L-Tryptophan as Fluorescent Probe for Determination of Folic Acid in Some Pharmaceutical Products

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***Abstract*—A new fluorescent probe L-Tryptophan was reported for the determination of folic acid (FA), based on its quenching effect of the fluorescence intensity of L-Tryptophan. The concentration of FA was proportional to the quenched fluorescence intensity of L-Tryptophan at excitation and emission wavelengths of 281 nm and 365 nm, respectively, in Britton–Robinson buffer solution of pH 7. Optimized conditions of pH, reaction time, potential interferences, concentrations of L-tryptophan, and buffer were investigated. FA was determined in a linear range of 2.0–16.0 µg ml with a correlation coefficient R^2 0.9974. The limit of detection and limit of quantification values were 0.09 µg/ml and 0.27 µg/ml, respectively. Relative standard deviation values for five replicated measurements of 2, 8, and 16 µg/ml FA were between 0.23 % and 1.07%. This method is efficient for routine analysis and quality control assay as it is relatively interferences free.**

***Index Terms*—Folic acid, L-Tryptophan, Pharmaceutical tablet, Spectrofluorimetry.**

I. Introduction

Vitamins are crucial for the growth and normal development of living beings. Vitamin B_9 that belongs to water-soluble B-group vitamin is essential for human body and has different chemical forms, folic acid (FA) and folate (Nasser, et al., 2005; Catharino, et al., 2006; Zare, et al., 2011). Folate is the general formula of Vitamin B_9 existed in food (Krishnaswamy and Nair, 2001) whereas FA is the synthetic form of this vitamin in the supplements and fortified foods (Bailey, 2000). FA has the chemical formula N-[4-[[(2-amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzoyl]-L-glutamic acid (Nagaraja, et al., 2002; Flores, et al., 2005; Deconinck, et al., 2011) (Fig. I). Human body is supplied with FA from its main dietary sources such as spinach, white beans, asparagus, dark-leaved vegetables, Brussels sprouts, soybean, oranges, and melons (Nasser, et al., 2005; Deconinck, et al., 2011). FA is vital for the formation and growth of red blood cells besides the prevention of anemia (Zhao, et al., 2006). This vitamin-like others cannot be synthesized by mammalian cells and must, therefore, be supplied in sufficient amounts in the diet (Aurora-Prado, et al., 2004).

Deficiency of Vitamin B_9 is resulted mainly from poverty, food preferences, drug use, and chronic alcoholism. In the case of the insufficient taking of Vitamin B_9, multivitamin supplements can be used to prevent the associated physiological problems (Aurora-Prado, et al., 2004; Deconinck, et al., 2011). FA limitation can lead to congenital malformations in the fetus (spina bifida, encephalocele, cleft palate, and hydrocephalus), as well as heart disease (Czeize and Dudas, 1992; Crane, et al., 1995; Oakley, et al., 1995).

FA is important for normal growth, reproduction (during gestation and lactation), and antibody formation. It functions as a coenzyme in the metabolism of amino acids (glycine), the synthesis of purines, pyrimidines, DNA, and RNA is crucial for cell division and protein synthesis. Its defect can modify DNA synthesis and induce chromosomal changes (Pacheco, et al., 2009). Lack of FA causes high risk for neural tube defects (Pacheco, et al., 2009). The extremity of these defects means; it is important to undertake genetic counseling, supplement diets with FA, and perform prenatal diagnosis of neural tube defects (Pacheco, et al., 2009).

Although FA is nontoxic, there is some concern that high doses may mask pernicious anemia. This result is only likely following ingestion of quantities >5 mg. Consuming medicines of FA levels above the approved value can be toxic to the patient (Kennedy, 2016). Therefore, there is a continuing necessity for the development of new analytical procedures for the determination of FA present in pharmaceutical formulations (Zhao, et al., 2006).

High-performance liquid chromatography (HPLC) is considered as a popular analytical technique for identification and quantification of FA in pharmaceutical formulations (Póo-Prieto, et al., 2006). In spite of the advantages of HPLC as it is a well-established technology in the pharmaceutical field, offering sensitivity and specificity, it has disadvantages include

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high operating costs, the need for large amounts of samples and solvents, and the generation of hazardous wastes such as organic solvents that are expensive to dispose of and can have remarkable environmental impacts (Deconinck, et al., 2011).

In the literature the reported methods for the determination of FA include hyphenated techniques of HPLC with different detectors (Aurora-Prado, et al., 2004; Nelson, et al., 2006; Chaudhary, et al., 2010), electrophoresis (Zhao, et al., 2006), electrochemical methods (Vaze and Srivastava, 2007; Prasad, et al., 2010; Ensafi and Karimi-Maleh, 2010), flow injection analysis (Nie, et al., 2000), and spectrophotometric methods (Pesce and Bodourian, 1986; Rao, et al., 1978).

The use of derivative spectrophotometry provides a simple and inexpensive solution for the determination of drugs in pharmaceutical formulations. Meanwhile, using zero-order spectrophotometry for the determination of FA in complex samples that contain large amounts of interferents has the disadvantage of prior extraction of the analyte and thus increasing the costs of routine analysis (Moura and Moita, 2012).

Molecular fluorescence has one of the most attractive characteristics, its inherent sensitivity, which is often one to three orders of magnitude better than absorption spectroscopy. Actually, single molecules of selected species have been detected by fluorescence spectroscopy under controlled conditions. Besides, the large linear concentration ranges of fluorescence methods, which are remarkably broader than linear concentration range in absorption spectroscopy. However, the smaller number of chemical systems that show appreciable fluorescence has been made fluorescence methods less widely applicable than absorption methods (Cruces, et al., 1994; Skoog, et al., 2013). The aim of the present work was, therefore, to use a simple, low cost but sensitive and selective analytical method as spectrofluorimetry for quantification of FA in pharmaceuticals based on its quenching to the fluorescence emission of L-Tryptophan (Fig. II). The results were compared with the recommended method described in the literature.

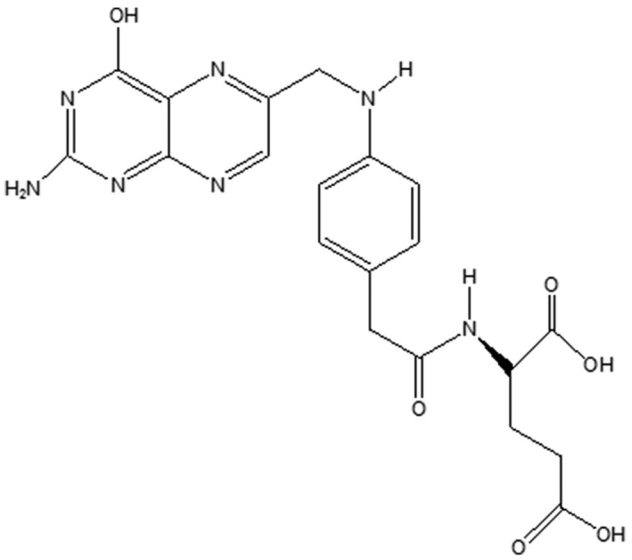

Fig. 1. Chemical structure of folic acid.

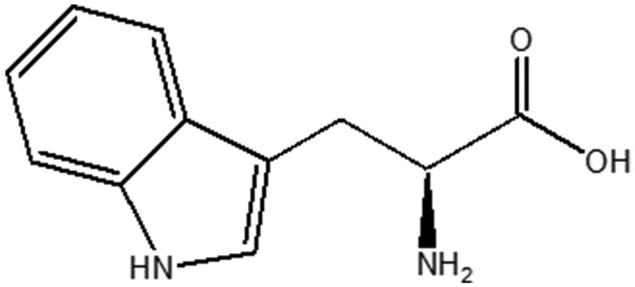

Fig. 2. Chemical structure of L-tryptophan.

II. Experimental

A. Apparatus

Fluorescence spectra measurements were carried out using Agilent Cary Eclipse Fluorescence Spectrophotometer, USA, with both slits width 5.0 nm and a quartz cell of 1.0 cm optimal path length. CyberScan pH 510 pH/mV meter was used for pH measurements of the solutions.

B. Material and Reagent

FA was obtained from Awamedica Pharmaceutical Company (Kurdistan Region-Iraq), used as received, the purity of which was 98%. A solution of 4.0×10^{-4} mol/L L-Tryptophan (from Sigma-Aldrich) was prepared by dissolving 0.0082 g of the solid in 50.0 ml of 0.1 mol/L NaOH solution. Different buffer solutions were prepared by mixing proper volumes of 0.1 mol/L sodium acetate (from Scharlau) and 0.1 mol/L acetic acid (from Scharlau) to prepare acetate buffer solution; 0.1 mol/L K_2HPO_4 (from Merck) and 0.1 mol/L KH_2PO_4 (from Sigma-Aldrich) to prepare phosphate buffer solution; and 0.04 mol/L phosphoric acid (from Merck), 0.04 mol/L boric acid (from Sigma-Aldrich), and 0.04 mol/L acetic acid (from Scharlau) to prepare Britton–Robinson (BR) buffer solution, respectively. A suitable volume of 0.2 mol/L sodium hydroxide (from Merck) was then added to adjust the pH using a pH meter.

C. Preparation of Standard and Sample Solution

A stock solution of standard FA (1.0 mg/ml) was prepared by dissolving 0.05 g in 40.0 ml of 0.1 mol/L NaOH with carefully stir, then completed to 50.0 ml with the same solvent and kept in a cool dark place. Working standard solutions were prepared daily by proper dilution of the stock standard solution with the same solvent.

All pharmaceutical products of FA in the local medical store are containing 5 mg and five different companies were used for the quantification; (Joriver, Jordan), (Wockhardt, UK), (Eipico, Egypt), (Actavis, US), and (Julphar, UAE). Ten tablets of FA were weighed and crushed for each pharmaceutical company, and then sample powder of the five companies was accurately weighed individually and placed in a 50.0 ml beaker and dissolved with 40.0 ml of 0.1 mol/L NaOH. The solution was stirred for 10 min to increase solubility. Insoluble excipient was removed by filtration using

Whatman No.41 membrane filter paper. The filtered solution was diluted to 50.0 ml with the same solvent.

D. Analytical Procedure

In 10.0 ml volumetric flask, 0.25 ml L-Tryptophan (4.0×10^{-4} mol/L), 0.8 ml BR buffer solution (pH 7.0), and adequate FA standard or sample 2.0–16.0 µg/L (0.2–1.6 ml) of 100 µg/L solution were added. This mixture was diluted to 10 ml with distilled water and mixed thoroughly for 10 min at 25 ± 2°C. The fluorescence intensity was measured against a blank with excitation wavelength at 281 nm and emission at 365 nm.

III. Results and Discussion

A. Optimization of Experiment

All parameters of the optimum conditions of fluorescence spectra of L-Tryptophan quenched by FA have been examined to obtain maximum sensitivity of the procedure. Different parameters such as pH, concentration of fluorescent probe L-Tryptophan, reaction time, and interferences were studied.

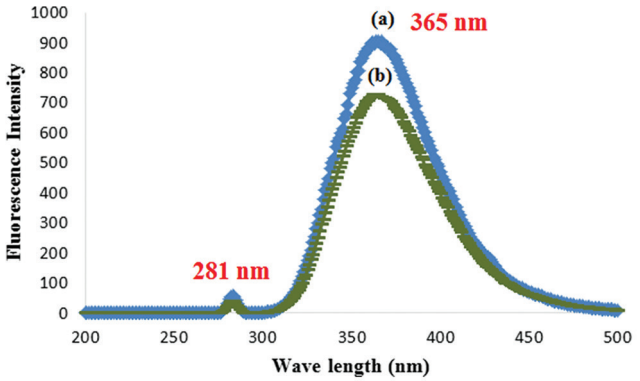

Fig. 3. Fluorescence excitation and emission spectra of (a) L-Tryptophan (4.0×10^{-4} mol/L), (b) L-Tryptophan (4.0×10^{-4} mol/L) with 5 µg/ml folic acid at pH 7.

Fluorescence emission spectra

The fluorescence spectrum of L-Tryptophan (4.0×10^{-4} mol/L) solution was recorded at excitation and emission wavelengths at 281 nm and 365 nm, respectively (Fig. IIIa). The intensity of the native fluorescence of L-Tryptophan marked quenches without any shift when 5 µg/ml FA was added to the solution containing (4.0×10^{-4} mol/L) L-Tryptophan. A new ion associated complex, produced during quenching of L-Tryptophan fluorescence (Fig. IIIb). Therefore, L-Tryptophan could be used as a fluorescent probe for FA.

pH buffer

Different buffer solutions of pH range (2–10) were examined for the reaction of 4.0×10^{-4} mol/L L-Tryptophan solution with 5 µg/ml FA. Acetate buffer, phosphate buffer, and BR buffer were carried out in the reaction, individually (Fig. IVa). It was observed that BR buffer of pH 7 has maximum intensity of quenched fluorescence spectrum of L-Tryptophan solution with FA due to the fact that FA acts as an acid and can quench the fluorescence spectrum of L-Tryptophan effectively in neutral medium. Therefore, this buffer was selected for next investigations.

To optimize the volume of BR buffer solution, different volumes (0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 ml) were added to the solution of L-Tryptophan with FA where 0.8 ml solution of BR buffer showed the maximum intensity of quenched fluorescence spectrum (Fig. IVb).

L-Tryptophan concentration and reaction time

Different volumes (0.1–25 ml) of 4.0×10^{-4} mol/L L-Tryptophan reagent were investigated with fixed (5 µg/ml) concentration of FA and BR buffer solution (pH 7) to obtain the maximum intensity of fluorescence spectrum. Fig. V showed that 0.25 mL volume of L-Tryptophan has maximum ΔF intensity.

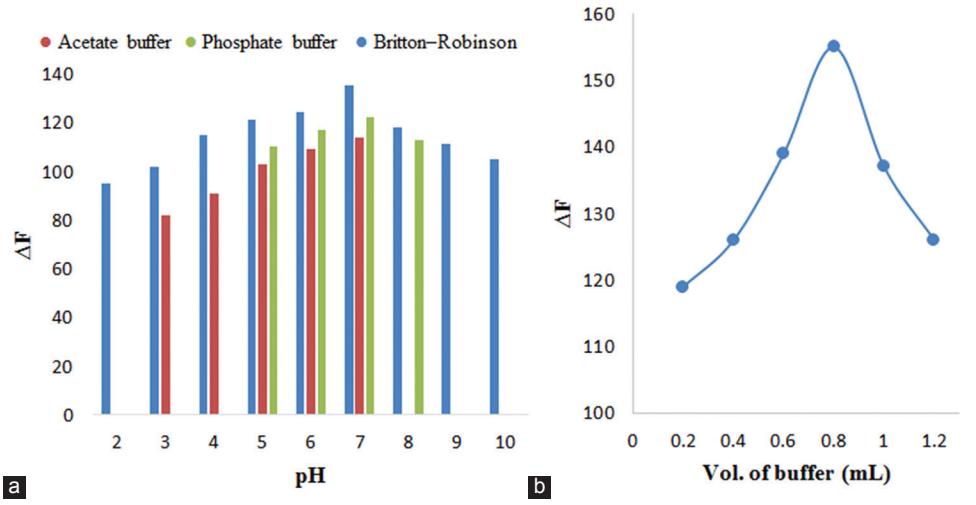

Fig. 4. (a) Effect of different pH buffer solutions on ΔF intensity of 4.0×10^{-4} mol/L L-Tryptophan solution with (5 µg/ml) folic acid; (b) different volumes of Britton–Robinson buffer versus ΔF intensity of L-Tryptophan solution with folic acid.

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The temperature had little influence on the fluorescence intensities of the system. The quenched fluorescence intensity (ΔF) slightly increased with temperature up to 20ºC and then remained constant up to 25°C. Therefore, room temperature (25°C) was selected for further study. The reaction time of mixing L-Tryptophan with FA at ambient temperature (25 ± 2°C) versus fluorescence intensity was also examined. It was found that quenching of the fluorescence intensity reached a maximum after 10 min from mixing the reagent with FA and remained constant for more than 100 min (Fig. VI).

Evaluation of selectivity

The excipients that are used in the pharmaceutical products in the form of tablets and capsules were used for selectivity assessment of FA under optimal experimental conditions (Manzoori, et al., 2011). The excipient solutions (500 µg ml) of lactose, fructose, glucose, starch, sucrose, and sodium chloride were mixed with 12 µg/ml FA in 10 ml of volumetric flask, individually. The spectra obtained were compared with the spectrum of (12 µg/ml) FA standard solution.

A level of interference was considered to be acceptable when the error is not higher than 3%. No significant levels of interferences were observed in the determination of FA in the presence of the common excipients (Table I).

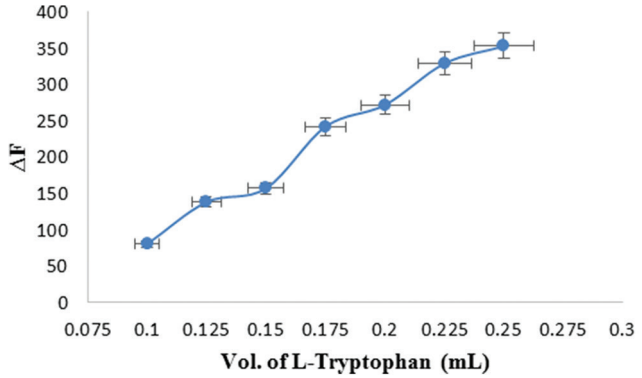

Fig. 5. Different volumes (0.1–25 ml) of 4.0 × 10^{-4} mol/L L-Tryptophan and fixed (5 µg/ml) folic acid versus ΔF intensity of the fluorescence.

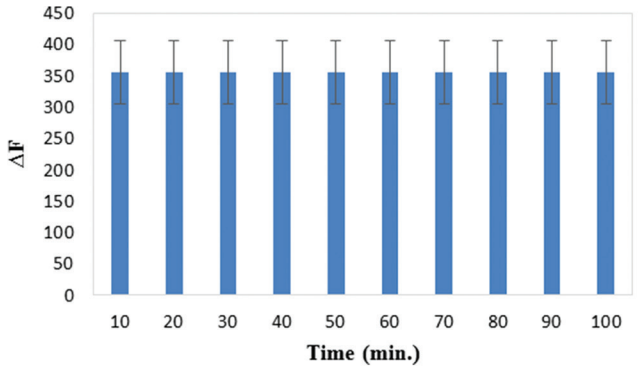

Fig. 6. Influence of reaction time of 4 × 10^{-4} mol/L L-Tryptophan with (5 µg/ml) folic acid at pH 7 on ΔF intensity of the fluorescence.

B. Analytical Figures of Merit

The determination of FA was investigated under optimum experimental conditions when the relative standard deviation (RSD%) was 1.07% as obtained from five replicated measurements of three different concentrations of FA. According to IUPAC guidelines of the validation of analytical method, the limit of detection (LOD) value = 3.3 SD/P and limit of quantification (LOQ) on value = 10 SD/P were adopted, in which SD is the standard deviation of five reagent blank measurements and P is the gradient of the calibration curve (Abd Ali, et al., 2019). The linear range was 2–16 µg/ml with correlation coefficient R^2 = 0.9974 and LOD 0.09 µg/ml (Fig. VII). The regression equation of standard solutions was $\Delta F = 40.119\ C_{FA} + 140.2$ in which C_{FA} is in µg/ml. The LOQ value of FA was 0.27 µg/ml.

The accuracy and precision of the proposed procedure was established by measuring the fluorescence intensity of three concentrations of standard FA in five replicates measurements (Table II). The values of RSD % and E % were between 1.10%–2.30% and −2.50%–1.10%, respectively, indicating that the method is valid and applicable.

C. Stoichiometry of the Reaction

The stoichiometry of the reaction between FA and L-Tryptophan was determined to adopt the limiting logarithmic method (Lakowicz, 2006). The proposed mechanism of the quenching reaction of FA and L-Tryptophan is the formation of an ion associated complex. Fig. VIII indicated a plot of log [FA] versus log ΔF at constant concentration of L-Tryptophan and log [L-Tryptophan] versus log ΔF at constant concentration of FA, respectively. Both plots were straight lines and depending on the gradients, the ratio of FA: L-Tryptophan complex is 1:1.

D. Quenching Mechanism of L-Tryptophan and FA

Fluorescence quenching is the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions, such as excited-state reactions, photoinduced electron transfer, fluorescence resonance energy transfer, ground-state complex formation, and collisional quenching (Bhattacharyya, et al., 1990). The physical origin of fluorescence quenching arising from the addition of a quenching agent, L-Tryptophan, to FA fluorescence can be

TABLE I
EVALUATION OF SELECTIVITY OF FA QUANTIFICATION USING L-TRYPTOPHAN TOWARDS SOME COMMON EXCIPIENTS PRESENTED IN THE PHARMACEUTICAL PRODUCTS

Coexisting materials	Allowance concentrations (µg/ml)	E %*
Lactose	500	2.10
Fructose	500	2.20
Glucose	500	1.80
Starch	500	−1.40
Sucrose	500	−2.10
Sodium chloride	500	−1.50

*Average of three determinations

interpreted in terms of the formation of ion-associated complex that enhanced fluorescence quenching. In this mechanism the fluorophore itself (FA) is fluorescent when the receptor site (amine group) is free. In the presence of L-Tryptophan, the fluorescence intensity is lowered due to the reaction between the carboxylic group of L-Tryptophan and receptor unit (amine group) of FA to form nonfluorescent ion-associated complex, and therefore, the net fluorescence is quenched. (Fig. IX).

Basically, this quenching process can be divided into two kinds of mechanisms: Static and dynamic quenching. Static and dynamic quenching can be distinguished by their different dependences on the temperature and excited-state lifetime. Dynamic quenching is diffusion controlled because the quencher (L-Tryptophan) must diffuse to the fluorophore (FA) during the lifetime of the excited state. Since high temperature will result in a large diffusion coefficient, the bimolecular quenching constants are expected to increase with temperature. Static quenching implies either the existence of a sphere of effective quenching or the formation of a ground-state nonfluorescent complex, whereas collisional or dynamic quenching involves the collision followed by the formation of a transient complex between an excited-state fluorophore (FA) and a ground-state quencher (L-Tryptophan).

TABLE II
PRECISION AND ACCURACY DATA OF THE PROPOSED METHOD

Contained amount (µg/ml)	Found by proposed method (µg/mL)	SD	RSD%	E%*
2	1.95	2.30	1.07	−2.50
8	8.09	1.10	0.24	1.10
16	16.08	1.80	0.23	0.50

*Average of five determinations

E. Application to the Pharmaceutical Tablets

The proposed procedure using L-Tryptophan as fluorescence probe for quantification of FA was applied to pharmaceutical tablets. The ingredients in the pharmaceutical tablets did not interfere in the quantification of FA. The applicability of the proposed procedure for the analysis of FA in pharmaceutical formulations was examined by investigating various pharmaceutical tablets, and the results are tabulated in Table III and were compared to the standard FA assay using HPLC method by means of t- and F-values at 95% confidence level. HPLC has the ability to separate and identify compounds that are present in any sample that can be

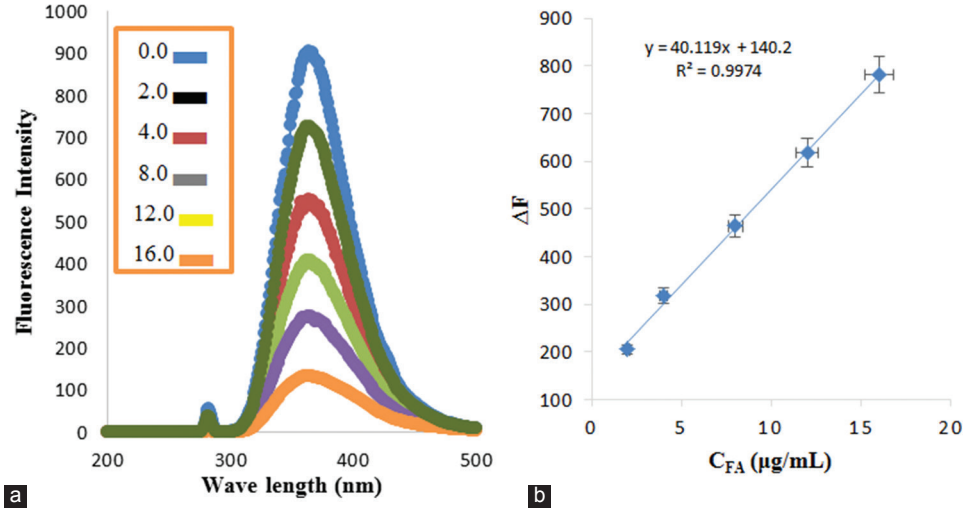

Fig. 7. (a) Different concentrations (2, 4, 8, 12, and 16 µg/ml) of folic acid versus fluorescence intensity of L-Tryptophan at pH 7. (b) Calibration curve of fluorometric quantitation of folic acid with L-Tryptophan under optimum condition.

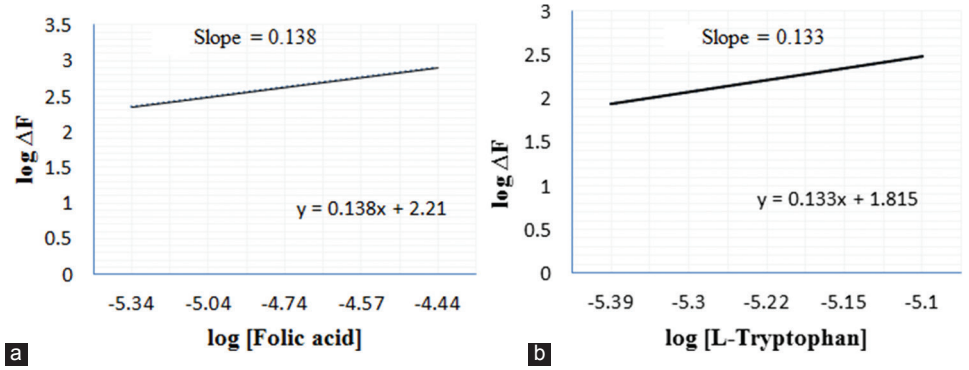

Fig. 8. Stoichiometric ratio of the fluorometric reaction of folic acid and L-Tryptophan reagent using a limiting logarithmic method. (a) Log[folic acid] versus log ΔF (b) Log [L-Tryptophan] versus log ΔF.

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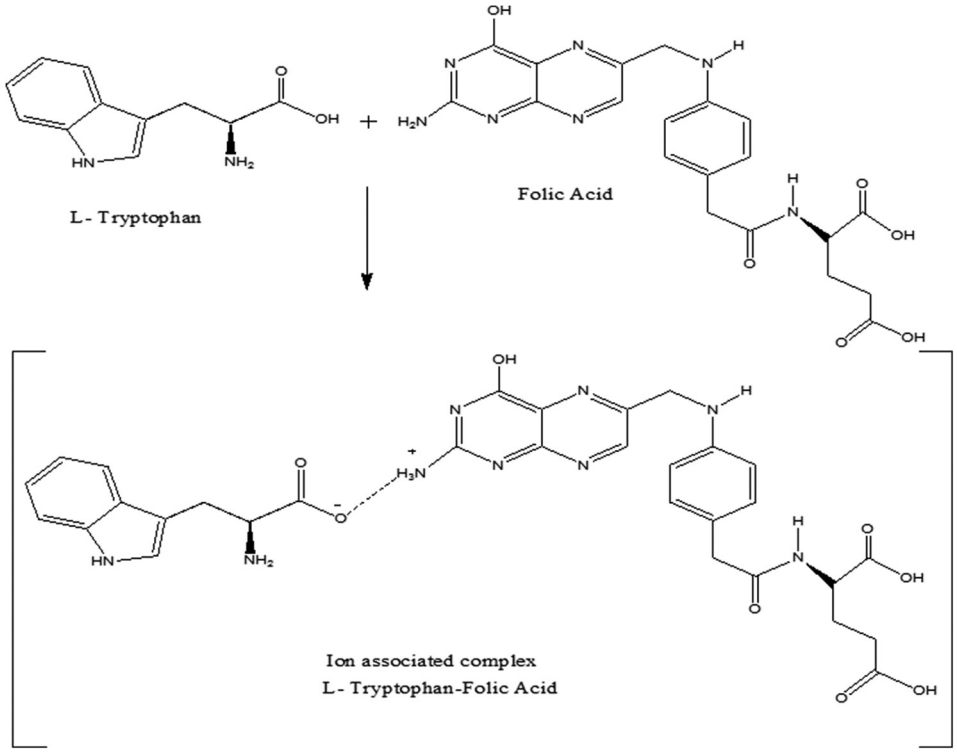

Fig. 9: Quenching mechanism of L-Tryptophan and folic acid due to the formation of ion associated complex.

TABLE III
Comparison of Fa Quantification in Pharmaceutical Tablets Using the Proposed Fluorometric L-Tryptophan Method and Standard Hplc Method

Pharmaceutical Tablets	Observed values (mg)	Values from proposed procedure	Values from HPLC	Recovery %	E %*	t and F values**
Joriver	5	4.86±0.19	4.98±0.02	97.6	−2.4	t=0.40, F=1.50
Wockhardt	5	5.10±0.12	5.01±0.10	101.8	1.8	t=1.95, F=1.00
Eipico	5	5.14±0.20	5.21±0.05	98.7	−1.3	t=0.73, F=2.20
Actavis	5	4.72±0.08	4.80±0.034	98.3	−1.7	t=0.80, F=1.07
Julphar	5	4.79±0.13	4.88±0.01	98.2	−1.8	t=0.23, F=3.20

*Average of five determinations. **Theoretical calculation of t and F at 95% confidence level (n=5) were 2.78 and 6.39 respectively

TABLE IV
Comparison of Determination of Folic Acid Using Different Analytical Techniques and the Proposed Method

Method's name	Ref.	LR	LOD	LOQ	pH
Spectrophotometer	Al-Araji, et al., 2017	0.75–10.50 µg/mL	0.159 µg/mL	0.531 µg/mL	11.0
Spectrofluorimeter	Manzoori, et al., 2011	0.01–1.10 mg/L	0.003 mg/L	-	6.2
Chromatography	Nelson, et al., 2006	0.02–73 ng	0.02 ng	0.06 ng	6.0
Electrochemistry	Mirmoghtadaie, et al., 2013	1.0×10^{-8}–1.0×10^{-6} mol/L	7.50×10^{-9} mol/L	-	12
HPLC	El-Leithy, et al., 2018	0.1–2 µg/mL	0.1 µg/mL	-	3.5–7.4
Proposed method	-	2.0–16.0 µg/mL	0.09 µg/mL	0.27 µg/ml	7.0

HPLC: High-performance liquid chromatography, LOD: Limit of detection, LOQ: Limit of quantification

dissolved in a liquid in trace concentrations as low as parts per trillion. The sample mixture or analyte is pumped with a solvent (mobile phase) in a column with chromatographic packing material (stationary phase) at high pressure. The sample is carried by a moving carrier gas stream of helium or nitrogen. Sample retention time will vary depending on the interaction between the stationary phase, the molecules being analyzed, and the solvent used. As the sample passes through the column it interacts between the two phases at different rates, primarily due to different polarities of analytes in the sample. Analytes that have the least amount of interaction with the stationary phase or the most amount of interaction with the mobile phase will exit the column faster. The HPLC system of Agilent 1100 controlled by ChemStation Data41 System and equipped with G 1311A quaternary pump and UV detector (VWD-G1314 A) was employed for the investigation. A reverse phase C18 column (Kromasil 100-5 Phenyl®, 300 mm × 4.6 mm, 5 µm) was used at 25°C. The experiment was run with mobile phase consisted of 0.1% v/v trifluoroacetic acid (TFA) and acetonitrile at ratio (80:20 v/v). It was injected with flow rate 1.5ml/min, and the elute was monitored at wavelength 290 nm.

In all cases, the average results obtained by the proposed procedure and standard method were statistically identical, and there were no significant differences between them at 95% confidence level. Therefore, the proposed methods are simple, sensitive, and reproducible and can be used for routine analysis of FA in pharmaceutical formulations.

Table IV shows the optimization results of the determination of FA using different analytical techniques and the proposed method. Although some analytical techniques have lower LOD value and wider linear range, the proposed method does not need pre-extraction of the sample besides the low cost of the technique compared with others such as HPLC.

IV. Conclusion

The proposed spectrofluorometry is simple, rapid, inexpensive, sensitive, and reproducible method for routine analysis of FA in pharmaceutical formulations without interference from common tablet excipients. The method has wide linear range with good accuracy and precision. The significance of the proposed method is direct measurement of FA in pharmaceutical formulations without need for pretreatment of the drug and extraction with organic solvent. Therefore, the method can be used successfully for quality control of FA in its dosage forms.

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