisation (ICH), *ICH Q14: Guideline on Analytical Procedure Development*, 2023.

[6] Nabeel, H. Shlair, and S. Kafia, "Indirect spectrophotometric determination of folic acid based on the oxidation reaction and studying some of the thermodynamic parameters," *Journal of Zankoy Sulaimani*, vol. 17, no. 1, 2015.

[7] J.-H. Choi, Z. Yates, M. Veysey, Y.-R. Heo, and M. Lucock, "Benefits and risks of folic acid to the nervous system," *Journal of Neurology, Neurosurgery & Psychiatry*, vol. 72, no. 5, pp. 567–571, 2002.

[8] M. A. A. Najma, K. S. Abd-Alrassol, Q. A. Qasim, H. H. Hussein, and H. N. K. Al-Salman, "Spectrophotometric determination of folic acid using 1,10-phenanthroline materials with ninhydrin reagent," *Materials Today: Proceedings*, pp. 1–8.

[9] A. Chaudhary, M. Rindhe, and S. Bhusari, "Development and validation of UV-visible spectrometry method for water soluble vitamin folic acid in pellet formulation," *International Journal of Research in Pharmacy and Pharmaceutical Sciences*, vol. 3, no. 2, pp. 107–110, Mar. 2018.

[10] R. R. Al-Araji, M. S. Mashkour, and E. A. J. Al-Mulla, "Spectrophotometric determination of vitamin folic acid B9 in some drugs using 1,2-naphthoquinone-4-sulphonate (NQS)," *Nano Biomedicine and Engineering*, vol. 9, no. 3, pp. 208–213, 2017.
[11] O. Modupe, J. B. Maurras, and L. Diosady, "A spectrophotometric method for determining the amount of folic acid in fortified salt," *Journal of Agriculture and Food Research*, vol. 2, Art. no. 100060, 2020.
[12] F. Olmo, A. Rodriguez, A. Colina, and A. Heras, "UV/Vis absorption spectroelectrochemistry of folic acid," *Journal of Solid State Electrochemistry*, vol. 26, pp. 29–37, 2022.
[13] N. S. T. Al-Awadi and R. A. K. Aldeen, "CFIA-colorimetric assessment and photometric determination of vitamin B9 (folic acid) using LEDs as a source of irradiation and two solar cells as an energy transducer," *IOSR Journal of Pharmacy and Biological Sciences*, vol. 12, no. 5, Ver. VII, pp. 10–22, Sep.–Oct. 2017.
[14] W. Wu *et al*., "Establishment and validation of HPLC methods for the determination of folic acid and parabens antimicrobial agents on folic acid oral solution," *BMC Chemistry*, vol. 19, Art. no. 19, 2025.
[15] Y. Albawarshi, A. Amr, K. Al-Ismail, M. Shahein, M. Majdalawi, M. Saleh, A. Khamaiseh, and B. El-Eswed, "Simultaneous determination of B1, B2, B3, B6, B9, and B12 vitamins in premix and fortified flour using HPLC/DAD: Effect of detection wavelength," *Journal of Food Quality*, vol. 2022, no. 1, Art. no. 9065154, 11 pages, 2022.
[16] D. Kahoun, P. Fojtíková, F. Vácha, M. Čížková, R. Vodička, E. Nováková, *et al*., "Development and validation of an LC-MS/MS method for determination of B vitamins and some of their derivatives in whole blood," *PLOS ONE*, vol. 17, no. 7, 2022.
[17] N. H. H. Hashim, R. Osman, N. A. Z. Abidin, and N. S. A. Kassim, "Recent trends in the quantification of vitamin B," *Malaysian Journal of Analytical Sciences*, vol. 25, no. 3, pp. 466–482, 2021.