A SIMULTANEOUS ESTIMATION, VALIDATION AND FORCED DEGRADATION STUDIES OF 5-FLUOROURACIL AND TEGAFUR IN A PHARMACEUTICAL DOSAGE FORM USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD

VAISHALI MISTRY*, AKSHAY YELWE, AMEY DESHPANDE

Department of Quality Assurance, Oriental College of Pharmacy, Navi Mumbai, Maharashtra, India. Email: vaishali.mistry@ocp.edu.in

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INTRODUCTION

5-Fluorouracil (Fig. 1) (5-fluoro-pyrimidine-2,4-dione) has been widely used in the chemotherapy of a variety of human carcinomas, including the head, neck, and gastrointestinal tract and breast using various schedules [1]. However, administration of the compound often caused severe gastrointestinal toxicity and myelosuppression. To overcome the toxicity, many derivatives and related compounds have been synthesized [4].

Tegafur (Fig. 2) [5-fluoro-1-oxolan-2-yl pyrimidine-2,4-dione] a 2-tetrahydrofuranyl derivative of 5-fluorouracil has been shown to have a broad spectrum of antitumor activity when administered intravenously or orally [1]. It acts as a prodrug of 5-fluorouracil and produces comparatively little myelosuppression [2,5].

Literature survey reveals HPLC for estimation 5-fluorouracil and tegafur is not available for pharmaceutical dosage form.

METHODS

5-fluorouracil and tegafur standard were provided by Yarrow Chem. Products, Dombivali, Mumbai, Maharashtra, India. Commercial tablet dosage form tegafur (5-fluorouracil 224 mg and Tegafur 100 mg) was purchased from local markets. The HPLC grade methanol and water were purchased from Thomas Fisher scientific Pvt. Ltd., Powai, Mumbai, Maharashtra, India. Hydrochloric acid, sodium hydroxide, and hydrogen peroxide were purchased from S.D. Fine chemicals.

HPLC instrument

The chromatographic separation was carried out by Shimadzu Prominence-i LC-2030 HPLC system equipped with UV detector and auto sampler. The lab solution software was used for signal monitoring and processing, UV chamber has been used for photolytic degradation. Hot air oven was used for thermal degradation.

Chromatographic conditions

The chromatographic separation of analytes was carried out using Shimadzu reversed-phase high-performance liquid chromatography (RP-HPLC) system with Shim-pack GIST C18 (250 × 4.6 mm) column. The mobile phase consists of water and methanol in the ratio 50:50 %v/v, and column temperature was maintained at 30°C the analytes were detected at 271 nm using UV detector. The runtime was set at 10 min at a flow rate of 1 ml/min.

Preparation of standard stock solution

Standard stock solution of 5-fluorouracil and tegafur was prepared separately by dissolving 22.4 mg of 5-fluorouracil and 10mg of tegafur in 100 ml of volumetric flask with water:methanol (50:50) as diluents and sonicated for 10 min. From the above solution, 1 ml of 5-fluorouracil and 1 ml of tegafur were transferred separately to 10 ml volumetric flask, made up the volume to 24 µg/ml and 10 µg/ml of stock solution of 5-fluorouracil and tegafur respectively.

Preparation of sample solution

Ten tablets (Tegafur capsules; 224 mg 5-fluorouracil and 10 mg tegafur) were weighed and the average weight of each tablet was calculated; then the weight equivalent to 10 tablets was transferred into 100 ml volumetric flask; 50 ml of diluents were added and sonicated for 30 min; further the volume made up with diluents and filtered. From the filtered solution, 1 ml was pipetted out into 10 ml volumetric flask and sonicated for 10 min, and volume made up to 10 ml with diluents.

Forced degradation studies

Forced degradation studies were carried out in the presence of acid, alkali, H₂O₂, and heat. With the sample bearing concentration 22.4 µg/ml and 10 µg/ml of 5-fluorouracil and tegafur, respectively, these studies help to know the inherent stability characteristic of the active molecule in drug product and possible degradation products [6,7].
Acid degradation was carried out by adding 5 ml of 1N HCl and after 60 min neutralizing the mixture by adding 5 ml of 1N NaOH.

Alkali degradation was carried out by adding 5 ml of 1N NaOH and after 60 min neutralizing the mixture by adding 5 ml of 1N HCl.

Oxidative degradation was performed by exposing the drug to 5 ml of 10% (v/v) H2O2 for 60 min.

Photolytic degradation was carried out by exposing the drug content to UV light inside a UV chamber for 1d.

Thermal degradation was performed by placing the drug in an oven at 105°C for 24 h to study dry heat degradation.

Statistical analysis
To evaluate the contribution of each factor with different levels of responses, two-way analysis of variance was performed using GraphPad Prism 7.04 Software.

RESULTS AND DISCUSSION

Method development
A number of trials were conducted with different columns, with various combination mobile phases to develop a suitable RP-HPLC method for estimation of 5-fluorouracil and tegafur in tablet dosage form. Then, finally a typical chromatogram was obtained with water and methanol in the ratio 50:50% v/v. The chromatographic separation was performed on SHIM-PACK C-18 (250 × 4.6 mm, 5 µ) column on injecting 20 µL, and the analysis were detected with UV detector at 271 nm. The retention time of 5-fluorouracil and tegafur was found to be 2.74 min and 3.66 min respectively. The force degradation study was also carried out using the developed method. The optimized conditions were given in Table 1.

Method validation
The validation was performed with an above developed RP-HPLC method for simultaneous estimation of 5-fluorouracil and tegafur according to ICH guidelines. Various parameters were evaluated such as system suitability, precision, accuracy, linearity, robustness, limit of detection (LOD), and limit of quantification (LOQ).

System suitability
System suitability was performed to verify the acceptability of the resolution and repeatability of the system. System suitability was
performed by injecting six replicate injections of the standard solution (100%) and parameters such as peak area, USP tailing, theoretical plates, retention time, and peak asymmetry were evaluated. The percentage RSD was determined and reported within limits (Table 2 and Fig. 3).

**Accuracy**

The accuracy of the proposed method was evaluated by calculating the recovery studies of the test drug at three different concentration levels (80%, 100%, and 120%) by the standard addition method. A known amount of 5-fluorouracil and tegafur was added to the pre quantified sample solution, and three replicates of each concentration were injected into developing chromatographic conditions. The mean percentage recovery of 5-fluorouracil and tegafur was varied between 99.5 and 100.4% indicating that the developed method was found to be accurate (Table 3).

**Precision**

The precision of an analytical procedure may be defined as the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The method precision and system precision studies were carried out by injecting six replicates of both standard and test solutions with the same concentration. The percentage RSD was calculated from the chromatograms and the results obtained were within the limits of 2%, and the proposed method was found to be precise (Table 4).

**Linearity**

The linearity of the method was determined at different concentration levels ranging from 13.44 μg/ml to 31.36 μg/ml of 5-fluorouracil and from 6 μg/ml to 14 μg/ml of tegafur. All the concentrations were prepared and injected into the system. The linearity curve was constructed by plotting peak area versus concentration of the analyte. From the results obtained that the proposed method was found to be linear. The regression coefficient was found to be 0.998 and 0.998 for 5-fluorouracil and tegafur, respectively (Figs. 4 and 5).

**LOD and LOQ**

In the present study, the LOD and LOQ 5-fluorouracil and tegafur were evaluated based on the standard calibration curve method. LOD is performed to know the lowest concentration level of the analysts that give a measurable response. LOD and LOQ for 5-fluorouracil are 10.97 μg/ml and 33.26 μg/ml and for tegafur are 4.89 μg/ml and 14.83 μg/ml, respectively.

**Robustness**

Robustness of the proposed method has been evaluated by small deliberate changes in the system parameters such as flow rate, inlet pressure, and column temperature. The method proved to be robust with RSD values of 0.05 for 5-fluorouracil and 0.05 for tegafur.

**Table 3: Percentage recovery results of 5-fluorouracil and tegafur**

| S. No | Spiked (%) | Percentage recovery | Mean percentage recovery | % RSD |
|-------|------------|---------------------|--------------------------|-------|
|       |            | 5-fluorouracil | Tegafur |                  | 5-fluorouracil | Tegafur |
| 1     | 80         | 99.56            | 99.67      | 99.66             | 0.05               | 0.05               |
|       |            | 99.61            | 99.71      |                   |                    |                    |
|       |            | 99.67            | 99.78      |                   |                    |                    |
| 2     | 100        | 99.86            | 99.84      | 99.90             | 0.06               | 0.04               |
|       |            | 99.92            | 99.92      |                   |                    |                    |
|       |            | 99.98            | 99.89      |                   |                    |                    |
| 3     | 120        | 99.98            | 100.26     | 100.35            | 0.49               | 0.24               |
|       |            | 100.46           | 99.98      |                   |                    |                    |
|       |            | 100.97           | 100.46     |                   |                    |                    |
It was found that none of the above parameters caused an alteration in the peak area, retention time, and USP tailing by small changes such as ±0.2 ml change in flow rate, ±2 nm wavelength, and ±2°C change in temperature. The percentage RSD was found to be within limits, and the method was found to be robust (Table 5).

**Assay of marketed formulation**

Analysis of the marketed formulation (tegafur capsule: 5-fluorouracil is 224 mg and tegafur is 100 mg) was purchased from the local pharma market. 10 capsules weighed and average weight were calculated; weight equivalent to 1 capsule was transferred into 100 ml volumetric flask, 50 ml of diluents was added and sonicated for 30 min, and further volume was made up with diluents and filtered. From filtered solution, 1 ml was a pipette out into 100 ml volumetric flask and made up to 100 ml with diluents. From the resulting solution, 20 µL was injected into HPLC system, and peak areas were recorded. The percentage assay of the marketed formulation was found to be 98.7 for 5-fluorouracil and 98.6 for tegafur (Table 6).
Forced degradation studies (Figs. 6-11)
ICH degradation was attempted to various stress conditions such as acid hydrolysis (using 1N HCl), base hydrolysis (using 1N NaOH), oxidative hydrolysis (using 5% H$_2$O$_2$), thermal degradation (heated at 100°C for 24 h), and photolytic degradation (using UV light inside a UV chamber for 48 h). The results of stress studies were shown in Table 7.

CONCLUSION
In the present study, a method has been developed using RP-HPLC and validated for simultaneous estimation with stability indication of 5-fluorouracil and tegafur in tablet dosage form. The validated method was successfully used for stress testing, analysis of 5-fluorouracil and tegafur. The proposed method was proved selective, accurate, precise, and rapid and it can be used for the routine analysis of 5-fluorouracil and tegafur in the formulation.

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AUTHORS’ CONTRIBUTIONS
We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this study.

Table 4: Results of method precision for 5-fluorouracil and tegafur

| S. No | Sample no | 5-fluorouracil | Tegafur |
|-------|-----------|---------------|---------|
|       |           | % Assay | % Assay |
| 1     | Injection 1 | 99.82   | 99.59   |
| 2     | Injection 2 | 99.83   | 99.60   |
| 3     | Injection 3 | 99.77   | 99.57   |
| 4     | Injection 4 | 99.88   | 99.61   |
| 5     | Injection 5 | 99.83   | 99.60   |
| 6     | Injection 6 | 99.85   | 99.61   |
| 7     | Average    | 99.83   | 99.60   |
| 8     | SD         | 0.038   | 0.015   |
| 9     | % RSD      | 0.038   | 0.015   |

Table 5: Results of robustness

| S. No | Parameters | 5-fluorouracil | Tegafur |
|-------|------------|---------------|---------|
|       |            | RT            | NTP     | TF | RT            | NTP     | TF |
| 1     | Flow rate 0.9 ml | 3.03   | 4138   | 1.21 | 4.04 | 4953   | 1.17 |
|       | Flow rate 1.1 ml | 2.50   | 3755   | 1.20 | 3.33 | 4568   | 1.15 |
| 2     | Temperature 28 | 2.75   | 3915   | 1.21 | 3.67 | 4755   | 1.16 |
|       | Temperature 32 | 2.74   | 3933   | 1.21 | 3.64 | 4739   | 1.16 |
| 3     | Wavelength 268 | 2.74   | 3948   | 1.21 | 3.65 | 4757   | 1.16 |
|       | Wavelength 273 | 2.74   | 3929   | 1.21 | 3.65 | 4754   | 1.16 |

RT: Retention time, NTP: Number of theoretical plate, TF: Tailing factor

Table 6: Percent content of marketed formulation

| S. No | Tablet                                      | Drug     | Amount taken (mg) | Amount found (mg) | % assay |
|-------|---------------------------------------------|----------|-------------------|-------------------|---------|
| 1     | Tegafi plus (5-fluorouracil 224 mg and tegafur 100 mg) | 5-fluorouracil | 224               | 223.61            | 99.83   |
|       |                                             | Tegafur  | 100               | 99.60             | 99.60   |

Table 7: Forced degradation studies of 5-fluorouracil and tegafur

| S. No | Stress condition | 5-fluorouracil | Tegafur |
|-------|------------------|---------------|---------|
|       |                  | % Assay | % Difference W.R.T control | % Assay | % Difference W.R.T control |
| 1     | Control          | 98.6    | NA                 | 99.4   | NA                 |
| 2     | Acid degradation | 91.52   | 8.47               | 90.19  | 9.80               |
| 3     | Base degradation | 94.37   | 5.62               | 93.29  | 6.70               |
| 4     | Oxidative degradation | 96.48 | 3.51               | 96.06  | 3.93               |
| 5     | Photolytic degradation | 97.24 | 2.75               | 96.58  | 3.41               |
| 6     | Thermal degradation | 98.87 | 1.12               | 98.56  | 1.43               |

Fig. 10: Chromatograph of thermal degradation
CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interests regarding the publication of this paper.

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