Stability Indicating RP-HPLC Method for In-Vitro Analysis of 5-Flurouracil

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Abstract

5-Flurouracil (FU), {5-fluoropyrimidines-2,4(1*H*,3*H*)-dione} is an anticancer drug that competitively inhibits thymidylate synthatase, an enzyme required for DNA synthesis that leads to programmed cell death of proliferating cells. It is used as topical cream for squamous cell carcinoma but a little information is available for its active drug targeting. Our attempt is to generate a validated stability indicating RP-HPLC method for the quantitation of 5-FU for active targeting to squamous cell carcinoma. In our study, RP-HPLC system used was (SHIMADZU (AT vp), Japan) and comprised of UV detector (SCL-10A vp), a pump system (LC-10 AT vp), and an injector. The separation was achieved on C_{18} LiChrospher®100 (250 x 4.6 mm, 5 µm) and elution was monitored gradiently with mobile phase consisting of HPLC water at a flow rate of 1ml/min. The injection volume was 20 µL previously filtered through 0.45µ membrane filter and elute was analyzed at the set wavelength of 269 nm. The proposed method was validated for various parameters such as specificity, linearity, precision, accuracy, and robustness. The calibration plot drawn was found to be linear over the concentration range of 1–10µg/mL and within the acceptable range (R^2 = 0.999). The developed method was found to be simple, precise, and accurate and can be reproduced with LOD and LOQ of 0.007108 and 0.021539µg/mL respectively. The degradation study at variable temperatures such as 0ºC; 8ºC, 25ºC, 37ºC and pH 1.2, 3.2, 4.2, 6.8 and 7.4 after storage for 24 h suggested that 5-FU was stable at aforementioned temperatures and pH. It further signifies that the method is suitable for in-process quality control analysis of new composition of 5- FU due to good accuracy and high degree of precision.

Keywords: 5-Fluorouracil, Active targeting, Assay validation, RP-HPLC, Squamous cell carcinoma, Solubility.

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Introduction

5-FU {5-fluoropyrimidines-2,4(1*H*,3*H*)-dione} is an anti-neoplastic agent that has fluorinated pyrimidines as shown in (Fig. 1). It also acts as anti-metabolite to uracil because of the presence of pyrimidine analogue. It competitively inhibits thymidylate synthatase, an enzyme required for DNA synthesis that leads to programmed cell death of proliferating cells. 5-FU inhibits DNA synthesis primarily by biochemical alteration forming an active metabolite 5-Fluoro-deoxyuridine monophosphate (FdUMP). The metabolite inhibits enzyme responsible for DNA synthesis. It also interferes with RNA synthesis by generating metabolite 5-Fluoro-deoxyuridine triphosphate (FUTP) to a lesser extent blocking protein synthesis and induces apoptosis[1,2].

5-FU is a white color powder, has low molecular weight 130.8g/mol, pKa value 8.0 and melting point 283ºC[3]. The drug is polar in nature and sparingly soluble in water but soluble in organic solvents such as dimethyl sulphoxide, dimethyl formamide and ethanol. It is widely used for treatment of solid tumor such as colorectal, breast, liver, brain, gastric cancer including invasive squamous cell carcinoma[4]. The parent drug is not suitable for therapy because of non-selectivity towards specific target and short biological half-life (8-20 min). Current chemotherapy requires targeting therapeutic agent at the site of tumor along with drug release in a sustained manner[5].

Several papers published in the past regarding reverse phase high performance liquid chromatography (RP-HPLC) were not appropriately validated as per the guidelines for method validation[6]. The methods either required large volume of sample or often were tedious to carry out routinely. They also used complex mobile phase composition[7-9]. Photodiode array detector used in the method[10,11] made the process draggy with long retention time[8,12,13]. Also the literature lacks the method validation of 5-FU in direct concern to squamous cell carcinoma of skin through active drug targeting. Thus, the aim of the study was to validate a simple, rapid, and a suitable RP-HPLC method for the quantitative estimation of 5-FU for therapeutic drug monitoring in proposed type of cancer.

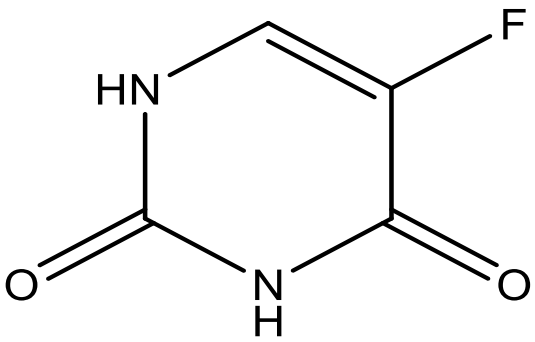

Fig. 1: Chemical structure of 5-FU

Materials and Method

Materials: 5-FU was a purchased from Avenscure (Bangalore, India). HPLC grade water, methanol, potassium dihydrogen phosphate, sodium hydroxide were purchased from Central Drug House (CDH) (New Delhi, India). All solutions and reagent were filtered through a 0.45µ membrane filter and degassed prior to use. All the samples were further filtered through MILLEX®GV Filter Unit, 0.22µ Durapore® PVDF membrane using syringe before injected into column. Stock solution was prepared every day and membrane filtered, stored in dark and in refrigerator. All chemicals and reagent used were of HPLC grade.

Instrumentation

Apparatus and Chromatographic condition: Configurationally, the HPLC system (SHIMADZU (AT vp), Japan) consisted of a UV detector (SCL-10A vp), a pump system (LC-10 AT vp), and an injector. The detection wavelength for the estimation of 5-FU was set at 269nm. The separation was achieved through the HPLC column lichrosphere® 100 PR-18 (5µ) 250×4.6 mm eluted gradiently with mobile phase HPLC grade water at a flow rate of 1 ml/min. The mobile phase was degassed before use. The HPLC system was operated at room temperature (25 ± 2°C).

Preparation of stock and calibration standard solution: The stock solution of 5-FU was freshly prepared by dissolving 10 mg of 5-FU in volumetric flask containing phosphate buffer of pH 7.4, sonicated for 2min and volume was made upto 100 ml. The final concentration of stock solution was 100µg/mL. From the stock solution of concentration 100µg/mL, a series of concentrations were prepared by diluting them with mobile phase. The concentration of different diluted calibration samples was 1, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10µg/mL.

Assay validation: The assay was validated in accordance with international conference on harmonization (ICH) and current Food and Drug Administration (FDA) guidelines for the biological method validation[6,14]. The different parameters such as linearity, accuracy, precision (intraday and interday), limit of detection (LOD) and limit of quantitation (LOQ) were studied.

The linearity of the method was established by injecting prepared calibration standard of drug in the concentration range of 1 to 10µg/mL and delineating peak area *vs* concentration. Each calibration sample was analyzed in triplicate *(n=3)* by injecting 20µL of sample solution. Calibrated samples were precisely validated for intraday and interday. For intraday precision, three different concentrations (1, 5, and 10µg/mL) of analyzing solutions were taken and each solution was injected in the chromatographic column in triplicate (*n=3*) on the same day. Similarly, interday precision was determined by repeating the experiment on three consecutive days. Before the next run of sample injection syringe was properly rinsed and injector loop was back-flushed with mobile phase at a flow rate of 1.0ml/min. The relative standard deviation (RSD) (%) values were calculated to determine the intraday and interday precision. The accuracy of the proposed analytical method was evaluated by adding three known concentrations of drug equivalent to 50%, 100% and 150% to the original concentration and determining the recovery of added drug. The experiment was performed in triplicate *(n=3)*. The robustness of the method was determined by varying the chromatographic parameters viz., flow rate to determine their influence on the quantitative analysis. The limit of detection (LOD) and limit of quantification (LOQ) were determined using standard deviation (SD) of the y-intercept and slope (S) of the calibration curve as per the given formulae:

$$LOD=3.3*\delta/S \text{ and } LOQ=10*\delta/S$$

The study was performed in triplicate *(n=3)*. All data was evaluated using statistical GraphPad Prism 4.0 (GraphPad Software, Inc., USA).

Solubility study: The solubility of 5-FU was performed by placing excess amount of 5-FU to Eppendorf tube (2 ml) containing different buffer solution and mixture was heated in water bath followed by solubilization using a vortex mixer (Tarson, India). Mixtures were shaken in a water-bath shaker (Remi, India) for 72 h

maintained at room temperature until equilibrium was attained. The equilibrated samples were centrifuged at 3,000rpm for 10min (Remi, India) to settle the undissolved 5-FU. The supernatant was separated and extracted in suitable medium for quantification of 5-FU at 269nm using HPLC.

Degradation under different pH: The stock solution of 5-FU was prepared in different pH 1.2; 3.2; 4.2; 6.8; and 7.4 and an appropriate volume was withdrawn and further diluted to prepare a solution of final concentration 5µg/mL. The diluted solution of each pH was analyzed for drug content. The experiment was performed in triplicate *(n=3)*.

Degradation under different temperature: The stock solutions were prepared in phosphate buffer of pH 7.4 at concentration of 100µg/mL and stored at 0ºC; 8ºC, 25ºC, 37ºC for 24h and then analyzed for drug content. The experiment was performed in triplicate *(n=3)*.

Results and Discussion

HPLC with UV detection was preferred as a simple, fast and effective separation method for the determination of 5-FU and its degradation under variable temperature and pH. The mobile phase selection was based on sensitivity, ease of preparation or availability, cost and suitability for drug content estimation. The mobile phase comprised of HPLC grade water and was found to be suitable as allowed complete separation of the drug using a C_{18} column at a flow-rate of 1.0mL/min. The HPLC grade water has advantage over buffered mobile phase because the later may choke and reduce the longevity of C_{18} column. The combination of mobile phase methanol: water (20:80) was also tried but because of high pressure generated it was omitted from the study. The chosen chromatographic conditions provided optimum resolution of 5-FU and retention time was 1.907 ± 0.020 min ever been reported for 5-FU. The set wavelength of UV-detection of 5-FU and sample run time was 269 nm and 10 min respectively. A typical HPLC chromatogram of 5-FU in which peak is well defined and free from tailing is shown in (Fig. 2**)**.

The calibration curve was found to be linear over the concentration range of 1-10µg/mL. This study signifies that method is able to analyze the sample solution in concentration range where analyte response is linearly proportional to concentration. The linear regression equation of this calibration curve was y=44077 x + 20311. The coefficient of correlation (R^2) was 0.999 (Fig. 3. and Table 1) and the RDS (%) was found to be < 1 %. There was no significant difference between the slopes of calibration plots constructed on different days ($p < 0.05$).

The accuracy of the analytical method is the intimacy towards the true value. The accuracy was determined in terms of recovery (%). The recovery of the assay method was 99.80–100.63% after spiking a previously analyzed test solution with additional standard drug solution. RSD (%) was found to be < 1% indicate that the proposed method was accurate. The values of recovery (%) and RSD (%) are shown in the Table 2.

As per ICH guidelines, precision is considered at two levels; repeatability (intraday) and intermediate (interday) precision. In fact, precision is the variation in the replicate value of homogenous sample. The intraday and interday variability or precision data are summarized in Table 3. It was calculated in terms of RSD (%). The low value (< 1 %) of RSD indicates the repeatability of the method.

The LOD and LOQ indicates the method can be used for the detection and quantitation of 5-FU in a very low concentration. The value of LOD and LOQ indicates the sensitivity of the validated method. The LOD and LOQ determined by standard deviation method were 0.007108 and 0.021539µg/mL respectively.

Robustness was analyzed to assess the influence of deliberate small changes in the chromatographic condition for the quantitation of 5-FU. It was estimated by applying small changes in the flow rate (1±0.2 ml/min) and mobile phase composition HPLC water: methanol in different proportion. The inclusion of methanol in mobile phase led to the problem of high pressure and therefore it was withdrawn. There was no significant change in the retention time of 5-FU was observed with changes in the flow rate. The RSD (%) value (< 1 %) indicated robustness of the method as shown in Table 4.

The solubility of drug over varied pH is required to estimate its potential for tissue targeting. Generally, the drug has to face variable pH of extracellular fluid before reaching to the target region. Therefore, we have estimated the solubility of drug at different pH likely to contribute to the microenvironment of squamous cell carcinoma. Solubility of 5-FU was estimated by developed RP-HPLC. The pH solubility profile of the drug was generated by precise content analysis of drug at different pH. The results are shown in the (Fig. 4). The method was found to be sensitive to generate pH solubility profile of the drug which has not been reported elsewhere in the literature.

The degradation study of 5-FU at 0ºC; 8ºC (refrigeration temperature); 25ºC; and 37ºC was carried to know the stability of drug at low temperature, room temperature and at physiological temperature of body. The result indicated that altering temperature would not affect the stability 5-FU. The stability of 5-FU at physiological temperature of body is an important aspect of the study. The HPLC chromatogram of 5-FU at this temperature is depicted in (Fig. 5).

Degradation study of 5-FU at different pH (1.2; 3.2; 4.2; 6.8 and 7.4) necessitates the active drug targeting to the squamous cell carcinoma of skin.

Following topical administration of therapeutic agent loaded in functionalized nanoparticles, it has to traverse a long journey before reaching to a destination. The acidic pH of skin poses some barrier to the drug followed by different diversified layer of skin and therefore, making hindrance to the active drug targeting. The trend of pH of skin changes from acidic to basic viz., physiological pH of blood. The degradation study of 5-FU at this pH makes system more robust towards stability. As we know that through active drug targeting therapeutic entity is delivered to specific region more efficiently than passive pathway. This study would help us to analyze the decomposed concentration of drug or concentration remained at the site of action. The HPLC chromatogram of 5-FU obtained by this study is shown in (Fig. 6).

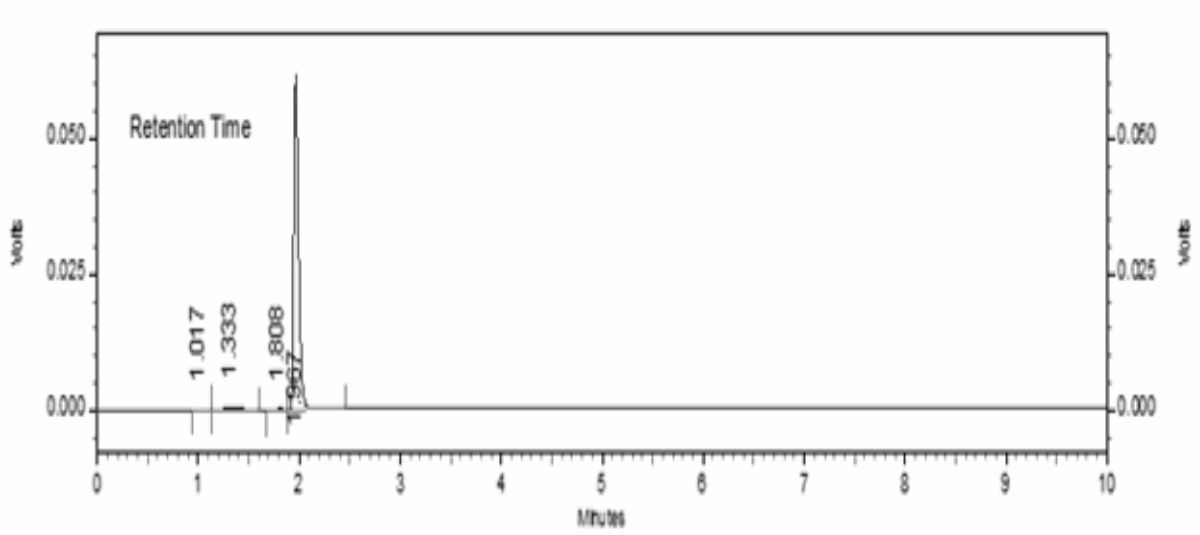

Fig. 2: A typical HPLC chromatogram of 5-Fluorouracil (5-FU)

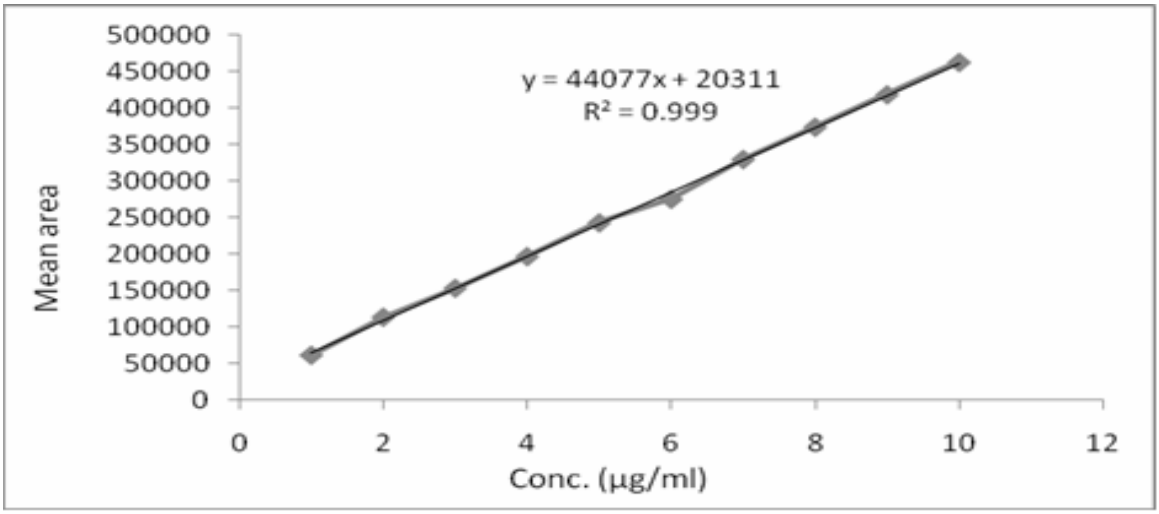

Fig. 3: Calibration curve of 5-FU in buffer medium

Table 1: Analytical parameters using RP-HPLC method

S. No.	Parameters	Numeral values
1	Linearity range	1-10µg/ml
2	Detection wavelength (λ_{max})	269nm
3	Regressed equation (Y^{β})	Y^{β}=44077x+20311
4	Slope (S)	44077
5	Intercept (c)	20311
6	Coefficient of correlation (R^2)	0.999

Table 2: Accuracy of the assay method

Conc. taken (µg/ml)	Excess amount added (µg/ml)	Total conc. (µg/ml)	Conc. detected (µg/ml) ± SD	% Recovery	% RSD
1	0%	1	0.998 ± 0.0089	99.80	0.89
2	50%	3	3.002 ± 0.0031	100.23	0.30
3	100%	6	6.00 ± 0.0035	99.87	0.17
4	150%	10	10.01 ± 0.0031	100.63	0.30

Table 3: Precision of the assay method (Intra and Interday)

Conc. (µg/ml)	Precision			
	Intraday		Interday	
	Mean conc. ± SD	RSD %	Mean conc. ± SD	RSD %
1	0.98 ± 0.025	0.25	1.00 ± 0.010	0.87
5	5.01 ± 0.040	0.8	5.01 ± 0.030	0.59
10	10.01 ± 0.053	0.39	10.03 ± 0.046	0.53

Table 4: Robustness of the assay method

Flow rate (mL/min)	Mean peak area ± SD	RSD %
0.8	3.00 ± 0.008	0.25
1.0	3.01 ± 0.026	0.88
1.2	3.01 ± 0.015	0.48

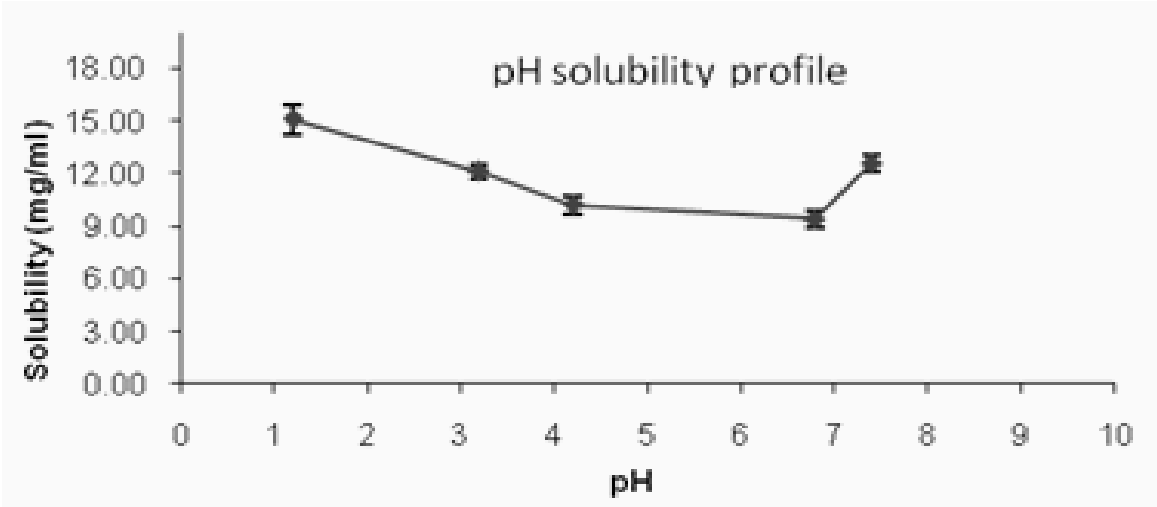

Fig. 4: pH solubility profile

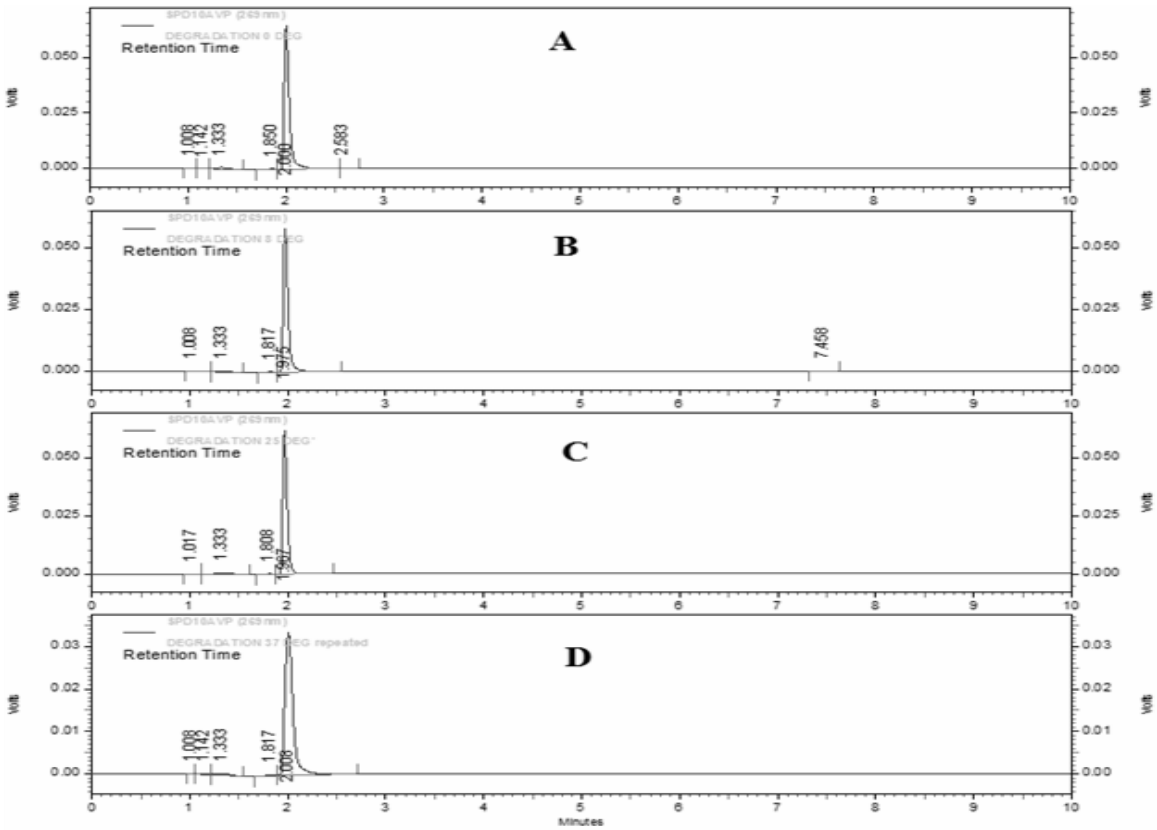

Fig. 5: HPLC chromatogram of 5-FU solution for their degradation at different temperature after 24h of storage at 0ºC (A), at 8ºC (B), at 25ºC (C), and at 37ºC (D)

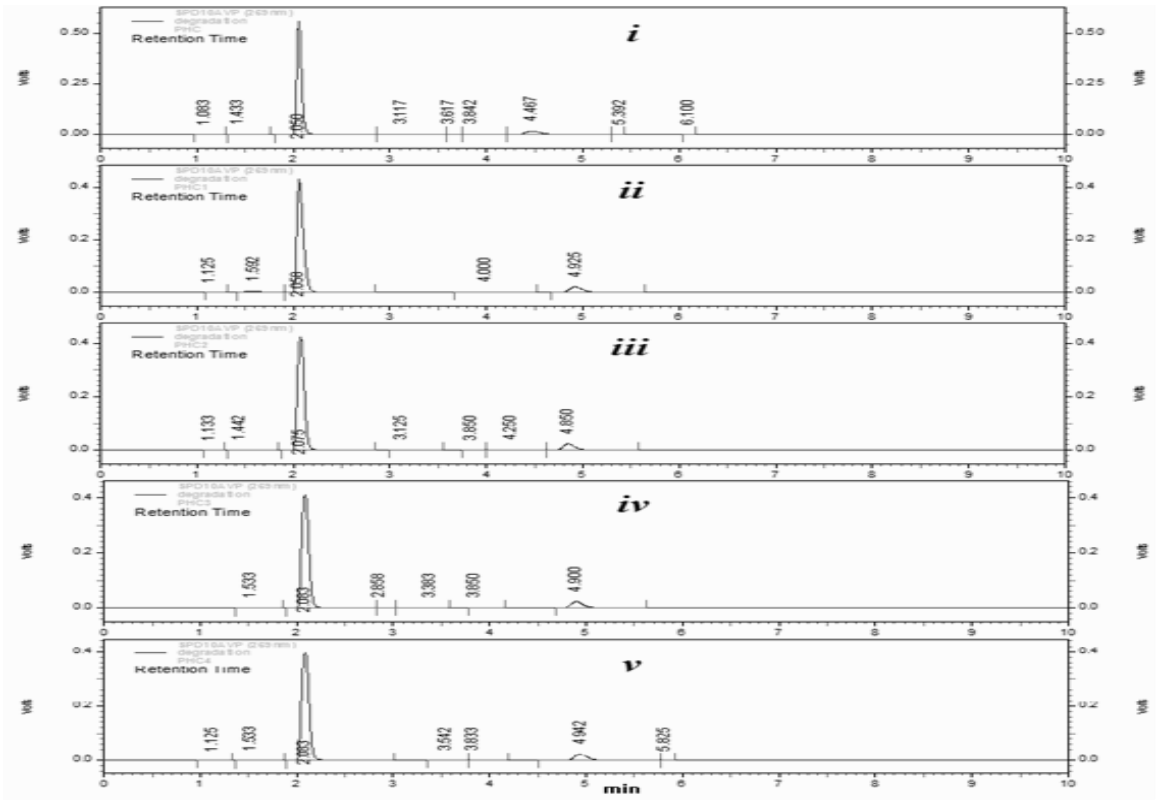

Fig. 6: HPLC chromatogram of 5-FU solution for their degradation under different pH after 24 h of storage at 1.2 (*i*); at 3.2 (*ii*); at 4.2 (*iii*); at 6.8 (*iv*), and at 6.8 (*v*)

Conclusion

A rapid, accurate, simple and precise analytical method was developed with RP-HPLC and validated as per ICH guidelines with respect to active drug targeting to squamous cell carcinoma of skin. The low retention time 1.907±0.020 min reported by using column LiChrospher®100 (250 x 4.6mm, 5µm) has never been reported for the said drug and this makes the method more economical and safe with respect to green solvent used. The developed method was highly sensitive and robust as the small but deliberate change in flow rate did not alter the peak area. The degradation study at variable temperature and pH suggested that 5-FU was stable. Therefore, developed method seemed to be suitable for routine analysis of 5-FU in biological fluids.

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Conflict of Interest: None

Source of Support: Nil

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