INTRODUCTION
Mitomycins are a class of aziridine-containing natural products isolated from Streptomyces caespitosus or Streptomyces Lavendulae [1, 2]. They include Mitomycin A, Mitomycin-based B, and Mitomycin C. If the Mitomycin appears alone, it commonly refers to Mitomycin C. Mitomycin C [3] is used to treat various diseases associated with the development and distribution 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 improves recombination during meiosis [12, 13] a crucial stage of the reproductive cycle [14]. In the plant Arabidopsis thaliana [15, 16] mutant strains defective in genes required 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 bacteria, to induce in the development of cancer therapy [20, 21]. Mitomycin C is used to alleviate symptoms of cancer of the pancreas and stomach and is under clinically tested for its application to gastrointestinal structures [22], wound healing from glaucoma surgery [23] corneal excimer laser surgery [24] and endoscopic dacryocystorhinostomy [25].

The drug Fluorouracil (S-FU) is used to treat cancer [26], marketed among others, under the brand name adrucil. Via injection into a vein for colon cancer, it is used [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 [32], basal cell carcinoma [33] and skin warts [34]. Many persons experience side effects when injected. Common side effects include inflammation of the mouth, loss of appetite, low blood cell counts, hair loss and inflammation of the skin. When used as a cream irritation, it usually takes place at the application’s site. In pregnancy, use of either type can injure the infant. Fluorouracil is in the antimetabolite [35] and pyrimidine analogue families of medications. How it functions is not entirely clear but believed to involve blocking the action of thymidylate synthase [36] and therefore preventing the development of DNA. The safest and most powerful medicines needed in a health system it is on the list of global health organizations [37]. fluorouracil has been given systematically for anal, breast, colorectal, esophageal and stomach, pancreatic and skin cancers (especially head and neck cancers). It has also been given topically (on the skin) for actinic Keratoses, scalp cancers and Bowen’s disease [38] and as eye droplets for the treatment of ocular surface neoplasia. Other applications include eye injections into a previously formed trabeculectomy [39] to prevent healing and induce tissue scarring while facilitating sufficient aqueous humour flow to decrease intraocular pressure [40].

MATERIALS AND METHODS
Chemicals
Acetonitrile, HPLC-grade orthophosphoric acid, water were purchased from Merck India Ltd, Mumbai, India. APIs of Mitomycin, Fluorouracil and their impurities as reference standards were procured from Spectrum solution for pharmacy research Pvt, Ltd, Hyderabad.

Instrumentation
Waters alliance liquid chromatography (model 2695) monitored with empower 2.0 data handling system and fitted with a Luna C18 (150x4.6 mm, 3.5 µ) and a detector of photodiode array (model 2998) was used for this study.

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

Chromatographic conditions
The HPLC analysis was performed on a reverse phase HPLC system with gradient elution mode using a mobile phase of acetonitrile and 0.1% OPA and Luna column C18 (150x4.6 mm, 3.5 µ) column with a flow rate of 1 ml/min.
Fig. 1: Chemical structures of (A) Mitomycin (B) Mitomycin impurity-A (C) Mitomycin impurity-B (D) Fluorouracil (E) Fluorouracil Impurity-A and (F) Fluorouracil impurity-B

Table 1: Gradient programmed

| Time (min) | Acetonitrile | Buffer |
|-----------|--------------|--------|
| 0         | 30           | 70     |
| 5         | 50           | 50     |
| 10        | 80           | 20     |
| 12        | 30           | 70     |
| 18        | 30           | 70     |

Table 1: Gradient programmed

Till today there are no HPLC methods reported in the literature, So, it has more interested to develop a novel and reliable HPLC strategy for the establishment of Mitomycin, Fluorouracil and their related impurities.

Diluents

Mobile phase was used as a diluent.

Preparation of regular stock solution

Accurately weighed and transfer 100 mg of Mitomycin, 50 mg of Fluorouracil working standards into a 100 ml clean dry volumetric flask and diluent was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. 1 ml of the above solution was taken into 10 ml volumetric flask and made up to the mark with diluents.

Impurities stock solutions

Accurately weighed and transferred 5 mg of impurity-A and impurity-B of Mitomycin and impurity-A and impurity-B of Fluorouracil working standards into a 100 ml clean dry volumetric flask and diluent was added and sonicated to dissolve completely and made volume up to the mark with the same solvent. 1 ml of the above solution was taken into 10 ml volumetric flask and made up to the mark with diluents.

Preparation of the standard solution

Pipetted 5 ml of the above standard stock solution and 5 ml of impurities stock solution into a 50 ml volumetric flask and diluted up to the mark with diluent.

Validation procedure

The analytical parameters [41-45] such as system suitability, precision, specificity, accuracy, linearity, robustness, LOD, LOQ, forced degradation and stability were validated according to ICH Q2 (R1) guidelines [46].

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.

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. It was tested by analyzing the blank sample and the samples spiked with fluorouracil and mitomycin.

Accuracy

Accuracy is the closeness to the true value of the test results produced by the process. The recovery trials were tested at three separate concentration levels. A minimum of three injections were given at each stage, measuring the amount of the drug present, the percentage of recovery and the associated standard deviation.

Precision

The degree of agreement among individual test results is the precision of analytical process. It was analyzed through multiple sampling analysis of a homogeneous sample in terms of repeatability, intraday and inter-day variations, the accuracy of the current system was evaluated. The sample was analysed at various time intervals on the same day as well as on different days.

Linearity

The linearity of the analytical approach is its capacity to generate outcomes within a definite scope. Peak area was directly proportional to the analytes concentration in the sample for the evaluation of the linearity spectrum; six series of standard solutions were chosen. Using the peak area versus the concentration of standard solution, the calibration curve was plotted and the regression equations were measured. The system of least squares was used to measure the slope, coefficient and intercept of correlation.

LOD and LOQ

LOD is the smallest analyte quantity in the sample that can be identified, LOQ is the smallest analyte quantity in the sample
which can be calculated with reasonable precision and accuracy. On the basis of calibration curves, LOD and LOQ were separately computed. LOD and LOQ were determined according to ICH guidelines as 3.3s/n and 10s/n, respectively, where s/n indicates the ratio of signal to noise.

Robustness

The robustness of an analytical procedure is a measure of its ability to remain unaffected by small but deliberate changes in method parameters of the system and provide an indication of its reliability during regular use. The robustness analysis was carried out by injecting the standard solution into the HPLC system and adjusting the flow rate (±0.2 ml/min), organic step (% of) chromatographic conditions. By evaluating the affect of the changed parameters, the separation factor, retention time and peak asymmetry were determined.

Forced degradation

Stress degradation should be no interference between the peaks obtained for a chromatogram of preparations. According to ICH guidelines, stress degradation studies were conducted. The peaks of degradation should be well apart from each other and the resolution between the peaks shall be at least 2.0 and the peak purity of the principal peaks shall pass. Forced degradation experiments were conducted to obtain the degradation of about 20 percent by various types of stress conditions.

RESULTS AND DISCUSSION

The main analytical challenge during development of a new method was to separate active pharma ingredients from their impurities. 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 accept achieveable retention times. Finally 0.1% OPA buffer and acetonitrile with gradient elution was selected because it results in a greater response of active pharmacy ingredient and their impurities. During the optimization of the method various stationary phases such as C8, C6, phenyl and amino columns were tested [47]. From these trials the peak shapes were relatively good with a column of Luna C18 150x4.6 mm, 3.5 µ with a PDA detector. The mobile phase flow rate has been done at 260 nm in order to obtain enough sensitivity. By using the above conditions, we get retention times of mitomycin and fluorouracil were about 2.984 and 10.383 min with a tailing factor of 1.05 and 1.03. The retention times of mitomycin impurity-A and impurity-B were impurities of 3.717, 4.770 min and the fluorouracil impurity-A and impurity-B were 5.800, 10.941 min, respectively. The number of theoretical plates for mitomycin and fluorouracil were 3102, 48107, which indicate the column’s successful output the % RSD for six replicate injections was around 0.94% the proposed approach suggests that it is extremely precise. According to ICH guidelines, the method established was validated.

Method validation

The optimized RP-HPLC validated method [48] according to ICH guidelines in terms of system suitability, linearity, consistency, precision and robustness.

System suitability

Device suitability parameters have been assessed, such as USP plate count, USP tailing and percent RSD.

| Suitability parameter      | Acceptance criteria | Mitomycin Mean | Mitomycin Std dev | Fluorouracil Mean | Fluorouracil Std dev |
|----------------------------|---------------------|----------------|-------------------|-------------------|----------------------|
| USP Plate count            | NLT 2000            | 3451           | 30.956            | 47555             | 385.738              |
| USP Tailing                | NMT 2.0             | 1.04           | 0.010             | 1.04              | 0.005                |
| USP Resolution             | NMT 2.0             | -              | -                 | 13.35             | 0.193                |

(n=6)

![Chromatogram of system suitability](image)

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 excipients and there was no interference of placebo with the principal peak. Hence the method is specific.

Linearity

The area of the linearity peak versus different concentrations has been evaluated for mitomycin, fluorouracil and their related substances. The test solutions are prepared for related substance method from impurity stock solution at various concentration levels. The spectrum of linearity has been found to be 10-
150μg/ml of mitomycin, 5-75 μg/ml fluorouracil and 0.5-7.5 μg/ml each impurity of mitomycin and fluorouracil. Under optimum chromatographic conditions, we get linear relations between the peak areas and the peak regions corresponding pitch concentrations. The correlation coefficients for all the components were under the limit.

Table 3: Linearity results of mitomycin, fluorouracil and their impurities

| Linearity  | Mitomycin | Fluorouracil |
|------------|-----------|--------------|
|            | Conc. (µg/ml) | Area | Conc. (µg/ml) | Area | Conc. (µg/ml) | Area |
| Linearity-1 | 10 | 995452 | 0.5 | 30930 | 0.5 | 50986 |
| Linearity-2 | 25 | 2647909 | 1.25 | 71994 | 1.25 | 155072 |
| Linearity-3 | 50 | 549861 | 2.5 | 146548 | 2.5 | 328439 |
| Linearity-4 | 100 | 10336275 | 5 | 272860 | 5 | 621459 |
| Linearity-5 | 125 | 12236122 | 6.25 | 342684 | 6.25 | 766171 |
| Linearity-6 | 150 | 14985674 | 7.5 | 405764 | 7.5 | 921159 |

Slope: 99103.67
Intercept: 103.95
CC: 0.99972

Table 3: Results of accuracy

| S. No | % Level | Mitomycin | Fluorouracil |
|-------|---------|-----------|--------------|
|       | % Recovery | Std dev | % Recovery | Std dev |
| 1     | 100.1 | 0.208 | 99.9 | 0.252 |
| 2     | 100.1 | 0.101 | 100.2 | 0.265 |
| 3     | 100.0 | 0.201 | 99.9 | 0.153 |

(n=3)

Accuracy

Accuracy was conducted in triplicate by analyzing active pharmaceutical 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.

Precision

The precision [49] of an analytical technique is the degree of closeness of a 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 (100 ppm) and fluorouracil
(50 ppm) spiked with that of each of 5% of imp-A and imp-B of mitomycin and imp-A and imp-B of fluorouracil. The % RSD was determined for each impurity and the results have shown that the technique is precise under the specified experimental conditions.

![Graphs](A, B, C, D, E, F)

**Fig. 4: Calibration plots of (A) Mitomycin (B) Mitomycin imp-A (C) Mitomycin imp-B (D) Fluorouracil (E) Fluorouracil imp-A (F) Fluorouracil imp-B**

| Sample number | % of related substances | Mitomycin | Fluorouracil |
|---------------|------------------------|-----------|--------------|
|               |                        | Spiked impurities | Total impurities | % Purity (100-total imp) | Spiked impurities | Total impurities | % Purity (100-total imp) |
| 1             |                        | 5.15       | 0.69         | 99.31                    | 5.06           | 0.55         | 99.45                    |
| 2             |                        | 5.16       | 0.62         | 99.38                    | 5.07           | 0.57         | 99.43                    |
| 3             |                        | 5.14       | 0.67         | 99.33                    | 5.09           | 0.51         | 99.49                    |
| 4             |                        | 5.13       | 0.63         | 99.37                    | 5.01           | 0.54         | 99.46                    |
| 5             |                        | 5.18       | 0.61         | 99.39                    | 5.03           | 0.52         | 99.48                    |
| 6             |                        | 5.17       | 0.65         | 99.35                    | 5.04           | 0.59         | 99.41                    |
| Average       |                        | 5.16       | 0.65         | 99.36                    | 5.05           | 0.55         | 99.45                    |
| Std Dev       |                        | 0.019      | 0.031        | 0.031                    | 0.029          | 0.030        | 0.030                    |
| % RSD         |                        | 0.36       | 4.78         | 0.03                     | 0.57           | 5.51         | 0.03                     |

(n=6)
Intermediate precision

Six replicates of the sample solution were analyzed on various analysts and different instruments were tested on separate days. The peak areas used to measure mean percent RSD values were measured. The following table gives the results.

LOD and LOQ

By steadily injecting the lower ones, LOD and LOQ of the compounds were carried out. The periodic solution concentrations of the LOD concentrations of Mitomycin and its impurities were 3.03, 0.15, 0.15 and their values for s/n are 8, 4, 4; Fluorouracil and its impurities were 1.52, 0.15, 0.15 and their s/n values were 6, 4, 4. The LOQ concentrations of Mitomycin and its impurities were 10, 0.5, 0.5 and their s/n values were 27, 22, 22; Fluorouracil and its impurities were 5, 0.5, 0.5 and their s/n values were 25, 23, 22, respectively. This method is validated as per the ICH guidelines [50-53].

Robustness

The conditions of the experiment were designed to test the robustness of the established system intentionally altered [54], such as flow rate, mobile phase in organic percentage in all these varied conditions [55, 56]. 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.

Table 5: Inter-day outcomes of accuracy of mitomycin and fluorouracil

| Sample number | % Related substances Mitomycin | Fluorouracil |
|---------------|--------------------------------|-------------|
|               | Spiked impurities | Total impurities | % Purity (100-total imp) | Spiked impurities | Total impurities | % Purity (100-total imp) |
| 1             | 5.06              | 0.71           | 99.29                  | 5.13              | 0.69           | 99.31                  |
| 2             | 5.07              | 0.75           | 99.25                  | 5.17              | 0.67           | 99.33                  |
| 3             | 5.08              | 0.73           | 99.27                  | 5.19              | 0.68           | 99.32                  |
| 4             | 5.03              | 0.74           | 99.26                  | 5.15              | 0.64           | 99.34                  |
| 5             | 5.04              | 0.72           | 99.28                  | 5.16              | 0.63           | 99.37                  |
| 6             | 5.06              | 0.74           | 99.26                  | 5.14              | 0.68           | 99.32                  |
| Average       | 5.06              | 0.73           | 99.27                  | 5.16              | 0.67           | 99.33                  |
| Std Dev       | 0.019             | 0.015          | 0.01                   | 0.022             | 0.024          | 0.02                   |

%(n=6)
of 1N NaOH was added and volume was made up to the mark with diluents.

After 15 min 1 ml of 1N HCl was added and left it for 15 min. After 15 min volume was made up to the mark with diluents. All the chromatographic impurities was well established. All the appropriate retention time, indicating that the proposed method to and peaks were well resolved from each other and separate with an related impurities of active pharma ingredients are well separated defined stability that shows gradient RP-HPLC methodology for the We present in this article simple, selective, validated and well-products of degradation formed during the stress conditions and the determination of Mitomycin and Fluorouracil as well as studies of forced degradation have their chromatographic impurities was well established. All the values are presented as mean±SD (n=3)

| Degradation condition       | Mitomycin | Fluorouracil |
|-----------------------------|-----------|--------------|
| % Assay                     | % Deg     | % Assay      | % Deg     |
| Acid degradation            | 86.8      | 13.2         | 85.2      | 14.8       |
| Alkali degradation          | 86.3      | 13.7         | 84.4      | 15.6       |
| Peroxide degradation        | 85.2      | 14.8         | 84.7      | 15.2       |
| Reduction degradation       | 87.1      | 12.9         | 85.9      | 14.1       |
| Thermal degradation         | 87.7      | 12.3         | 87.5      | 12.5       |
| Hydrolysis degradation      | 88.4      | 11.6         | 87.1      | 12.9       |

CONCLUSION
We present in this article simple, selective, validated and well-defined stability that shows gradient RP-HPLC methodology for the quantitative determination of Mitomycin and Fluorouracil as well as their chromatographic impurities was well established. All the products of degradation formed during the stress conditions and the related impurities of 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 RS 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.

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AUTHORS CONTRIBUTIONS
All the authors have contributed equally.

Table 6: Robustness data of mitomycin and fluorouracil

Table 8: Forced degradation results of mitomycin and fluorouracil

| Parameter name                          | % RSD  |
|-----------------------------------------|--------|
| Flow minus (0.8 ml/min)                 | 0.64   |
| Flow plus (1.2 ml/min)                  | 0.38   |
| Organic minus (-10%)                    | 0.59   |
| Organic plus (+10%)                     | 0.52   |

RSD-Relative standard deviation; All the values are presented as mean±SD (n=3)

Degradation studies
Mitomycin and Fluorouracil sample was subjected into various forced degradation conditions [57-59] to effect partial degradation of the drug. Studies of forced degradation have carried out to find out that the method [60] is suitable for products of degradation [61-63]. 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 [64, 65].

Acid degradation
1 ml of sample stock solution was taken into 10 ml volumetric flask and 1 ml of 1N HCl was added and left it for 15 min. After 15 min volume was made up to the mark with diluents.

Alkali degradation
In 1 ml of sample stock solution (10 ml volumetric flask), 1 ml of 1N NaOH was added and left it for 15 min. After 15 min 1 ml of 1N HCl was added and made up to the mark with diluents.

Peroxide degradation
In 1 ml of sample stock solution in a 10 ml volumetric flask and 1 ml of 30% sodium bisulphate solution was added and left it for 15 min. After 15 min, volume was made up to the mark with diluents.

Reduction degradation
In 1 ml of sample stock solution was transferred into 10 ml volumetric flask and 1 ml of 30% sodium bisulphate solution was added and left it for 15 min. After 15 min, volume was made up to the mark with diluents.

Thermal degradation
Take 1 ml of sample stock solution into 10 ml volumetric flask make up to the mark with diluents. After that keep the sample solution in an oven for 6 hr at 105 °C.

Degradation of hydrolysis
1 ml of sample stock solution was taken into 10 ml volumetric flask and 1 ml of HPLC grade water was added and left it for 15 min. After 15 min volume was made up to the mark with diluents.

CONFLICTS OF INTERESTS
Declared none

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