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Original Article

A NEW RELATED SUBSTANCES METHOD DEVELOPMENT AND VALIDATION OF TWO ANTI-CANCER DRUGS BY USING EFFECTIVE LIQUID CHROMATOGRAPHIC METHOD

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ABSTRACT

Objective: The present study was aimed at developing and successively validating novel, simple, responsive and stable RP-HPLC method for the measurement of active pharmaceutical ingredients of mitomycin and fluorouracil and their related substances.

Methods: Using the impurity-spiked solution, the chromatographic approach was optimized. The chromatographic method used Luna C18 column of dimensions 150x4.6 mn, 3.5 µm, using gradient elution with a mobile phase of acetonitrile and 0.1 percent orthophosphoric acid. A flow rate of 1 ml/min and a detector wavelength of 260 nm using the PDA detector was provided in the instrumental settings. Recovery, specificity, linearity, accuracy, robustness, ruggedness was determined as a part of method validation and the results were found to be within the acceptable range.

Results: According to the ICH guidelines, the developed approach was validated. The calibration charts plotted were linear with a regression coefficient of R^2>0.999.

Conclusion: The method developed was found to be applicable to routine analysis and to be used for the measurement of active pharmaceutical ingredients (i. e, Mitomycin and Fluorouracil and its related impurities). Since there is no HPLC method reported in the literature for the estimation of Mitomycin, Fluorouracil and its related impurities; there is a need to develop quantitative methods under different conditions to achieve improvement in specificity, selectivity etc.

Keywords: Mitomycin, Fluorouracil, RP-HPLC, Development, Validation

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INTRODUCTION

Mitocycins 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 borealis burgdorferi, a factor in lyme disease [20, 21]. Mitomycin C is used to alleviate symptoms of cancer of the pancreas and stomach and is under clinically tested for its potential to treat gastrointestinal strictures [22], wound healings from glaucoma surgery [23] corneal exciter laser surgery [24] and endoscopic dacryocystorhinostomy [25].

The drug Fluorouracil (5-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 keratosis [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 pyramiding 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 Kerasotes, scalp cancers and Bowen's disease [38] and as eye drops for the treatment of ocular surface summons neoplasia. Other applications include eye injections into a previously formed trabeculectomy [39] bleb to prevent heeling 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 C_{18} (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.

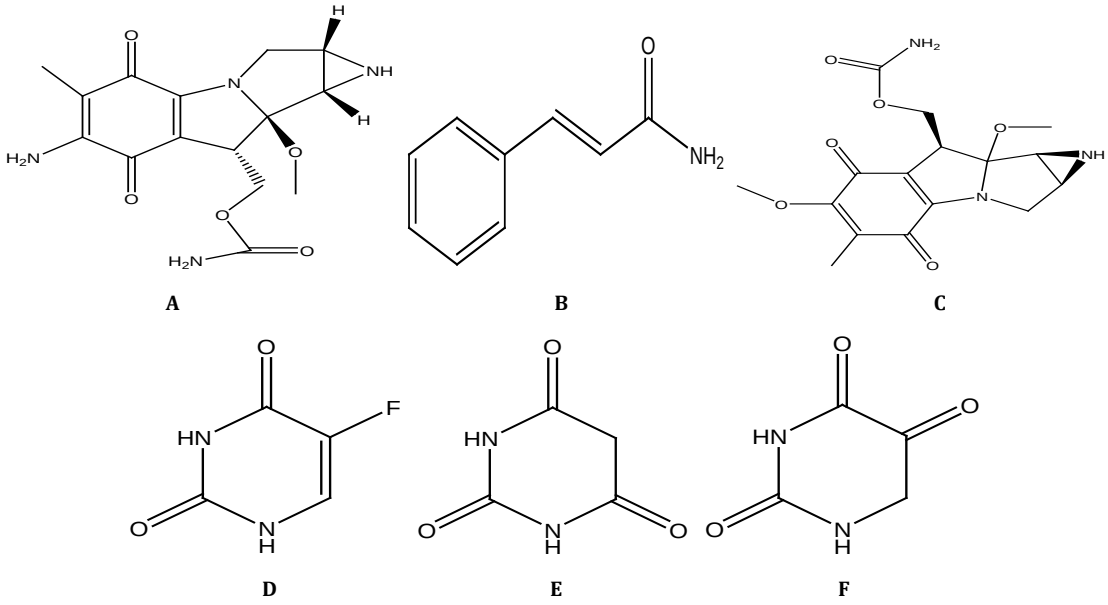


Fig. 1: Chemical structures of (A) Mitomycin (B) Mitomycin impurity-A (C) Mitomycin impurity-B (D) Fluorouracil (E) Fluorouracil Impurity-A and (F) Fluorouracil impurity-B

Table 1: Gradient programmed

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

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 transferred5 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 volume was 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 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 10 s/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 (±percent) 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 should 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 achieve acceptable 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 C_8, C_{18} phenyl and amino columns were tested [47]. From these trials the peak shapes were relatively good with a column of Luna C_{18} 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.

Table 2: Results of system suitability

Suitability parameter	Acceptance criteria	Mitomycin		Fluorouracil	
		Mean	Std dev	Mean	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)

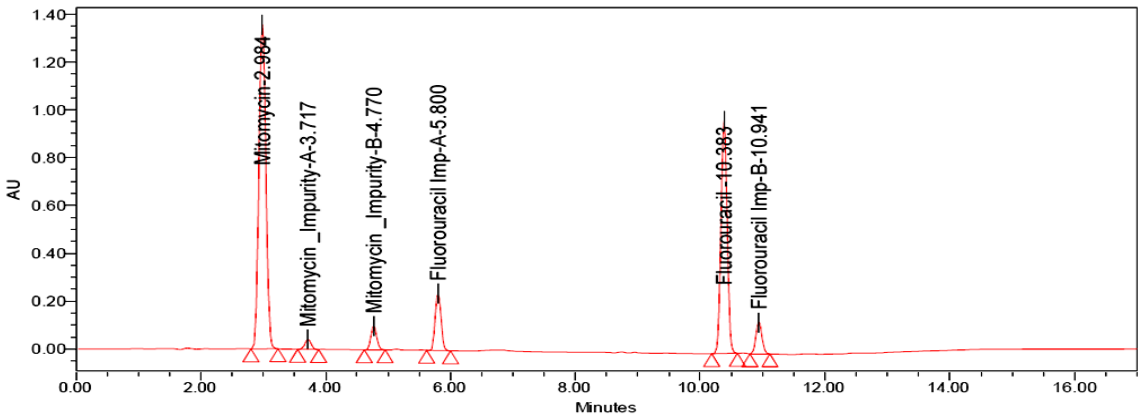


Fig. 2: Chromatogram of system suitability

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.

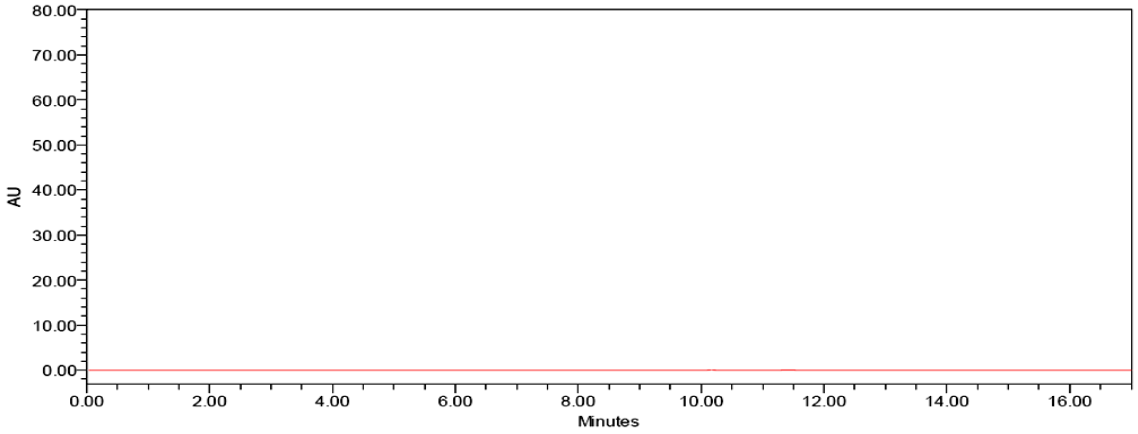


Fig. 3: Chromatogram of blank

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

A

Linearity	Mitomycin		Imp-A		Imp-B	
	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	5498961	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		53892.42		123150.03	
Intercept	158957.55		4464.89		1547.91	
CC	0.99912		0.99972		0.99964	

B

Linearity	Fluorouracil		Imp-A		Imp-B	
	Conc. (µg/ml)	Area	Conc. (µg/ml)	Area	Conc. (µg/ml)	Area
Linearity-1	5	703856	0.5	140060	0.5	92975
Linearity-2	12.5	1677472	1.25	372964	1.25	226087
Linearity-3	25	3521197	2.5	779597	2.5	458160
Linearity-4	50	6555366	5	1524653	5	868924
Linearity-5	62.5	8097411	6.25	1877405	6.25	1095114
Linearity-6	75	9515457	7.5	2240095	7.5	1315810
Slope	127263.01		300307.34		174264.37	
Intercept	114323.82		3957.87		6998.51	
CC	0.99945		0.99984		0.99988	

Table 3: Results of accuracy

S. No	% Level	Mitomycin		Fluorouracil	
		% Recovery	Std dev	% Recovery	Std dev
1	50	100.1	0.208	99.9	0.252
2	100	100.1	0.101	100.2	0.265
3	150	100.0	0.201	99.9	0.153

(n=3)

Accuracy

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.

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.

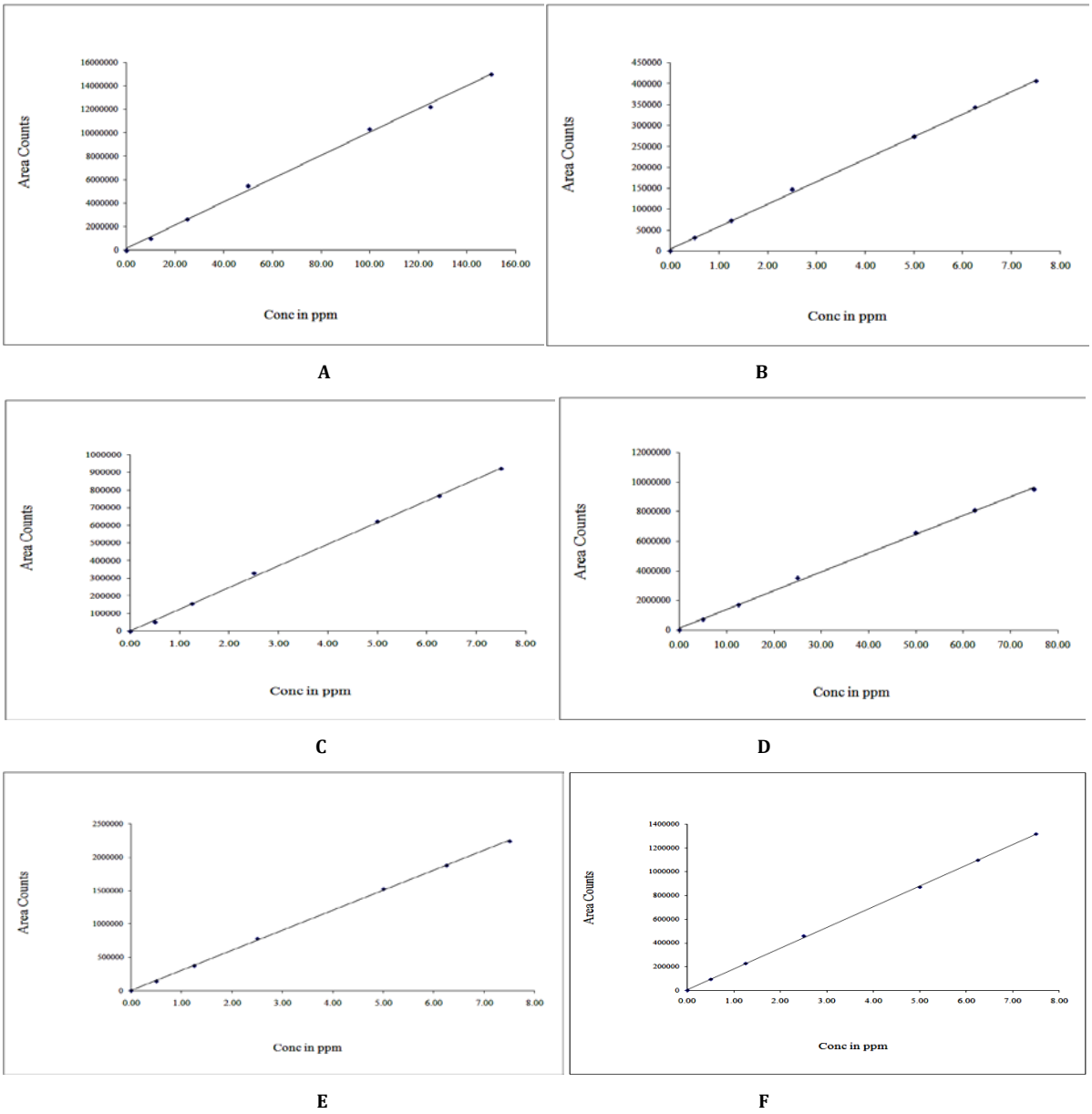


Fig. 4: Calibration plots of (A) Mitomycin (B) Mitymycin imp-A (C) Mitomycin imp-B (D) Fluorouracil (E) Fluorouracil Imp-A (F) Fluorouracil imp-B

Table 4: Intraday precision results of mitomycin and fluorouracil

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)

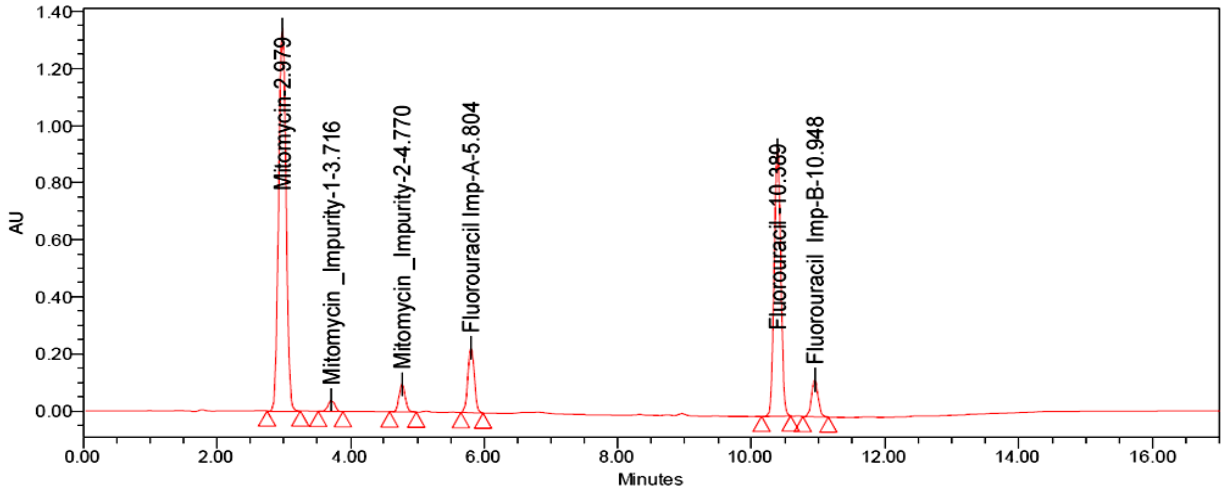


Fig. 5: Chromatogram of sample

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].

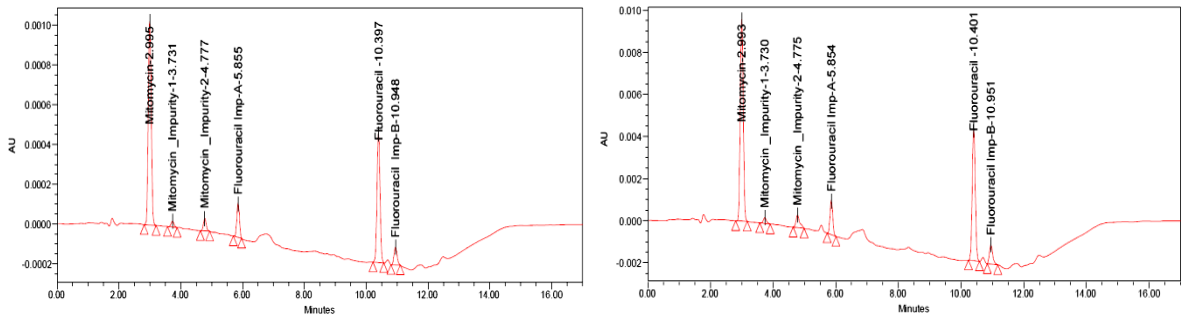
Robustness

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	0.015	0.022	0.024	0.021
% RSD	0.37	2.01	0.01	0.42	3.65	0.02

(n=6)



A **B**

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

Table 6: Robustness data of mitomycin and fluorouracil

Parameter name	% RSD	
	Mitomycin	Fluorouracil
Flow minus (0.8 ml/min)	0.64	0.72
Flow plus (1.2 ml/min)	0.38	0.68
Organic minus (-10%)	0.59	0.29
Organic plus (+10%)	0.52	0.64

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 ml of 1N NaOH was added and 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 leaft 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, 0.3 ml of 30% hydrogen peroxide was added and leaft it for 15 min. After 15 min, volume was made up to the mark with diluents.

Reduction degradation

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 for 15 min. After 15 min, volume was made up to the mark with diluents.

Table 8: Forced degradation results of mitomycin and fluorouracil

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.

CONFLICTS OF INTERESTS

Declared none

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