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Analytical Method Development And Stability Indicating Studies Of Gemcitabine Hcl

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1. INTRODUCTION

Method development should be based on several considerations. It is preferable to have maximum sample information to make development fast and desired for intended analytical method application, physical and chemical properties are most preferable as primary information. Moreover, separation goal needs to defineat beginning so appropriate method can be developed for the purpose. An LC method development is very huge area for even pharmaceuticals with regulatory requirement of international standards. So, prior to method validation and usage at quality control many aspects needs to focus as per ICH guidelines. In recent time, there is incrased tendency towards the development of stability indicating assays, using the approach of stress testing as enshrined in the International Conference on Harmonization (ICH) guidelines. Even this approach is being extended to drug combinations to allow accurate and precise quantitation of multiple drugs in presense of these degredent products and interaction product if any. Various publications are available regarding the determination method of individual drugs and combinational drugs in bulk and pharmaceutical dosage forms by spectrophotometric, HPTLC, LC-MS, UPLC and HPLC but very limited publications are available for stability indicating methods for determination of drugs in combined dosage forms.[1-7]

The author undertaken in this studies in the area of pharmaceutical analysis and mainly addresses stability indicating method development and validation for determination of selected drugs and in active pharmaceutical substances as well as in pharmaceutical dosage forms by RP-HPLC. The developed methods are simple, precise, accurate, specific, selective and more economical for the routine analysis in pharmaceutical laboratories. [8-13]

2. DRUG PROFILE

Gemcitabine hydrochloride **Fig: 1** is an analogue of cytarabine. Which is metabolised intracellular to active diphosphate and triphosphate nucleosides, which inhibit DNA synthesis and induce apoptosis. It is primarily active against cells in S phase. It is given in management of solid tumours including those of the bladder, lungand pancreases; it is also being tried in cancer of the breast, cervix and ovary. [14-16]

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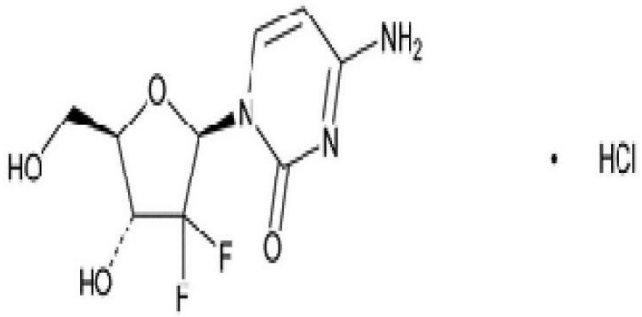


Fig: 1: Chemical Structure of Gemcitabine Hydrochloride

Molecular formula : $C_9H_{11}F_2N_3O_4.HCl$

Molecular weight : 299.7

Chemical name : Cytidine, 2-deoxy-2, 2-difluro-, monohydrochloride;

2-Deoxy-2, 2-difluorocytidine monohydrochloride (β-isomer).

Solubility : Soluble in water and sparingly soluble in methanol.Description : An off- white to white crystalline powder.

Pharmacokinetic Data:

Protein binding : <10%

Half-life : Short infusions 32-94 minutes for long infusions 245- 638 min.

Combinational dosage form of these drugs is not available. The in house formulation of bilayer tablets were prepared based on the individual strengths of these drugs in the laboratory. (200 mg of Gemcitabine hydrochloride). [17-19]

Experimental methods[20-28]

Materials:

The reference sample of Gemcitabine hydrochloride were obtained as gift samples from Mars Therapeutic Ltd., India. Potassium dihydrogen orthophosphate, ortho phosphoric acid and used were AR grade from Hi-media. Aceonitrile was HPLC grade (Merk). Milli Q (Millipore India (Pvt.) Ltd.) water purification system was used to obtain HPLC grade water.

Instrumentation:

The author attempted to develop a liquid chromatographic method for determination of Gemcitabine hydrochloride using an isocratic Waters HPLC instrument on a Inertsil-ODS-3v (250 × 4.6 mm, 5µm). Separation was performed with Waters model alliance 2695 auto sampler, photo diode arrays (PDA) detector (996 model) and a Waters model alliance 2690 auto sampler, photo diode arrays (PDA) detector. Ultrasonic bath (Spincotech Pvt. Ltd., Mumbai). It was operated by Empower-2 software. A Shimadzu balance was used for weighing the materials and Systronics-361 pH meter was used for pH measurement.

The Mobile Phase:

The mobile phase consisting of 50 mM orthophosphoric acid adjusted to pH 5.50. Mix the buffer and acetonitrile in the ratio of 40 : 60 v/v.

The Diluent:

Mobile phase was used as a diluent.

Preparation of Standard Solution:

An accurately weighed quantity of 20 mg Gemcitabine hydrochloride in to 50 ml volumetric flask and dilute to volume with diluent. Transferred 5.0 ml of standard stock in to 50 ml volumetric flask and dilute tovolume with diluent. 40 µg/ml of Gemcitabine hydrochloride). From this serial dilutions were prepared for construct the calibration curve.

Preparation of Sample (tablet) Solution:

Twenty tablets were weighed and crushed to fine powder. The sample powderwas taken equivalent to 20 mgof Gemcitabine hydrochloride into a 50 ml volumetric flask and to this 20 ml of diluent was added to dissolve. Then solution was

ultrasonicated for 20 min, filtered through a 0.45 µm membrane filter final volume of the solution was made up to 50 ml with diluent. From this 5 ml of aliquot was pipetted out and transferred into 50 ml volumetric flask made up to the mark by using diluents.

Forced degradation studies: [29-35]

Forced degradation studies of the drugs were carried out under conditions of hydrolysis, oxidation, neutral, photolytic and thermal.

Alkali Hydrolysis:

Forced degradation in basic media was performed by dissolving 10 mg drug in 1 mL acetonitrile in 10 mL volumetric flask and diluted up to mark with 1N NaOH. Then this solution was kept at 80°C for reflux. Sample was withdrawn at 0 min, after 1,2,3,4 and 5 Hours. Pipetted out 0.3 mL of this solution in 10 mL volumetric flask and neutralized with 0.3 mL 1 N HCl and diluted up to the mark with acetonitrile.

Acid Hydrolysis:

Forced degradation in acidic media was performed by dissolving 10 mg drug in 1 mL acetonitrile in 10 mL volumetric flask and diluted up to mark with 1N HCl. Then this solution was kept at 80°C for reflux. Sample was withdrawn at 0 min, after 1, 2, 3 and 4 Hours. Pipetted out 0.3 mL of this solution in 10 mL volumetric flask and neutralized with 0.3 mL 1 N NaOH and diluted upto the mark with acetonitrile.

Hydrolysis at neutral pH (water degradation)

Forced degradation at neutral pH in water was performed by dissolving 10 mg drug in 1 mL acetonitrile in 10 mL volumetric flask and diluted up to mark with water. Then this solution was kept at 80°C for reflux. Sample was withdrawn at 0 min, after 10 min, 30 min, 1, 2, 3 and 4 Hours. Pipetted out 0.3 mL of this solution in 10 mL volumetric flask and diluted up to the mark with acetonitrile.

Oxidative Hydrolysis:

Oxidative stress degradation was performed by dissolving 10 mg drug in 1 mL acetonitrile in 10 mL volumetric flask and diluted up to mark with 30 % H_2O_2. Then this solution was kept at room temperature. Sample was withdrawn at 0 min. and after 48 Hours. Pipetted out 0.3 mL of this solution in 10 mL volumetric flask and diluted up to the mark with ACN.

Thermal Hydrolysis:

For thermal degradation, Drug (Gemcitabine) kept in Petri dish into the oven at 80° for 3 hr. Thereafter, 10 mg of Gemcitabine was weighed and transferred to 10 mL volumetric flasks and diluted up to the mark with acetonitrile. Pipetted out 0.3 mL of this solution in 10 mL volumetric flask and diluted upto the mark with acetonitrile.

Photolytic Degradation:

The photo stability was also studied by exposing drug at direct sunlight for 6 Hour. Thereafter, 10 mg of Gemcitabine was weighed and transferred to 10 mL volumetric flasks and diluted up to the mark with acetonitrile. Pipetted out 0.3 mL of this solution in 10 mL volumetric flask and diluted upto the mark with acetonitrile.

Results & discussion:

Method Development:

For developing the method, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all other conditions constant. Method development consists of selecting the appropriate wave length and choice of stationary and mobile phases. The following studies were conducted for thispurpose.

Detection of Wavelength: The spectra of diluted solutions of the Gemcitabine and Gemcitabine hydrochloride in mobile phase were recorded separately on UV spectrophotometer. The peaks of maximum absorbance wavelengths were observed. The spectra of the both Gemcitabine and Gemcitabine hydrochloride were showed balanced wavelength at 298 nm.

Choice of Stationary Phase: Preliminary development trials were performed with C18, C8 and C3 with different types of configurations and from different manufacturers. Finally the expected separation and shapes of peak was succeeded in C8 column Inertsil-ODS. (250 × 4.6 mm, 5 µm).

Selection of the Mobile Phase:

In order to get sharp peak and base line separation of the components, the author has carried out a number of experiments by varying the composition of various solvents and its flow rate. To effect ideal separation of the drug under isocratic conditions, mixtures of solvents like water, methanol and acetonitrile with or without different buffers in different combinations were tested as mobile phases on a C8 stationary phase. A mixture of 50 mM orthophosphoric acid adjusted to pH 5.50 as which buffer : acetonitrile (40 : 60 v/v) (pH was adjusted to 5.50 with 0.1% tritely amine) the most suitable

of all the combinations since the chromatographic peaks obtained were better defined and resolved and almost free from tailing. [36-39]

Flow Rate:

Flow rates of the mobile phase were changed from 0.5-2.0 ml/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents. It was found from the experiments that 1.2 ml/min flow rate was ideal for the successful elution of the analyte.

Optimized Chromatographic Conditions:

Chromatographic conditions were optimized in below table. These optimized conditions were used for the simultaneous determination of Gemcitabine and Gemcitabine hydrochloride in bulk drug and its combined tablet formulations. The chromatograms of blank, standard and sample were showed in **Tables.**

Table: 1 Optimized Chromatographic Conditions

Mobile phase	Ortho phosphoric acid buffer : acetonitrile (40 : 60 v/v)
Pump mode	Isocratic
pH	5.50 (adjusted with 0.1% triethylamine)
Diluent	Mobile phase
Column	Inertsil-ODS-3v (250 × 4.6 mm, 5 µm).
Column temperature	Ambient
Wavelength	298 nm
Injection volume	20 µl
Flow rate	1.2 ml/min
Run time	6 min
Gemcitabine hydrochloride	3.84 min

Validation of the Proposed Method: [40-45]

The proposed method was validated as per ICH (51) guidelines. The parameters studied for validation were specificity, linearity, precision, accuracy, robustness, system suitability, limit of detection, limit of quantification and solution stability. All the parameters were validated by using target concentration.

Specificity:

The specificity of method was performed by comparing the chromatograms of blank, standard and sample. It was found that there is no interference due to excipientsin the tablet formulation and also found good correlation between the retention times of standard and sample. The specificity of results was showed in **Table: 2.**

Table: 2 Specificity Study

Name of the solution	**Retention time (min)**
Blank	No peaks

Placebo	No peaks
Gemcitabine hydrochloride	4.797

Specificity by Forced Degradation Study:

In order to determine the analytical method and assay for the study of stability indicating method in formulation of Gemcitabine hydrochloride, studied under various stressed conditions to conduct forced degradation studies. The used forced degradation conditions, stress agent concentration and times of stress, were found to effect degradation, preferably not lessthan 10% and not complete degradation of active materials. The discovery of such conditions was based on trial and error.

Linearity:

Linearity was performed by preparing Gemcitabine hydrochloride at different concentration levels. Twenty microlitres of each concentration was injected into the HPLC system. The mean peak areas were calculated and linearity plots of concentration over the mean peak areas were constructed individually. The regressions of the plots were computed by least square regression method. Linearity results were presented in **Tables.**

Table: 2 Linearity Study of Gemcitabine Hydrochloride

S.No	**Concenration (µg/ml)**	**Peak area**
1	24	1618.059
2	32	2221.812
3	40	2630.866
4	48	3273.848
5	56	3796.983
Correlation coefficient		0.996
Slope		67.6
Intercept		43.3

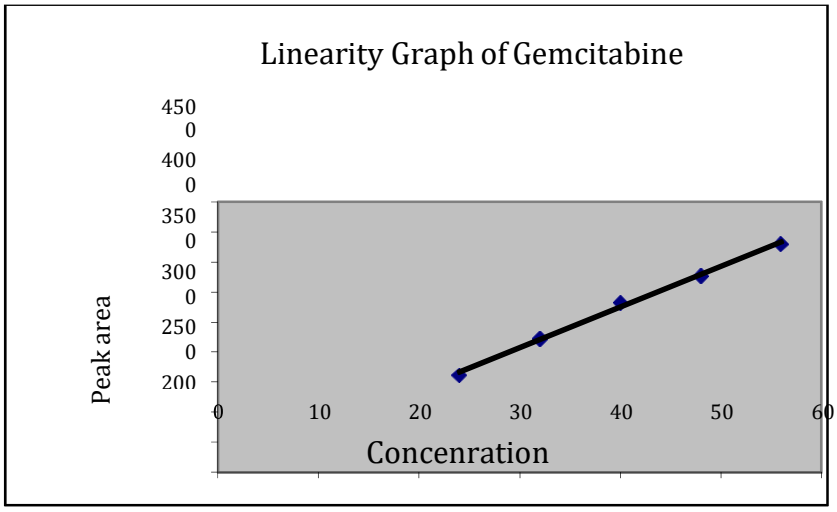


Fig: 2 Linearity Graph of Gemcitabine Hydrochloride

Precision:

Precision is the degree of repeatability of an analytical method under normal operational conditions. Precision of the method was performed as system precision, method precision and intermediate precision.

System Precision:

To study the system precision, six replicate standard solutions of Gemcitabine hydrochloride were injected. The percent relative standard deviation (%RSD) was calculated and it was found to be 0.47 for Gemcitabine hydrochloride respectively, which were well within the acceptable criteria of which %RSD should not more than 2.0**.**

Table: 3 System Precision

Sam.No	**Peak area of Gemcitabine hydrochloride**
1	2613.551
2	2607.979
3	2634.570
4	2626.944
5	2607.003
6	2608.425
Mean	2612.412
%RSD	0.47

Method Precision:

The method precision study was carried out on six preparations from the same tablet sample Gemcitabine hydrochloride and percent amount of both were calculated. The %RSD of the assay result of six preparations in method precision study was found to be 0.47 for Gemcitabine hydrochloride respectively, which were well within the acceptance criteria not more than 2.0.

Table: 4 Method Precision

Sam.No	**%Assay**
	Gemcitabine hydrochloride
1	99.69
2	99.24
3	98.64
4	98.93
5	98.91
6	99.81
Mean	99.203

%RSD	0.47

Intermediate Precision:

The intermediate precision study was carried out by different analysts, from the same tablet of Gemcitabine hydrochloride. The %RSD of the assay result of six preparations for intermediate precision study was 0.17 for Gemcitabine hydrochloride respectively, which were well within theacceptance criteria of not more than 2.0.

Table: 5 Intermediate Precision Study of Gemcitabine Hydrochloride

Sam.No	%Assay	Mean	%RSD
1	99.25	99.25	0.17
2	99.38		
3	99.54		
4	99.69		
5	99.42		
6	99.66		

Accuracy:

The accuracy of the method was determined by standard addition method. A known amount of standard drug was added to the fixed amount of pre-analysed tablet solution. Percent recovery was calculated by comparing the area before and after the addition of the standard drug. The standard addition method was performed at 80%, 100% and 120% level. The solutions were analysed in triplicate at each level as per the proposed method. The percent recovery and %RSD at each level was calculated and results were presented. Satisfactory mean recoveries ranging from 97.8 to 100.3 for Gemcitabine hydrochloride respectively were obtained by the proposed method. This indicates that the proposed method was accurate.

Table: 6 Recovery Study for Gemcitabine Hydrochloride

% Level	Amount of API added (µg/ml)	Peak area(mV)	Amount recovered (µg/ml)	% Recovery	Mean of recovery	% RSD
80%	32	2100.973	32.10	100.3	98.99	0.45
	32	2042.067	32.21	97.8		
	32	2042.067	31.31	98.8		
100%	40	2624.580	40.19	100.2	99.90	0.15
	40	2614.107	39.94	99.7		

	40	2614.107	39.98	99.8		
120%	48	3115.461	47.60	99.1	99.7	0.13
	48	3155.096	47.69	99.8		
	48	3148.187	48.10	100.2		

Robustness:

The robustness study was performed by slight modification in flow rate of the mobile phase, wavelength and pH of the mobile phase. It was observed that no marked changes in chromatograms, which demonstrated that the developed method was robust in nature.

Table: 6 Robustness Study for Gemcitabine Hydrochloride

Condition	**%Assay**	**%Difference**
Unaltered	100	-
Flow rate at 1.0 ml/min	99.24	0.76
Flow rate at 1.2 ml/min	100.56	0.56
Wavelength		
296 nm	99.96	0.4
300 nm	100.50	0.50
Mobile phase		
pH at 5.3	101.04	1.04
pH at 5.7	98.6	1.4

System Suitability:

System suitability was studied under each validation parameters by injecting six replicates of the standard solution.

Table: 7 System Suitability for Gemcitabine Hydrochloride

Parameter	**Tailing factor**	**Theoretical plates**
Specificity study	1.89	6983
Linearity study	1.86	6975
Precision study	1.88	6982

Robustness study Flow rate		
Flow rate at 1.0 ml/min	1.83	7142
Flow rate at 1.2 ml/min	1.82	6789
Mobile phase		
pH at 5.3	1.81	6921
pH at 5.7	1.82	6668
Wavelength		
296 nm	1.71	6854
300 nm	1.73	6243

Solution Stability:

To determine the stability of Gemcitabine hydrochloride, the sample solution was observed under room temperature. Any change in the retention time, peak shape and variation in response was compared to the pattern of chromatogram of freshly prepared solution.

Table: 8 Solution Stability of Gemcitabine Hydrochloride

Drug name	**Stability**	**Peak area (mV)**	**%Assay**	**%Deviation**
Gemcitabine hydrochloride	Initial	2618.035	100.0	0.0
	6 hr	2624.580	100.2	0.2
	12 hr	2614.107	99.7	0.3
	18 hr	2614.107	99.8	0.2
	24 hr	2614.107	99.8	0.2

Limit of Detection and Limit of Quantification:

The limit of detection and limit of quantification were evaluated by serial dilutions of Gemcitabine hydrochloride stock solution in order to obtain signal to noise ratio of 3 : 1 for LOQ and 10 : 1 for LOD. The concentration of the drugs was listed given table. The LOD and LOQ values were calculated from the calibration curve.

Table: 9 LOQ and LOD of Gemcitabine Hydrochloride

Drug name	LOQ	LOD
Gemcitabine hydrochloride	8.4 µg/ml	2.77µg/ml

Forced degradartion studies[46-50]

The degradation study indicated that the drug degrades as shown by the decreased areas in the peaks when compared with peak areas of the same concentration of the non degraded drug, without giving any additional degradation peaks. Percent degradation was calculated by comparing the areas of the degraded peaks in each degradation condition with the corresponding areas of the peak of the drug under non degradation condition. The % degradation was found to be 4 to 100% of Gemcitabine in the given condition using developed HPLC method**.** Summary of degradation studies of the drug is given in Table**.** Present study indicates the suitability of reversed-phase column procedure for the analysis of Gemcitabine in Pharmaceutical dosage form. The percentage of Gemcitabine was found to be satisfactory, which is comparable with the corresponding claim amount results. The labeled amount of Gemcitabine 40 mg/tablet recovered 100.18%. It can be concluded that the method is simple, accurate, and precise.

Table-10:Forced Degradation studies

Sr. No	Parameters (Stress condition /duration/state)	% of undergrad	% of individual Degradation products		Total %Deg.
1	Neutral/H2O at pH7/4h	82	18	-	18
2	Acidic/1NHCl/4h/R	07	83	17	93
3	Alkali/1NNaOH/5.30 h/ Ref.	00	80	20	100
4	Oxidative/30%H2O2/48h	95	-	-	5
5	Photo/sunlight/6 h	91	-	-	9
6	Thermal/80^{0}C/3h	96	-	-	4

Formulation	Label claim (mg)	Amount found (mg)	% Assay±SD
Gembin 1000	40	40.04	100.18±0.77

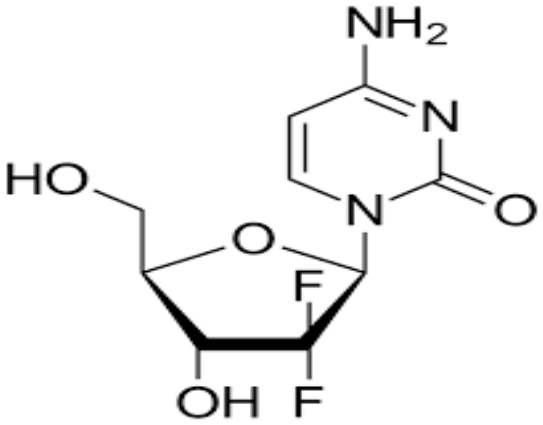


Fig-3:Chemical structure of Gemcitabine

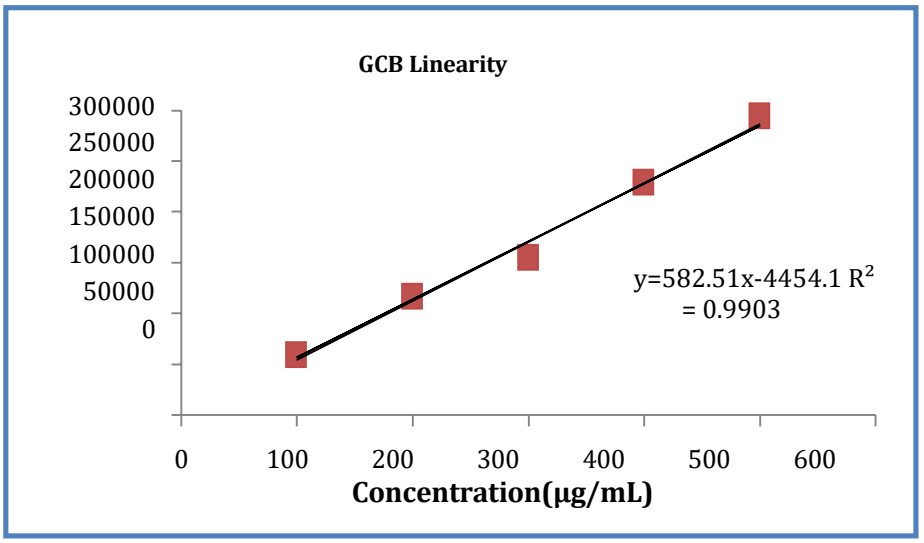


Fig-4: Calibration curve of Gemcitabine

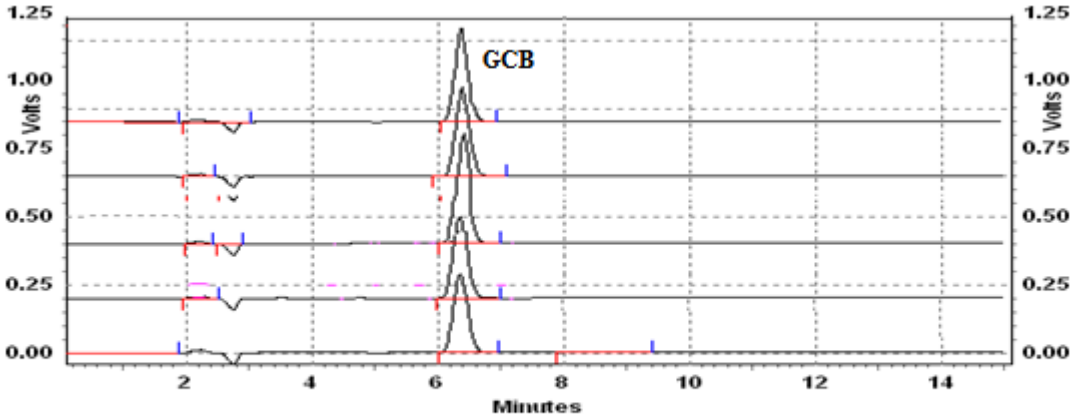
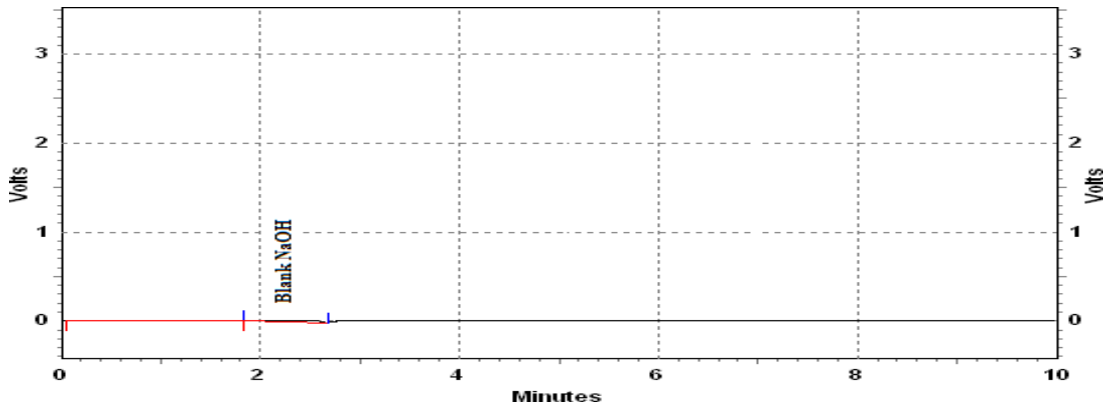
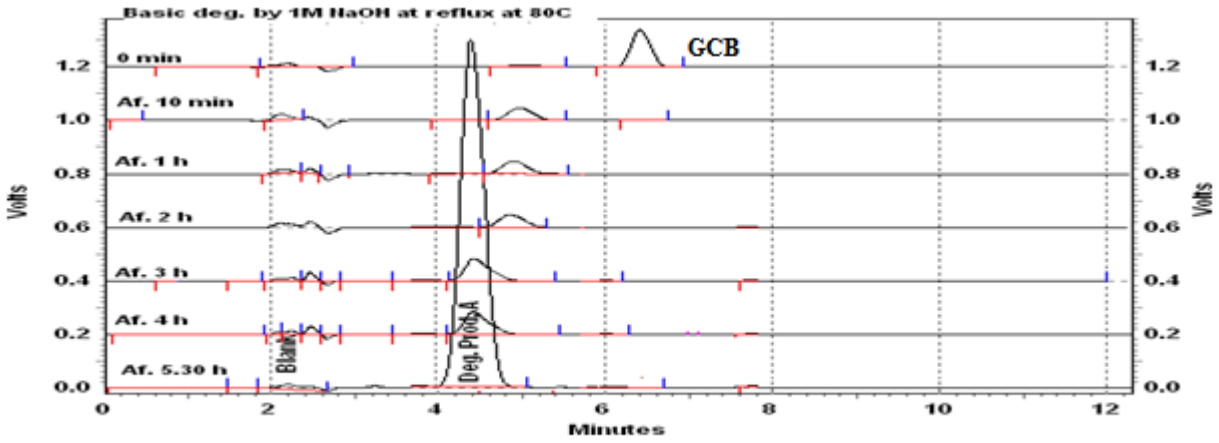


Fig-5: Linearity of Gemcitabine

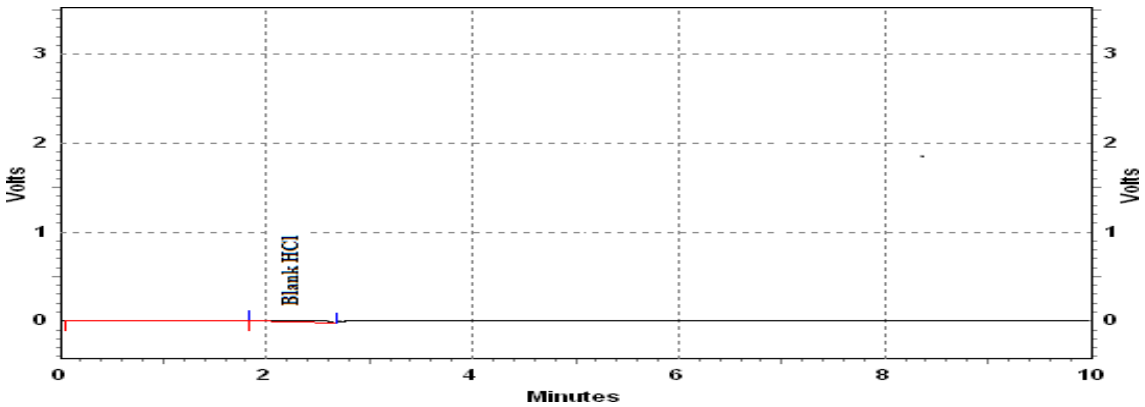


(a)



(b)

Chromatograms of (a) Blank and (b) GCB in 1 N NaOH at 0 min, after 10 min, 1, 2, 3, 4 and 5.30 hrs reflux at 80° C



(a)

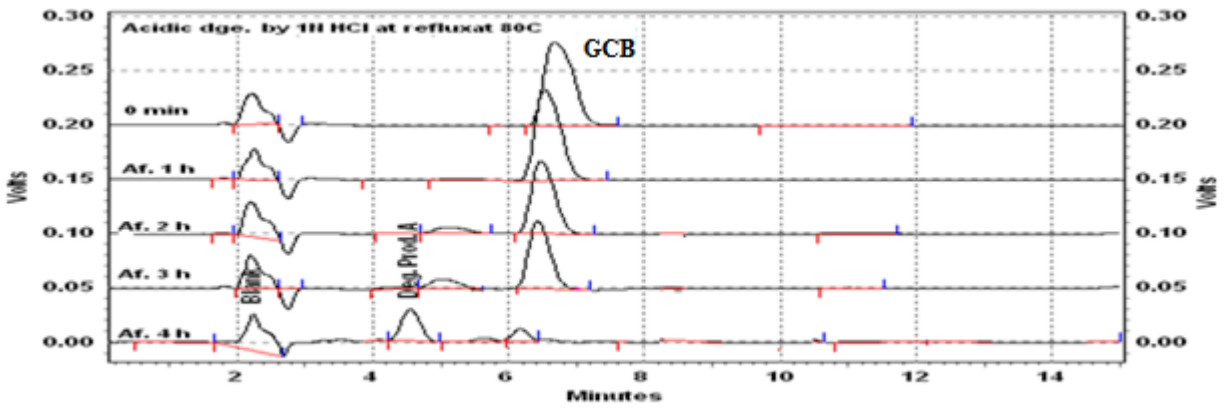


Fig-6: Chromatograms of (a) Blank and (b) GCB in 1 N HCL at 0min, after1, 2, 3and4hrsreflux at 80º C.

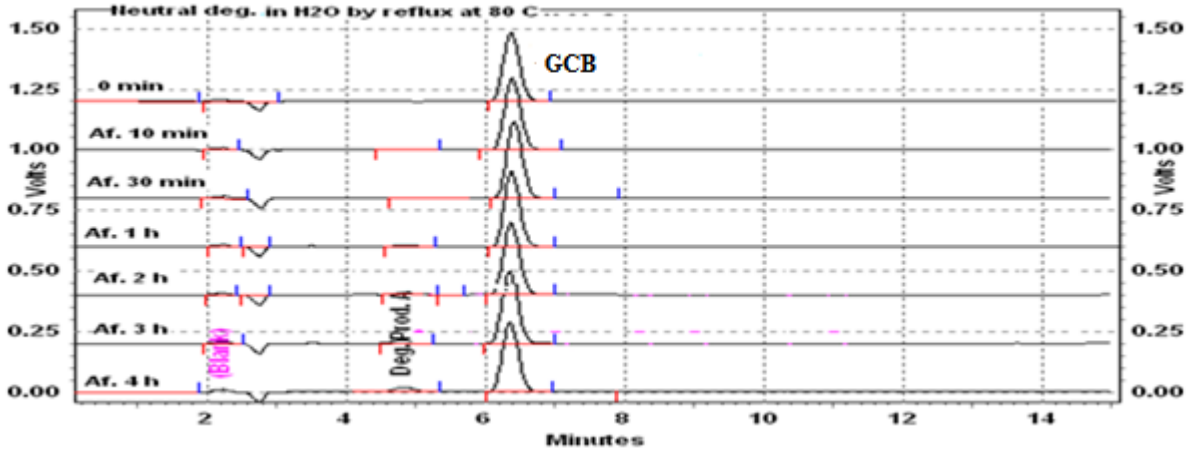
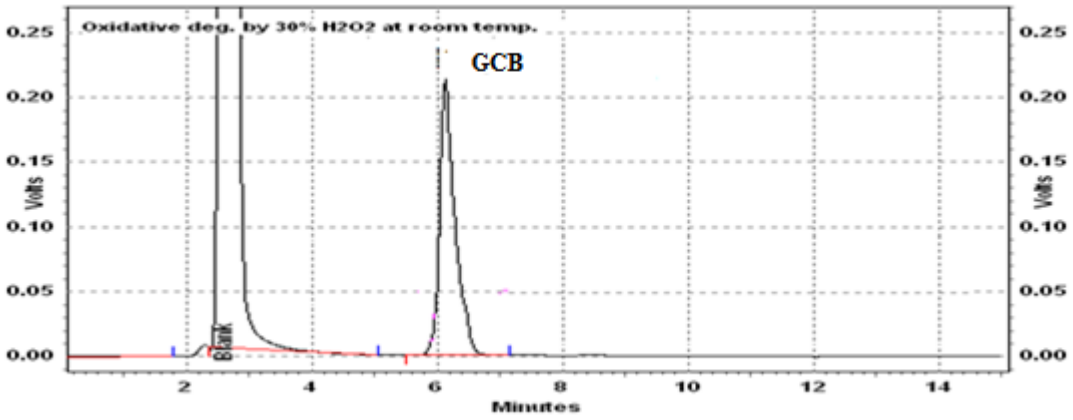


Fig-7: Chromatograms of GCB in Neutral condition at 0 min, after 10min, 30min,1hr, 2hr, 3hr and 4 hr reflux at 80º C



Chromatograms of GCB in 30%H_2O_2 after48 hrs

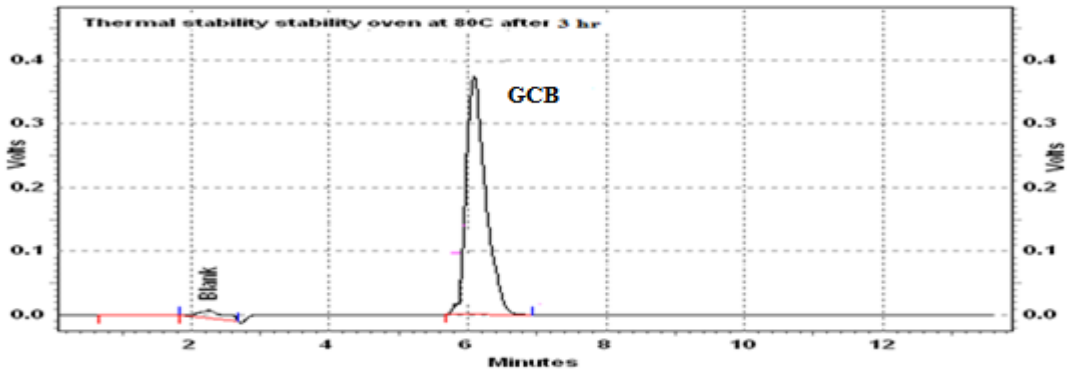


Fig-8: Chromatograms of thermal degradation of GCB after3 hrs

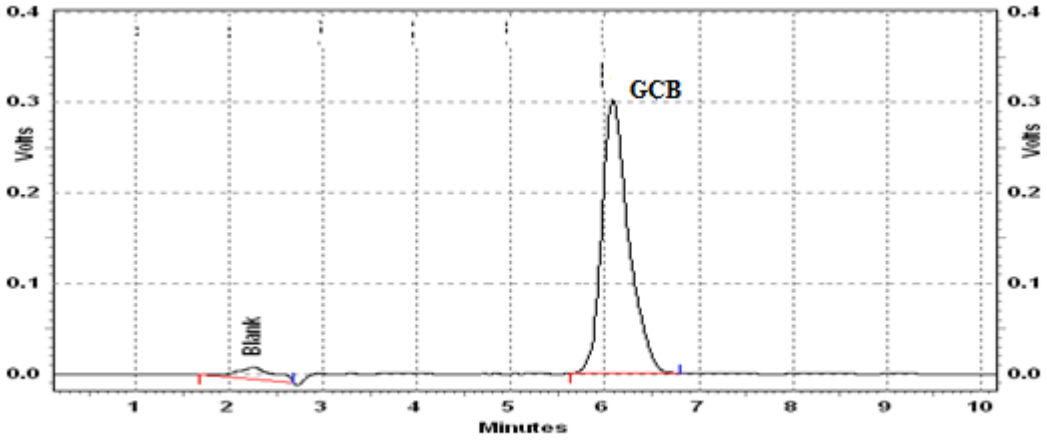


Fig-9: Chromatograms of Photolytic degraded GCB

3. CONCLUSION:

The author developed a new stability indicating RP-HPLC method for the determination of Gemcitabine hydrochloride in bulk and combined dosage form by using column Inersil ODS-3V (250 × 4.6 mm, 5 µm**)** with mobile phase containing mixture of orthophosphoric acid buffer and acetonitrile (40 : 60% v/v) in an isocratic pump mode. The detection of the drugs was monitored at 298 nm. The retention time for Gemcitabine hydrochloride was 4.797 min respectively. The method was found to be linear in the range of 24-56 µg/ml for Gemcitabine hydrochloride with correlation coefficient r^2=0.996 respectively. The developed method was stability indicating method, by conducting forced degradation studies at extreme conditions.

The author attempted method was simple, precise, rapid, and sensitive and economical and validated as per ICH guidelines. It was used for routine analysis in quality control laboratories.

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