

Indirect spectrophotometric determination of folic acid based on the oxidation reaction and studying some of the thermodynamic parameters

Nabeel S. Othman¹, Shlear H. Hasan² & Kafia M. Surchi³

¹ Chemistry Department, College of Science, Mosul University, Mosul, Iraq.

² Department of food technology, College of Agriculture, University of Salahaddin, Erbil, Iraq.

³ Chemistry Department, College of Science, University of Salahaddin, Erbil, Iraq.

E-mail: E-mail: blndmuh@yahoo.co.uk

Article info

Original: 02 Oct. 2014
Revised: 14 Nov.. 2014
Accepted: 10 Dec. 2014
Published online:
20 March 2015

Keywords:

Spectrophotometry
Folic acid
2,2'-Bipyridyl/FeCl₃
Thermodynamic parameters

Abstract

A simple and sensitive indirect spectrophotometric method was developed for the determination of folic acid in pure form and in its pharmaceutical formulations. The method was based on the oxidation of folic acid by iron (III) in acidic medium, then reaction of liberated iron (II) with 2, 2'-bipyridyl to produce a red complex which is water-soluble, stable, and has a maximum absorption at 520 nm against the reagent blank with a molar absorptivity of $2.8338 \times 10^5 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. Beer's law is valid over the concentration range of 0.5-18.0 $\mu\text{g/ml}$ folic acid. The values of some thermodynamic parameters [free energy change (ΔG), enthalpy change (ΔH), and entropy change (ΔS)] were estimated from the values of stability constants (K) at different temperatures. It was found that colored product formation was spontaneous, exothermic and accompanied by an increase of random. The proposed method has been applied successfully to determine folic acid in different pharmaceutical preparations.

Introduction:

Folic acid (FA) or pteroyl-L-glutamic acid, chemically known as N-[4-[(2-amino-1, 4-dihydro-4-oxo-6-pteridiny] methyl] amino] benzoyl]-L-glutamic acid is a water soluble vitamin B (Fig. 1). Folic acid can promote the formation of red blood cells and is identified as an anti-anemia and growth factor. Folic acid is routinely prescribed to pregnant women and widely used in the treatment and prevention of megaloblastic anemia's^[1].

The proposed work aims mainly to develop a sensitive spectrophotometric method for the determination of folic acid in pure form and in pharmaceutical formulations. The method is based on the oxidation of folic acid by iron (III) in acidic medium, then reaction of liberated iron (II) with 2,2'-bipyridyl to produce a red complex which is water-soluble, stable, that has been applied successfully to the assay of folic acid in pharmaceutical preparations. Also estimation of some thermodynamic parameters which are found to be effective on the value of stability constant of the color complex formed.

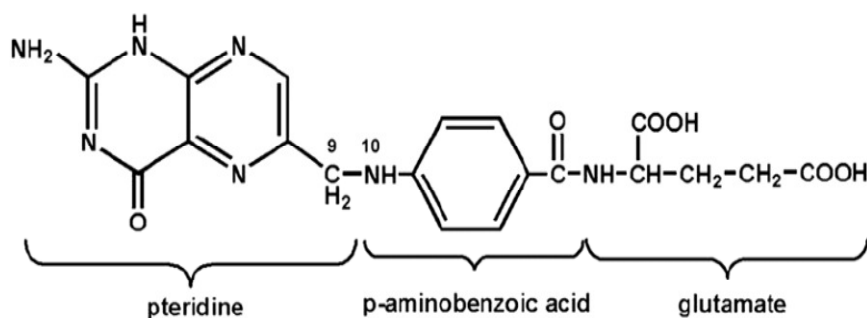


Fig.1: Structural formula of folic acid.

Different methods have been reported for the determination of folic acid in various matrixes. The most common methods include chromatographic [2-7], electrochemical techniques [8-12], fluorimetry [13]. These methods are time consuming, high cost and hard operation. Spectrophotometric method [14-18] is a powerful analytical technique that has excellent sensitivity, sufficient accuracy and requires relatively simple and inexpensive instrumentation. These advantages make it as an attractive method for the determination of different species in various samples at trace levels.

Experimental

• Apparatus

A (Cecil CE3021-England) UV-VIS spectrophotometer was used for all spectral and absorbance measurements with matched 1-cm quartz cells. Water bath (Lab. companion shaking BS-11, Korea) was used since the reaction requires heat control. A pH-meter type (Korea) is used for pH reading. Electronic balance (Sartorius AG Gottingen B2- 2105 Germany).

• Materials and reagents

All chemicals and reagents used were of analytical reagent grade and freshly prepared in bidistilled water. Folic acid pure was obtained from Company for drug Industries and medical applications SDI, Samarra, Iraq. Stock standard solution of folic acid (1000 µg/ml) was prepared by dissolving 0.1000 g of folic acid in 10 ml of 0.1 M sodium hydroxide and then the volume was made up to 100 ml in a volumetric flask with the same solvent and kept in the dark at 5°C, this solution was stable for one week, and working standard solutions were prepared by suitable dilution of the stock standard solution. Ferric chloride hexahydrate (0.02 M) was freshly prepared by dissolving 0.5406 g of ferric chloride hexahydrate (Fluka) in 5 ml of hydrochloric acid (1 M) and then the volume was completed to 100 ml in a dark volumetric flask with distilled water. 2,2'-Bipyridyl solution (0.01 M) was prepared by dissolving 0.1561 g of 2,2'-bipyridyl (BDH) in 5 ml of ethanol (Analar) and the solution was made up to 100 ml in a volumetric flask with distilled water. Concentrated acetic acid (Fluka) was appropriately diluted with distilled water to get the required concentration (0.02 M acetic acid). 0.1 M Sodium hydroxide solution was prepared by dissolving 1.0 g of sodium hydroxide (Fluka) in distilled water and diluting to the mark with the same solvent in 250 ml volumetric flask. And a stock standard solution of each interfering species was prepared by dissolving 0.1 g of the compound in distilled water then the volume was completed to 100 ml in calibrated flask. Other solutions were prepared by serial dilutions of the stock solution.

• Preparation of tablets solution.

Weigh and finally powdered an enough number of tablets followed by extraction of an accurately weighed portion of the powder equivalent to about 0.1 g of FA dissolving in 0.1 M sodium hydroxide solution. Shake and filter the solution in to a 100 ml volumetric flask. Wash the residue on the filter with 10 ml portions of 0.1 M sodium hydroxide solution and dilute the combined filtrate and washings to the mark with the same reagent, to obtain (1000 µg/ml) of FA. Working solution was prepared by appropriate dilution of 10 ml of this solution to 100 ml by distilled water, to obtain (100 µg/ml) of FA^[19]. Other solutions were prepared by serial dilutions of the stock solution.

- Preliminary investigation.

To a 10 ml volumetric flask, 1.0 ml of folic acid (100 µg/ml) with 0.3 ml of ferric chloride solution (0.02 M) was added, followed by 2.0 ml of 2,2'-bipyridyl solution (0.01 M), the solution left for 15 minutes in water bath adjusted at 60°C, then the solution cooled, before addition of 0.5 ml of 0.02 M hydrochloric acid. After that volume was completed to the mark with distilled water, left to stand for 5 minutes at room temperature before the absorbance of the red colored product was measured, that has a maximum absorbance at λ_{max} 520 nm against the reagent blank (Fig. 2).

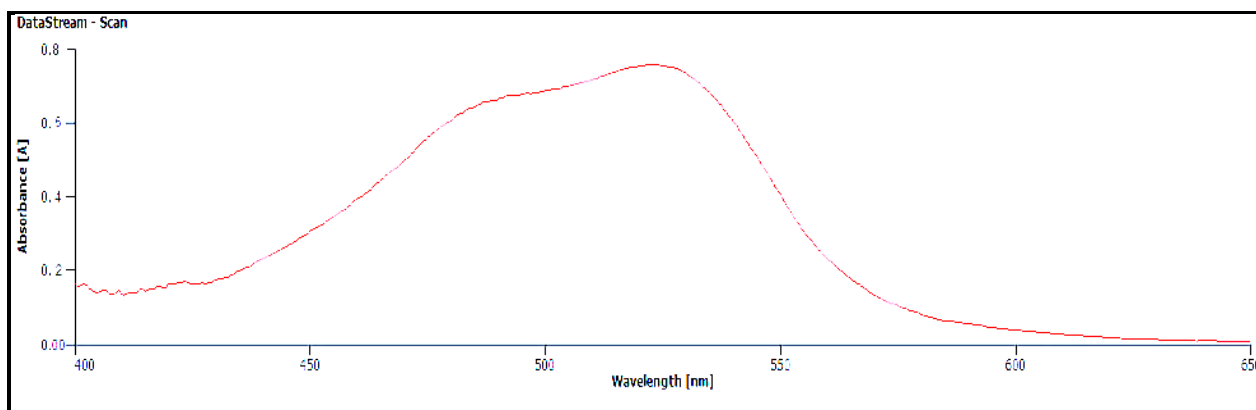


Fig. 2: Absorption spectra of 100 µg folic acid /10 ml.

Results and discussion

Optimization of variables:

- Effect of pH

The effect of pH on color intensity is examined firstly. The extent of complex formation, and hence the absorbance of the final solution, is often a function of hydrogen ion concentration. Oxidation of folic acid by Fe^{+3} and subsequent chelation of Fe^{+2} with 2,2'-bipyridyl was found to occur in neutral medium, but the presence of acid was necessary to increase the stability of the developed red color chelate by maintaining the desired pH^[20]. So different acids have been used and the results indicate that 1.0 ml of acetic acid solution (0.02 M) is the most suitable acid solution (pH= 3.74) to produce the highest color intensity (Table I).

Table I: Effect of pH on absorbance.

Acid used, (0.02M)	Absorbance/ml concentration of acid added.					pH-range
	0.2	0.5	0.7	1.0	1.5	
Without acid			0.306			3.62
HCl	0.233	0.287	0.301	0.300	0.281	2.92-2.78
H ₃ PO ₄	0.216	0.239	0.284	0.294	0.210	2.78-2.55
H ₂ SO ₄	0.182	0.190	0.201	0.216	0.198	2.68-2.53
CH ₃ COOH	0.280	0.302	0.315	0.343	0.297	3.85-3.67

- Effect of ferric chloride concentration.

The effect of the concentration of $FeCl_3$ (0.2–1.5 ml) of 0.02 M on the absorbance of the red complex containing different concentration of folic acid (25–150 µg /10 ml) has been investigated. The results illustrated in Table II indicated that the absorbance of red colored chromogen at 520 nm was increased with increasing volume of ferric chloride and became constant at 0.5 ml; above this volume, the absorbance decreased. Therefore, 0.5 ml of 0.02 M ferric chloride solution gives the highest absorbance with a determination coefficient ($R^2=0.9989$) over a range of determined concentration of 25–150 µg folic acid/10 ml therefore it was recommended for the subsequent experiments.

Table II: The effect of ferric concentration.

Ml of Fe ⁺³ (0.02M) solution	Absorbance/ μg of folic acid in 10 ml					R ²
	25	50	75	100	150	
0.2	0.079	0.161	0.201	0.313	0.391	0.9713
0.3	0.085	0.168	0.221	0.332	0.422	0.9792
0.5	0.097	0.179	0.232	0.345	0.456	0.9989
1.0	0.073	0.153	0.210	0.301	0.432	0.9970
1.5	0.041	0.125	0.187	0.272	0.391	0.9954

- *Effect of 2, 2'-bipyridyl concentration.*

The influence of different amounts of 2,2'-bipyridyl solution on the absorbance of solution containing different concentration of folic acid (25-150 μg /10 ml) was studied. The results indicated that smaller amounts resulted in incomplete complex formation. Increased concentration increases the absorbance and reached maximum on using a volume of 2.5 ml of 0.01 M 2,2'-bipyridyl solution which also gives the highest value of determination coefficient (Table III).

Table III: The effect of 2, 2'-bipyridyl concentration on absorbance.

ml of 2,2'-bipyridyl (0.01M) solution	Absorbance/ μg of folic acid in 10 ml					R ²
	25	50	75	100	150	
1.0	0.064	0.11	0.178	0.245	0.388	0.9953
1.5	0.091	0.171	0.224	0.301	0.451	0.9974
2.0	0.118	0.221	0.366	0.543	0.778	0.9941
2.5	0.157	0.317	0.468	0.618	0.895	0.9991
3.0	0.103	0.212	0.356	0.487	0.689	0.9952

- *Effect of temperature.*

The effect of temperature on the color intensity of the resulting complex was investigated. The formation of colored complex was slow at room temperature and required longer time for completion. Hence, efforts were made to accelerate the reaction by carrying out the reaction at higher temperatures. It was observed that the maximum absorbance was obtained when heating the reaction mixture on a water bath at 60°C, however, 60°C was recommended. Further heating the absorbance was decreased (Table IV).

Table IV: Effect of temperature.

Temperature °C	Room* T	30	50	60	70	80	90
Absorbance	0.050	0.082	0.520	0.896	0.820	0.722	0.693

*Room Temperature= 19 °C.

- *Effect of heating time on oxidation-reduction reaction.*

The effect of time needed to complete the oxidation-reduction reaction was studied by allowing the solutions to stand in water bath at 60°C for different times, after adding the component of reaction (folic acid, ferric chloride hydrated, and 2,2'-bipyridyl), then the absorbance was measured against the reagent blank. The results in Table V indicated that high absorbance of complex at 520 nm occurred after 35 minutes and there was no significant difference in the intensity of complex above 35 minutes and also instability results indicate. Therefore, the standing time 35 minutes was recommended for the subsequent experiments.

Table V: Effect of heating time on oxidation-reduction process.

Time (min.)	0	5	10	15	20	25	30	35	40	45	50
A	0.062	0.158	0.382	0.569	0.696	0.893	0.917	0.976	0.755	0.586	0.501

- *Effect of order of addition of reactants.*

After fixing all other parameters, other experiments were performed to ascertain the influence of the order of addition of reactants [folic acid (FA), Fe^{+3} (O), 2,2'-bipyridyl (R), acetic acid (A)] was examined. The results (shown in Table VI) indicated that order (I) of addition of reagents was the optimum order due to the high intensity of the formed color and hence the same order of addition was followed throughout the investigation.

Table VI: Effect of order of addition.

Order of addition	Order no.	Absorbance
FA + O + R+A	I	0.975
FA+A + R + O	II	0.524
O + R + FA+A	III	0.605
O + FA+A + R	IV	0.853
R + FA +A + O	V	0.541
R +A+ O + FA	VI	0.517

- *Effect of time and stability of colored complex.*

The effect of time on the development and stability period of the red complex was investigated under optimum experimental conditions cited above. The absorbance measured from 0 to 180 minute after emerging the flasks from water bath and adds the acid then completed the volume to 10 ml. The results in Table VII indicated that the red complex was stable for at least 3 hours.

Table VII: Effect of time on absorbance.

Folic acid ($\mu\text{g}/10\text{ml}$)	Absorbance/minutes									
	0	5	10	20	30	40	50	60	120	180
50	0.481	0.478	0.475	0.470	0.461	0.458	0.456	0.445	0.427	0.413
100	0.668	0.668	0.667	0.665	0.664	0.662	0.662	0.658	0.652	0.640
150	0.974	0.972	0.973	0.971	0.970	0.966	0.957	0.939	0.917	0.893

- *Effect of surfactant.*

The effect of surfactants with different orders of addition on the absorbance was studied; the addition of 2 ml of each of cetylpyridinium chloride (CPC, 1×10^{-3} M), sodium dodecyl sulphate (SDS, 1×10^{-3} M) and Triton X-100 (1%) was examined. The investigations showed that there was no remarkable change in the absorbance of CPC. On the other hand, both SDS and Triton X-100 give a turbid solution; therefore, they were omitted in this study (Table VIII).

Table VIII: Effect of surfactant on absorbance.

Surfactant solution	Absorbance* / order** of addition			
	I	II	III	IV
CPC (1×10^{-3} M)	0.626	0.585	0.615	0.632
SDS (1×10^{-3} M)	Turbid	Turbid	Turbid	Turbid
TritonX-100 1%	Turbid	Turbid	Turbid	Turbid

*The absorbance without surfactant= 0.973 at 520 nm.

** Surfactant (S), folic acid (FA), Fe^{+3} (O), 2,2'-bipyridyl (R), acetic acid (A).

I= FA + S+ O + R+A; II= FA + O + S + R+A; III= FA + O + R+ S +A; IV= FA + O + R +A+S.

Recommended procedure and calibration graph

To a series of 10 ml calibrated flasks, 0.05-1.8 ml of 100 $\mu\text{g}/\text{ml}$ folic acid solution were transferred, then 0.5 ml of 0.02 M ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) solution was added, followed by 2.5 ml of 0.01 M 2,2'-bipyridyl solution, the solutions left for 35 minutes in water bath adjusted at 60°C , then the solution

cooled, before addition of 1.0 ml of 0.02 M acetic acid. After that volume were completed to the mark with distilled water, left to stand for 5 minutes at room temperature before the absorbance of the red colored product were measured at 520 nm against the reagent blank. Employing the conditions described in the procedure, a linear calibration curve for FA was obtained (Figure 3), which shows that Beer's law was obeyed over the concentration range of 0.5 – 18 $\mu\text{g/ml}$.

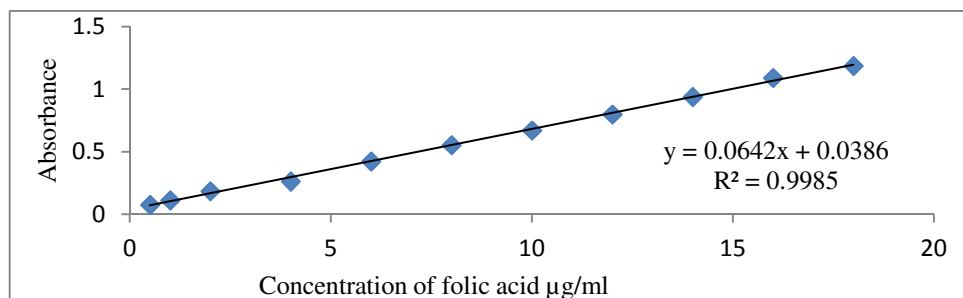


Fig. 3: Calibration graph of folic acid.

Analytical characteristics

Analytical characteristics such as regression equation, linear range, relative standard deviation, relative error, molar absorptivity and Sandell's sensitivity values of each method were determined under the optimized conditions as shown in Table IX. The limits of detection (LOD) and quantitation (LOQ) were calculated according to the International Union of Pure and Applied Chemistry (IUPAC) definition^[21] using the formula:

$$\text{LOD} = 3S/b \text{ and } \text{LOQ} = 10S/b$$

Where:

S is the standard deviation of blank absorbance values and b is the slope of the calibration plot, are also presented in Table IX. The high values of molar absorptivity and low values of Sandell's sensitivity and LOD indicate the high sensitivity of the proposed methods.

Table IX: Optical, regression characteristics, the stability constants and thermodynamic parameters of the proposed method for folic acid.

Parameter	The proposed method
Medium	CH_3COOH (0.02 M)
λ_{max} , nm	520
Beer's law range ($\mu\text{g/ml}$)	0.5-18
Detection limits ($\mu\text{g/ml}$)	0.06
Quantitation limit ($\mu\text{g/ml}$)	0.2
Molar absorptivity (L/mole. cm)	2.8338×10^5
Sandell's sensitivity ($\mu\text{g/cm}^2$)	1.55
Regression equation ($Y = a + bC$)*	
Intercept (a)	0.0386
Slope (b)	0.0642
Determination coefficient (R^2)	0.9985
Relative standard deviation, %	0.31
Relative error, %	0.0231
K ($\text{L}^3 \cdot \text{mole}^{-3}$)	0.095×10^{12}
ΔH (KJ/mole)	-43.648
ΔS (J/mole. K)	77.610
ΔG (KJ/mole)	-70.656

* In $Y = a + bC$, Y is absorbance and C is concentration

Stability constant and thermodynamic parameters of the product at different temperatures

The Van't Hoff equation provides information about the temperature dependence of the stability constant. The Van't Hoff equation may be derived from the Gibbs Helmholtz equation, which gives the temperature dependence of the Gibbs free energy [22]. The stability constant K of the product was evaluated at four temperatures for the reaction of Fe⁺² (from oxidation-reduction reaction) with 2,2'-bipyridyl solution in a ratio of 1:3 as shown in Fig (4) [23].

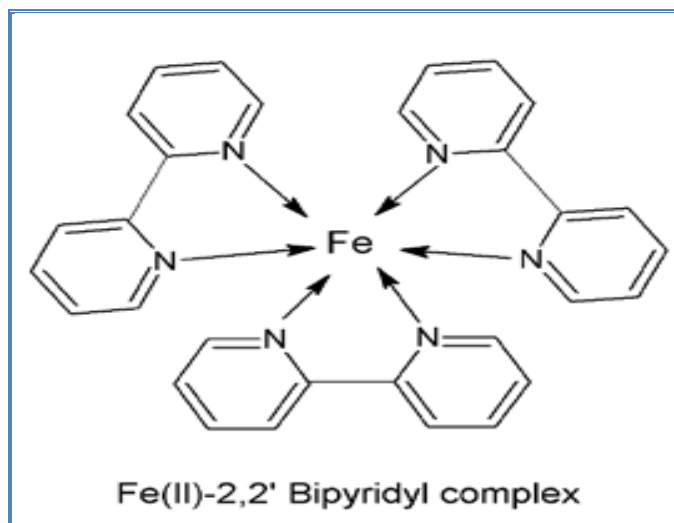


Fig. 4: The red complex of Fe⁺² with 2, 2'-bipyridyl.

Then α degree of dissociation of the color complex is determined spectrophotometrically by equation 1 [24]:-

$$\alpha = (A_m - A_s) / A_m \dots\dots (1)$$

Where:

A_m: is the absorbance of the color complex at optimal conditions.

A_s : is the absorbance of the color complex at stoichiometric ratio of 1:3 donor – acceptor.

$$K_{1:3} = \frac{1 - \alpha}{27\alpha^4 C^3} \dots\dots (2)$$

Where: C is concentration of complex formed.

The stability constant K of the color complex was evaluated [25] at four temperatures in a range as shown in Table X using equation 1 and 2. The heat of color complex formation ΔH was calculated using Van't Hoff equation (also known as the Vukancic-Vukovic equation) [26] of a form:-

$$\ln K = \text{constant} - \Delta H / RT \dots\dots (3)$$

Table X: The stability constant (K) of the color complex.

Temp (k)	A _m	A _s	α	K (L ³ .mol ⁻³)
333	0.060	0.037	0.380	0.099 *10 ¹²
343	0.038	0.021	0.44	0.049 *10 ¹²
353	0.027	0.014	0.481	0.030 *10 ¹²
363	0.023	0.011	0.522	0.20 *10 ¹²

A plot between lnK versus the inverse of absolute temperature from equation (3) shows a straight line of R² (0.9859) as shown in Fig. (5).

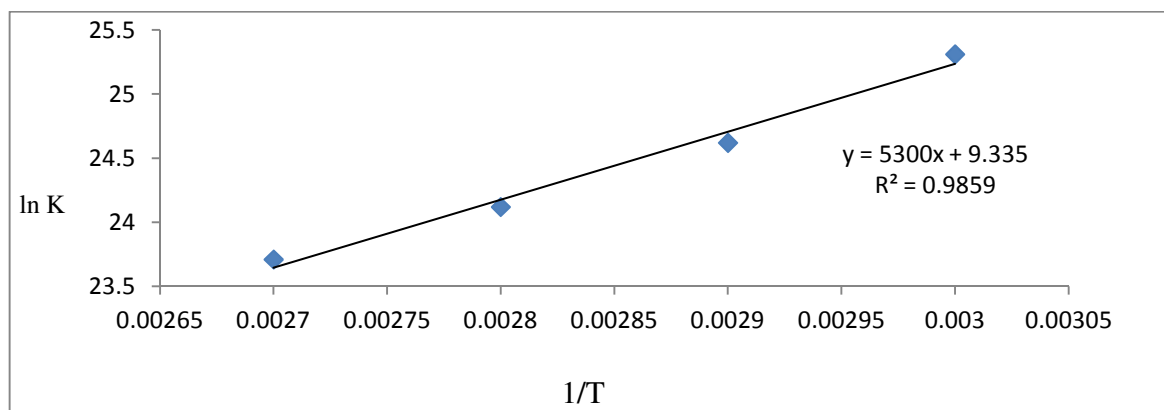


Fig. 5: Van't Hoff plot of lnK of color complex versus 1/T.

From the variation of stability constants values with temperature of the color complex, the thermodynamic function, ΔG , ΔH and ΔS can be evaluated. The Gibbs free energy of color complex formation was calculated from equation (4)^[27] of the form:-

$$\Delta G = -RT \ln K \dots\dots\dots (4)$$

The entropy change (ΔS) for the color complex formation was represented by using equation (5)^[28]:-

$$\Delta G = \Delta H - T\Delta S \dots\dots\dots (5)$$

The thermodynamic parameters of the color complex formed were given in Table XI. It was clear from the thermodynamic parameters of complex that:

- a) The stability constants (K) decreased with increased of temperature, confirming that complex was not stable at higher temperature^[28, 29].
- b) Sufficiently large negative value of ΔG showed that spontaneous formation of the complexes. Spontaneity increased with temperature^[30].
- c) Negative values of ΔH indicated the exothermic nature of the color complex formation^[30].
- d) The ΔS values for the complex were positive, confirming that the complex formed is entropically favorable, and so it is responsible for the complexing process^[30]. The results of this work are mainly responsible for complex formation

Table XI: The stability constants and thermodynamic parameters for associated species at different temperatures.

Temp (k)	K (L ³ .mole ⁻³)	ΔH (KJ/mole)	ΔS (J/mole. k)	ΔG (KJ/mole)
333	0.099 *10 ¹²	-44.228	77.609	-70.072
343	0.049 *10 ¹²	-43.588	77.612	-70.209
353	0.030 *10 ¹²	-43.392	77.609	-70.788
363	0.20 *10 ¹²	-43.383	77.611	-71.556
Average	0.095*10 ¹²	-43.648	77.610	-70.656

Interference studies

In order to assess the possible analytical applications of the proposed analytical method which described above to the assay of commercial folic acid formulations, the effect of some common excipients used in pharmaceutical preparations were investigated by carrying out the determination of FA in the presence of different excipients. Experimental results showed that sodium chloride, glucose, sucrose, fructose, lactose, galactose, and starch had no effect on the determination of FA. Interestingly, only vitamin B1 and vitamin B2 has serious effect on the determination of folic acid.

Application of the method

The proposed method was successfully applied to the determination of FA in tablets and the results are summarized in Table XII. The results obtained were statistically^[31] compared with those of the official BP method, HPLC method^[32] by applying the Student's t-test for accuracy and F-test for precision. The results

indicate that there was no significant difference between the proposed method and the reference method with respect to accuracy and precision at 99% confidence level.

Table XII: Application of the proposed method for the determination of folic acid in different brands of tablets.

Tablet FA sample	Label amount (mg)	Obtained value* (mg)	Official BP method*	Recovery ± SD,%	Calculated t-value **	Calculated F-value ***
Awamedica Folic Awa-Erbil, Iraq.	5.0	4.85	4.82	99.4±1.04	1.80	4.79
Julphar Folicum- U.A.E.	5.0	4.88	4.85	99.4±0.39	1.91	0.98
Joswe Folic acid-Naur, Jordan.	5.0	4.77	4.96	103.9±0.10	1.98	1.43

*Average of five determinations.

** Tabulated t-value for four degrees of freedom; and p=0.01 is 3.365.

*** Tabulated F-value for four degrees of freedom; and p=0.01 is 15.97.

Conclusion

The proposed method is simpler, less time consuming and more sensitive than those previously published. The proposed method was more advantageous than other reported visible spectrophotometric ^[14-18] methods with respect to sensitivity, simplicity, reproducibility, precision, accuracy and stability of the colored species. The proposed method is suitable for the determination of folic acid in pure form and pharmaceutical formulations without interference from excipients such as starch and glucose, suggesting potential applications in bulk drug analysis.

References

- [1] A.A. Al-Warthan, "Flow injection chemiluminometric determination of folic acid in pharmaceutical formulations.", *Anal. Sci.*, 10, pp. 919–922, (1994).
- [2] B. Klejduš, J. Petrova, D. Potesil, V. Adam, R. Mikelova, J. Vacek, R. Kizek, and V. Kuban, "Simultaneous determination of water- and fat-soluble vitamins in pharmaceutical preparations by high-performance liquid chromatography coupled with diode array detection", *Anal. Chim. Acta*, 520, pp. 57–67, (2004).
- [3] D. Fruhauf and M. Juza, "Development, optimization and validation of a sub-minute analytical enantioselective high performance liquid chromatographic separation for a folic acid precursor in normal phase mode", *J. Chromatography, A* 1269, pp. 242–254, (2012).
- [4] B.C. Nelson, K.E. Sharpless and L.C. Sander, "Quantitative determination of folic acid in multivitamin-multielement tablets using liquid chromatography/ tandem mass spectrometry.", *J. Chromatogr. A*, 1135, pp. 203–211, (2006).
- [5] D.E. Breithaupt, "Determination of folic acid by ion-pair RP-HPLC in vitamin fortified fruit juices after solid-phase extraction.", *Food Chem.*, 74, pp. 521–525, (2001).
- [6] M.S.A. Pradao, C.A. Silva, M.F. Tavares and K.D. Altria, "Determination of folic acid in tablets by microemulsion electrokinetic chromatography.", *J. Chromatogr. A*, 1051, pp. 291–296, (2004).
- [7] M.J. Akhtar, M.A. Khan and I. Ahmad, "High-performance liquid chromatographic determination of folic acid and its photodegradation products in the presence of riboflavin", *J. Pharm Biomed Anal.*, 16, pp. 95–99, (1997).
- [8] H.X. Guo, Y.Q. Li, L.F. Fan, X.Q. Wu, M.D. Guo, "Voltammetric behavior study of folic acid at phosphomolybdic-poly pyrrole film modified electrode", *Electrochim Acta*, 51, pp. 6230–6237, (2006).
- [9] J. Amezdelopez, A. Costagarica, A. Mirandaordieres and P. Tunonblanco, "Phase-selective alternating-current adsorptive stripping voltammetry of folic acid on a mercury thin-film electrode", *Electroanalytes*, 4, pp. 87–92, (1992).
- [10] A.C. Le Gall, C. Vandenberg, "Determination of folic acid in sea water using adsorptive cathodic-stripping voltammetry", *Anal. Chim. Acta*, 282, pp. 459–70, (1993).
- [11] D.B. Luo, "Determination of folic acid by adsorptive stripping voltammetry at the static mercury drop electrode", *Anal. Chim. Acta.*, 189, pp. 277–283, (1986).
- [12] S.W. Szczepaniak and M. Ren, "Adsorptive stripping-voltammetric determination of folic acid in pharmaceutical preparations", *Electroanalytes*, 6, pp. 505–507, (1994).

- [13] J.L. Manzoori, A. Jouyban, M. Amjadi, and J. Soleymani, "Spectrofluorimetric determination of folic acid in tablets and urine samples using 1, 10-phenanthroline-terbium probe", *Luminescence*, 26(2), pp.106–111, (2011).
- [14] A. Pathak and S.J. Rajput, "Simultaneous determination of a ternary mixture of doxylamine succinate, pyridoxine hydrochloride, and folic acid by the ratio spectra-zero-crossing, double divisor-ratio spectra derivative, and column high-performance liquid chromatographic methods", *J. AOAC Int.*, 91(5), pp.1059–1069, (2008).
- [15] P. Nagaraja, R.A. Vasantha, and H.S. Yathirajan, "Spectrophotometric determination of folic acid in pharmaceutical preparations by coupling reactions with iminodibenzyl or 3-aminophenol or sodium molybdate-pyrocatechol.", *Anal. Biochem.*, 307(2), pp.316–321, (2002).
- [16] K. V. S. S. Murty, C. S. P. Sastry and B. G. Rao, "Spectrophotometric determination of aromatic amines using catechol and iodine", *J. Ind. Chem. Soc.*, 59, pp. 1107–1109, (1982).
- [17] M.N. Reddy, N. Viswanadham, and C.S.P. Sastry, "Spectrophotometric methods for the determination of folic acid", *Ind. Drugs*, 21, pp.460–462, (1984).
- [18] F. Buhl, and U. Hachula, "Spectrophotometric determination of folic acid and other reductants using a couplet redox complexation reaction with cerium (IV) and arsenazo III", *Chemia Analyczna*, 36, pp. 27–34, (1991).
- [19] M.Q. Al-Abachi and R.S.Al-Abaidi, "Spectrophotometric assay of folic acid in pharmaceutical tablets via oxidative coupling.", *Iraqi J. of Sci.*, 45(1), pp.24-29, (2004).
- [20] D. L. Massart, B. G. M. Vandeginste, S. N. Deming, Y. Michotte and L. Kaufman, "Chemometrics: Data Handling in Science and Technology", Elsevier, Amsterdam, Vol. 2, (1988).
- [21] IUPAC, "Analytical Chemistry Division. Nomenclature, symbol, units and their usage in spectrochemical analysis. II. Data interpretation.", *Spectrochim. Acta. B*, 33, pp.241-246, (1978).
- [22] B.C. Karlsson, A.M. Rosengren, P.O. Andersson and I.A. Nicholls, "The spectrophysics of warfarin: Implications for protein binding.", *J. Phys. Chem., Part B*, 111, pp. 10520–10528, (2007).
- [23] D.S. Foster, "photometric and fluorometric methods of analysis metals", part 1, John Wiley & Sons, Inc. ; New York, pp.750,(1978).
- [24] L. G. Hargis, "Analytical chemistry: Principles and techniques", Prentice-Hall, Inc., Englewood Cliffs, New Jersey, Ch. 23, pp. 549-568, (1988).
- [25] C. Rossotti, H. Rossotti, "The Determination of stability Constant", McGraw – Hill book company, Inc, USA, pp.23-78, (1961).
- [26] P. Atkins, J. De Paula, "Physical Chemistry", 8th Edn. W. H. Freeman and Company, pp.212, (2006).
- [27] J. Hine, "Physical Organic Chemistry", 2nd ed., McGraw-Hill, New York, pp. 486-487, (1962).
- [28] F. Khan, and L. Tantuway, "Thermodynamics in [Mn(II)-antibiotics-bacitracin] mixed system: a polarographic approach.", *J. Pharm. Biomed. Anal.*, 27, pp. 933-944, (2002).
- [29] A. A. Al-sarawy, "Potentiometric and thermodynamic studies of 4-sulfamethoxazoleazo-3-methyl-2-pyrazolin-5-one and its metal complexes.", *Chem. Pap.*, 58(2), pp.109-112, (2004).
- [30] E. G. Akgemci and T. Atalay, "Determination of thermodynamic parameters of some complexes of 9-ethyl-3-carbazolecarboxaldehyde 4-phenyl-3-thiosemicarbazone.", *Turk. J. Chem.*, 24(1), pp.89-94, (2000).
- [31] J.N. Miller and J.C. Miller, *Statistics and chemometrics for analytical chemistry*, 5th Ed., Pearson Education Limited, Harlow, England, pp.107-149, (2005).
- [32] British Pharmacopoeia, H.M. Stationery Office, London, UK, 1, pp.575, (2013).