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Batch andflow injection spectrophotometric methods fordetermination of folic acid inpharmaceutical preparationsusing N-bromosuccinimide as an oxidant

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Abstract: A simple, rapid and sensitive batch and flow injection spectrophotometric methods have been developed for the determination of folic acid (FA) in pure form andin its pharmaceutical preparations. The proposed methods involve the addition of a measured excess of N-bromosuccinimide (NBS) in acid medium followed by determination of unreacted NBS by reacting with a fixed amount of methyl orange (MO) and measuring the absorbance at 509 nm.The optimum reaction conditions and other analytical parameters have been evaluated. Linearity was observed from 0.5-6.0 and 1.0-8.0µg/mlfolic acid by batch and flow injection procedures, respectively. Statistical analysis of the results and comparison with results by the British Pharmacopoeia method are also reported.
Key words: Folic acid, Spectrophotometric, Flow Injection,Methyl orange, N-bromosuccinimide.

1. Introduction

Folic acid (FA) (N (4-((2-amino-l,4-di-hydro-4-oxo-6-pteridinylmethyl) amino)-benzoyl)-L-glutamic acid) (Figure1) is a member of the vitamin-B group. It is reduced in the body to tetrahydrofolate, which is a coenzyme for various metabolic processes including the synthesis of purine and pyrimidine nucleotides, and hence in the synthesis of DNA. FA plays a major role in the synthesis of red blood cells, in the formation of RNA and DNA, in the development of tissues and the brain of the fetus and the growth of a baby.[1]There are various methods described for the determination of folic acid are based on high-performance liquid chromatography using different detectors.[2-6]Other methods use derivative spectrophotometry[7] spectrophotometry,[8-10] Spectrofluorimetric,[11]IR Spectroscopy[12]and adsorptive stripping voltammetry.[13]

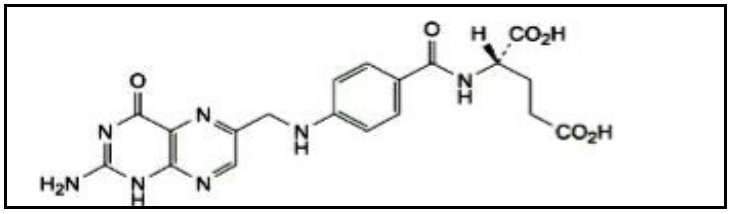

Figure 1 Structure of folic acid.

However, some of these procedures suffer from oneor another disadvantage such as narrow range of determination; require heating or extraction, long time for the reaction to complete, and also instability of the

colored product produced. The use of NBS as an analytical reagent offered adequate sensitivity and accuracy, also the simplicity and low cost of the analytical method. The use of NBS for the determination of FA has not been reported yet. Therefore, the present study was undertaken to evaluate NBS as an analytical reagent for the spectrophotometric determination of FA. Flow injection (FI) system is adequate procedure to use in routine analysis in pharmaceutical laboratories control due to their simplicity, high analytical frequency and capacity to reduce reagent consumption when compared with batch procedure.[14, 15]

The purpose of the present investigation was to develop, two simple, rapid and sensitive batch and FI methods using spectrophotometric detection weredescribed for the determination of folic acid was presented. The method was based on the oxidation of folic acid by a known excess of N-bromosuccinimide (NBS) in acidic medium followed by a reaction of the excess oxidant with methyl orange (M.O) to bleach its red color. The proposed methods have been successfully applied to the determination of FA in different brands of tablets.

2. Experimental section

2.1. Materials

All chemicals were of analytical reagents grade.

2.1.1. Folic acid stock standard solution.

A solution of 1000 $\mu g.ml^{-1}$ was prepared by dissolving 0.1000 g of FA (provided by Company for Drug Industries and Medical Applications SDI, Samarra, Iraq) in 10 ml of 0.1 M sodium hydroxide and then the volume is made up to 100 ml in a volumetric flask with the same solvent and kept in the dark at 5°C in plastic container, this solution is stable for one week. Working standard solutions were prepared by suitable dilution of the stock standard solution.

2.1.2. N-bromosuccinimide.

A stock solution of 0.01 M NBS (Fluka) [1-Bromo-2,5-pyrrolidinedione], ($C_4H_4BrNO_2$, M.Wt. 177.99 g /mole) was freshly prepared by dissolving 0.445 g of NBS in a least amount of warm water in a 250 mL measuring flask and then completed with distilled water to the mark. More dilute solutions were prepared by simple dilution with distilled water.

2.1.3 Methyl orange.

A stock solution of 1.52 X 10^{-3} M methyl orange (E. Merck) [4-dimethylaminoazobenzene-40-sulfonic acid sodium salt], ($C_{14}H_{14}N_3NaO_3S$, M.Wt. 327.33 g /mole) was prepared by dissolving 50 mg of dye (99% purity) in distilled water and diluting to 100 mL in a volumetric flask with distilled water. More dilute solutions were prepared by simple dilution with distilled water.

2.1.4. Hydrochloric acid.

A 1.0 M of HCl was prepared by diluting 8.33 ml of concentrated acid (Merck, Darmstadt, Germany, sp.gr. 1.18%, 37%) to 100 mL with distilled water. More dilute solutions were prepared by simple dilution with distilled water.

2.1.5. Solutions of interferences.

A stock standard solution of each interfering species (sodium chloride, glucose, sucrose, fructose, lactose, galactose, and starch) was prepared by dissolving 0.1 g of the compound in distilled water then the volume is completed to 100 ml in calibrated flask. Other solutions were prepared by serial dilutions of the stock solution.

2.2. Preparation of tablets solutions.

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 mole/l sodium hydroxide. 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.[21]Other solutions were prepared by serial dilutions of the stock solution.

2.3. Instrumentation

All spectral and absorbance measurements were performed on a (Cecil CE3021-England) UV-VIS spectrophotometer was used for all spectral and absorbance measurements with matched 1 cm quartz cells. The FI system comprised a multi-channel peristaltic pump (DESAGA Heidelberg-England) provided with silicon pump tubes of (0.8 mm i.d.). A 3-way loop injection valve with various sample loops (Rheodyne, Altex 210, Supelco-USA), and Jenway UV-VIS 6305 spectrophotometer (Tokyo, Japan) as the detector with 100 µl and 1.0 cm path length quartz flow cell was used in the flow of the FI system. Flexible Teflon tubes of 0.5 mmi.d. were used for reaction coils and to transport reagents solutions. T-link was also used to mix two streams of reagents. Two- Channel recorder (LKB-BROMMA 2210) was connected with the spectrophotometer.

2.4. Procedures

2.4.1. Recommended batch procedure.

In to a series of 10 ml volumetric flasks an increasing volume of FA solution (100 µg/ml) were transferred to cover the range of the calibration graph (0.5 - 6.0 µg/ ml). Then 0.6 ml of HCl (0.5 M) and 0.8 ml of NBS (5.6 X 10^{-4} M) were added. The solutions were lifted for 15 min at room temperature (25˚C), finally adding 1.0 ml of MO (5 X 10^{-4} M) then dilution to the mark with distilled water. The absorbance was measured after 5 min at 509 nm versus the reagent blank, prepared in the same manner but containing no drug.

2.4.2. Recommended FI procedure.

A volume of 100 µl of prepared sample solution (FA) was loaded into the sample loop by means of a syringe. Sample was injected into a 0.2 M HCl carrier stream pumped at a rate of 0.70 ml/min. The NBS solution (3 X10^{-4} M) was added to the carrier stream at a rate of 0.70 ml/min in a confluence manner downstream to ensure rapid and adequate mixing. After that, the M.O solution (3.5 X10^{-4} M) was added to a stream containing the unreacted NBS at a rate of 0.70 ml/min. After injection, the valve was returned to the load position when the maximum change in absorbance value had been reached. The absorbance was monitored at 509 nm in a quartz flow cell, at which the maximum absorption occurred and connected to a recorder, at 0.5 mV and with a chart speed of 30 cm/h. FI system is shown in Figure9.

3. Results and discussion

NBS has been used as an analytical reagent for many organic compounds[16].NBS has beenused as an oxidizing agent for several spectrophotometric andchemiluminescence reactions[17].In the present work, it wasfound that NBS can oxidizeFAin an acidic medium. In addition, it reacts immediatelywith M.O in an acidic medium to bleach outits red color. Therefore, after the oxidation of the drug underinvestigation by NBS, the excess NBS was reacted with the M.O (Scheme1). Theabsorption spectrum of the M.Owhich has maximumabsorption at 509 nm shown in Figure2. Therefore, the different parametersaffecting the oxidation reaction, and hence the subsequentdetermination of these drugs were optimized.

FA+ NBS (excess) → FA (oxidized product) +NBS (unreacted)

NBS (unreacted) + MO → Oxidized product of MO + Unreacted MO (indicator bleaching)

↓

(Measured spectrophotometrically at λ_{max}=509 nm)

Scheme 1 Reactions of indirect determination of folic acid by oxidation with N-bromosuccinamid

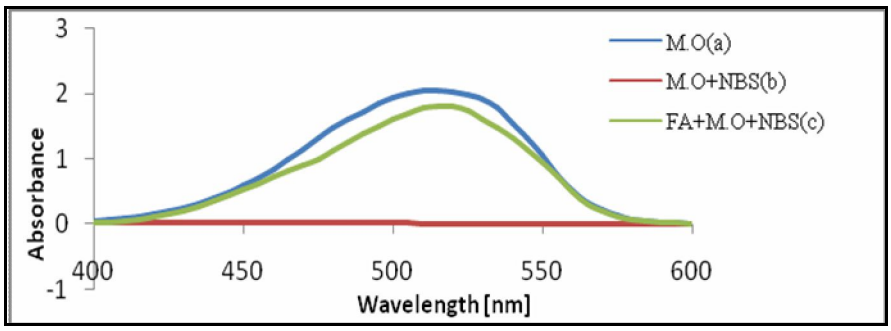

Figure 2 Absorption spectra of solution containing 0.5 M HCl and (a) 5 X 10^{-4} M MO; (b) 5.6 X 10^{-4} M NBS + a; and (c) 50 µg/10 mlFA+ b.

3.1. Optimization of variables

3.1.1. For spectrophotometric method

3.1.1.1. The chosen of dye and concentration

The preliminary experiments were performed to optimize the useful and optimum concentration of dye (methyl orange, phenol redand crystal violate) that can be determined spectrophotometrically.The results indicated that methyl orange was found to be a useful reagent for thereaction. Then selection of methyl orange concentration was studied, which indicated that 1.0 ml of methyl orange dye was the best volume, it gave stablehighest intensity.

3.1.1.2. The effect of oxidant reagents

N-bromosuccinamid was found to be a useful oxidizing agent, other oxidizing agents such as ($K_3Fe(CN)_6$,KIO_3 and NBS) have also been tested, but none offered real advantages over NBS as inFigure3. Also the effect of different volumes (0.2 to 2) ml of 5.6 X 10^{-4} M of NBS on the color of the dye was studied the results indicated that0.8 ml of NBS solution was enough to obtain a maximum bleaching of the color of methyl orang dye therefor it was recommended in the subsequent experiments.

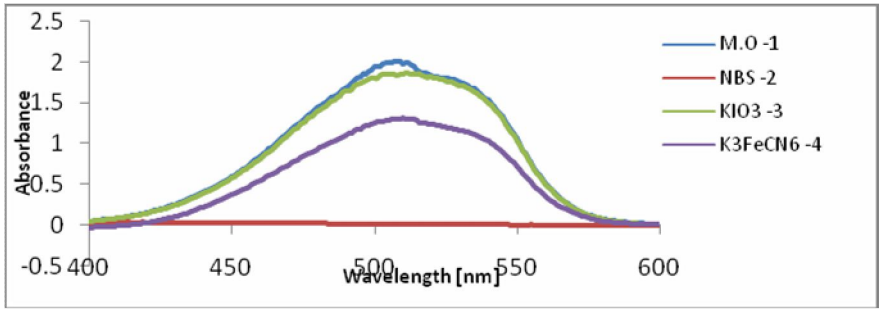

Figure 3 The effect of oxidant on bleaching the dye when number 1- is methyl orange dye MO), 2- is MO with NBS, 3- is MO with potassium iodate and 4- is MO with potassium hexacyanoferrate (III).

3.1.1.3.Nature and amount of acid

The reactions were tested in HCl, H_2SO_4, HNO_3, and CH_3COOH solutions. The results indicated that the reaction is suitable in hydrochloric acid medium. A 0.5 M HCl was found to be adequate for the oxidation of the drugs. The variation in HCl amount from 0.2 to 1.0 ml has been studied. The maximum absorbance was achieved when the HCl was 0.6 ml of 0.5 M, which was used in all subsequent experiments.

3.1.1.4. Effect of temperature.

The effect of temperature on the color intensity of methylorange color studied. In practice a maximum absorbance was obtained when the color was developed at room temperature (25˚C), decrease in color intensity

and stability was observed in low or high temperature, therefor room temperature is recommended for subsequent experiments.

3.1.1.5. Order of addition.

To obtain optimum results the order of addition of components should be studied.The results indicated that the order of addition of reagents should be followed as given by the procedure, otherwise, a loss in color intensity and stability are observed.

3.1.1.6. Effect of time on oxidation.

It was observed that if methyl orange was added immediately to the solution containingFA and NBS in acidic medium the resulted solution is bleached rapidly and the absorbance is very low. This can be explained by the fact that the drug oxidation by NBS is a time developing reaction and thus the influence of the reaction time was studied. In this respect, solutions containing 1.0 ml of 50 µg/10FA, 0.6 ml of 0.5M HCl and 0.8ml of 5.6 X 10^{-4} M NBS have been let to react at darkness in different times before adding the indicator and measuring the absorbance at 509nm. The results indicated that 15 minute was the optimum time to give complete oxidation of FA and 5 minutes was chosen as standing time to bleaching of the color of the dye MO, the color was remains constant for another 55 minutes.

3.1.2.Optimization of FI method

3.1.2.1.Optimization of chemical parameters

Batch method for the determination of FA was adopted as a basis to develop FI procedure. The manifold used for the determination of folic acid is shown in Figure9. The parameters of flow in thedetermination of FA were optimized. According to the results of preliminary spectrophotometric studies concerning the effect of acidic medium on the absorbance of the product, hydrochloric acid was used for the FI method.

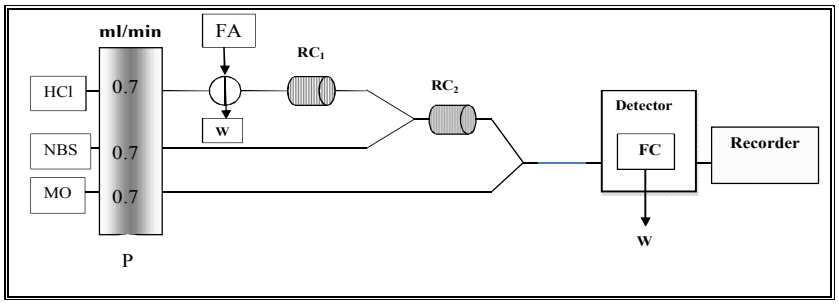

Figure 9 FIA manifold for the determination of folic acid in pharmaceutical preparations. P, peristaltic pump; RC_1, reaction coil 1 (90 cm); RC_2, reaction coil 2 (70 cm); W, waste; FC, flow cell.

3.1.2.1.1. The effect of HCl concentration

The effect of the concentration of hydrochloric acid was studied in the range (1to 10)X10^{-1} M with fixed FA concentration of (20 µg/10 ml). A gradual increase in the analytical signal was observed with increasing the HCl concentration (Figure4). Therefore, 0.2 M HCl was used as a carrier as it gives reasonable sensitivity and baseline stability.

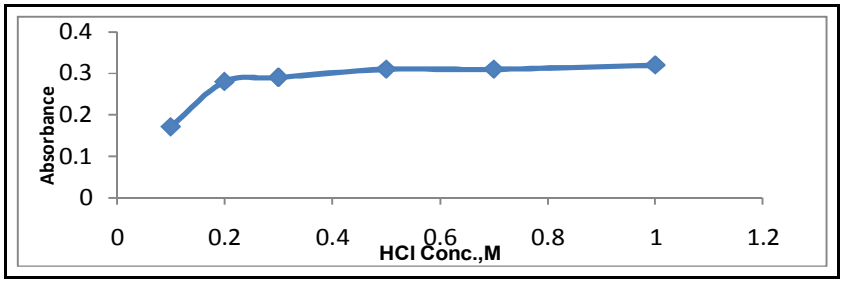

Figure 4 Effect of HCl concentration by FIA method.

3.1.2.1.2. The effect of NBS concentration

The influence of the NBS concentration on the absorbance was studied at constant concentrations of FA. Fixed volumes (100 µl) of FA was used and injected into the HClstream. The effect of changing the concentration of NBS in the range ($0.5X10^{-4}$to $6X10^{-4}$) M on the absorption peak height was shown in Figure5. The figure shows that, a maximum analytical signal was achieved when the NBS concentration reached to 3 $X10^{-4}$ M,and was chosen for further use.

3.1.2.1.3. The effect of methyl orange concentration

The effect of different concentrations of methyl orange in the range ($0.5\ X10^{-4}$to $6\ X10^{-4}$) M on the absorption peak height was shown in Figure5. The figure shows that, a maximum analytical signal was achieved when the M.O concentration reached to $3.5\ X10^{-4}$, and was chosen for further use.

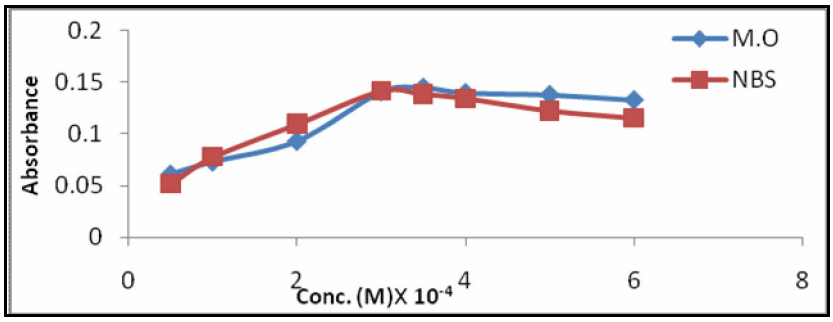

Figure 5 Effect of NBS and M.O concentration by FIA method.

3.1.2.2.Optimization of physical parameters

3.1.2.2.1. Effect of flow rate

Flow-rates of the carrier stream and the reagent streams were studied over the range 0.5 to 8.0 ml/min under the same experimental conditions as mentioned above. The peak height increased nonlinearly when the flow-rates were increased (Figure6). The maximum peak height was obtained at 0.7 ml/min for each channel (i.e., the total flow rate for three channels was2.1 ml/min). Above 2.1 ml/min, the peak heights again decreased, which may have been due to an insufficient reactions time. A flow-rate of 0.7 ml/min for each channel was chosen as a compromise between the peak shape, the sensitivity and the sampling time.

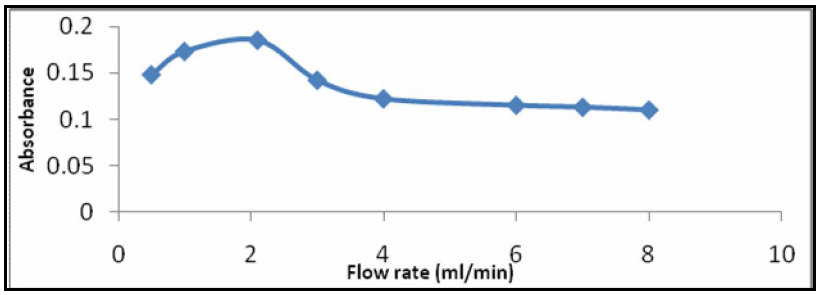

Figure 6 Effect of the flow rate (ml/min).

3.1.2.2.2.Effect of reaction coil length

The coil length which is an essential parameter that affected on the sensitivity of the color reaction product was investigated. Two coils were used in the manifold, as shown in Figure9. The first coil (RC_1), in which the oxidation ofthe drug by NBS took place, was changed over the range 50 to 200 cm. A considerable increase in the analytical signal wasobserved upon increasing the coil length up to 90 cm, andstarted to decrease after that (Figure7).This indicates that thereaction between the drug under investigation and NBS wasfast, and a further increase in the reaction coil length resulted insignificant peak broadening and a longer time to go back to thebaseline. Therefore, RC_1 was chosen to be 90 cm to ensurehigh sensitivity and a high measurement rate.Similarly, a significant change in the analytical signal wasobserved when RC_2 was increased from 50 to 200 cm, as shownin Figure7. A gradual increase in the peak height was observedwhen the length of RC_2

increased from 50 to 70 cm anddecreased after that. The maximum change in peak height wasobserved when the coil length was 70 cm. Therefore, a 70 cmreaction coil was chosen.

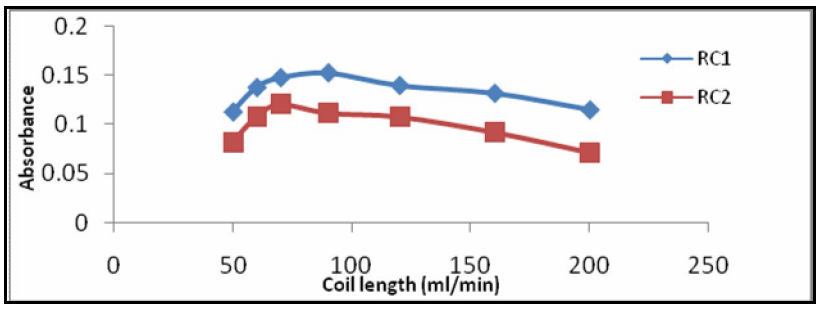

Figure 7 Effect of the coil length (ml/min).

3.1.2.2.3. Effect of the volume of injected sample

The effect of the sample volume on the peak height was investigated by injecting different volumes using 25to 160 µl. As expected, an increase in the volume of injected sample solution led to an increase in the peak height, then it been constant, as shown in Figure8. Consequently, the sensitivity of measurement could be improved by using 100 µl of sample volume.

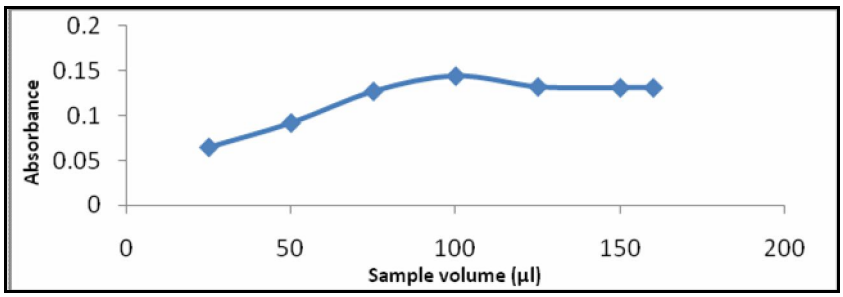

Figure 8 Effect of the sample volume.

3.2. 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 Table1.The limits of detection (LOD) and quantitation (LOQ) were calculatedaccording to the International Union of Pure and Applied Chemistry (IUPAC) definition[18]using the formula:

LOD = 3S/b andLOQ = 10S/b

Where: S is the standard deviation of blank absorbancevalues and b is the slope of the calibration plot, are also presented in Table 1.The high values of molar absorptivity and low values of Sandell's sensitivity and LOD indicate the high sensitivity of the proposed methods.

Table1 Analytical characteristics of the proposed method for folic acid.

Parameter	**Batch method**	**FI- method**
Beer's law range (µg / ml)	0.5-6.0	1.0-8.0
Detection limits (µg / ml)	0.018	0.2
Quantitation limit (µg/ml)	0.06	0.66
Molar absorptivity (l /mole. cm)	7.283×10^5	3.266×10^4
Sandell's sensitivity (µg /cm^2)	0.006	0.013
Regression equation (Y = a + bC)*		
Intercept (a)	0.020	0.0167
Slope (b)	0.165	0.0074
Determination coefficient (R^2)	0.9950	0.9968

Relative standard deviation, %	0.15	0.78
Relative error, %	-0.25	-1.54

* In Y = a + b C, Y is absorbance and C is concentration.

3.3. Interference studies

In order to assess the possible analytical applications of the proposed analytical method described to the assay of commercial FA formulations, the effect of some excipients frequently present in the 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 with concentration (10 µg/ ml) which having the recovery% by batch and FI- spectrophotometric methods thatranged from 98.5 to 100.0% and 98.0 to 100.0%, respectively for three replicates.

3.4. Application to analysis of tablets

The proposed batch and FIA spectrophotometric methods were successfully applied to the determination of FA in different brands of tablets. The results were summarized in Table 2. In comparison of the batch and the FIA procedure with standard method, HPLC method[19]using t- and F-statistical tests[20], the later method is more convenient than the former method because of its speed (sample through-put of 20 injection. h^{-1}), wider linear range of calibration graph, and good recovery were obtained. Also the results indicated 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 2 Application of the proposed method for the determination of folic acid in different brands of tablets.

Tablet FA sample	**The proposed method**						
	Label value tablet / (mg)	**Obtained value batch method (mg)**	**Recovery ± SD,%*, t ** and F-value *****	**Obtained value FI method (mg)**	**Recovery ± SD,%*, t ** and F-value *****	**Official BP method**	**Recovery ± SD,% ***
Awame dica Folic Awa– Erbil, Iraq.	5.0	4.92	98.4±0.76 t=1.98 F=0.67	4.78	95.6± 0.66 t=1.82 F=8.56	4.82	96.4±1.14
Actavis Folic acid- Barnsta ple,UK	5.0	4.90	98.0±0.92 t=1.96 F=1.47	4.89	97.8±0.79 t=2.00 F=1.47	5.01	100.2± 0.32
JulpharF olicum- U.A.E.	5.0	4.79	95.8±0.98 t=1.96 F=0.68	4.80	96.0±0.81 t=1.79 F=4.22	4.85	97.0±0.46

***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.**

4. Conclusion

Two simple, accurate and sensitive batch and FI- spectrophotometric methods have been developed for determination of FA in pharmaceutical preparations. The developed procedures based on addition of a measured excess of NBS in acid medium followed by determination of unreacted NBS by reacting with a fixed amount of methyl orange and measuring the absorbance at 509 nm. The proposed methods needs neither temperature nor pH controland nor long time for the reaction to complete. The methods were successfully applied in different brands of tablets.

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