A SIMPLE STABILITY INICATING ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANOUS ESTIMATION OF GEMCITABINE, IRINOTECAN IN API AND TABLET DOSAGE FORM BY RP-HPLC.

A.SOWNDARYA[1], DR.SUBHAS SAHOO[2]*

Department of Pharmaceutical Analysis and Quality Assurance, Pulla Reddy Institute of Pharmacy, Gummadidala (M), Hyderabad.

ABSTRACT : *The objective of the present research work was to develop an innovative, simple, and economic method for estimation of Gemcitabine, Irinotecan in bulk and dosage form by RP-HPLC.The chromatographic conditions were performed on Symmetry ODS RP C_{18},5µm, 15mm x 4.6mm i.d..as stationary phase and mobile phase was prepared with a mixture of ACN : Methanol with (90 : 10), flow 1.0 ml/min, Injection Volume 20µl, at detection wavelength 247 nm and run time at 10.0 mins.The analytical method is valid for estimation of Gemcitabine, Irinotecan over a range of 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.A specific, sensitive, economic method estimation of Gemcitabine, Irinotecan has been developed based on ICH Guidelines with bulk and dosage forms.*

Key Words: *Gemcitabine, Irinotecan HPLC, Method Development, ICH, Validation, Accuracy, Precision.*

I.INTRODUCTION

Gemcitabine inhibits thymidylate synthetase, leading to inhibition of DNA synthesis and cell death. Gemcitabine is a prodrug so activity occurs as a result of intracellular conversion to two active metabolites, gemcitabine diphosphate and gemcitabine triphosphate by deoxycitidine kinase. Gemcitabine diphosphate also inhibits ribonucleotide reductase, the enzyme responsible for catalyzing synthesis of deoxynucleoside triphosphates required for DNA synthesis. Finally, Gemcitabine triphosphate (diflurorodeoxycytidine triphosphate) competes with endogenous deoxynucleoside triphosphates for incorporation into DNA.

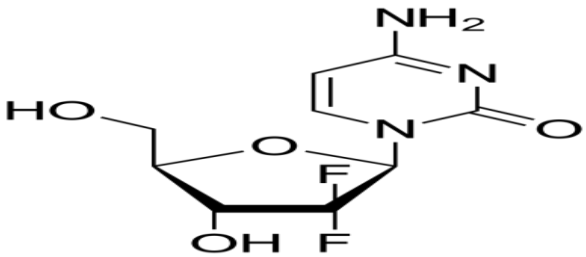


Fig-1: Structure of Gemcitabine

Irinotecan is an antineoplastic enzyme inhibitor primarily used in the treatment of colorectal cancer. It is a derivative of camptothecin that inhibits the action of topoisomerase I. Irinotecan prevents religation of the DNA strand by binding to topoisomerase I-DNA complex, and causes double-strand DNA breakage and cell death. It is a derivative of camptothecin. Irinotecan was approved for the treatment of advanced pancreatic cancer in October, 2015 (irinotecan liposome injection, trade name Onivyde).

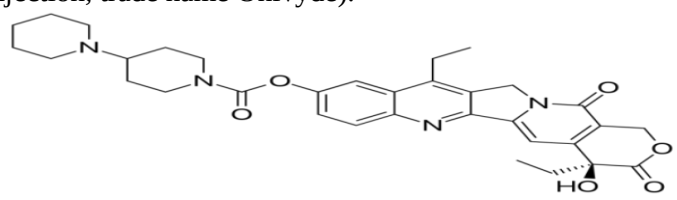


Fig-2: Structure of Irinotecan

II.EXPERIMENTAL

2.1 Materials and Methods:

Pharmaceutical grade working standard Gemcitabine, 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 (T60-LAB INDIA), analytical balance 0.1mg Sensitivity (SHIMADZU), pH meter (Labindia), ultra sonicator. The column used is Symmetry ODS RP C_{18},5µm, 15mm x 4.6mm i.d. (as Stationary phase) with the flow rate 1.0ml/min (isocratic).

2.3 Sample & Standard Preparation for the Analysis:

10 mg of Gemcitabine, Irinotecan standard was transferred into 10 ml volumetric flask, dissolved & make up to volume with Methanol. A further dilution was done by transferring 0.1ml of the above solution into a 10ml volumetric flask and make up to volume with methanol to get 10ppm concentration. It is scanned in the UV in the range of 200 to 400nm. This has been performed to know the maxima of Gemcitabine, Irinotecan, so that the same wave length can be utilized in HPLC UV detector for estimating the Gemcitabine, Irinotecan **.**

2.4 Selection of wavelength: 25 mg of Gemicitabine 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.

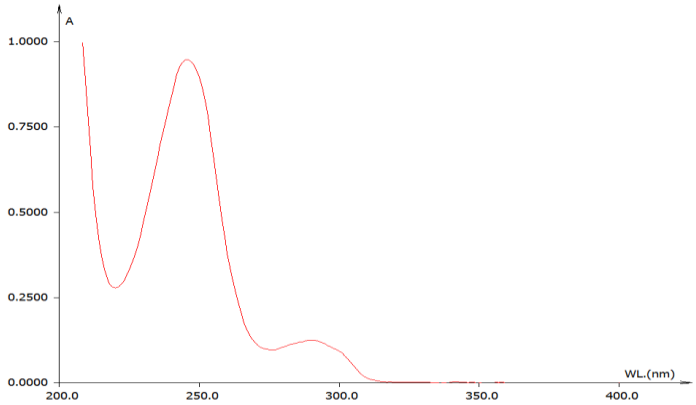


Fig-1:UV spectrum for Gemcitabine

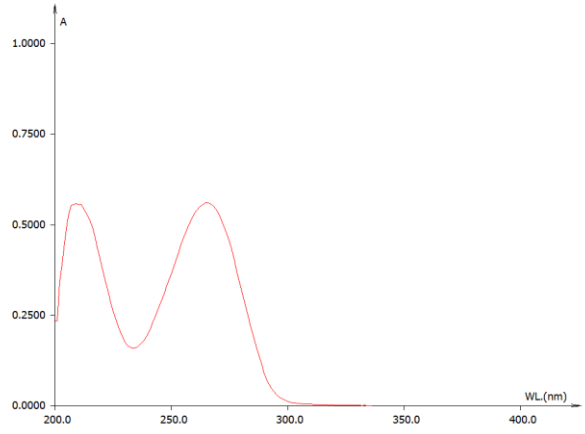


Fig-2: UV Spectrum for Irinotecan

While scanning the Gemcitabine solution we observed the maxima at 240nm and for the Irinotecan solution we observed the maxima at 269nm. The UV spectrum has been recorded on T60-LAB INDIA make UV – Vis spectrophotometer model UV-2450.

2.5 Method Development

2.5.1 Preparation of Mobile Phase:

The mobile phase was prepared with the combination of 750ml of Methanol and 250ml of Phosphate Buffer (0.2 M, pH=3) was made upto the volume of 1000ml and were mixed well and degassed in ultrasonic water bath for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration.

2.5.2 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 (0.2 M, pH=3) = 75:25
Column	Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d.
Flow rate	1.0 ml/ min.
Wavelength	259 nm
Sampling System	Automatic
Temp. of Auto sampler	Ambient
Volume of injection	20µl
Run time	0.8
Mode of Separation	Isocratic

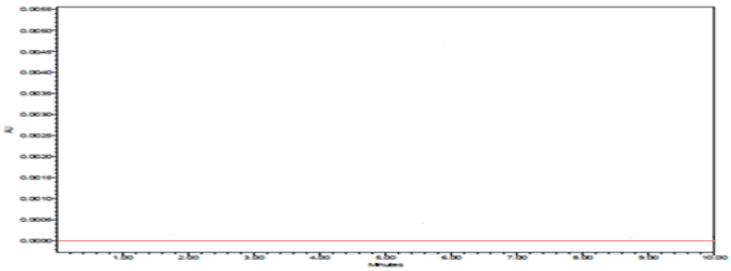
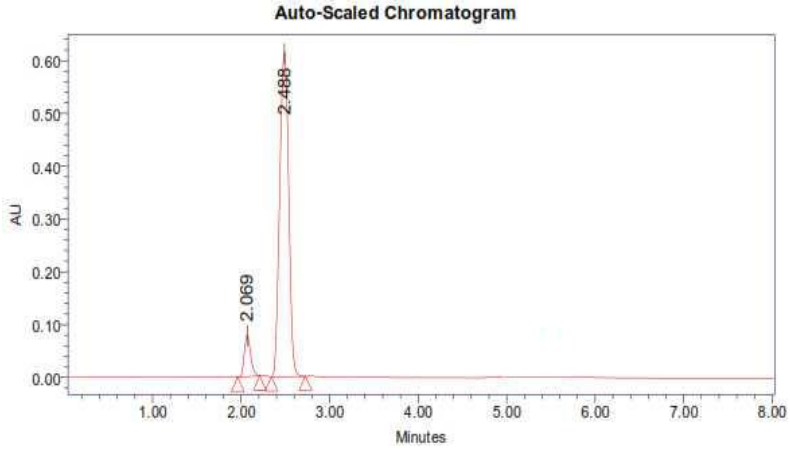


Fig-3: Chromatogram for Blank Preparation



Fig–4: Chromatogram of Gemcitabine and Irinotecan in Optimized Condition

2.6 Method validation:

2.6.1 Linearity & Range:

Linearity range was found to be 5-30 µg/ml for Gemcitabine .The correlation coefficient was found to be 0.999, the slope was found to be 15716 and intercept was found to be 3648 for Gemcitabine .

Linearity range was found to be 10-60 µg/ml for Irinotecan .The correlation coefficient was found to be 0.999, the slope was found to be 10995 and intercept was found to be 36657 for Irinotecan .

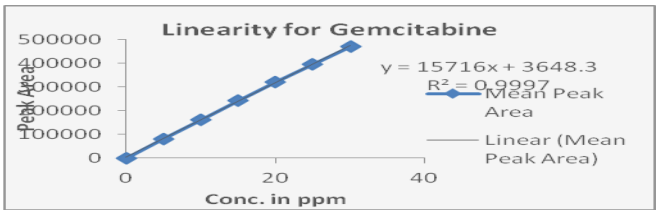


Fig-5: Standard curve for Gemcitabine

Table-2: Linearity Results for Gemcitabine

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
5	82442
10	161724
15	242754
20	321606
25	396371

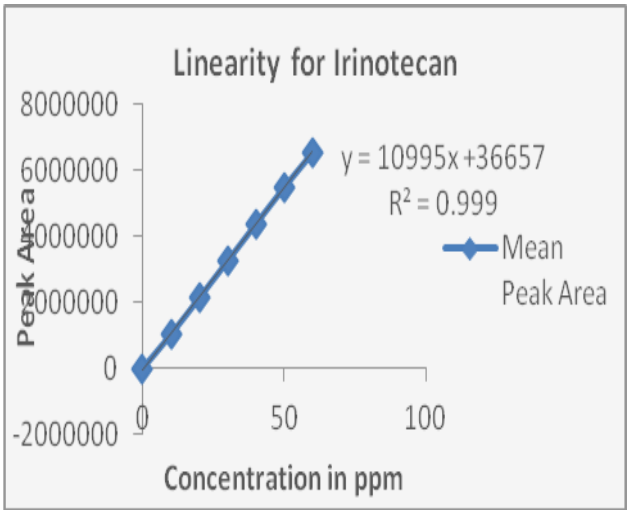


Fig-6: Standard curve for Irinotecan

Table-2: Linearity Results for Irinotecan

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
10	1031032
20	2135302
30	3255282

40	4379382
50	5493754
60	6539365

2.6.2. Accuracy:

Table-3: Accuracy Readings of Gemcitabine

Sample ID	Concentration (µg/ml)		Peak Area	%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered			
S_1 : 80 %	12	11.974	191834	99.783	Mean=99.76% S.D. = 0.296248% R.S.D.= 0.296934
S_2 : 80 %	12	11.936	191235	99.466	
S_3 : 80 %	12	12.007	192358	100.058	
S_4 : 100%	15	15.243	243212	101.62	Mean= 100.8887% S.D.= 1.044048% R.S.D.= 1.034852
S_5 :100%	15	15.203	242581	101.353	
S_6 :100%	15	14.954	238673	99.693	
S_7 : 120%	18	17.899	284962	99.438	Mean= 100.9737% S.D. = 1.331212% R.S.D. = 1.318376
S_8 :120%	18	18.324	291643	101.8	
S_9 :120%	18	18.303	291312	101.683	

Table-4: Accuracy Results for Irinotecan

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	24	24.186	191834	100.775	Mean= 100.779% S.D. = 0.406015 % R.S.D.= 0.402876
S_2 : 80 %	24	24.090	191235	100.375	
S_3 : 80 %	24	24.285	192358	101.187	
S_4 : 100 %	30	29.932	365768	99.773	Mean= 99.45533% S.D. = 0.293933 % R.S.D.= 0.295542
S_5 : 100 %	30	29.820	364532	99.40	
S_6 : 100 %	30	29.758	363851	99.193	
S_7 : 120 %	36	35.696	429135	99.155	Mean= 99.57733% S.D. = 0.366784 % R.S.D. = 0.368341
S_8 : 120 %	36	35.914	431534	99.761	
S_9 : 120 %	36	35.934	431756	99.816	

2.6.3. Precision:

2.6.3.1. Repeatability

Table-5: Data showing repeatability analysis for Gemcitabine &Irinotecan

HPLC Injection Replicates of Gemcitabine &Irinotecan	AUC for Gemcitabine	AUC for Irinotecan
Replicate – 1	249684	3233700
Replicate – 2	249696	3241323
Replicate – 3	246325	3245927
Replicate – 4	249816	3245927
Replicate – 5	249892	3222194
Replicate – 6	249793	3212863

Average	249201	3233655.667
Standard Deviation	1411.088941	13591.6592
% RSD	0.56624	0.420318

2.6.3.2. Intermediate precision:

Table-6: Data for Gemcitabine analysis

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
12	12.67	0.35	12.34	0.45
15	15.23	0.47	15.89	0.78
18	18.68	0.47	18.34	0.46

Table-7:Data for Irinotecan analysis

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
24	24.87	0.46	24.76	0.24
30	30.57	0.27	30.74	0.57
36	36.27	0.87	36.44	0.29

2.6.4. Method Robustness:

Table-8: Result of Method Robustness Test for Gemcitabine

Change in parameter	% RSD
Flow (0.8 ml/min)	0.45
Flow (1.2 ml/min)	0.38
More Organic	0.87
Less Organic	0.76
Wavelength of Detection (242 nm)	0.99
Wavelength of detection (238 nm)	0.95

Table-9: Result of Method Robustness Test for Irinotecan

Change in parameter	% RSD
Flow (0.8 ml/min)	0.57
Flow (1.2 ml/min)	0.44
More Organic	0.86
Less Organic	0.75
Wavelength of Detection (271 nm)	1.03
Wavelength of detection (267 nm)	0.94

2.6.5. LOD & LOQ:

Limit of detection and limit of quantification

Observations : The LOD was found to be 0.05µg/ml and LOQ was found to be 0.15µg/ml for Gemcitabine respectively which represents that sensitivity of the method is high.

The LOD was found to be 0.09µg/ml and LOQ was found to be 0.27µg/ml for Irinotecan respectively which represents that sensitivity of the method is high.

2.6.6. System Suitability Parameter

Table-10: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs> 2	2.97
2	Asymmetry	T ≤ 2	Gemcitabine = 0.25 Irinotecan = 0.28
3	Theoretical plate	N > 2000	Gemcitabine = 2978 Irinotecan = 3067

2.6.7 Assay of Gemicitabine & Irinotecan in Dosage Form

Estimation of GEMICITABINE & IRINOTECAN by making SYNTHETIC MIXTURE
GEMICITABINE & IRINOTECAN 200 mg & 400 mg

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.

ASSAY:

Table-11: Assay of GEMCITABINE & 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	200/400	199.1(±0.59)/399.2(±0.88)	99.55 (±0.384)/99.8 (±0.316)

Results and Discussion: The assay of Synthetic Mixture containing Gemcitabine was found to be 99.55 % and Irinotecan was found to be 99.8%.

2.6.8 Stability Studies:

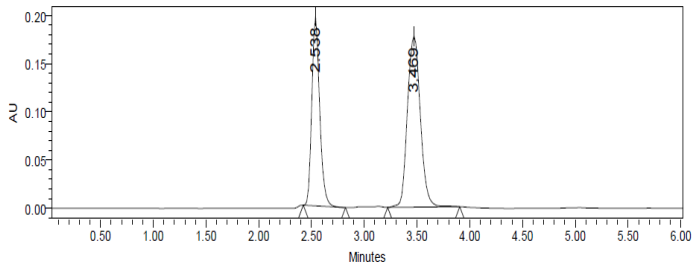


Fig-7: Chromatogram for Acid Degradation

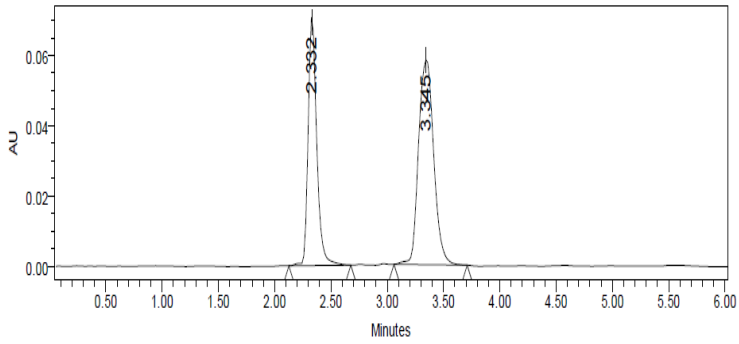


Fig-8: Chromatogram for Basic Degradation

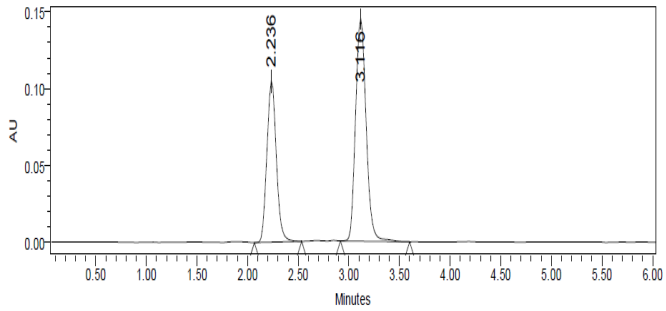


Fig-9: Chromatogram for Thermal Degradation

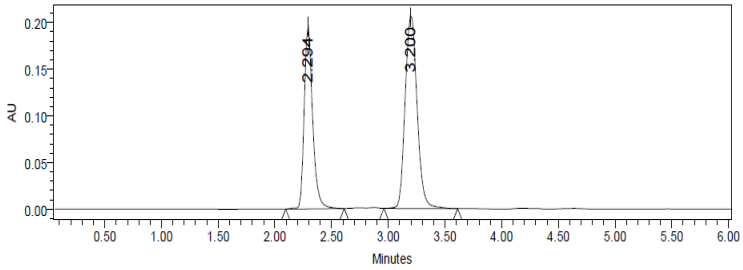


Fig-10: Chromatogram for Photolytic Degradation

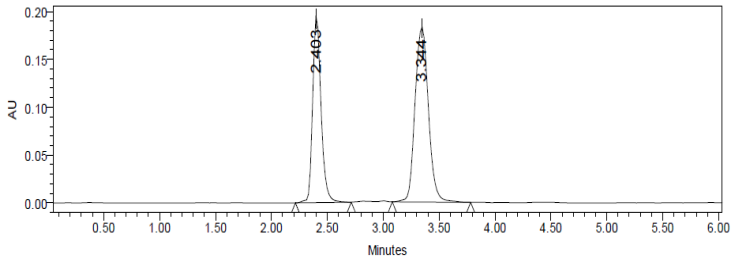


Fig-11: Chromatogram for Oxidation with 3% H_2O_2 Degradation

Table-11: Forced Degradation Studies of Gemicitabine & Irinotecan API.

Stress condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	95.95	4.07	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	59.41	40.51	100.00
Thermal Degradation (50 ^{0}C)	24Hrs.	79.23	20.42	100.00
UV (254nm)	24Hrs.	97.21	2.63	100.00
3% Hydrogen peroxide	24Hrs.	96.36	3.24	100.00

III. RESULTS AND DISCUSSION

The results obtained in method validation were :

Linearity & Range: Linearity range was found to be 5-30 µg/ml for Gemcitabine .The correlation coefficient was found to be 0.999, the slope was found to be 15716 and intercept was found to be 3648 for Gemcitabine .

Linearity range was found to be 10-60 µg/ml for Irinotecan .The correlation coefficient was found to be 0.999, the slope was found to be 10995 and intercept was found to be 36657 for Irinotecan .

Accuracy: From the Accuracy Method, we observed that the mean %Recovery of the drug are 99.769%, 100.8887% and 100.9737% which is within the range of 98-102% and %RSD is within the range <2 i.e. 0.296934%, 1.034852% and 1.318376% respectively.

From the Accuracy Method, we observed that the mean %Recovery of the drug are 100.779%, 99.45533% and 99.57733% which is within the range of 98-102% and %RSD is within the range <2 i.e. 0.402876%, 0.295542% and 0.368341% respectively.

Repeatability: The repeatability study which was conducted on the solution having the concentration of about 15µg/ml for Gemcitabine and 30µg/ml for Irinotecan (n =6) showed a RSD of 0.56624% for Gemcitabine and 0.420318% for Irinotecan. It was concluded that the analytical technique showed good repeatability.

LOD & LOQ:The LOD was found to be 0.09µg/ml and LOQ was found to be 0.27µg/ml for Irinotecan respectively which represents that sensitivity of the method is high.

Assay: The assay of Synthetic Mixture containing Gemcitabine was found to be 99.55 % and Irinotecan was found to be 99.8%.

Degradation studies: The results of the forced degradation studies indicated the specificity of the developed method that has been developed. Gemcitabine and Irinotecon were stable only in oxidative stress conditions and photolytic stress conditions. The results of stability studies are given in the following Table.

IV.CONCLUSION

A sensitive & selective stability indicting RP-HPLC method has been developed & validated for the analysis of Irinotecan and Gemcitabine 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 Irinotecan and Gemcitabine indicated that the developed method is specific for the estimation of Irinotecan and Gemcitabine. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility.

Antimicrobial agents are the natural or synthetic compounds which at certain concentrations inhibit the growth of or kill microorganisms completely. The term antimicrobials are collective for antiviral, antibacterial, antifungal and antiprotozoal. Due to the rapid development of microorganism's resistance to antimicrobial agents, it is necessary to discover new synthetic compound to help in the battle against pathogenic microorganisms.

Thienopyrimidines are a class of fused heterocycles which are common sources for thedevelopment of new potential therapeutic agents. There are three isomeric thienopyrimidines corresponding to the three possible types of annulation of thiophene to the pyrimidine ring:thieno[2,3-d]pyrimidine, thieno[3,4-d]pyrimidine, and thieno[3,2-d]pyrimidine.

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