

NEW VALIDATED REVERSE PHASE ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR MITOMYCIN AND FLUOROURACIL IN ACTIVE PHARMACEUTICAL INGREDIENT AND TABLET FORM AND ITS STRESS STUDIES

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ABSTRACT

Objective: The current investigation was pointed at developing and progressively validating novel, simple, responsive and stable RP-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 250x4.6 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 were given in the instrumental settings. Validation of the proposed method was carried out according to an international conference on harmonization (ICH) guidelines.

Results: The two active ingredients LOD and LOQ were established with respect to test concentration. The calibration charts plotted were linear with a regression coefficient of $R^2 > 0.999$, 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.

Key words: Mitomycin, Fluorouracil, RP-UPLC, Development, Validation.

INTRODUCTION

The mitomycins are a family of aziridine-containing natural products isolated from *Streptomyces caespitosus* 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 dacryocystorhinostomy [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 keratosis [32], basal cell carcinoma [33], and skin warts [34]. When used by injection most people develop side effects. 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 at the site of application usually occurs. Use of either form in pregnancy may harm the baby. Fluorouracil is in the antimetabolite [35] and pyrimidine analog families of medications. How it works is not entirely clear but believed to involve blocking the action of thymidylate synthase [36] and thus stopping the production of DNA. It is on the World Health Organization's List of Essential Medicines, the safest and most effective medicines needed in a health system [37]. Fluorouracil has been given systemically for anal, breast, colorectal, oesophageal, stomach, pancreatic and skin cancers (especially head and neck cancers). It has also been given topically (on the skin) for actinic keratoses, skin cancers and Bowen's disease [38] and as eye drops for treatment of ocular surface squamous neoplasia. Other uses include ocular injections into a previously created trabeculectomy [39] bleb 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.

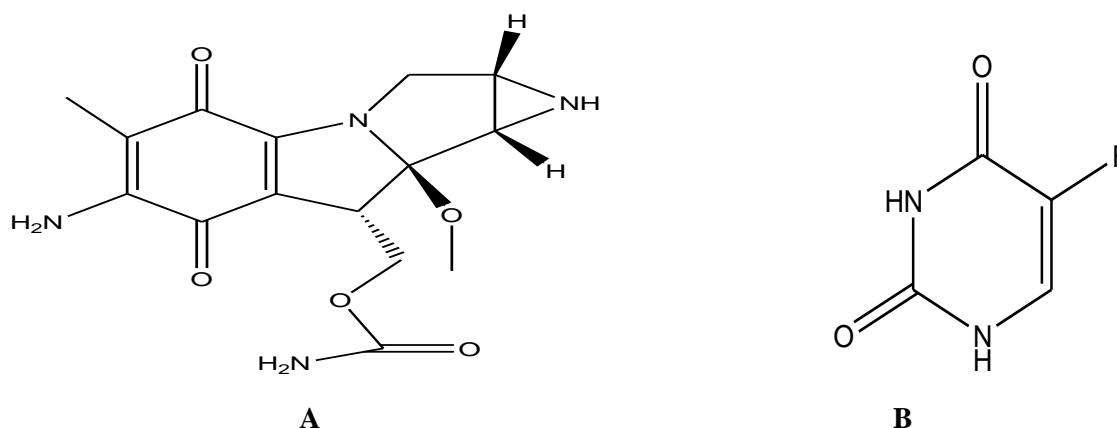


Fig. 1: Structure of (A) Mitomycin and (B) Fluorouracil

MATERIALS AND METHOD

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 lt 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 70ml of diluents to 100mg of Mitomycin and 100 mg of Fluorouracil taken in a 100 ml volumetric flask and sonicate for 10 minutes to fully dissolve the contents and then make up to the mark with diluent.

Preparation of Standard solution: 1 ml of solution is drawn from the above normal stock solution into a 10ml 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 minutes to fully dissolve the contents and then make up the mark with diluent. This solution is filtered into a device using a 0.45 μ 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 4.6mm, 5 μ. The mobile phase flow rate has been done at 255nm 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 & 1.11. The number of theoretical plates for Mitomycin and Fluorouracil were 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.

There are no UPLC and HPLC methods reported in the literature. So, it is interesting to develop a UPLC method for the estimation of the combined drugs in vitro method.

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].

Table 1: Results of system suitability

System suitability parameter	Acceptance criteria	Drug name	
		Mitomycin	Fluorouracil
USP Plate Count	NLT 2000	3628	5487
USP Tailing	NMT 2.0	1.02	1.11
USP Resolution	NLT 2.0	-	8.64
% RSD	NMT 2.0	0.17	0.50

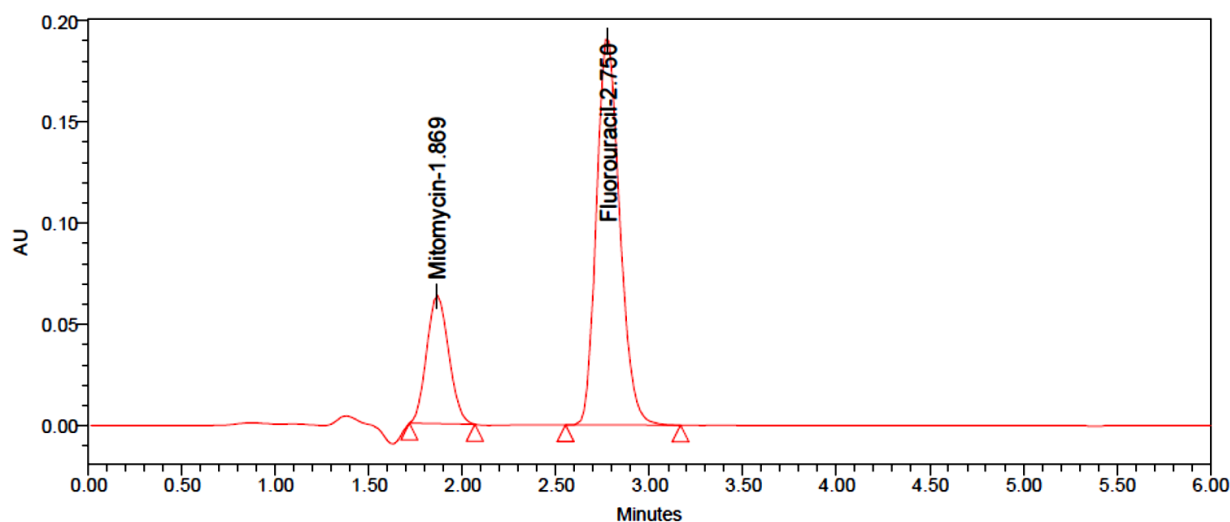


Fig. 2: Chromatogram of system suitability

Specificity: The capacity to test the analyte unequivocally in the presence of other elements, such as impurities, Excipients 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 figure 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.

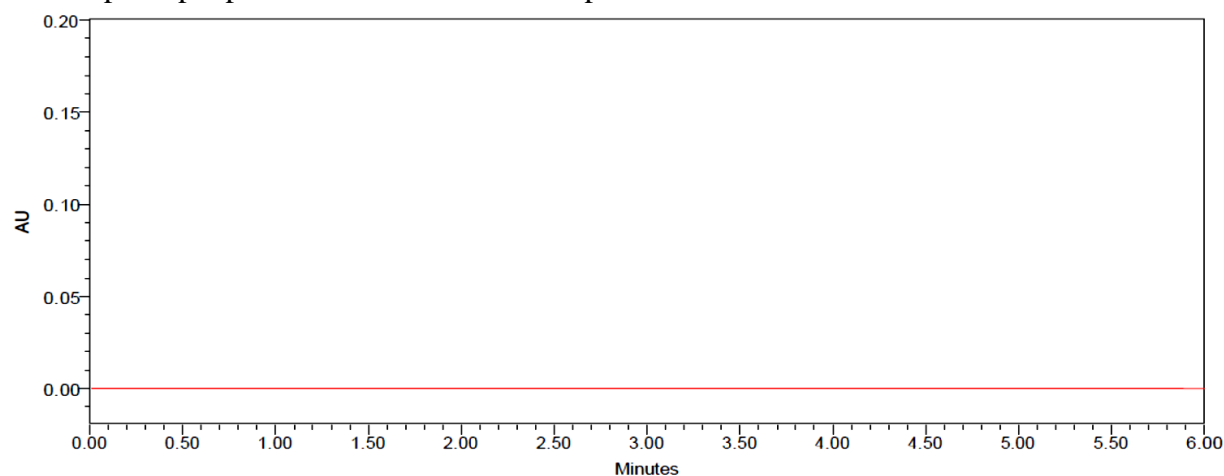
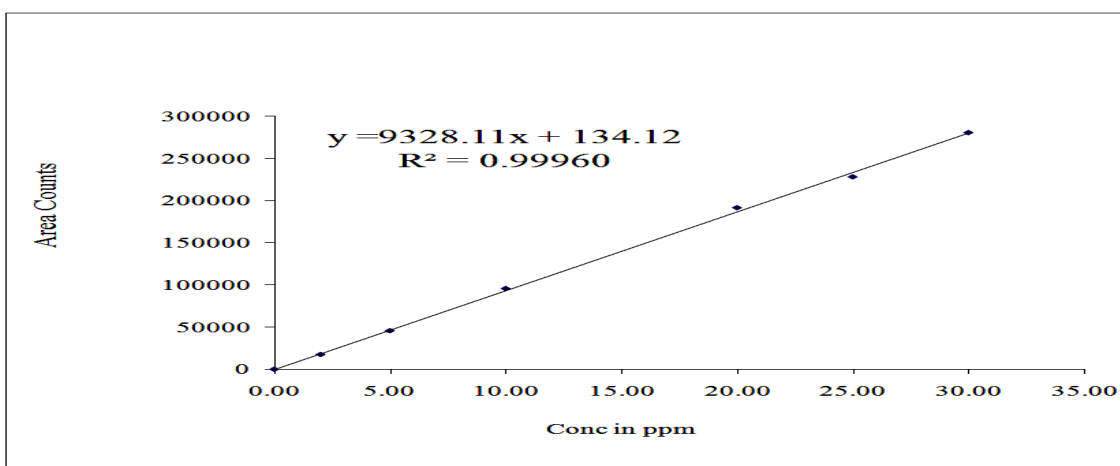


Fig. 3: Chromatogram of blank

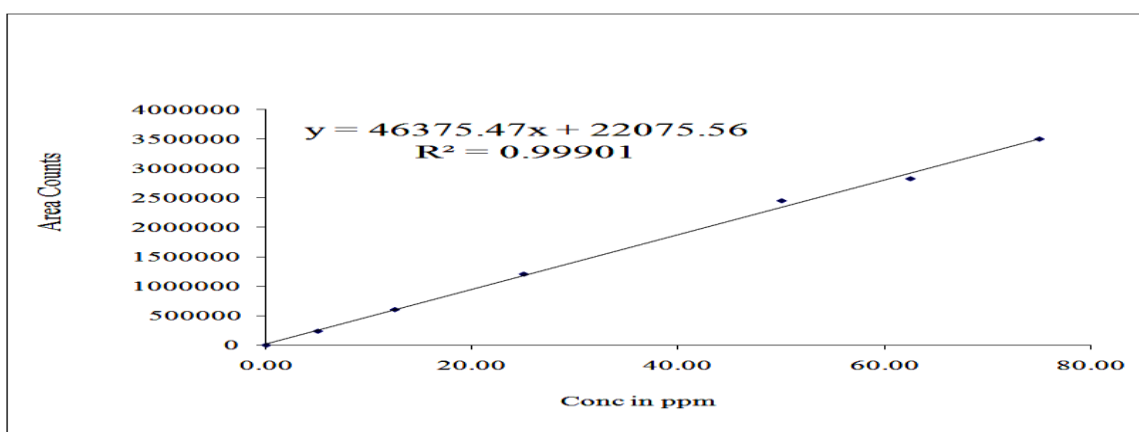
Linearity: The area of the linearity peak versus different concentrations has been evaluated for Mitomycin, Fluorouracil, as 10, 25, 50, 100, 125, 150 percent respectively. The linear regression analysis was plotted with the peak area versus concentration data. The correlation coefficients of regression,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

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



A



B

Fig. 4: Calibration plots of (A) Mitomycin (B) Fluorouracil

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.

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)

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 (20ppm) and Fluorouracil (50ppm).

Table 4: Intraday precision results of Mitomycin and Fluorouracil

Mitomycin				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)

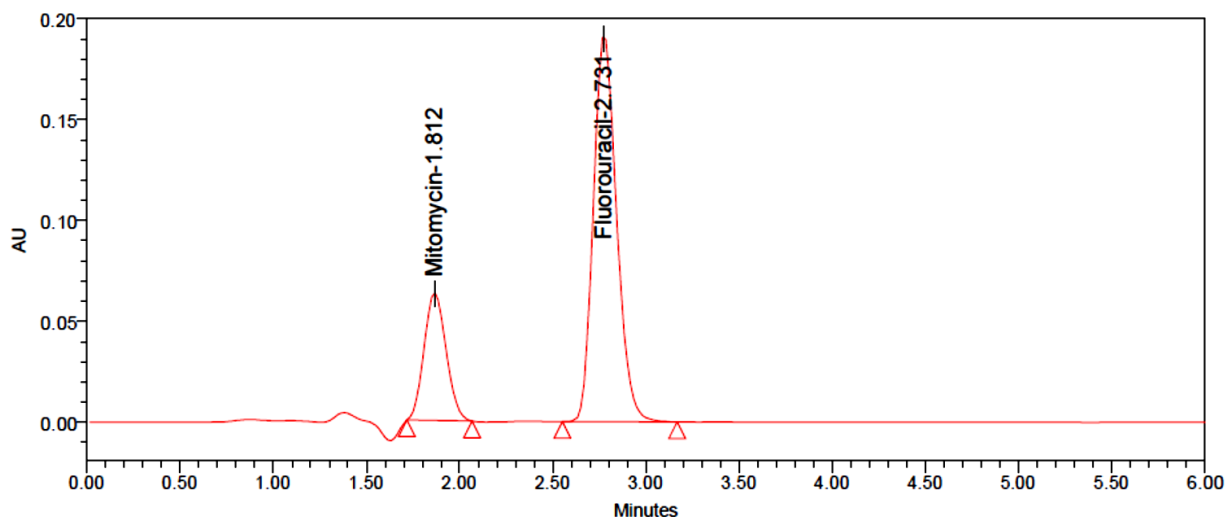


Fig. 5: Chromatogram of sample

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 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 (20 μ g/ml) and Fluorouracil (50 μ g/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 (20 μ g/ml) and Fluorouracil (50 μ g/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].

Table 5: Inter-day outcomes of accuracy of Mitomycin and Fluorouracil

Mitomycin				Fluorouracil		
S.No.	Conc.(μ g/ml)	Area counts	% assay as is	Conc.(μ g/ml)	Area count	% assay as is
1	20	191884	100.2	50	2451206	100.1
2		191327	100.0		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		0.48	0.49
Mean			100.08			99.85
SD			0.24833			0.48477

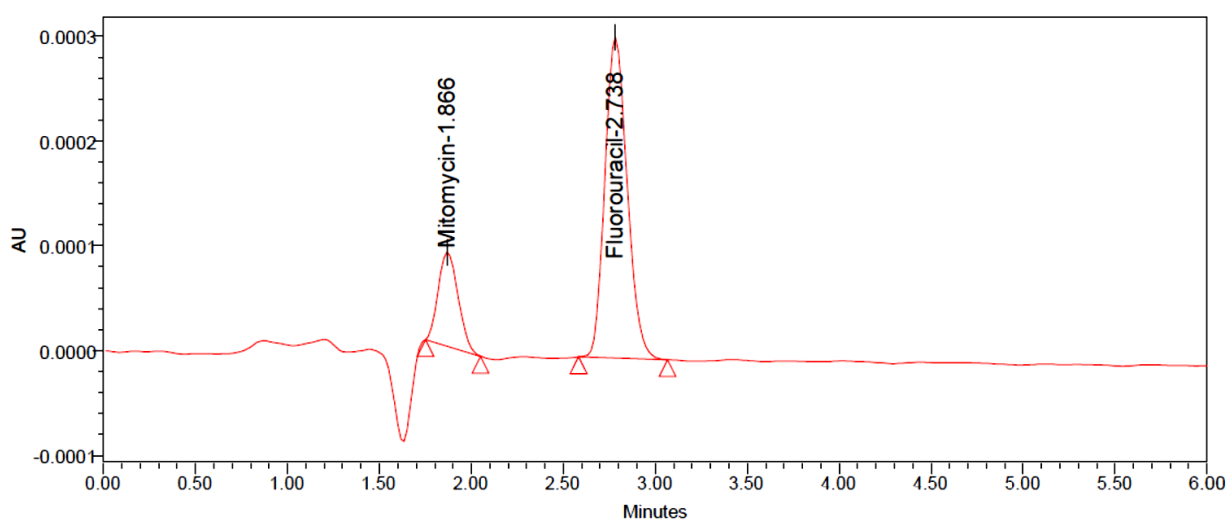
mean \pm SD (n=6)

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-

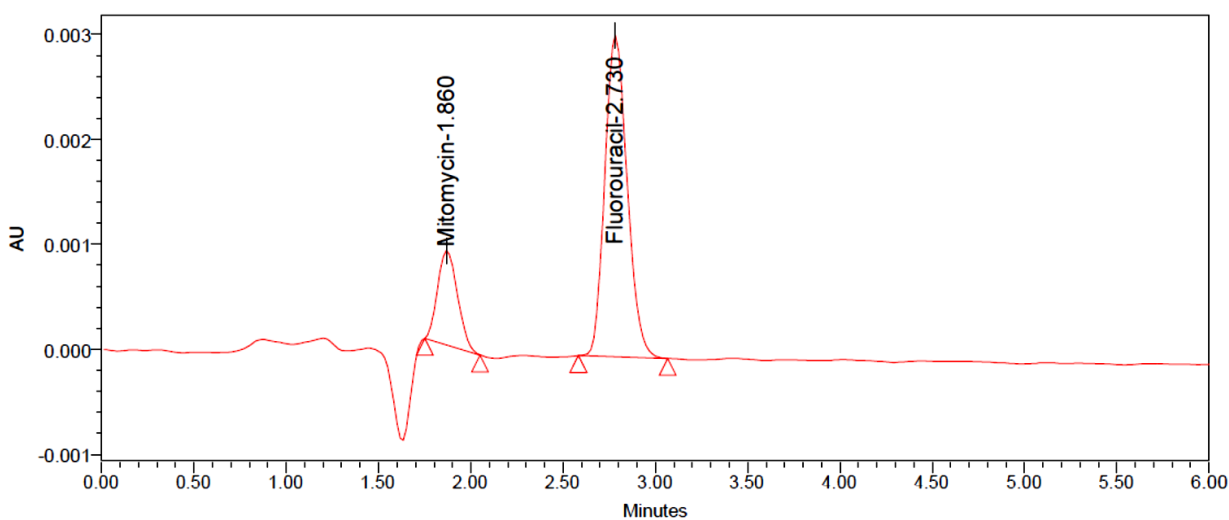
HPLC method by injecting increasingly lower concentrations of the standard solution. The LOD and LOQ concentrations and their S/N values were shown in the following table. The method is validated as per the ICH guidelines [50]. LOD and LOQ results were tabulated in table 6.

Table 6: LOD and LOQ for Mitomycin and Fluorouracil

Mitomycin				Fluorouracil			
LOD		LOQ		LOD		LOQ	
Concentration	s/n	Concentration	s/n	concentration	s/n	Concentration	s/n
0.025 μ g/ml	4	0.083 μ g/ml	28	0.063 μ g/ml	7	0.208 μ g/ml	25



A



B

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

Robustness: The conditions of the experiment were designed to test the robustness of 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].

Table 7: Robustness data of Mitomycin and Fluorouracil

Parameter name	% RSD	
	Mitomycin	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

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.

Table 8: Stability results of Mitomycin and Fluorouracil at RT

Stability	Mitomycin		Fluorouracil	
	Purity	% of deviation	Purity	% of deviation
Initial	100	0.00	100	0.00
6 Hrs	99.9	-0.10	99.9	-0.10
12 Hrs	99.8	-0.20	99.2	-0.80
18 Hrs	99.7	-0.30	98.8	-1.20
24 Hrs	99.6	-0.40	98.4	-1.60

Table 9: Stability results of Mitomycin and Fluorouracil at 2-8°C

Stability	Mitomycin		Fluorouracil	
	Purity	% of deviation	Purity	% of deviation
Initial	100.2	0.00	100	0.00
6 Hrs	100.1	-0.10	99.8	-0.20
12 Hrs	99.9	-0.30	99.3	-0.70
18 Hrs	99.8	-0.40	98.9	-1.10
24 Hrs	99.7	-0.50	98.4	-1.60

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, 1 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.

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

Table 10: Forced degradation results of Mitomycin and Fluorouracil

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

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.

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CONFLICTS OF INTEREST

None

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