IJPSR (2021), Volume 12, Issue 11 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 15 October 2020; received in revised form, 09 February 2021; accepted, 09 October 2021; published 01 November 2021

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

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Keywords:

Mitomycin, Fluorouracil, HPLC, Development and Validation

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ABSTRACT: A simple, sensitive and rapid chromatographic method was developed and validated for quantification of Mitomycin and Fluorouracil in bulk and pharmaceutical dosage form using Symmetry C18 (4.6 × 150 mm, 3.5 µ) column. The mobile phase consists of buffer, 0.1% Ortho Phosphoric acid (OPA), and acetonitrile in the ratio of 50:50 v/v. The flow rate is maintained as at 1.0 ml/min; detection was carried out by absorption at 260 nm using photodiode array detector. The calibration curve was linear and the regression coefficient (R2) value was found to be 0.999 and concentrations ranging from 10-150 µg/ml of mitomycin and 5-75 µg/ml of fluorouracil, respectively. The LOD and LOQ of the method were found to be 0.1 µg/ml, 0.05 µg/ml and 1 µg/ml, 0.5 µg/ml for Mitomycin and Fluorouracil. The number of theoretical plates and tailing factors for Mitomycin and Fluorouracil were not less than 2000 and were not more than 2, respectively. The developed method was found to be simple, economical, suitable and validated according to ICH guidelines.

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, 5, 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.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.12(11).6077-82

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.12(11).6077-82

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 pancreatic and stomach cancer symptoms 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]. **Fig. 1** shows the structure of mitomycin. Fluorouracil (5-FU), sold under the brand name Adrucil among others, is a medication used to treat cancer [26]. Injection into a vein 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 skin inflammation. 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 systematically for anal, breast, colorectal, oesophageal and 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 to treat ocular surface squamous neoplasia. Other uses include ocular injections into a previously created trabeculectomy [39] blebs to inhibit healing and cause scarring of tissue, thus allowing adequate aqueous humor flow to reduce intraocular pressure [40]. **Fig. 2** shows the structure of fluorouracil.

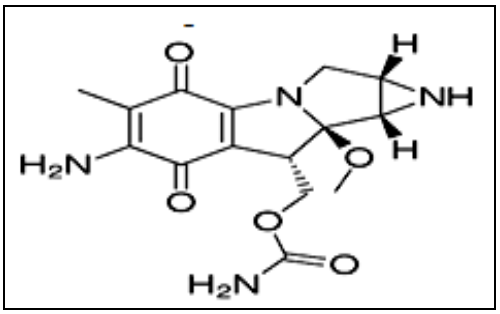

FIG. 1: STRUCTURE OF MITOMYCIN

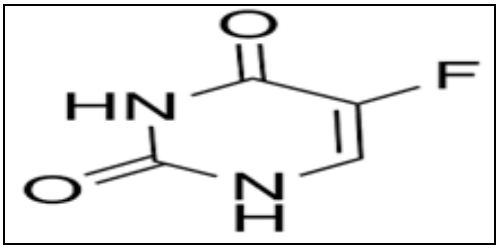

FIG.2: STRUCTURE OF FLUOROURACIL

MATERIALS AND METHODS: Acetonitrile, Orthophosphoric acid and water (HPLC grade), were purchased from Merck Ltd. Worli, Mumbai, India. APIs of Mitomycin and Fluorouracil as reference standards were procured from Spectrum Pharma research solutions Pvt. Ltd, Hyderabad.

Instrumentation: Waters Alliance liquid chromatography (model 2695) monitored with Empower 2 data handling system and fitted with a symmetry C18 (150 × 4.6 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 1lt of HPLC grade water and filtered through 0.45 µ filter paper.

Preparation of Mobile Phase: Add buffer and acetonitrile in 50:50 ratio, mixed thoroughly, sonicated for 5 min, filtered through 0.22 µm membrane filter and used as mobile phase. The HPLC analysis was performed on a reversed-phase HPLC system with isocratic elution mode using a mobile phase of acetonitrile and 0.1% OPA (50:50) on Symmetry C18 column (150 × 4.6 mm, 3.5 µ) with 1ml/min flow rate at 260 nm using PDA detector.

Diluent: Mobile phase was used as a diluent.

Preparation of Standard Solution (Mitomycin 100 µg/ml and Fluorouracil 50 µg/ml): Accurately weigh and transfer 100 mg of mitomycin and 50 mg of fluorouracil working standard into 100 ml volumetric flask and add app. 70 ml of diluent, sonicate to dissolve it for 30 min and made up to the mark with diluent. This is used as a stock solution. Take 5 ml of the stock solution and transferred it into 50 ml volumetric flask and made upto the mark with diluent.

Optimization of Chromatographic Conditions: Various combination of mobile phase was screened with respect to resolution, theoretical plate count, tailing and other system suitability parameters. Finally, the separation was performed with a freshly prepared mobile phase consists of buffer: acetonitrile in the ratio of 50:50 with a flow rate of 1.0 ml/min. 260 nm wavelength, injection volume of 10 µl and ambient temperature was maintained during the entire process to obtain a symmetric peak of mitomycin and fluorouracil.

RESULTS AND DISCUSSION: To obtain the best chromatographic condition, different columns

like C18, C8 and CN-, propyl, and mobile phases were tested. The best chromatographic separation occurred on Symmetry C18 column with a mobile phase consisting of acetonitrile and 0.1% OPA in (50:50) at a flow rate of 1ml/min and PDA detection at 260 nm.

Finally, the following conditions were found to be optimum after evaluating the column efficiency by parameters. PDA spectrum of Mitomycin and Fluorouracil was shown in **Fig. 3** and optimized chromatographic conditions were shown in **Table 1**.

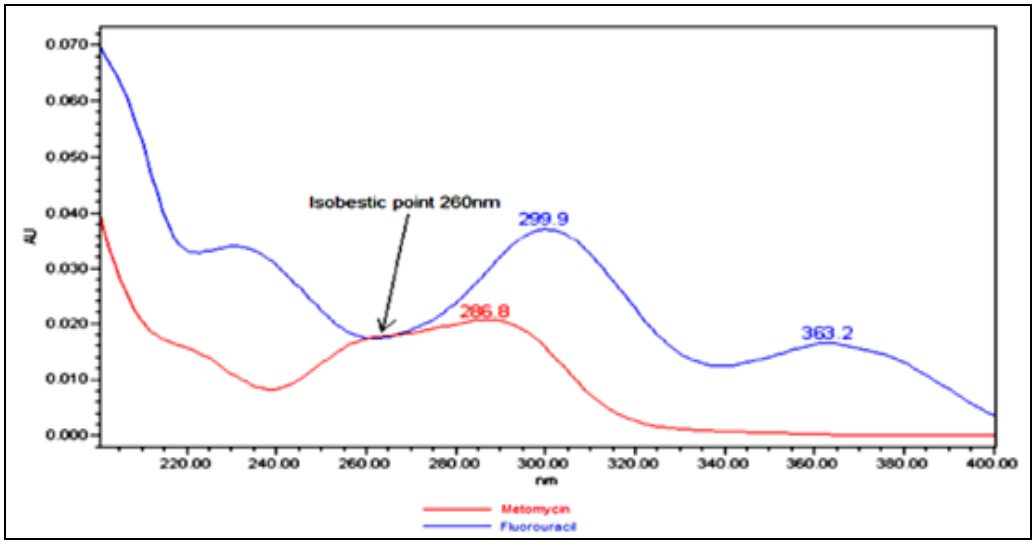

FIG. 3: PDA SPECTRUM OF MITOMYCIN AND FLUOROURACIL

TABLE 1: OPTIMIZED CHROMATOGRAPHIC CONDITIONS

Stationary Phase	Symmetry C_{18} (150x4.6mm, 3.5µ)
Mobile Phase	Acetonitrile : 0.1 OPA (50:50)
Injection volume	10 µl
Flow rate	1.0 ml/min
Column temperature	25°C
Wavelength	260 nm
Run time	8 min.
Retention time of Mitomycin	2.770 min.
Retention time of Fluorouracil	5.118 min.

System Suitability: The system suitability was performed by injecting a standard solution containing 100 µg/ml of mitomycin and 50 µg/ml of fluorouracil in six replicates. The result indicates that the system suitability parameter is within the limit. The results are shown below. The results of system suitability were represented in **Table 2** and the standard chromatogram as shown in **Fig. 4**.

TABLE 2: SYSTEM SUITABILITY RESULTS

Parameter	Mitomycin	Fluorouracil
Theoretical plate count	3661	9074
Tailing factor	1.05	0.99
Resolution	-	11.75
Retention time	2.770	5.118

Linearity: The method's linearity was established by plotting a graph between concentration and corresponding peak area for mitomycin and fluorouracil over a concentration range from 100-15 µg/ml and 5-75 µg/ml, respectively. The correlation coefficient was found to be 0.999 for both drugs. The calibration curves are shown in **Fig. 5** and the linearity results are shown in **Table 3**.

TABLE 3: RESULTS OF LINEARITY

S. no.	Mitomycin		Fluorouracil	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	10	416890	5	121716
2	25	761356	12.5	311818
3	50	1305500	25	588822
4	75	1951265	37.5	917344
5	100	2586456	50	1205546
6	125	3271404	62.5	1510828
7	150	3825525	75	1811815

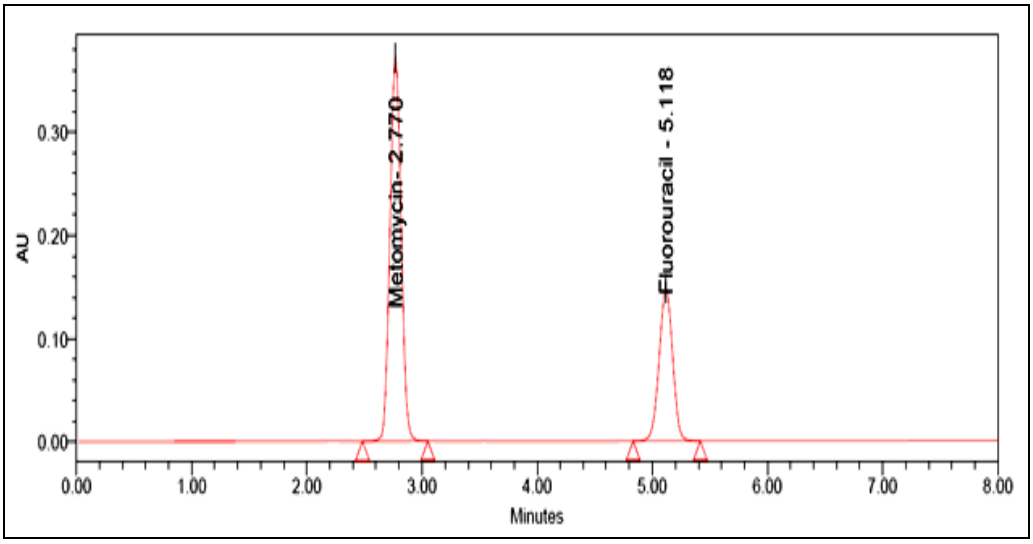

FIG. 4: CHROMATOGRAM OF SYSTEM PRECISION

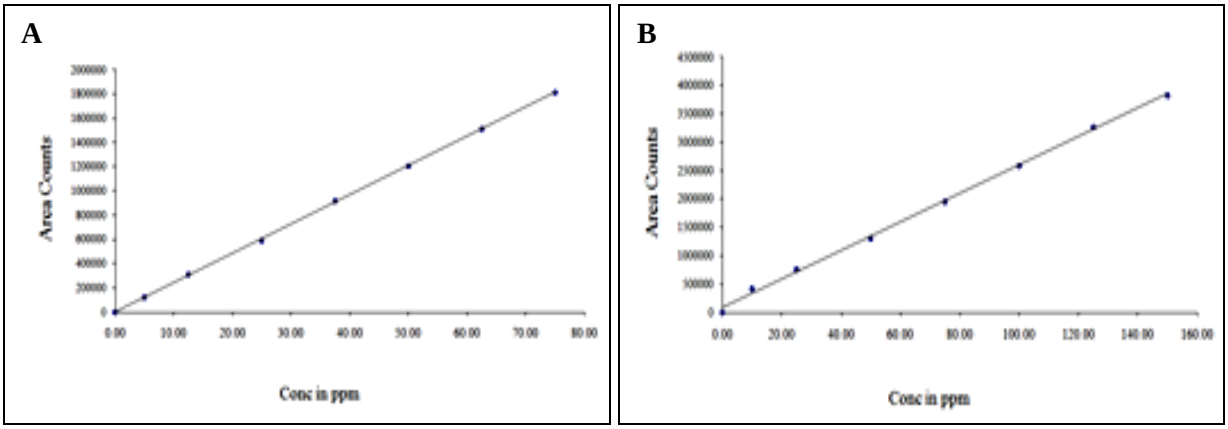

FIG. 5: CALIBRATION CURVE OF (A) FLUOROURACIL (B) MITOMYCIN

Limit of Detection (LOD) and Limit of Quantification (LOQ): The LOD and LOQ were found to be 0.1 µg/ml, 0.05µg/ml and 1 µg/ml, 0.5 µg/ml for Mitomycin and Fluorouracil, respectively. The results are given in **Table 4**.

TABLE 4: RESULTS OF LOD AND LOQ

Drug	LOD	LOQ
Mitomycin	0.1	1
Fluorouracil	0.05	0.5

Precision:

Method Precision (or) Repeatability: The % RSD value for six replicate injections of known concentration of Mitomycin and Fluorouracil carried out on the same day was <2%, indicating that the method was repeatable. Method precision results are represented in **Table 5**.

Accuracy: The concentrations of Mitomycin and Fluorouracil were prepared in three levels of 50%, 100%, and 150%. The percentage recovery obtained was found to be in the acceptable limit of 98%-102%. From this it was found that the developed method is precise and accurate. Accuracy results were represented in **Table 6**.

Robustness: The robustness of the chromatographic method was determined by varying flow rate and mobile phase composition.

% RSD was found to be within the acceptable limit. Robustness results were tabulated in **Table 7**.

TABLE 5: RESULTS OF METHOD PRECISION

S. no.	Area of Mitomycin	Area of Fluorouracil
1	2541286	116977
2	2546871	117774
3	2542783	117354
4	2546874	116856
5	2537136	117669
6	2536784	116983
Mean	2541955	117268
Std Dev	4460.017	389.8406
%RSD	0.18	0.33

TABLE 6: ACCURACY RESULTS

Accuracy	Amount of Mitomycin	% Recovery	Amount of Fluorouracil	% Recovery
50%	50	99.26	25	100.01
100%	100	98.54	50	99.47
150%	150	99.11	75	99.36

TABLE 7: RESULTS OF ROBUSTNESS

Parameter	% RSD of Mitomycin	% RSD of Fluorouracil
Flow (1.2ml/min)	0.44	0.55
Flow (0.8ml/min)	0.41	0.83
Organic phase (55:45)	0.56	0.39
Organic phase (45:55)	0.51	0.74

CONCLUSION: Till today, there is no HPLC method to estimate the combination of Mitomycin and Fluorouracil. For the estimation of these two drugs simultaneously HPLC method was developed and validated according to ICH guidelines. All the validation parameters, including system suitability, accuracy, method precision, LOD, LOQ and robustness are within acceptable limits. The proposed method can be used to routine Mitomycin and Fluorouracil in the combined dosage form.

ACKNOWLEDGEMENT: I am thankful to my faculty lecturers for supporting and encouragement to complete this research work.

CONFLICTS OF INTERESTS: Nil

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How to cite this article:
Jaison Jose T, Subbareddy Y and Sankar K: A new assay method development and validation of two anti-cancer drugs by using effective liquid chromatographic method. Int J Pharm Sci & Res 2021; 12(11): 6077-82. doi: 10.13040/IJPSR.0975-8232.12(11).6077-82.