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Determination of Folic Acid in both Pure and Pharmaceutical Preparations via Oxidative Coupling Reaction

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Abstract

In this study, the development of an indirect spectrophotometric method for the determination of folic acid in pure and pharmaceutical preparations is described. The method is based on the oxidation of pyrocatechol with iron (III) in an acidic medium, followed by the reaction with folic acid (FA) to produce a stable, water-soluble orange compound with maximum absorption at 350 nm versus the blank reagent. The complex of charge transfer was studied under optimal conditions; the titration graph was linear over the range of 0.5-25 µg/mL with a relative error of 1.2-2.8 and a relative standard deviation of 2.43-1.45 depending on the concentration level.

Keywords: Folic acid, Pyrocatechol, Oxidative coupling, Vitamin B9, Beer's law.

تقدير حامض الفوليك في المستحضرات النقية والصيدلانية من خلال تفاعل الازدواج الاوكسجيني

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الخلاصة

في هذا الدراسة, تم تطوير طريقة طيفية غير مباشرة لتقدير حامض الفوليك في المستحضرات النقية والصيدلانية. تعتمد الطريقة على اكسدة البيروكاتيكول بالحديد (III) و في وسط حامضي و من ثم التفاعل مع حامض الفوليك FA لإنتاج مركب برتقالي مستقر قابل للذوبان في الماء بأقصى امتصاص عند 350 نانومتر مقابل البلانك. تمت دراسة معقد انتقال الشحنة في ظل الظروف المثلى . فأعطى منحني المعايرة علاقة خطية عند المدى 0.5−25 مايكروغرام /مل بخطأ نسبي 1.2−2.8 و انحراف معياري نسبي من 2.43 الى 1.45 اعتمادا على مستوى التركيز .

1. Introduction

One of the B vitamins is folic acid, also referred to as vitamin B9 and folacin [1,2]. The folate molecule is made up of three connected compounds: A methylene (CH_2) group links 2-amino-4-hydroxy-pteridine to a *p*-amino benzoyl group, which is subsequently linked to glutamic acid (or poly-glutamate) *via* an amide (O=CNH) linkage. The compound 4-(((2-Amino-4-oxo-3,4-dihydropyridine-6-yl)methyl)amino)benzoyl)glutamic acid is the chemical

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name of folic acid [3,4]. Over 150 different molecules can be considered folates [5]. Folic acid has a low solubility in water and is very soluble in saline media. Folic acid solubility increased with an increase in pH scale; it's synthesized by plants and microorganisms; therefore humans get vitamins from different sources of food or supplements [6,7]. The supplementation was shown to decrease the danger of neural birth defects. On the other hand, vitamin B9 is critical for synthesized red cells, RNA, DNA, and amino acid formation like methionine and homocysteine and is used to treat anemia caused by a deficiency of folic acid [8-10].

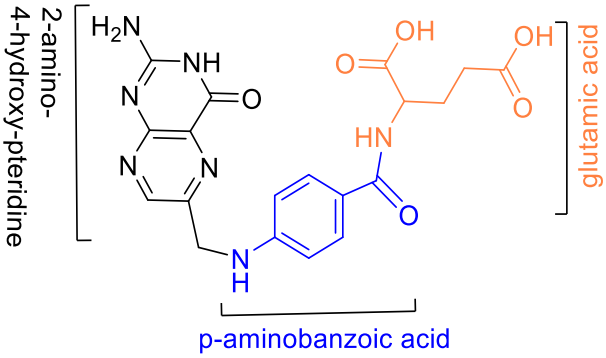


Figure 1: Structure of Folic acid.

The spectrophotometric method is a powerful analytical methodology with high sensitivity, sufficient accuracy, and very simple and inexpensive instrumentation. Because of these characteristics [11], it is a preferred method. The oxidative coupling reaction is one of the simpler methods of determining compounds in drugs because it is cheap and does not require the expertise of the analyst. This reaction does not require the use of an organic solvent and is of green reaction chemistry [12]. Various analytical methods have been used in the literature, such as microbiological assay [immunoassay, UV spectrophotometric (UV-visible) liquid chromatography-mass spectrometry (LC-MS) [12-14], capillary electrophoresis (CE), ultra-high performance liquid chromatography (UHPLC) [14], and ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) for the determination of compounds in drug preparations [2,15]. The microbiological method was used as a standard method for the determination of folic acid. This method has high sensitivity, but it cannot separate the different forms of folate [16,17]. The method is tedious and time-consuming. On the other hand, high-performance liquid chromatography (HPLC) is one of the methods for the determination of folic acid that has been documented in the literature, as it is more accurate, easier, and can determine individual folate forms when compared to the microbiological method [17-21]. While liquid chromatography-mass spectrometry provides many advantages [22,23], this instrument is very expensive and requires the expertise of the analyst in the laboratory. The aim of this study is to use the method (UV-visible spectrophotometer) *via* oxidative coupling. This method features a reaction with high sensitivity, accuracy, and very simple, inexpensive instrumentation and does not require the high expertise of the analyst in the laboratory.

2. Experimental part

Chemicals

All the chemicals utilized were of analytical grade. Folic acid was provided by a state company for drug industries and medical appliances (SDI) in Samarra, Iraq. Folic acid capsules were obtained from a local source or pharmacy. The HDL company provided pyrocatechol, hydrochloric acid (HCl), ethanol, and iron (III) sulfate, nonahydrate $Fe_2(SO_4)_3.9\ H_2O$. The water used was double-distilled.

Instrumentation

A UV-visible spectrophotometer (SHIMADZU 1800 double beam region 190-1000 nm, Japan) was used for all absorption measurements. Infrared spectral data were recorded using a Shimadzu 8400S FT-IR spectrometer. A pH meter (a Jenway 3020 pH meter) was used to measure pH.

Preparation of samples

FA solution (500 mg/ml) Preparation

A solution of folic acid (50 mg, 0.1 mmol) in absolute EtOH (3 mL), and solicited for three minutes. This solution was then added slowly over 5 minutes to distilled water (22 mL). The solution was stirred and heated to increase its solubility. The volume of this solution was then completed to 100 mL in a volumetric flask. This solution was stable for approximately 10 days.

Preparation of iron(III)sulfate hydrate $Fe_2(SO_4)_3.9H_2O$ solution (10mM)

In a volumetric flask, $Fe_2(SO_4)_3.9H_2O$ (500 mg, 0.88 mmol) was dissolved in double-distilled water (10 mL), and the volume was completed to 100 mL.

Preparation of pyrocatechol (PC) Solution (5 mM)

A solution of PC (110 mg, 0.1mmol) was dissolved in double-distilled water (20 mL). The volume of this solution was then completed to 200 mL in a volumetric flask. The solution was stable for approximately a week.

Preparation of capsule solution (50 mg/mL)

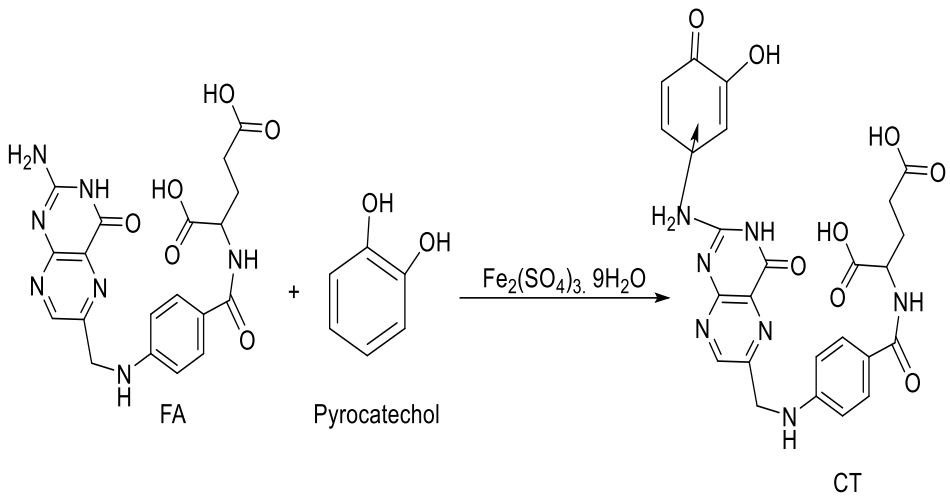
Twenty-five capsules (containing 50 mg FA) were dissolved in EtOH (3 mL) and double-distilled water (22 mL). The volume of this solution was then completed to 200 mL in a volumetric flask.

Calibration graph

Folic acid solution was added to a set of volumetric flasks (20 mL) at a concentration of 1-40µg/mL to PC solution (1.5 mL, 5 mM), followed by $Fe_2(SO_4)_3$ solution (4 mL, 10 mM). In a volumetric flask, the volume of this solution was then completed to 200 mL with double-distilled water. The solution was left in a water bath at 80 °C for 35 minutes, after which it was left too cold for 15 minutes at room temperature. The absorbance of the orange compound was measured at 350 nm against the blank.

3. Results and discussion

In this study, we used the property of oxidative coupling reaction in the react amino group in folic acid and the system aromatic in pyrocatechol. The CT complex was prepared by mixing equimolar amounts of folic acid with the acceptor (pyrocatechol) in ethanol, as shown in Scheme 1.



Scheme 1: The charge transfers folic acid with pyrocatechol.

The charge transfer complex was characterized by a UV-visible spectrophotometer (Figure 2) and gave maximum absorption at 350 nm, explaining the shifting between the FA, CT-complex, and pyrocatechol. This gives evidence that a new complex was formed. The FT-IR spectrum of the complex formed (Figure 3) shows a characteristic peak of the amino group (NH_2) at 3417-3326 cm^{-1}, which shifts to 3391-3244 cm^{-1} in FA complex with pyrocatechol, confirming that the interaction has occurred at the NH_2 site [24]. ^1H NMR spectrum of the FA-PC complex in DMSO-d_6 (Figure 4) showed signals between 6.8 and 8.6 ppm for aromatic protons, a signal at 9.14 ppm belonging to the pyrazine ring proton, and a signal at 6.85 ppm attributed to the NH_2 group [5,25].

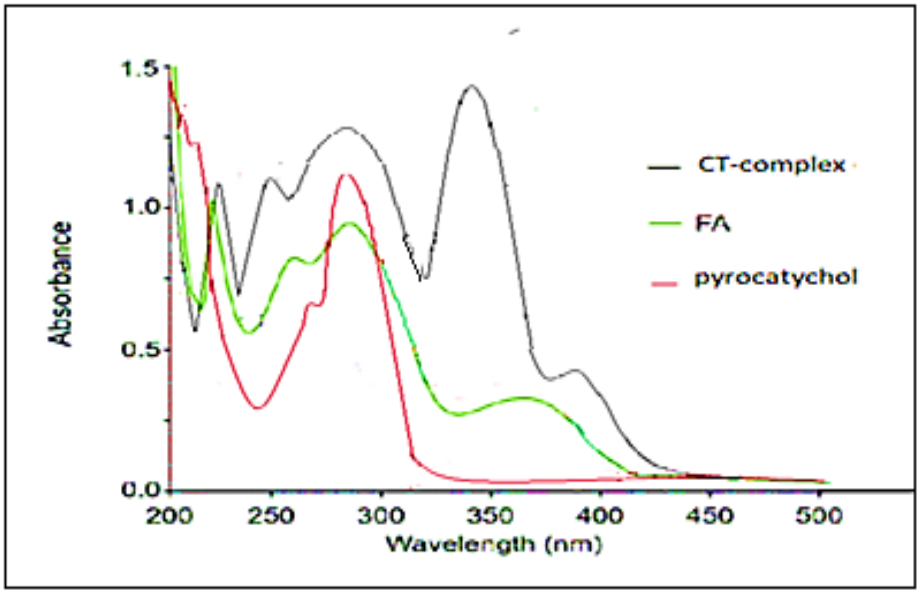


Figure 2: UV-visible spectrum of charge transfer complex (CT-complex), folic acid (FA) and pyrocatechol

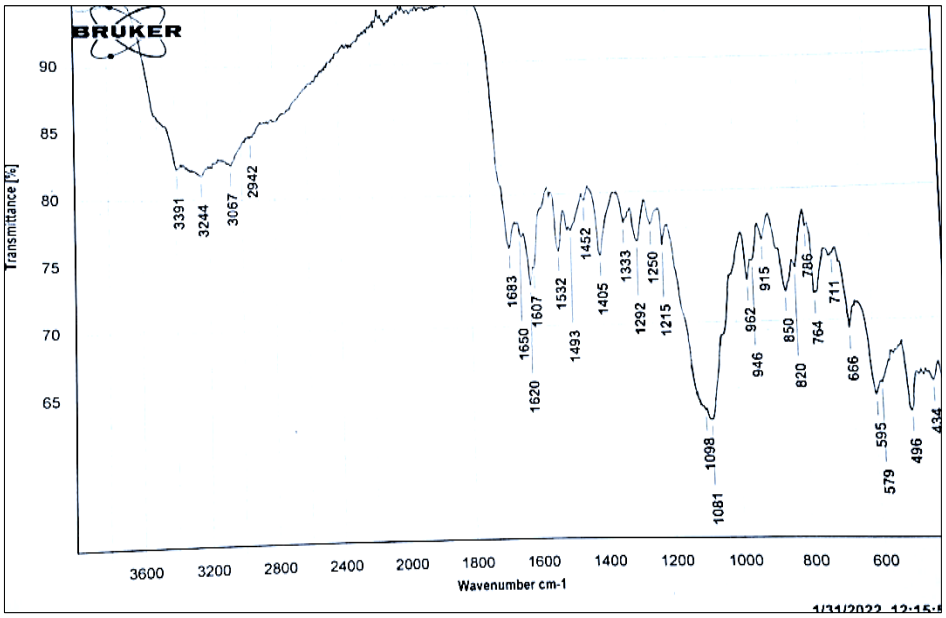


Figure 3: The FT-IR spectrum of FA-PC complex.

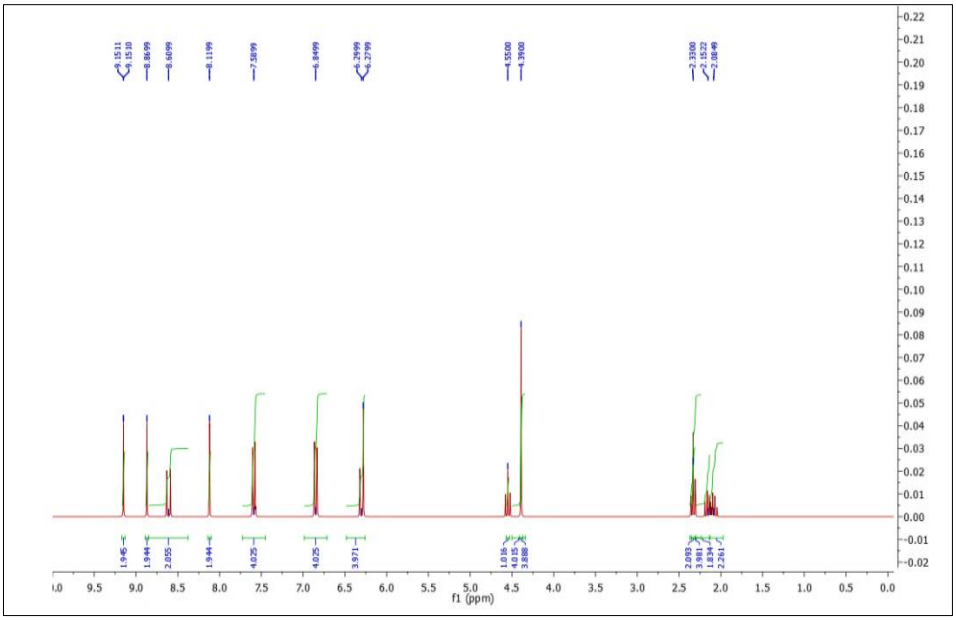


Figure 4: ^{1}H NMR spectrum of FA-PC complex.

The Influence of pH on reaction

The effect of pH on colour intensity was examined. The amount of complex formation, and hence the final solution's absorbance, is often a function of H-ion concentration. By Fe^{+3}, oxidation of the pyrocatecoland reaction with FA was found to occur in a neutral medium, but the presence of acid was necessary to increase the stability of the newly developed orange-color CT by maintaining the desired pH. Various acids and different volumes of these acids were studied, and the results indicated that HCl solution (1.0 mL, 0.1 M) is the most suitable acid solution (pH = 9.88) to produce the highest absorption (Table 1).

Table 1 : Influence of pH on absorption

Acid (0.1M)	Abs/mL concentration of acid and base						pH range
	0.2	0.5	1	1.5	2.0	2.5	
Without acid	-	-	0.306	-	-	-	3.32
H_2SO_4	0.142	0.161	0.203	0.20	0.197	0.182	2.98-2.63
HCl	0.152	0.198	0.362	0.189	0.187	0.180	6.39-3.44
NaOH	0. 139	0.137	0.129	0.130	0.131	0.122	6.39-10.5

The influence of iron (III) sulfate concentration

In this work, a study was conducted to show the influence of different oxidative reagents, including KlO_3, NH_4VO_3, *N*-bromocuccimimed, KlO_4, and $Fe_2(SO_4)_3.9H_2O$ solution, on the absorbance of solutions containing different concentrations of folic acid. The Fe compound gives a yield of charge transfer, while other compounds don't give any result. The effect of a 10 mM concentration of $Fe_2(SO_4)_3.9H_2O$ (0.2-5 mL) on the absorption of the orange complex with various solution of FA (0.2-5 mL) was then investigated. Table 2 shows that the complex's absorbance at 350 nm increased with increasing oxidative reagent volume until it reached a constant at 4 mL, after which the absorbance decreased. Therefore, the solution of $Fe_2(SO_4)_3$ volume (4 mL, 10 mM) gave the highest absorbance with $R_2 = 0.99$, and it was recommended for the subsequent experiments [26].

Table 2: The effect of iron(III) sulfate concentration

Ml of 10mM $Fe_2(SO_4)_3$	Abs.of folic acid and concetration								
	0.2	**0.5**	**1.5**	**3**	**3.5**	**4**	**4.5**	**5**	**R_2**
0.2	0.015	0.141	0.152	0.136	0.157	0.267	0.132	0.047	0.987
0.5	0. 140	0.195	0.284	0.299	0.369	0.629	0.198	0.031	0.993
1	0.121	0.230	0.194	0.189	0.221	0.288	0.213	0.12	0.434
1.5	0.031	0.171	0.142	0.163	0.176	0.221	0.192	0.172	0.545
2	0.04	0.032	0.110	0.032	0.137	0.216	0.021	0.152	0.512

The influence of pyrocatechol concentration

Pyrocatechol is one of the organic compounds that can participate in the coupling reaction, and the formed CT complex causes the hydroxyl group and the π-system to have hydroxyl groups as well, giving the CT complex stability and color. Folic acid doesn't give a CT complex with picric acid in THF or benzene, and folic acid doesn't give yields with bromonayl, resosenol, or phenol. The influence of different amounts of pyrocatechol on the absorbance of a solution containing folic acid (4 mL) and Fe^{+3} (0.5 mL) was studied. The results indicated that smaller amounts resulted in incomplete complex formation. Increased concentration increases the absorbance, which reaches its maximum when using pyrocatechol solution (2.5 mL, 5 mM), which also gives the highest value of the determination coefficient.

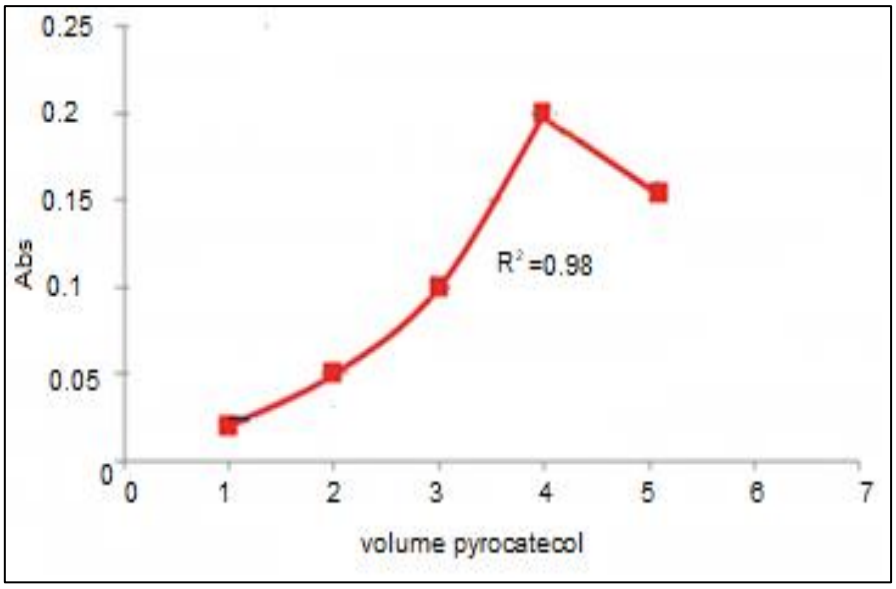


Figure 5: The effect of pyrocatechol concentration

The influence of temperature

The temperature had an effect on the color intensity of the resultant complex, which was studied. The formation of the colored complex was slow at room temperature and required a longer time for completion. As a result, efforts were made to accelerate the reaction by performing it at higher temperatures. The maximum absorbance was achieved when the reaction mixture was done in a water bath at 80 °C, as can be seen in Figure 6, and the absorbance dropped as the temperature rose.

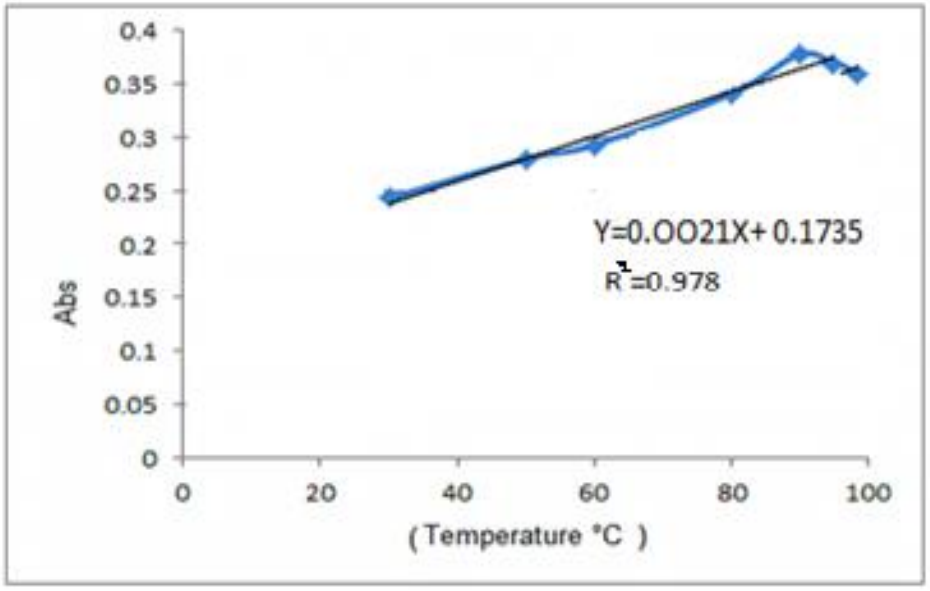


Figure 6: Effect of temperature on oxidative coupling reactions

The influence of heating time on the oxidative coupling reaction

The effect of the time needed to complete the reaction was studied by allowing the solutions to stand in a water bath at 80 °C for different times. After adding the components of the reaction (folic acid, ferric sulfate, and pyrocatechol), the absorbance was measured against the reagent blank .The results in Table 3 indicated that the high absorbance of CT at 350 nm occurred after 35 minutes and that there was no significant difference in the intensity of the complex above

35 minutes. Therefore, a standing time of 35 minutes was recommended for the subsequent experiments.

Table 3: The influence of heating time on absorbance

Time (min)	5	10	15	20	25	30	35	40	45	50	55
Abs	0.336	0.385	0.388	0.388	0.380	0.374	0.410	0.373	0.395	0.38	0.343

The influence of time

Under the ideal experimental circumstances of concentration, sequence of addition, and temperature indicated above, the influence on the stability of the orange complex was examined. The absorbance was measured from 5 to 145 minutes, and the results in Figure 7 indicated that the orange complex was stable for at least 80 minutes.

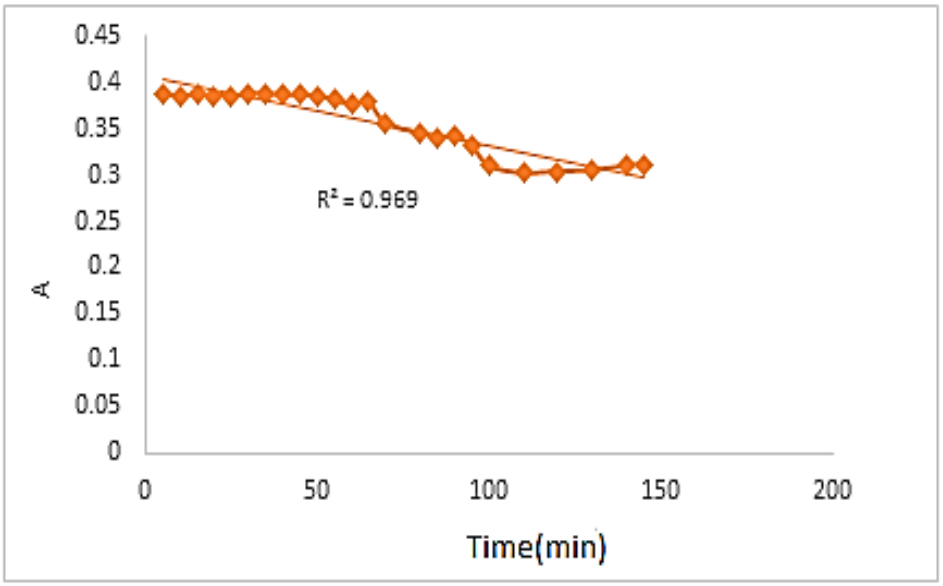


Figure 7: Time and the stability of the color complex

The influence of the order addition

The order of the addition of reactants plays an important role in obtaining the high intensity of the formed charge transfer complex. Table 4 shows the order of addition of reagents and the conditions for optimum performance. This order [FA + (O + A + R)] was followed throughout the study.

Table 4: The influence of the order addition

Order of addition	Color of solution	Absorbance
***R + (FA + O + A)**	No change in color	0.215
O + (FA + R + A)	No change in color	0.195
FA + (R + O + A)	Orange	0.582
FA + (O + A + R)	Deep orange	0.673
FA + (O + R + A)	Yellow	0.483

*[folic acid [FA], $Fe_2(SO_4)_3.9H_2O$[O], pyrocatecol [R], hydrochloric acid [A].

The calibration graph

After fixing the optimum experimental conditions, folic acid solution (0.05-2.5 mL, 100 µg/mL) was transferred to a series of volumetric flasks (20 mL), followed by $Fe_2(SO_4)_3.9H_2O$ solution (4 mL, 10 mM), and pyrocatechol solution (2.5 mL, 5 mM). The solutions were left in a water bath adjusted to 80 °C for 35 minutes. The solution was then cooled to room temperature before adding HCl acid (1.0 mL, 0.1 M) in a series of volumetric flasks. The volume was filled with double-distilled water (20 mL) and left at room temperature for 5 minutes. The absorbance was measured at 350 nm, and a linear calibration curve for folic acid (FA) was obtained (Figure 7). As shown in Figure 8, Beer's law was obeyed over a concentration range of (0.5-25 µg/mL). The limits of detection and quantitation were calculated according to the equations [3]:

The limits of detection

$$LOD = 3S/b \quad \ldots\ldots\ldots 1$$

The limits of quantitation

$$LOQ = 10S/b \quad \ldots\ldots\ldots 2$$

Where S = standard deviation, b = the slope.

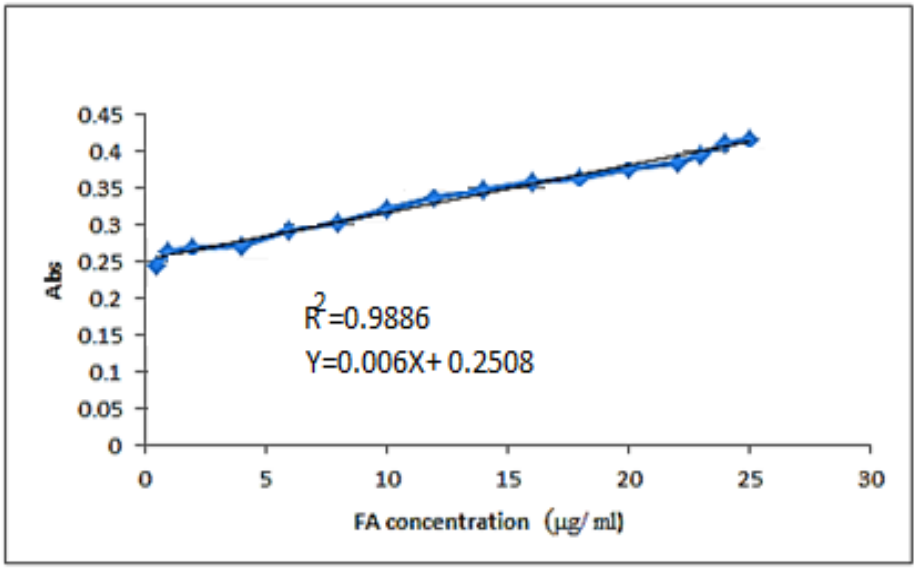


Figure 8: Calibration curve for estimation of FA

Table 5: Analytical values of statistical treatments for the proposed method for folic acid

Parameter	Value
Determination coefficient (R2)	0.9886
Coefficient Correlation, R	0.994
Linearity (µg.mL^{-1})	0.5-25
Slope, b (mL.µg^{-1})	0.0064
Intercept, a	0.2508
Sensitivity Se	0.0064
Sdyx	0.00617
LOD (µg.mL^{-1})	2.89
LOQ (µg.mL^{-1})	9.647
Medium	HCl (0.01 M)
Measurement wavelength (nm)	350 nm

Accuracy and precision

The precision and accuracy of the method were evaluated using a pure drug (folic acid) solution at three different concentrations. Table 6 shows the statistical treatment of the study for the suggested method.

Table 6: The accuracy and precision of the proposed method

Folic acid concentration (µg.mL^{-1})		Rec%	RE%	RSD% (n=6)
Present	Found			
6	6.077	+1.2	101.283	2.43
12	12.082	+0.68	100.68	1.91
16	16.045	+0.28	100.28	1.455

Interference studies

In order to assess the effectiveness of this method in the determination of folic acid in pharmaceuticals, we studied some common excipients used in drug preparations. These were investigated by carrying out the determination of folic acid in the presence of different excipients, such as, glucose, sucrose, lactose, dextrose, and starch. Table 7 shows the experimental results that showed the excipients had no effect on the determination of folic acid.

Table 7: The influence of excipients on the determination of folic acid (12 µg.mL^{-1})

Excipients	FA (µg.mL^{-1})	E%	Rec%
Starch	12.02	+0.02	100.02
Lactose	11.858	-1.24	98.75
Dextrose	12.00	0	100
Glucose	11.997	+0.975	99.975
sucrose	12.00	0	100

Table 8: The application of the method in determination of folic acid in capsule

Folic acid concentration (µg ml^{-1})		E%	Rec%	RSD% (n=6)
Present	Found			
6	6.071	+1.18	101.18	2.4
12	12.072	+0.6	100.6	1.19
16	16.045	+0.28	100.28	1.45

The continuous variation method (Job's method)

The stoichiometry of the coupling reaction between folic acid and pyrocatechol in acid media was investigated using Job's method under optimized conditions. Figure 9 shows a ratio of 1:1 of folic acid to pyrocatechol, which gives the complex. Figure 10 shows the structure of the complex that was formed.

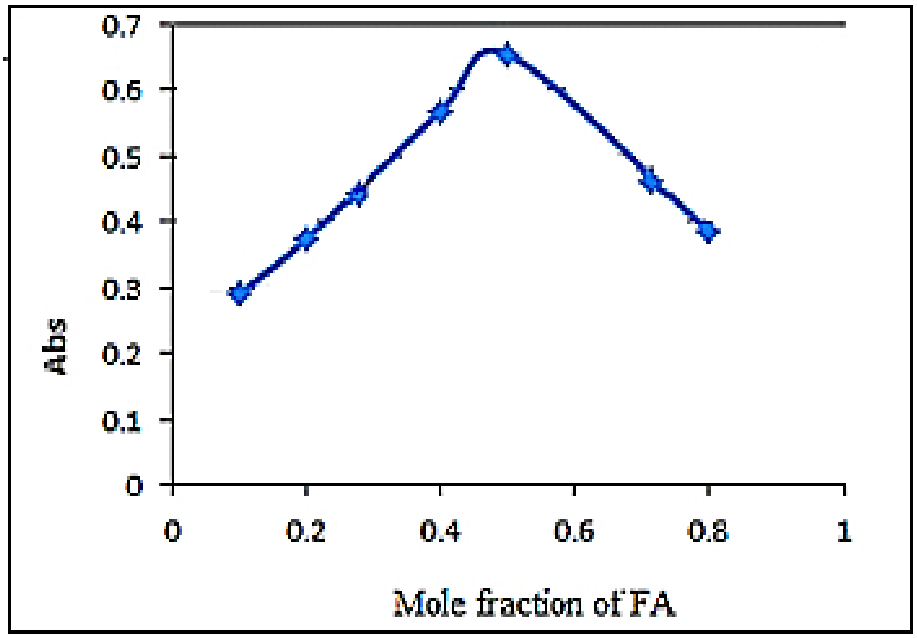


Figure 9: The continuous variation method

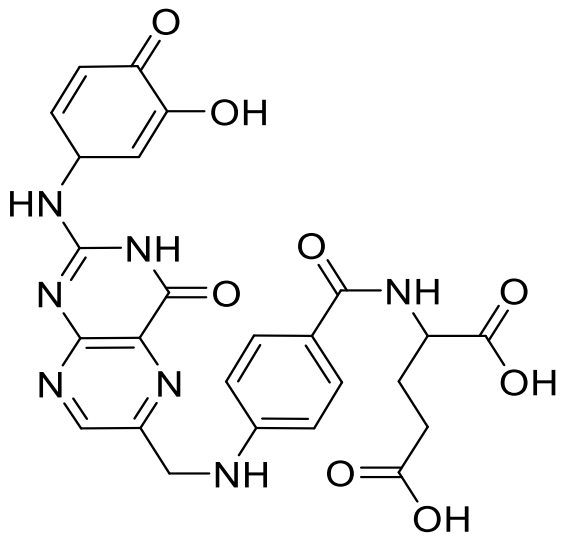


Figure 10: The structure of the complex CT

4. Conclusion

The method in this study described the evaluation of pyrocatechol as a color reagent in the development of simple, cheap, quick-sensitive, and accurate spectrophotometric methods for the determination of vitamin B9 in pharmaceutical formulations (capsules). The conditions in terms of concentrations of the reagents (pyrocatechol, folic acid, and ferric sulfate), time reaction, temperature, and pH of the solution were studied and complete.

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