# NEW METHOD FOR THE SIMULTANEOUS ESTIMATION OF METHOTREXATE AND FOLIC ACID BY USING RP-HPLC IN BULK AND PHARMACEUTICAL DOSAGE FORM

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## *Abstract*

*The aim of this study is to create and validate a fast, easy-to-use, affordable, sensitive, and accurate method for measuring Methotrexate and Folic acid in bulk and pharmaceutical products using Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). A inerstsil ODS column was used with a running phase composed of 0.1% formic acid: methanol (60:40 v/v) at a flow rate of 1.0 ml/min. UV detection was used at a wavelength of 270 nm. Folic acid and Methotrexate correlation coefficients were found to be 0.999 over a concentration range of 2.5-15 µg/ml and 18.75-112.5 µg/ml, respectively. Methotrexate and Folic acid have respective retention times of 2.709 and 7.337 minutes. The run time for separating Folic acid and Methotrexate peaks was 10 minutes. This method, proposed as a regular analysis and quality control tool for medications that contain these active drugs either individually or in combinatio, was evident to be a suitable one.*

***Keywords:*** *HPLC, Folic acid, Methotrexate, Development, Validation.*

## 1. Introduction

Methotrexate (MTX), formerly known as amethopterin, is a chemotherapy agent and immune-system suppressant[1]. It is used to treat cancer, autoimmune diseases, and abortion of ectopic pregnancies[2]. Types of cancers it is used for include breast cancer, leukemia, lung cancer, lymphoma, gestational trophoblastic disease[3], and osteosarcoma.[4] Types of autoimmune diseases it is used for include psoriasis, rheumatoid arthritis, and Crohn's disease[5]. Common side effects include nausea, feeling tired, fever, increased risk of infection, low white blood cell counts, and breakdown of the skin inside the mouth. Other side effects may include liver disease, lung disease, lymphoma[6], and severe skin rashes. People on long-term treatment should be regularly checked for side effects. It is not safe during breastfeeding. In those with kidney problems, lower doses may be needed. It acts by blocking the body's use of folic acid.

Folate, also known as vitamin $B_9$ and folacin, is one of the B vitamins. Manufactured folic acid, which is converted into folate by the body, is used as a dietary supplement and in food fortification as it is more stable during processing and storage.[7] Folate is required for the body to make DNA and RNA and metabolise amino acids necessary for cell division. As humans cannot make folate, it is required in the diet, making it an essential nutrient. It occurs naturally in many foods. Folate in the form of folic acid is used to treat anemia[8] caused by folate deficiency. Folic acid is also used as a supplement by women during pregnancy to reduce the risk of neural tube

defects (NTDs)[9] in the baby [10]. Low levels in early pregnancy are believed to be the cause of more than half of babies born with NTDs. Long-term supplementation with relatively large amounts of folic acid is associated with a small reduction in the risk of stroke and an increased risk of prostate cancer[11-12]. There are concerns that large amounts of supplemental folic acid can hide vitamin $B_{12}$ deficiency. Not consuming enough folate can lead to folate deficiency. This may result in a type of anemia in which red blood cells become abnormally large. Symptoms may include feeling tired, heart palpitations[13], shortness of breath[14], open sores on the tongue, and changes in the color of the skin or hair. Folate deficiency in children may develop within a month of poor dietary intake. Simultaneous determination of Methotrexate and Folic acid using HPLC was found in the current study.

## 2. Experimental Study

### Solutions and Reagents

The pure Folic acid and Methotrexate used in this study was provided by Glenmark Pharmaceutical Private Ltd., located in Andheri (E), Mumbai, India (99.8-99.9 percent purity). Other reagents, including acetonitrile, formic acid, and water, were obtained from Merck (India) Ltd. in Worli, Mumbai, India, and were of HPLC grade.

### Collection of instruments

Waters alliance liquid chromatography (model 2695) monitored with empower 2.0 data handling system and a detector of photo diode array (model 2998) was used for this study.

### Buffers are chosen

0.1 percent formic acid.

### Step of mobility

For Standard review, the mobile step was 0.1% formic acid buffer in a 60:40 (v/v) methanol mixture and was degassed beforehand. A mobile phase chosen to produce well-defined peaks with a low tailing factor (2.0) and a plate count of over 2000 was selected.

### Prepare the diluent

The diluent's mobile process was set up.

### Conditions of Chromatography

For the HPLC experiments, an inertsil ODS column (150 x 4.6 mm, 3.5 µm) was used. The elution was conducted with isocratic conditions using methanol: formic acid (0.1% volume) (40:60 by volume) at a flow rate of 1.0 ml/min. The injection volume was 10 µl, and the run time was 10 minutes, with the column temperature set to room temperature and the absorbance measured at 270 nm (Because maximum absorbance was observed at this wavelength. So, this was selected as wavelength).

### Standard Solution Preparation

To dilute 10 mg of Folic acid and 75 mg of Methotrexate, measure out the drug and transfer it to a 100 mL volumetric flask, then add approximately 70 mL of a diluent and sonicate for 30 minutes to dissolve it, after that add more diluent to the total amount. Further dilute 5 mL to 50 mL with diluents.

### Sample Solution Preparation

Measure out 81 mg of sample and transfer it to a 100 mL volumetric flask, then add approximately 70 mL of a diluent and sonicate for 30 minutes to dissolve it, after that add more diluent to the total amount.

### Validation Process[15-23]

### System Precision

The system's performance has been validated through assessment of device suitability parameters. Limits were found to be met for a variety of parameters, including plate count, tailing, and RSD percentage.

**Specificity**

Being able to identify and test a given analyte in the presence of other elements required to be combined in the Standard and the standard solution is known as specificity. Blank Standards and those with Folic acid and Methotrexate will be tested using chromatograms.

**Accuracy**

Being close to the real meaning of the technique is what defines accuracy. Three concentrations will be used to test the recovery trials. The drug's quantity, percentage of recovery, and standard deviations were calculated after every injection at each level.

**Precision**

It is the level of agreement between the various test results that determines the precision of the analytical methodology. Researchers examined the effects of sampling a homogeneous population more than once. The current process was evaluated in terms of its ability to provide repeatable, intraday, and inter-day results. It was examined by sampling the materials on the same day and over the course of different days.

**Linearity**

Linearity is the feature of analytical process which allows for a direct proportion of analytical results in response to a certain concentration of the analyte in the Standard. A total of seven series of standard solutions were selected for the assessment of the linearity spectrum. The calibration curve was drawn by comparing regular solution concentration with peak area. Using the least square method, the slope, intercept, and coefficient of correlation were calculated.

**Forced Degradation**

The peaks in the chromatogram should agree. ICH guidance Q1 (A) R2 was performed in conjunction with stress degradation experiments. The peaks of degradation should be well distanced and at least 1.0 resolution between peaks. For the largest peaks to go over, a separation must occur. A degradation of around 20 percent has been attained via several various stress conditions like acid, alkali, peroxide, reduction, thermal and photo in what is known as a forced degradation experiment.

**Robustness**

Robustness refers to a procedure's resistance to small process parameter changes, as well as its reliability in normal operation. An organic solution was introduced into the HPLC system for a robustness analysis, and the chromatographic settings (such as flow rate and mobile-phase organic content) were modified. The separation factor, retention time, and peak asymmetry were determined by evaluating the effects of altered parameters.

## 3. Results and Discussion

The aim of this study is to establish a single isocratic HPLC method for the simultaneous quantification of Folic acid and Methotrexate in bulk and pharmaceutical dosage forms that is reliable, precise, and cost effective. According to the UV spectra of these compounds, an appropriate wavelength for simultaneous estimation of two drugs was chosen.

**Optimization of the method**

Using buffers (0.1% orthophosphoric acid, 0.1% formic acid, 0.1% triethylamine) and acetonitrile, methanol as mobile phase different trials were conducted in isocratic and gradient modes. Various stationary phases including phenyl, biphenyl, amino, C4, and C8, were used to test the system. The resolution and retention times were improved by changing the mobile step composition at each trial. In the end, the separation was achieved using an inertsil ODS column (250mm x 4.6mm, 5µm) and a mobile phase of 0.1% formic acid : methanol (60:40 v/v) with a flow rate of 1.0 ml/min and UV detection

at a wavelength of 270 nm. The entire performance lasted ten minutes. Conditions for optimized chromatography are provided in table 1.

**System Suitability**

To attain results, the following device suitability parameters were established after six consecutive injections of normal solution: theoretical plate number, time, peak area, tailing factor, and resolution. The chromatogram in Figure 1 was the representative of the suitability results detailed in table 2.

**Table 1. Method suitability conditions**

| Parameter | Suitable conditions |
|---|---|
| Column | Inertsil ODS (250 x 4.6 mm, 5 µ) |
| Moving Phase | 0.1% formic acid: Acetonitrile (60:40 v/v) |
| Volume of injection | 10 µl |
| Stream rate | 1.0 mL/min |
| Temperature of column | 25°C |
| Wavelength | 270 nm |
| Time duration | 10 minutes |
| Retention time of Folic acid | 7.337 min |
| Retention time of Methotrexate | 2.709 min |

**Table 2. Results of system suitability**

| Parameter | Methotrexate | Folic acid |
|---|---|---|
| Number of plates | 5847 | 3652 |
| Tailing | 1.02 | 0.98 |
| Resolution | | 19.45 |
| Peak elution time | 2.709 | 7.337 |
| % RSD | 0.45 | 0.29 |

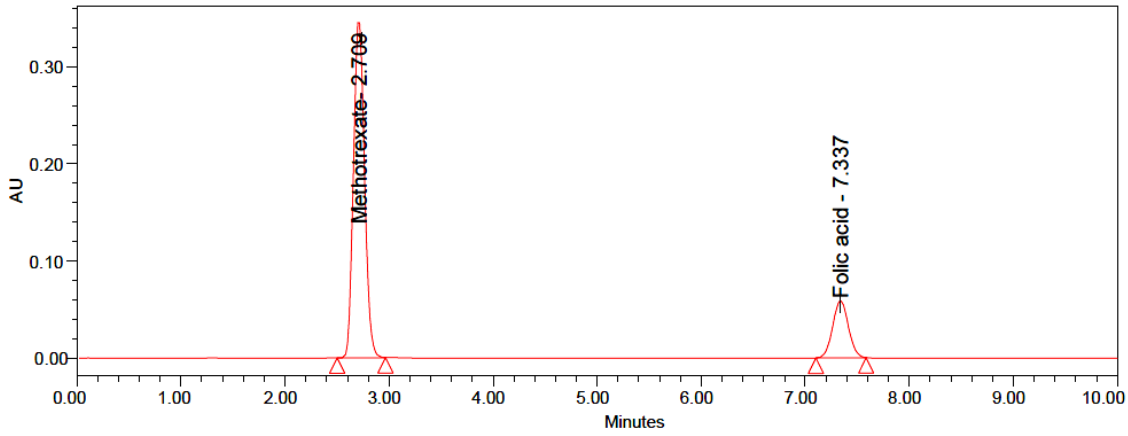


**Figure 1. Chromatogram of standard**

**Specificity**

There was no participation from Folic acid and Methotrexate at the elution time. As seen in Figure 2, the blank chromatogram is present.

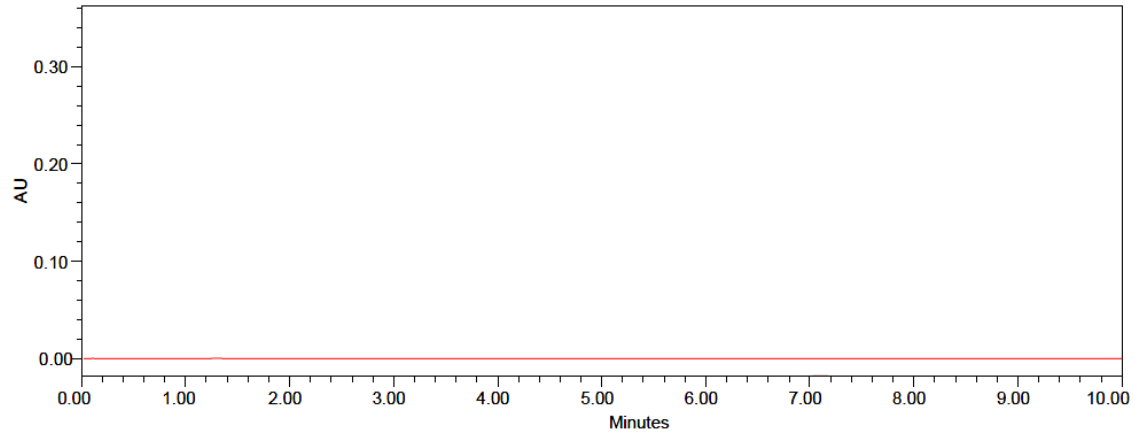


**Figure 2. Chromatogram of blank**

## Linearity

By using a calibration curve to determine the linearity of the area of peak, its corresponding concentration was discovered. From this graph, it appears that the range of 18.75-112.5 µg/mL of Methotrexate and 5-30 µg/mL of Folic acid had a straight line. Linearity results were demonstrated in table 3.

**Table 3. Results of linearity**

| S. No | Methotrexate | | Folic acid | |
|---|---|---|---|---|
| | Concentration (µg/mL) | Area | Concentration (µg/mL) | Area |
| 1 | 18.75 | 896531 | 2.50 | 152478 |
| 2 | 37.50 | 1726354 | 5.00 | 345821 |
| 3 | 56.25 | 2574581 | 7.50 | 502302 |
| 4 | 75.00 | 3458214 | 10.00 | 658945 |
| 5 | 93.75 | 4263215 | 12.50 | 806542 |
| 6 | 112.50 | 5102563 | 15.00 | 981523 |

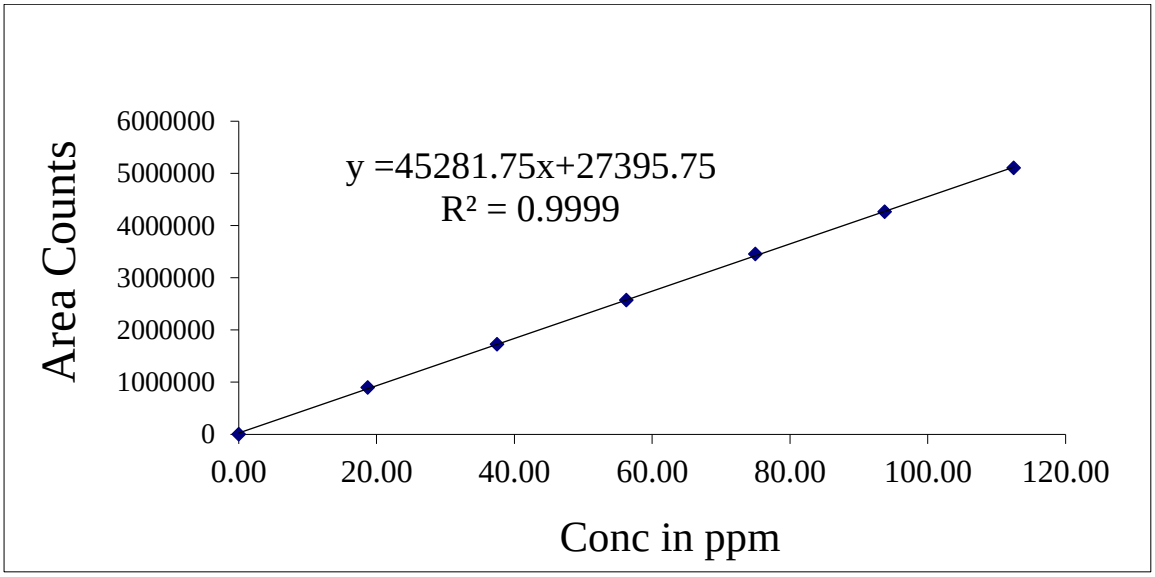


**Figure 3. Calibration plot of Methotrexate**

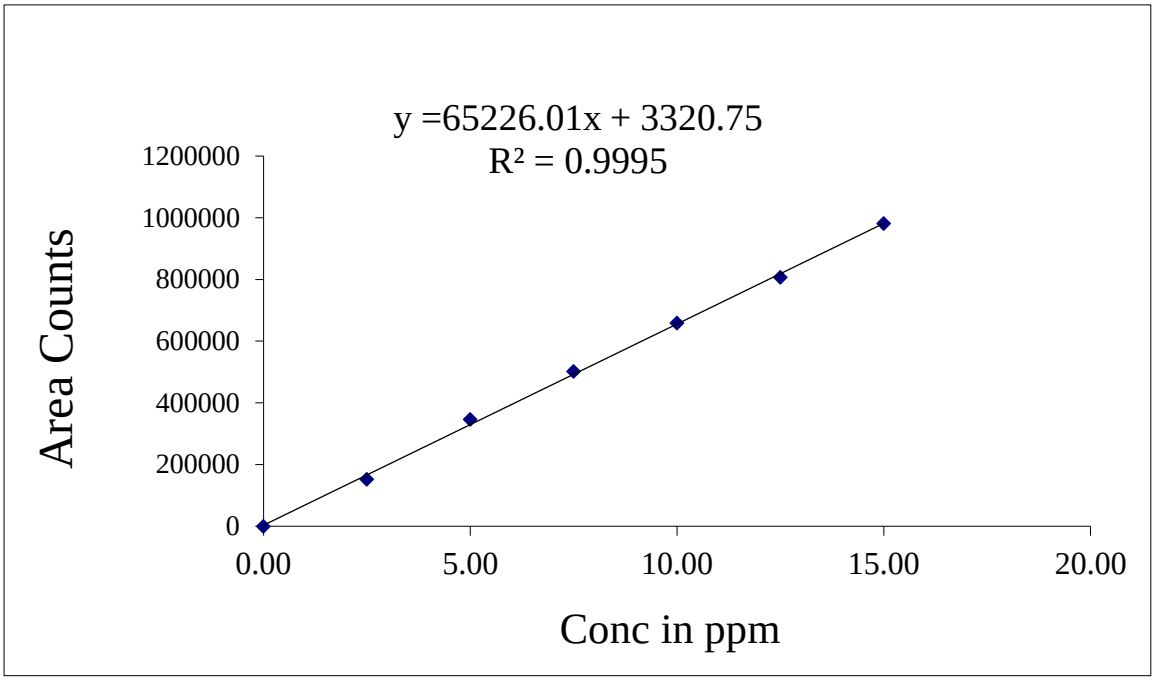


**Figure 4. Calibration plot of Folic acid**

**Precision**

Intraday and intermediate precision variances were assessed in relation to the procedure's accuracy. The samples were examined six times on the same day to obtain intraday results for Folic acid and Methotrexate. The system's intermediate precision was explored by analyzing data in the same laboratory using a variety of examiners and tools. It is very accurate, with an RSD percentage of less than 2%. The process was precise, yielding the best drug recoveries at each additional concentration. Table 4 shows the method precision results.

**Table 4. Outcomes of method precision**

| S. No. | Methotrexate | | Folic acid | |
|---|---|---|---|---|
| | Area | % Assay | Area | % Assay |
| 1 | 3392891 | 98.5 | 651314 | 99.5 |
| 2 | 3453789 | 100.3 | 650341 | 99.4 |
| 3 | 3435810 | 99.7 | 659739 | 100.8 |
| 4 | 3414608 | 99.1 | 656352 | 100.3 |
| 5 | 3459396 | 100.4 | 652343 | 99.7 |
| 6 | 3441312 | 99.9 | 650561 | 99.4 |
| Mean | 3432968 | 99.7 | 653442 | 99.9 |
| Std. dev | 25111.38 | 0.731 | 3788.74 | 0.575 |
| % RSD | 0.731 | 0.73 | 0.58 | 0.58 |

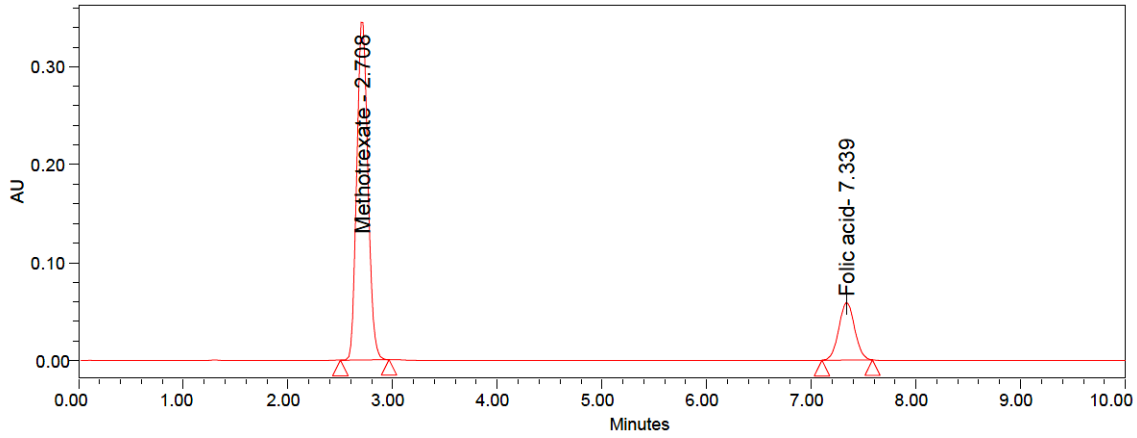


**Figure 5. Chromatogram of method precision**

## Intermediate Precision (Ruggedness)

Intermediate precision results were shown in table 5.

**Table 5. Results of intermediate precision**

| S.No. | Methotrexate | | Folic acid | |
|---|---|---|---|---|
| | Area | % Assay | Area | % Assay |
| 1 | 3452889 | 100.2 | 653320 | 99.8 |
| 2 | 3413794 | 99.1 | 651346 | 99.5 |
| 3 | 3435815 | 99.7 | 651747 | 99.6 |
| 4 | 3454611 | 100.3 | 655359 | 100.1 |
| 5 | 3439397 | 99.8 | 650540 | 99.4 |
| 6 | 3441324 | 99.9 | 656556 | 100.3 |
| Mean | 3439638 | 99.8 | 653145 | 99.8 |
| Std dev | 14732.33 | 0.427 | 2389.32 | 0.354 |
| % RSD | 0.428 | 0.43 | 0.366 | 0.35 |

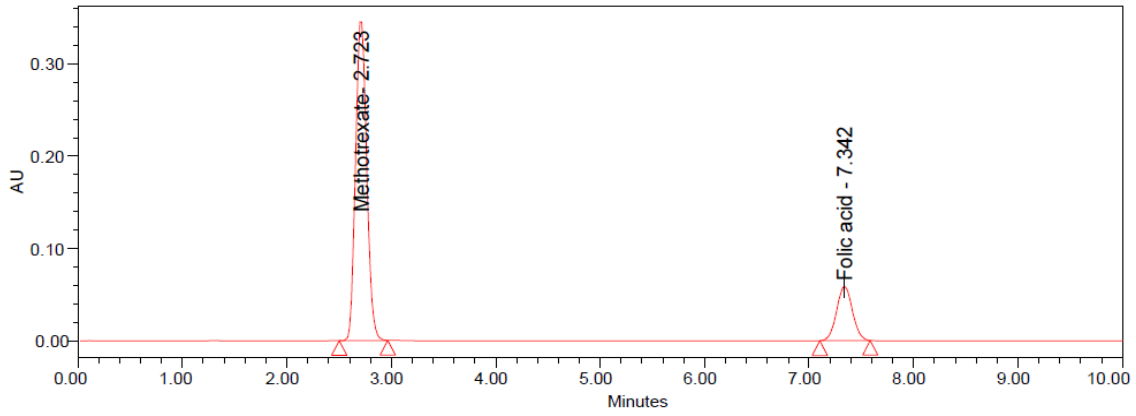


**Figure 6. Chromatogram of intermediate precision**

## Accuracy

By measuring the recovery experiments at three stages, the method's precision was reached (50 percent, 100 percent, and 150 percent). APIs were made with concentrations of Folic acid of 5, 10, and 15 micrograms/mL and Methotrexate of 37.5, 75 and 112.5 micrograms/mL. For each stage of the spike, the test solution was injected three times, and the assay was performed in accordance with the test process. In addition to being able to determine the percentage of recovered data, the mean and relative standard deviations have also been found. The strategy was effective because the recovery values fell within the target range. Table 6 presents the accuracy results.

**Table 6.Results of accuracy**

| Accuracy | Amount of Methotrexate | % Recovery | Amount of Folic acid | % Recovery |
|---|---|---|---|---|
| 50***** | 33.8 | 99.1 | 0.5 | 99.1 |
| 100* | 67.5 | 100.2 | 1 | 99.8 |
| 150* | 101.3 | 98.6 | 1.5 | 99.4 |

* Results are mean recovery of three sample preparations

## LOD and LOQ

The concentration level at which the analytes are reliably detected and quantified is the limit of detection and quantification. Methotrexate and Folic acid had a LOD concentrations of 0.225 µg/ml, 0.03 µg/ml and their S/N values of 3, 3. The LOQ concentrations of Folic acid and Methotrexate were 0.75 µg/ml, 0.1 µg/ml, and their S/N values were 10, 10. (S/N is the ratio of signal to noise).

## Robustness

To ensure the robustness of the chromatographic technique, the researchers evaluated flow rate and the composition of the mobile phase. By changing the flow rate and mobile phase ratio, the area of drugs changes. So, the percentage of relative standard deviation changes. Here in Table 7 (robustness results) the %RSD values are in within the acceptable limit.

**Table 7. Outcomes of robustness**

| Parameter | % RSD of Methotrexate | % RSD of Folic acid |
|---|---|---|
| Flow (0.8 mL/min) | 0.27 | 0.38 |
| Flow (1.2 mL/min) | 0.25 | 0.21 |
| Organic phase (44:56) | 0.26 | 0.24 |
| Organic phase (36:64) | 0.1 | 0.18 |

## Forced Degradation

The proposed approach can be used for successful evaluations of release and stability tests, and it can be called a stability preferable technique. Acid, Alkali, oxidation, reduction, photo, and thermal degradation are all included in the ICH-required forced degradation analysis. The chromatograms show that the selected drugs remained stable under the stress conditions, despite the presence of degraded peaks. Results of forced degradation were given in table 8.

## Acid degradation

A volume of 1 ml sample stock solution was transferred to a volumetric flask with a capacity of 10 ml, to which 1 ml of 1N HCl was added and left to stand for 15 minutes. After 15 min add 1 ml of 1N NaOH and make up to the diluent mark. Filter the solution using syringe filter and injected into HPLC system.

## Alkali degradation

A volume of 1 ml sample stock solution was transferred to a volumetric flask with a capacity of 10 ml, 1 ml of 1N NaOH was added, and the mixture was left to stand for 15 minutes. After 15 minutes, add 1 mL of 1N HCl to bring the solution up to the required concentration. Use a syringe filter to filter the solution, which will then be injected into the HPLC system.

## Peroxide degradation

A volume of 1 ml sample stock solution was moved to a volumetric flask of 10 ml, add 1 ml of 30% hydrogen peroxide solution and make up to the mark with diluents. Filter the solution using syringe filter and injected into HPLC system.

**Reduction degradation**

Using a volumetric flask with a capacity of 10 ml, transfer 1 ml of sample stock solution and add 1 ml of 30% hydrogen peroxide solution, then dilute to the required concentration with diluents. Use a syringe filter to filter the solution, which will then be injected into the HPLC system.

**Thermal degradation**

During the 6-hour baking period, the sample solution was kept at 105°C. The resulting solution was injected into an high-performance liquid chromatography system.

**Photolytic degradation**

A weight of 100mg sample was exposed to sunlight for 6 hrs. and the exposed sample was analyzed. Prepare the sample solution by using this sample and inject into HPLC system.

**Table 8. Forced degradation results**

| Stress Parameter (24 hrs) | % Degradation | |
|---|---|---|
| | Methotrexate | Folic acid |
| Acid degradation (1N HCl) | 13.54 | 14.0 |
| Alkali degradation (1N NaOH) | 12.9 | 13.8 |
| Peroxide degradation (30% Peroxide) | 12.8 | 14.6 |
| Reduction degradation (30% sodium bi sulphate) | 9.47 | 10.5 |
| Thermal (sample, 70°C, 6 Hrs) | 2.5 | 4.2 |
| Photo (UV-Vis light- (200 W h/$m^2$) and fluorescent light (1.2 milliion lux-h) | 2.1 | 3.5 |

## 4. Conclusion

In this study, a novel, quick, sensitive, and easy-to-use HPLC method was developed for the simultaneous estimation of Methotrexate and Folic acid in API and pharmaceutical dosage types. Because there are no HPLC methods published, this approach is the most practical option. Shorter run time, low cost, and all the other characteristics are benefits. All the parameters were verified and were found to be within the acceptable range, including linearity, accuracy, specificity, robustness, and process precision. According to our research, the RSD values for all the parameters came in at less than 2%, showing that the procedure is accurate and that the results we found are consistent. Therefore, it's possible to use the current approach in QC laboratories for routine study and manufacturing Folic acid and Methotrexate pharmaceuticals without having to separate the substances first.

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