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Validated High Performance Liquid Chromatographic Techniques for the Determination of Gemcitabine and Irinotecan with Applications to Stability Studies

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Abstract

Objective: The objective of the present research work was to develop an innovative, simple, and economic method for estimation of Gemcitabine and Irinotecan in bulk and dosage form by RP-HPLC. **Methods:** The chromatographic conditions were performed on Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d. as stationary phase and mobile phase was prepared with a mixture of Methanol : Phosphate buffer (0.02M, pH-2.6) = (55:45), flow 1.0 ml/min, with Injection Volume 10µl, at detection wavelength 255 nm and run time at 7.0 mins. **Results:** The analytical method is valid for estimation of Gemcitabine and Irinotecan over a range of 6 µg/ml–14 µg/ml and 12 µg/ml–28 µg/ml. The results of system suitability test, linearity, precision and accuracy, robustness, specificity, LOD and LOQ and stabilities presented in this report are within the acceptance range. **Conclusion:** A specific, sensitive, economic method estimation of Gemcitabine and Irinotecan has been developed based on ICH Guidelines with bulk and dosage forms.

Keywords
Gemcitabine and Irinotecan, HPLC, Method Development, ICH, Validation, Accuracy, Precision.

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1. INTRODUCTION:

Gemcitabine, sold under the brand name Gemzar, among others, is a medication used to treat a number of types of cancer. These cancers include breast cancer, ovarian cancer, non-small cell lung cancer, pancreatic cancer, and bladder cancer. It is given by slow injection into a vein.[1-3] Common side effects include bone marrow suppression, liver and kidney problems, nausea, fever, rash, shortness of breath, mouth sores, diarrhea, neuropathy, and hair loss.[4-6] Use during pregnancy will likely result in harm to the baby. Gemcitabine is in the nucleoside analog family of medication. It works by blocking the creation of new DNA, which results in cell death.[7] Gemcitabine is used in various carcinomas. It is used as a first - line treatment alone for pancreatic cancer, and in combination with cisplatin for advanced or metastatic bladder cancer and advanced or

metastatic non-small cell lung cancer. [8-10] It is used as a second-line treatment in combination with carboplatin for ovarian cancer and in combination with paclitaxel for breast cancer that is metastatic or cannot be surgically removed. It is commonly used off-label to treat cholangiocarcinoma and other biliary tract cancers. It is given by injection into a vein at a chemotherapy clinic. [11]

The IUPAC Name of Gemcitabine is 4-amino-1-[(2R,4R,5R)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl) oxolan-2-yl]-1,2-dihydropyrimidin-2-one. [12]

Irinotecan, sold under the brand name Camptosar among others, is a medication used to treat colon cancer, and small cell lung cancer. For colon cancer it is used either alone or with fluorouracil. For small cell lung cancer, it is used with cisplatin. [13] It is given by slow injection into a vein. Common side effects include diarrhea, vomiting, bone marrow suppression, hair loss, shortness of breath and fever. Other severe side effects include blood clots, colon inflammation, and allergic reactions. [14-16] Those with two copies of the UGT1A1*28 gene variant are at higher risk for side effects. Use during pregnancy can result in harm to the baby. Irinotecan is in topoisomerase inhibitor family of medication. It works by blocking topoisomerase 1 which results in DNA damage and cell death. Irinotecan-associated diarrhea is severe and clinically significant, sometimes leading to severe dehydration requiring hospitalization or intensive care unit admission. [17] This side - effect is managed with the aggressive use of antidiarrheals such as loperamide or co-phenotrope with the first loose bowel movement.

The IUPAC Name of Irinotecan is (19S)-10,19-diethyl-19-hydroxy-14,18-dioxo-17-oxa-3,13-diazapentacyclo [11.8.0.0^ {2,11}.0^ {4,9}.0^ {15,20}] henicosa-1(21),2,4(9),5,7,10,15(20)-heptaen-7-yl[1,4'-bipiperidine]-1'-carboxylate. [18]

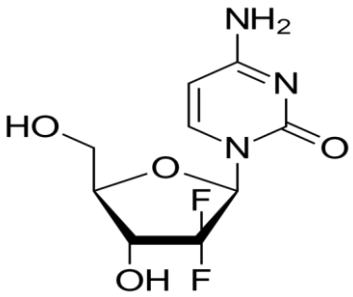


Fig-1: Structure of Gemcitabine

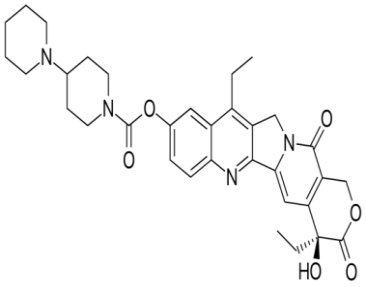


Fig-2: Structure of Irinotecan

A survey of literature reveals that good analytical methods are not available for Gemcitabine and Irinotecan. The present research manuscript describes innovative, simple, economical, accurate, specific, robust, rugged and rapid RP-HPLC method developed in selected solvent system (Mobile Phase) and validated in accordance with International Conference on Harmonization (ICH) Guidelines Q2 (R1), for the estimation of Gemcitabine and Irinotecan in bulk drug and in its dosage forms.

2. EXPERIMENTAL:

2.1 Materials and Methods:

Pharmaceutical grade working standard Gemcitabine and Irinotecan were obtained from Syncorp Pvt. Laboratories, Hyderabad, India. All chemicals and reagents were HPLC grade and were purchased from S D Fine-Chem Limited & Loba Chemie Pvt Ltd, Mumbai, India.

2.2 Instrumentation:

The analysis was performed using HPLC (Waters-717 series) with PDA detector and data handling system EMPOWER2 software, UV-Visible double beam spectrophotometer (T-60 LABINDIA), analytical balance 0.1mg Sensitivity (SHIMADZU), pH meter (Labindia), ultra sonicator. The column used is Phenomenex Luna C_{18}, 100A, 5µm, 250mmx4.6mm i.d. (as Stationary phase) with the flow rate 1.0ml/min (isocratic).

2.3 Sample and Standard Preparation for the Analysis

25 mg of Gemcitabine standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 0.5 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

25 mg of Irinotecan standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 0.5 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

2.4 Selection of wavelength

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV analysis. It scanned in the UV spectrum in the range of 200 to 400nm. While scanning the Gemcitabine and Irinotecan solution we observed the maxima at 260 nm and 247 nm. The isobestic point for the drugs was found at 255nm.

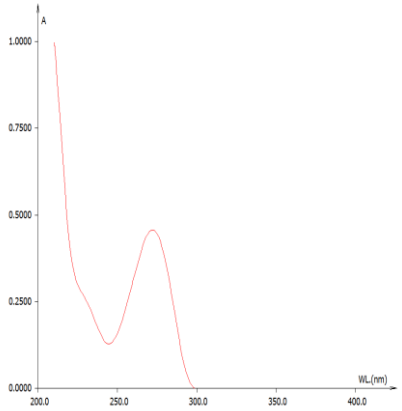


Fig – 3. UV Spectrum for Gemcitabine

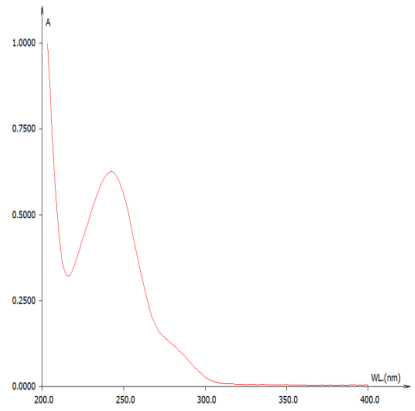


Fig-4. UV Spectrum for Irinotecan

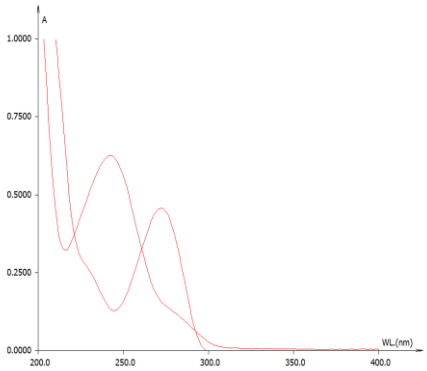


Fig -5. Isobestic Point for Gemcitabine and Irinotecan

2.5 Method Development

2.5.1 Preparation of 0.02M Phosphate Buffer Solution:

Weighed 0.50 grams of di-sodium hydrogen phosphate and 0.301 grams of potassium dihydrogen phosphate was taken into a 1000 ml beaker, dissolved and diluted to 1000 ml with HPLC water, adjusted the pH to 2.6 with orthophosphoric acid.

2.5.2 Preparation of Mobile Phase:

The mobile phase was prepared with the combination of Methanol and Phosphate buffer (0.02 M, pH-2.6) at the volume of 1000 ml. 550 ml of Methanol and 450 ml of Phosphate buffer were mixed well and degassed in ultrasonic water bath for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration.

2.5.3 Summary of Optimized Chromatographic Conditions:

The Optimum Chromatographic conditions obtained from experiments can be summarized as below:

Table-1: Summary of Optimized Chromatographic Conditions

Mobile phase	Methanol: Phosphate buffer (0.02M, pH-2.6) = 55:45
Column	Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d.
Column Temperature	Ambient
Detection Wavelength	255 nm
Flow rate	1.0 ml/ min.
Run time	07 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	10µl
Type of Elution	Isocratic

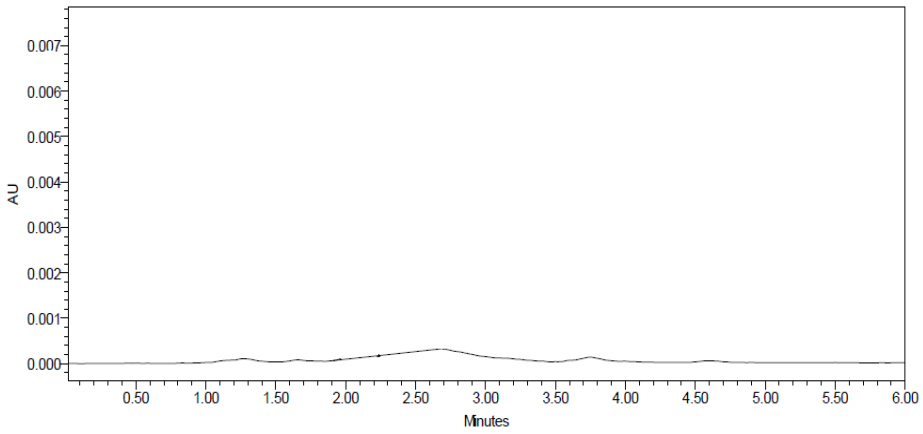
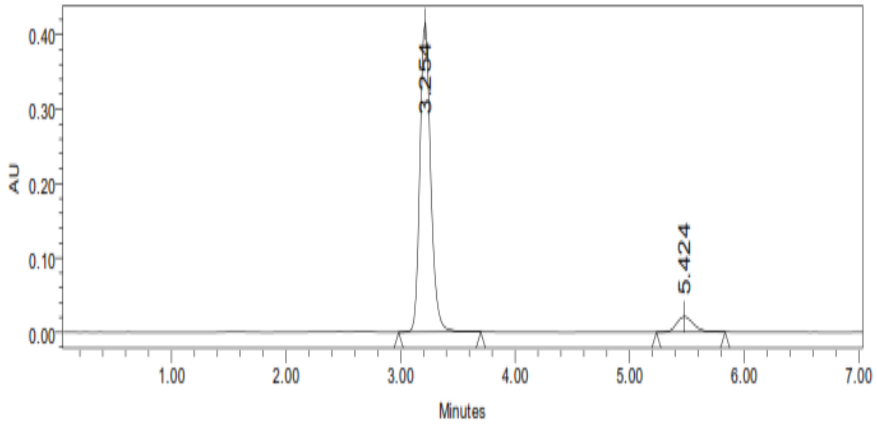


Fig-6: Chromatogram for Blank Preparation



Fig–7: Chromatogram of Gemcitabine and Irinotecan in Optimized Condition

2.6 Method validation:

2.6.1 Linearity & Range:

Calibration standards at five levels were prepared by appropriately mixed and further diluted standard stock solutions in the concentration ranges from 6-14 µg/ml and 12-28 µg/ml for Gemcitabine and Irinotecan. Samples in triple injections were made for each prepared concentration. Peak areas were plotted against the corresponding concentration to obtain the linearity graphs. Chromatograms of each solution were recorded.

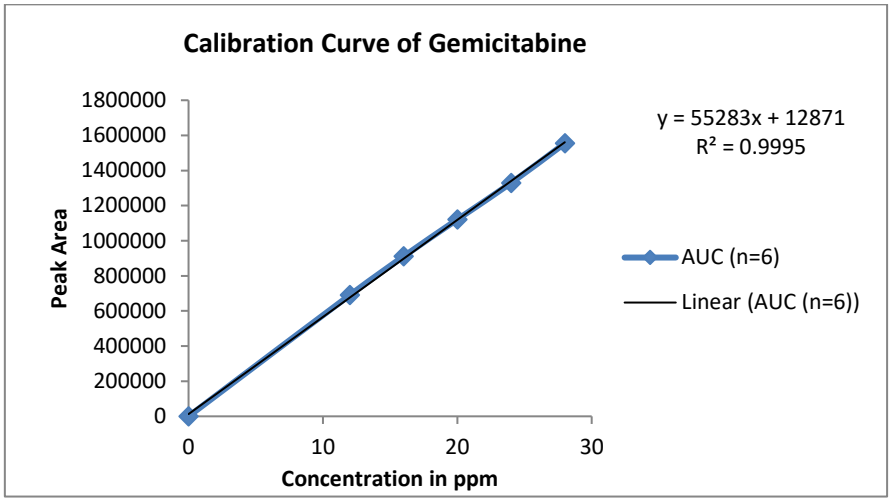


Fig-8: Standard curve for Gemcitabine

Table-2: Linearity Readings for Gemcitabine

CONC. (µg/ml)	MEAN AUC (n=6)
0	0
6	192164
8	247293
10	306089
12	370481
14	447930

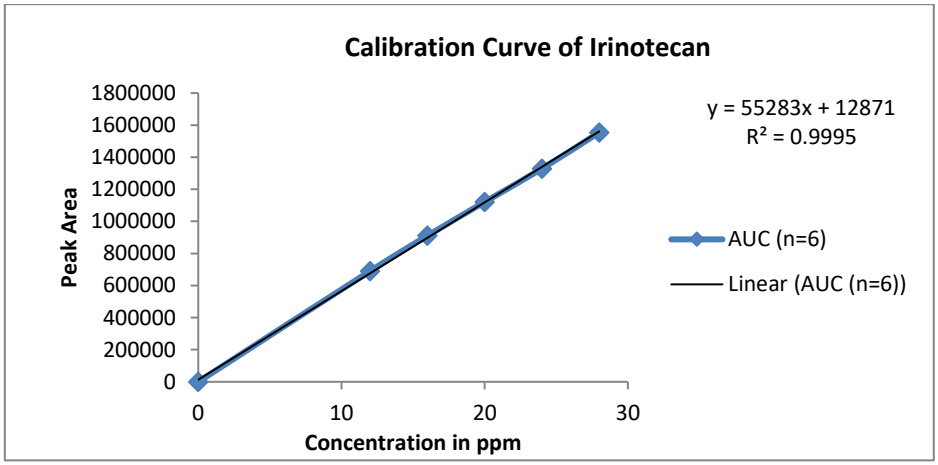


Fig-9: Standard curve for Irinotecan

Table-3: Linearity Readings for Irinotecan

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
12	690316
16	910621
20	1121057
24	1328903
28	1554666

2.6.2. Accuracy:

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80 %, 100 % and 120 %) of pure drug of Gemcitabine and Irinotecan were taken and added to the pre-analyzed formulation of concentration 10 µg/ml and 20 µg/ml. From that percentage recovery values were calculated. The results were shown in table - 4 and 5.

Table-4: Accuracy Readings of Gemcitabine

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	8	8.064107	458679	99.867	Mean= 100.4113%
S_2 : 80 %	8	7.843532	446485	100.637	S.D. = 0.473694346
S_3 : 80 %	8	8.19449	465887	100.73	% R.S.D.= 0.471753
S_4 : 100 %	10	9.892661	559767	99.41	Mean= 100.6646667%
S_5 : 100 %	10	9.978655	564521	100.868	S.D. = 1.166369295 R.S.D.=
S_6 : 100 %	10	10.19623	576549	101.716	1.158667
S_7 : 120 %	12	11.85907	668476	99.878	Mean= 100.4637%
S_8 : 120 %	12	12.16785	685546	100.69	S.D. = 0.51154309
S_9 : 120 %	12	12.18644	686574	100.823	% R.S.D. = 0.509181

Table-5: Accuracy Readings of Irinotecan

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	16	15.71861	881843	98.24132	Mean= 98.66425667%
S_2 : 80 %	16	15.75267	883726	98.4542	S.D. = 0.558426265% R.S.D.=
S_3 : 80 %	16	15.88756	891183	99.29725	0.565996
S_4 : 100 %	20	20.00427	1118767	100.0213	Mean= 100.8802%
S_5 : 100 %	20	20.37881	1139473	101.8941	S.D. = 0.945972362
S_6 : 100 %	20	20.14504	1126549	100.7252	% R.S.D.= 0.9377182
S_7 : 120 %	24	23.69705	1322915	98.73771	Mean= 98.87614%
S_8 : 120 %	24	23.73053	1324766	98.87722	S.D. = 0.137893172
S_9 : 120 %	24	23.76324	1326574	99.01349	% R.S.D. = 1.401528

2.6.3. Precision:

2.6.3.1. Repeatability

The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug. Gemcitabine and Irinotecan (API). The percent relative standard deviation was calculated for Gemcitabine and Irinotecan are presented in the Table - 6.

Table-6: Repeatability Readings of Gemcitabine and Irinotecan

HPLC Injection Replicates	AUC for Gemcitabine	AUC for Irinotecan
Replicate – 1	623568	1113214
Replicate – 2	613241	1105241
Replicate – 3	625408	1113424
Replicate – 4	617412	1105987
Replicate – 5	612541	1104216
Replicate – 6	622546	1113219
Average	615786	1109216.833
Standard Deviation	5510.431332	4493.157884
% RSD	0.890043	0.405074

2.6.3.2. Intermediate precision:
The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Gemcitabine and Irinotecan revealed that the proposed method is precise.

Table-7: Results of Intra-Assay & Inter-Assay for Gemcitabine

Conc. Of Gemcitabine (API) (µg/ml)	Observed Conc. Of Gemcitabine (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
8	8.21	0.76	8.23	0.46
10	10.37	0.33	10.36	0.57
12	12.56	0.23	12.56	0.75

Table-8: Results of Intra-Assay & Inter-Assay for Irinotecan

Conc. Of Irinotecan (API) (µg/ml)	Observed Conc. of Irinotecan (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
16	16.12	0.65	16.34	0.55
20	20.43	0.54	20.67	0.27
24	24.33	0.76	24.37	0.51

2.6.4. Method Robustness:
Influence of little changes in optimized chromatographic conditions like changes in flow rate (± 0.1 ml/ min), mobile phase ratio (±2 %), Wavelength of detection (±2 nm) and organic phase (±5 %) studied to measure the robustness of the method are also in favour of (Table - 9, % RSD < 2 %) the developed RP-HPLC method for the analysis of Gemcitabine and Irinotecan (API).

Table-9: Results of Method Robustness Test for Gemcitabine

Change in parameter	% RSD
Flow (0.8 ml/min)	0.55
Flow (1.2 ml/min)	0.86
More Organic	0.88
Less Organic	0.81
Wavelength of Detection (261 nm)	0.81
Wavelength of detection (257 nm)	0.79

Table-10: Results of Method Robustness Test for Irinotecan

Change in parameter	% RSD
Flow (0.8 ml/min)	1.03
Flow (1.2 ml/min)	0.68
More Organic	0.77
Less Organic	0.63
Wavelength of Detection (233 nm)	1.09
Wavelength of detection (229 nm)	0.92

2.6.5. LOD & LOQ:
The detection limit (LOD) and quantitation limit (LOQ) may be expressed as:
L.O.D. = 3.3(SD/S).
L.O.Q. = 10(SD/S)
Where, SD = Standard deviation of the response
S = Slope of the calibration curve
The LOD was found to be 0.06 µg/ml and 0.09 µg/ml for Gemcitabine and Irinotecan respectively. The LOQ was found to be 0.18 µg/ml and 0.27 µg/ml for Gemcitabine and Irinotecan respectively.

2.6.6. System Suitability Parameter
System suitability testing is an integral part of many analytical procedures. The tests are based on the

concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established. The data are shown in Table-11.

Table-11: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs> 2	3.57
2	Asymmetry	T ≤ 2	Gemcitabine = 0.12 Irinotecan = 0.24
3	Theoretical plate	N > 2000	Gemcitabine = 2987 Irinotecan = 3014

2.6.6 Estimation of Gemcitabine and Irinotecan in Tablet Dosage Form

Twenty tablets/Capsules were taken and the I.P. method was followed to determine the average weight. Finally, the weighed tablets are powdered and triturated well by using mortar and pestle. A quantity of powder which is equivalent to the 100mg of drugs were transferred to a clean and dry 100ml of volumetric flask and add 70 ml of mobile phase and the resulted solution was sonicated for 15 minutes by using ultra sonicator, Then the final volume was make up to the mark with the mobile phase. The final solution was filtered through a selected membrane filter (0.45 µm) and in order to sonicated to degas the mobile phase (Solvent system). From this above stock arrangement (1 ml) was exchanged to five distinctive 10 ml volumetric flagons and volume was made up to 10 ml with same dissolvable framework (Mobile stage). The readied arrangements were infused in five repeats into the HPLC framework and the perceptions were recorded. A duplicate injection (Blank Solution) of the standard arrangement likewise infused into the HPLC framework and the chromatograms and peak zones were recorded and figured.

Table-12: Assay of GEMCITABINE and IRINOTECAN Tablets

This Combination is not Available	Labelled amount of Drug (mg) Gemcitabine /Irinotecan	Mean (±SD) amount (mg) found by the proposed method (n=6)	Mean (± SD) Assay (n = 6)
Synthetic mixture of Gemcitabine and Irinotecan	100/200	99.2(±0.56)/199.5(±0.63)	99.2 (±0.284)/99.75 (±0.396)

2.6.7 Stability studies:

The API (Gemcitabine and Irinotecan) was subjected to stress conditions in various ways to observe the rate and extent of degradation that is likely to occur in the course of storage and/or after administration to body. The various degradation pathways studied are acid hydrolysis, basic hydrolysis, thermal degradation, photolytic degradation and oxidative degradation.

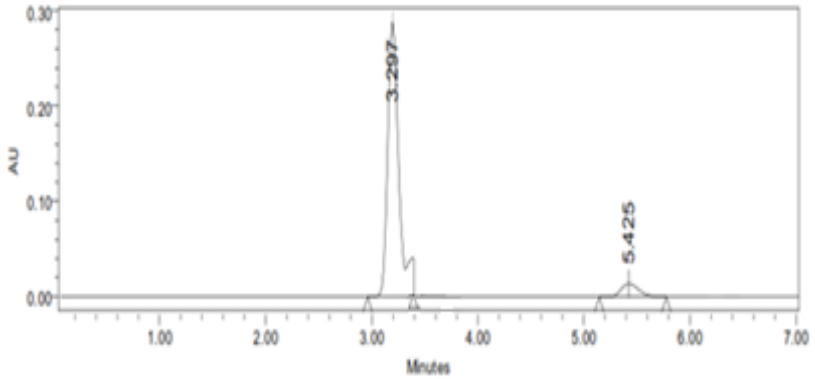


Fig-8: Chromatogram for Acid Degradation

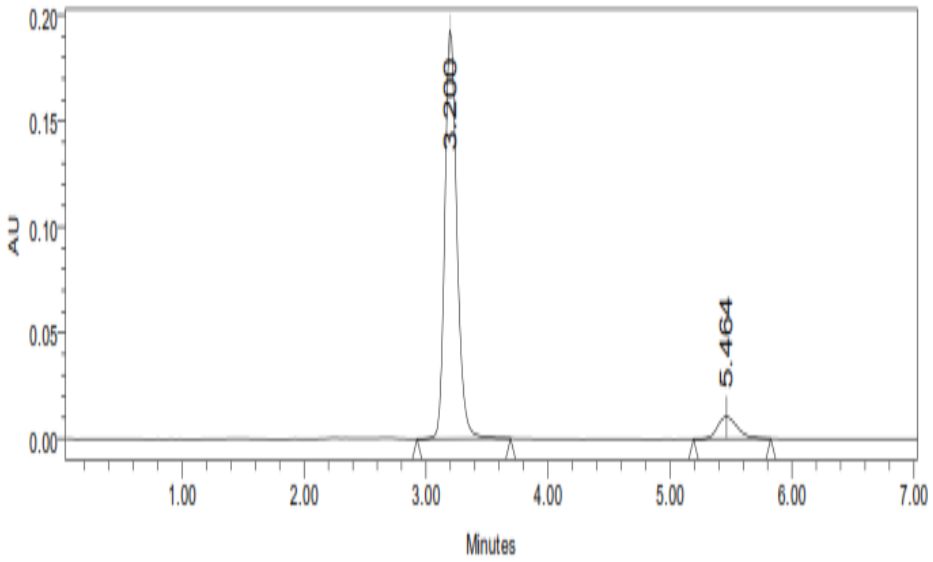


Fig-9: Chromatogram for Basic Degradation

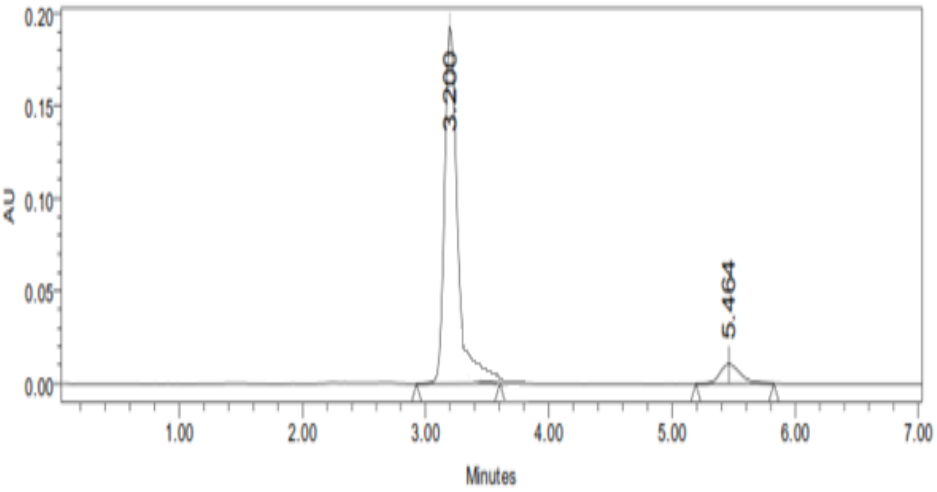


Fig-10: Chromatogram for Thermal Degradation

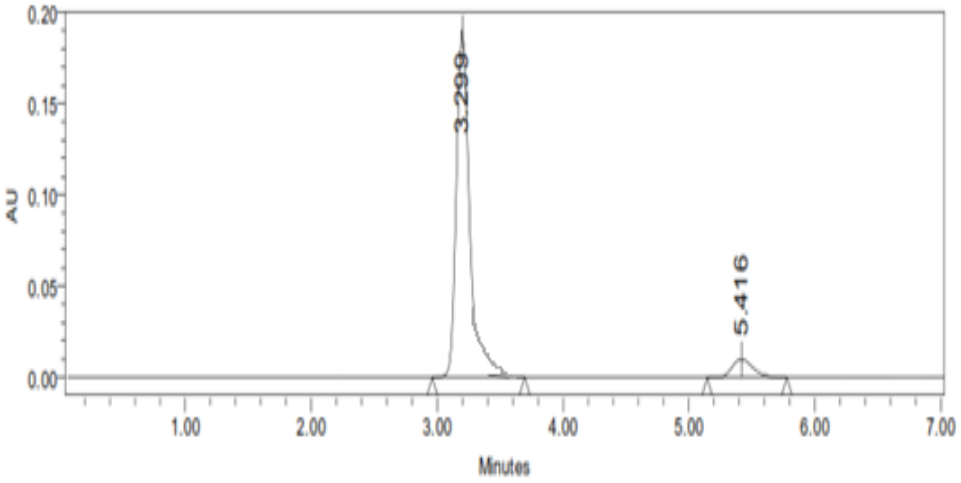


Fig-11: Chromatogram for Photolytic Degradation

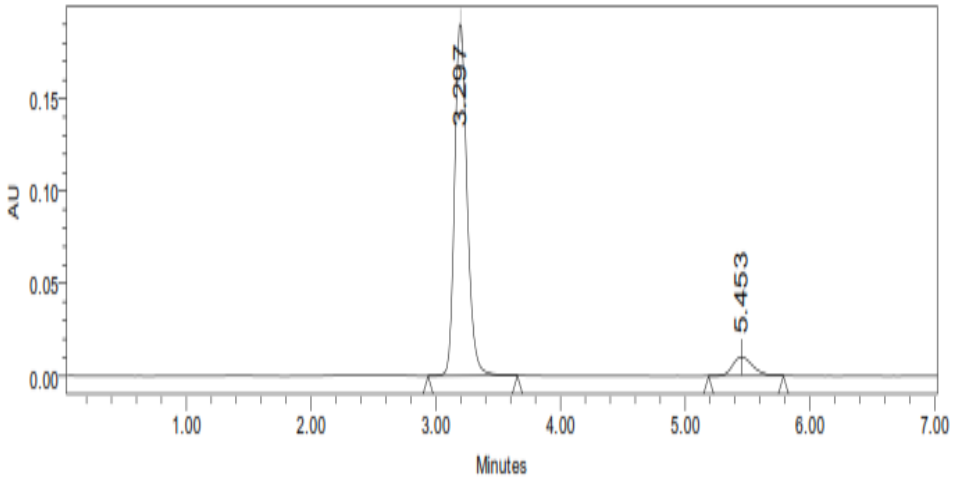


Fig-12: Chromatogram for Oxidation with 3% H_2O_2 Degradation

Table-12: forced degradation studies of Gemcitabine and Irinotecan API.

Stress condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	95.62	4.38	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	97.13	2.87	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	96.24	3.76	100.00
UV (254nm)	24Hrs.	95.43	4.57	100.00
3% Hydrogen peroxide	24Hrs.	96.16	3.84	100.00

3. RESULTS:

The optimized chromatographic conditions were Develosil ODS HG-5 RP C_{18}, 5µm, 15 cm x 4.6 mm i.d. as stationary phase and mobile phase was prepared with a mixture of Methanol : Phosphate buffer (0.02 M, pH-2.6) = (55:45), flow 1.0 ml/ min, with Injection Volume 10 µl, at detection wavelength 255 nm and run time at 7.0 min. In these chromatographic conditions the peak was pure, sharp, symmetric and found a greater number of theoretical plates.

The results obtained in method validation were:

Linearity & Range: The calibration curve showed good linearity in the range of 6-14 µg/ml and 12-28 µg/ml, for Gemcitabine and Irinotecan (API) with correlation coefficient (r^2) of 0.999 and 0.999. A typical calibration curve has the regression equation of y = 55283x + 12871 and y = 55283x + 12871 for Gemcitabine and Irinotecan.

Accuracy: The mean recoveries were found to be 100.4113, 100.6646667, 100.4637% for Gemcitabine and 98.66425667, 100.8802, 98.87614% Irinotecan. The limit for mean % recovery is 98-102% and as both the values are within the limit, hence it can be said that the proposed method was accurate.

Repeatability: The repeatability study which was conducted on the solution having the concentration of about 10 µg/ml and 20 µg/ml for Gemcitabine and Irinotecan showed % RSD of 0.890043 % and 0.405074 %. It was concluded that the analytical technique showed good repeatability.

LOD & LOQ: The Minimum concentration level at which the analyte can be reliable detected (LOD) are 0.06 µg/ml and 0.09 µg/ ml for Gemcitabine and Irinotecan. The quantified (LOQ) were found to be 0.18 µg/ml and 0.27 µg/ml respectively.

Assay: The assay in Vogipax M Tablet containing Gemcitabine and Irinotecan was found to be 99.2% and 99.75%.

Degradation studies: The results of the stress studies indicated the specificity of the method that has been developed. Gemcitabine and Irinotecan were stable only in acidic, basic and thermal stress conditions and photolytic stress conditions.

4. DISCUSSION:

To develop a precise, linear, specific RP-HPLC method for analysis of Gemcitabine and Irinotecan, different chromatographic conditions were applied & the results observed were compared with the methods available in literatures.

Kuna Mangamma, et al. achieved separation by using acetonitrile and phosphate buffer in the ratio of 60:40v/v as mobile phase.[19] Koduru Swathi, et al developed method by using a mobile phase in

combination of Acetonitrile: Phosphate buffer pH adjusted to 3.80 orthophosphoric acid in the ratio of 80:20 v/v but we have used Methanol : Phosphate buffer (0.02 M, pH-2.6) = (55:45).[20] As per B. Siddartha, et al. used Hypersil BDS C18 column (250 x 4.6 mm x 5 µ) with a mobile phase composed of buffer and acetonitrile in the ratio of 93:7 v/v in isocratic mode, maintained at ambient temperature, is used as stationary phase applied for pharmaceutical dosage form.[21]

The result shows the developed method is yet another suitable method for assay which can help in the analysis of Gemcitabine and Irinotecan in formulations.

5. CONCLUSION:

A sensitive & selective stability indicting RP-HPLC method has been developed & validated for the analysis of Gemcitabine and Irinotecan API. Based on peak purity results, obtained from the analysis of samples using described method, it can be concluded that the absence of co-eluting peak along with the main peak of Gemcitabine and Irinotecan indicated that the developed method is specific for the estimation of Gemcitabine and Irinotecan. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility.

6. REFERENCES:

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