ABSTRACT

Objective: The current investigation was pointed at developing and progressively validating novel, simple, responsive and stable UPLC method for the measurement of active pharmaceutical ingredients of Mitomycin and Fluorouracil.

Methods: A simple, selective, validated and well-defined stability that shows isocratic UPLC methodology for the quantitative determination of Mitomycin and Fluorouracil. The chromatographic strategy utilized Inertsil ODS column of dimensions 250×4.6 mm, 5 micron, using isocratic elution with a mobile phase of acetonitrile and 0.1 percent formic acid (70:30). A flow rate of 1 ml/min and a detector wavelength of 255 nm utilizing the PDA detector was given in the instrumental settings. Validation of the proposed method was carried out according to an international conference on harmonization (ICH) guidelines.

Results: LOD and LOQ for the two active ingredients were established with respect to test concentration. The calibration charts plotted were linear with a regression coefficient of R²>0.999, means the linearity was within the limit. Recovery, specificity, linearity, accuracy, robustness, ruggedness were determined as a part of method validation and the results were found to be within the acceptable range.

Conclusion: The proposed method to be fast, simple, feasible and affordable in assay condition. During stability tests, it can be used for routine analysis of production samples and to verify the quality of drug samples during stability studies.

Keywords: Mitomycin, Fluorouracil, UPLC, Development, Validation

INTRODUCTION

The mitomycins are a family of aziridine-containing natural products isolated from Streptomyces caesipitosus or Streptomyces lavendulae [1, 2]. They include mitomycin A, mitomycin B, and mitomycin C. When the name mitomycin occurs alone, it usually refers to mitomycin C, its international nonproprietary name. Mitomycin C is used as a medicine [3] for treating various disorders associated with the growth and spread of cells. In the bacterium Legionella pneumophila [4-6], mitomycin C induces competence for transformation [7]. Natural transformation is a process of DNA transfer [8, 9] between cells and is regarded as a form of bacterial sexual interaction. In the fruit fly Drosophila melanogaster [10, 11], exposure to mitomycin C increases recombination during meiosis [12, 13], a key stage of the sexual cycle [14]. In the plant Arabidopsis thaliana [15, 16], mutant strains defective in genes necessary for recombination during meiosis and mitosis [17, 18] are hypersensitive to killing by mitomycin C [19]. Mitomycin C has been shown to have activity against stationary phase persisters caused by Borrelia burgdorferi, a factor in lyme disease [20, 21]. Mitomycin C is used to treat symptoms of pancreatic and stomach cancer and is under clinical research for its potential to treat gastrointestinal strictures [22]. Wound healing from glaucoma surgery [23] corneal excimer laser surgery [24] and endoscopic dacrocystorhinostomy [25].

Fluorouracil (5-FU), sold under the brand name Adrucil among others, is a medication used to treat cancer [26]. By injection into a vein it is used for colon cancer [27], esophageal cancer [28], stomach cancer, pancreatic cancer [29], breast cancer [30], and cervical cancer [31]. As a cream, it is used for actinic keratoses, skin cancers and Bowen’s disease [32] and as eye drops for the treatment of ocular surface squamous neoplasia. Others uses include ocular injections into a previously created trabeculectomy [33] to inhibit healing and cause scarring of tissue, thus allowing adequate aqueous humor flow to reduce intraocular pressure [40]. The present study aims the development and validation of Mitomycin and Fluorouracil using UPLC.

Fig. 1: Structure of (A) Mitomycin and (B) Fluorouracil

MATERIALS AND METHODS

Chemicals

Acetonitrile (HPLC grade), formic acid, water (HPLC grade), were purchased from Merck India Ltd, Mumbai, India. APIs of Mitomycin, Fluorouracil standards were procured from Glen mark, Mumbai.
The instrumentation
Waters Acquity model UPLC with quaternary pump, PDA detector 
with empower 2.0 software was used [41].

Preparation of buffer
1 ml of formic acid is dissolved in 1 L of HPLC grade water and filter 
through 0.45 µ filter paper.

Chromatographic conditions
The analysis was performed on reverse phase UPLC system with 
isocratic elution mode using a mobile phase of acetonitrile and 0.1%
formic acid (70:30) and Inertsil ODS column (250x4.6 mm, 5 µ)
column with a flow rate of 1 ml/min.

Diluent
Water and Acetonitrile in the ratio (50:50) is used as diluent.

Validation procedure
The analytical parameters such as system suitability, precision, 
specificity, accuracy, linearity, robustness, LOD, LOQ, forced 
degradation and stability were validated according to ICH Q2 (R1) 
guidelines [42-47].

Preparation of the standard stock solution
For standard stock solution preparation, add 70 ml of diluents to 
100 mg of Mitomycin and 100 mg of Fluorouracil taken in a 100 ml 
volumetric flask and sonicate for 10 min to fully dissolve the 
contents and then makeup to the mark with diluent.

Preparation of standard solution
1 ml of solution is drawn from the above normal stock solution into 
a 10 ml volumetric flask and diluted up to the level.

Preparation of sample solution
Take 130 mg of the sample drug Mitomycin and 100 mg of the 
sample drug Fluorouracil into a 100 ml volumetric flask and add 70 
ml of diluents and sonicate for 10 min to fully dissolve the contents 
and then make up the mark with diluent. This solution is filtered into 
a device using a 0.45µ nylon syringe in a vial.

RESULTS AND DISCUSSION
The main analytical challenge during development of a new method 
was to separate active Pharma ingredients. In order to provide a 
good performance the chromatographic conditions were optimized.

Method optimization
To optimize the chromatographic conditions, different ratios of 
phosphate buffer and the acetonitrile in the mobile phase with 
isocratic and gradient mode was tested. However, the mobile phase 
composition was modified at each trial to enhance the resolution 
and also to achieve acceptable retention times. Finally, 0.1% formic 
acid buffer and acetonitrile with isocratic elution was selected 
because it results in a greater response of active pharmacy 
ingredients. During the optimization of the method, various 
stationary phases such as C8, C18 phenyl and amino, inertsil ODS 
columns were tested. From these trials the peak shapes were 
relatively good with a inertsil ODS column of 250 x 4.6 mm, 5 µ. The 
mobile phase flow rate has been done at 25 5 nm in order to obtain 
enough sensitivity. By using above conditions, we get retention times 
of Mitomycin and Fluorouracil were about 1.869 min. and 2.750 min 
with a tailing factor of 1.05 and 1.11. The number of theoretical plates 
for Mitomycin and Fluorouracil was 3624,5748, which indicate the 
column’s successful output the % RSD for six replicate injections was 
around 0.17% (Mitomycin) and 0.50% (Fluorouracil); the proposed 
approach suggests that it is extremely precise. According to ICH 
guidelines, the method established was validated.

System suitability
System suitability parameters have been calculated to check the 
performance of the system. The parameters can be measured and 
found to be within the limit, including USP plate count, USP tailing, 
and percent RSD. Results of system suitability were given in the 
following table 1 [48].

| System suitability parameter | Acceptance criteria | Drug name | Mitomycin | Fluorouracil |
|-----------------------------|---------------------|-----------|-----------|--------------|
| USP Plate Count             | NLT 2000            | 36.28     | 5.467     |
| USP Tailing                 | NMT 2.0             | 1.02      | 1.11      |
| USP Resolution              | NLT 2.0             | -         | 8.64      |
| % RSD                       | NMT 2.0             | 0.17      | 0.50      |

Specificity
The capacity to test the analyte unequivocally in the presence of 
other elements, such as impurities, Excitements that might be 
assumed in order to be present in the sample solution and norm 
solution, is specificity.

According to the test method placebo, sample and standard 
solutions were analyzed individually to examine the interference. 
The below fig. shows that the active ingredients were well separated 
from blank and their exipients and there was no interference of 
placebo with the principal peak. Hence the method is specific.

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Table 1: Results of system suitability

Fig. 2: Chromatogram of system suitability
Fig. 3: Chromatogram of blank

Table 2: Linearity of mitomycin and fluorouracil

| S. No. | Conc. µg/ml | Mitomycin area count | Conc. µg/ml | Fluorouracil area count |
|--------|-------------|----------------------|-------------|------------------------|
| 1      | 2.00        | 17504                | 5.00        | 236501                 |
| 2      | 5.00        | 45653                | 12.50       | 603257                 |
| 3      | 10.00       | 95687                | 25.00       | 1205746                |
| 4      | 20.00       | 191546               | 50.00       | 2451068                |
| 5      | 25.00       | 228167               | 62.50       | 2825715                |
| 6      | 30.00       | 280568               | 75.00       | 3498601                |
|        | Correl coef | 0.9996               |             | 0.9990                 |
|        | Slope       | 9328.11              |             | 46375.47               |
|        | intercept   | 134.12               |             | 22075.56               |

Fig. 4: Calibration plots of (A) Mitomycin (B) Fluorouracil
Linearity
The area of the linearity peak versus different concentrations has been evaluated for Mitomycin, Fluorouracil, as 10, 25, 50, 100, 125, 150 percent respectively. The linear regression analysis was plotted with the peak area versus concentration data. The correlation coefficients of regression, Percent, y-intercept and slope of the calibration curves were calculated. The correlation coefficients achieved greater than 0.999 for all.

Accuracy
In this method, Accuracy was conducted in triplicate by analyzing active pharma ingredient sample solution spiked with known amounts of all the impurities at three kinds of concentration levels of 50, 100 and 150% of each at a specified limit. For all impurities, percentage recoveries were measured and found to be within the limit. The accuracy and reliability of the developed method were established. The percentage recovery values were found to be in the range of 100.13-100.59% for Mitomycin and 99.81-99.95% for Fluorouracil. The results are given in table 3, 4 and 5.

Precision
The precision of an analytical technique is the degree of closeness of series of measurements derived from multiple homogeneous mixture samplings. The exactness of the process of related substances was performed by injection of six individual injection determinations of Mitomycin (20 ppm) and Fluorouracil (50 ppm).

Table 3: Results of accuracy

| S. No. | % Level | Mitomycin % recovery | Fluorouracil % recovery |
|-------|---------|----------------------|------------------------|
| 1     | 50      | 100.24               | 99.98                  |
| 2     | 100     | 100.59               | 99.81                  |
| 3     | 150     | 100.13               | 99.95                  |
| mean  |         | 100.32               | 99.91                  |
| SD    |         | 0.240                | 0.091                  |

Mean±SD (n=3)

Table 4: Intraday precision results of mitomycin and fluorouracil

| S. No. | Conc. (µg/ml) | Area counts | % Assay as is | Conc. (µg/ml) | Area counts | % Assay as is |
|--------|---------------|-------------|---------------|---------------|-------------|---------------|
| 1      | 20            | 191365      | 100           | 50            | 2451991     | 100.2         |
| 2      | 191143        | 99.9        | 2451387       | 100.1         |
| 3      | 191650        | 100.2       | 2435647       | 99.5          |
| 4      | 191554        | 100.1       | 2458475       | 100.4         |
| 5      | 190546        | 99.6        | 2455305       | 100.3         |
| 6      | 193341        | 101         | 2461250       | 100.5         |

% RSD 0.49 0.47   0.37 0.36
Mean 100.13 100.17
SD 0.47188 0.35590

Mean±SD (n=6)

Intermediate precision
Six replicates of the sample solution were studied by various researchers, and on separate days different instruments were tested. The peak regions used to determine to mean percent RSD values have been calculated. The results are given in the following table.

Intraday precision
Six replicates of a sample solution containing Mitomycin (20µg/ml) and Fluorouracil (50µg/ml) were analysed on the same day. Peak areas were calculated, which were used to calculate mean, SD and % RSD values.

Interday precision
Six replicates of a sample solution containing Mitomycin (20µg/ml) and Fluorouracil (50µg/ml) were analysed on a different day. Peak areas were calculated which were used to calculate mean, SD and % RSD values. The present method was found to be precise as the RSD values were less than 2% and also the percentage assay values were close to be 100%. The results are given in table 5 [49].

Fig. 5: Chromatogram of sample
Table 5: Inter-day outcomes of accuracy of mitomycin and fluorouracil

| S. No. | Conc. (µg/ml) | Area counts | % assay as is | Conc. (µg/ml) | Area count | % Assay as is |
|--------|---------------|-------------|---------------|---------------|------------|---------------|
| 1      | 20            | 191884      | 100.2         | 50            | 2451206    | 100.1         |
| 2      | 20            | 191327      | 100.0         | 50            | 2451954    | 100.2         |
| 3      | 191009        | 99.8        |               | 2434567       | 99.4       |               |
| 4      | 191567        | 100.1       |               | 2454877       | 100.3      |               |
| 5      | 191256        | 99.9        |               | 2448512       | 100        |               |
| 6      | 192368        | 100.5       |               | 2425457       | 99.1       |               |

%RSD 0.26 0.25
Mean 100.08 99.85
SD 0.24833 0.48477

Mean±SD (n=6)

LOD and LOQ

LOD for LOD and LOQ were calculated separately using the calibration curve process. The LOD and LOQ of the compound were calculated using the developed RP-HPLC method by injecting increasingly lower concentrations of the standard solution. The LOD and LOQ concentrations and their S/N values were shown in the following table. The method is validated as per the ICH guidelines [50]. LOD and LOQ results were tabulated in table 6.

Table 6: LOD and LOQ for mitomycin and fluorouracil

| LOD          | Mitomycin | Fluorouracil |
|--------------|-----------|--------------|
| LOD          | LOD       | LOD          |
| Concentration| s/n       | Concentration| s/n       |
| 0.025 µg/ml  | 4         | 0.0083 µg/ml | 28        |
| 0.208 µg/ml  | 2         | 0.065 µg/ml  | 7         |
| 0.208 µg/ml  | 25        |              |           |

Fig. 6: Chromatogram of (A) LOD and (B) LOQ

Table 7: Robustness data of mitomycin and fluorouracil

| Parameter name       | % RSD Mitomycin | % RSD Fluorouracil |
|----------------------|-----------------|---------------------|
| Flow minus (0.8 ml/min) | 0.32            | 0.26                |
| Flow plus (1.2 ml/min) | 0.24            | 0.40                |
| Organic minus (-10%) | 0.21            | 0.68                |
| Organic plus (+10%)  | 0.10            | 0.85                |
Robustness
The conditions of the experiment were designed to test the robustness of the established system intentionally altered, such as flow rate, mobile phase in organic percentage in all these varied conditions. The resolution between active Pharma ingredients from impurities was not significantly affected and there was no significant influence on the time of retention, plate count and tailing factor. Hence this method was robust [51].

Stability
The standard and sample solution was kept at room temperature and at 2-8 °C up to 24 h. Then these solutions were pumped into the device and calculate the % of deviation from initial to 24 h [52]. There was no significant deviation observed and confirmed that the solutions were stable up to 24 h percentage of the assay was not quite 2%. There is no effect in storage conditions for Mitomycin and Fluorouracil drugs.

Degradation studies
The Fluorouracil and Mitomycin sample was subjected into various forced degradation conditions to effect partial degradation of the drug. Studies of forced degradation have carried out to find out that the method is suitable for products of degradation [53, 54]. In addition, the studies provide details about the conditions during which the drug is unstable in order that the measures are often taken during formulation to avoid potential instabilities.

Acid degradation
1 ml of standard stock solution passed on to a volumetric flask of 10 ml of 1N HCl and leaves it for 15 min. After 15 min add 1 ml of 1N NaOH and made up to the mark with diluents.

Alkali degradation
1 ml of standard stock solution was put in a 10 ml volumetric flask and add 1 ml of 1N NaOH and leave it for 15 min. After 15 min add 1 ml of 1N HCl and made up to the mark with diluents.

Peroxide degradation
In a 10 ml volumetric flask, 1 ml of standard stock solution was transferred, add 0.3 ml of 30% hydrogen peroxide and made up to the mark with diluents.

Reduction degradation
In a 10 ml volumetric flask, 1 ml standard stock solution was transferred and add 1 ml of 30% sodium bi sulphate solution and made up to the mark with diluents.

Table 8: Stability results of mitomycin and fluorouracil at RT

| Stability | Mitomycin | Fluorouracil |
|-----------|-----------|--------------|
| Initial   | 100       | 100          |
| 6 H       | 99.9      | 99.9         |
| 12 H      | 99.8      | 99.2         |
| 18 H      | 99.7      | 98.8         |
| 24 H      | 99.6      | 98.4         |

| Stability | Mitomycin | Fluorouracil |
|-----------|-----------|--------------|
| Initial   | 100.2     | 100          |
| 6 H       | 100.1     | 99.8         |
| 12 H      | 99.9      | 99.3         |
| 18 H      | 99.8      | 98.9         |
| 24 H      | 99.7      | 98.4         |

Table 9: Stability results of mitomycin and fluorouracil at 2-8 °C

| Degradation condition | Mitomycin | Fluorouracil |
|-----------------------|-----------|--------------|
| % assay               | % Deg     | % assay      | % Deg     |
| Acid degradation      | 84.7      | 15.2         | 83.2      | 16.5      |
| Alkali degradation    | 86.9      | 13.1         | 83.3      | 16.7      |
| Peroxide degradation  | 86.3      | 13.7         | 87.7      | 12.3      |
| Reduction degradation | 88.5      | 11.5         | 85.4      | 14.6      |
| Thermal degradation   | 89.1      | 10.9         | 88.9      | 11.1      |

The standard solution was set in an oven at 105° for 6 h. The resultant solution was injected into HPLC.

CONCLUSION
We present in this article simple, selective, validated and well-defined stability that shows gradient RP-UPLC methodology for the quantitative determination of Mitomycin and Fluorouracil. All the products of degradation formed during the stress conditions and the related active pharma ingredients are well separated and peaks were well resolved from each other and separate with an appropriate retention time, indicating that the proposed method to be fast, simple, feasible and affordable in assay condition. Therefore the developed method during stability tests, it can be used for routine analysis of production samples and to verify the quality of drug samples during stability studies.

ACKNOWLEDGEMENT
The authors are grateful to the management of Shree Icon Pharmaceutical Laboratory, Labbipeta, Vijayawada, Andhra Pradesh, India, for providing the necessary facilities and assistance in carrying out this study.

FUNDING
Nil

AUTHORS CONTRIBUTIONS
All authors have contributed equally.
CONFLICTS OF INTERESTS
Declared none

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