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A Validated RP-HPLC Method for the Estimation of Related Substances of Gemcitabine in Gemcitabine Injection 38 mg/mL

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Abstract

A novel, simple, sensitive and stability-indicating high-performance liquid chromatography method was developed and validated for the related substances of Gemcitabine in Gemcitabine Injection 38mg/vial. Reversed-phase chromatography was performed on Agilent 1100 series with Software Empower-2 and Agilent 1100 series with PDA 1100 series Software Empower-2 photodiode array detector using ZorbaxRx C8 (250 mm × 4.6 mm, 5 µm particle size) column with pH 3.0 (adjusted with Ortho Phosphoric acid) of monobasic sodium phosphate buffer as mobile phase-A and Methanol as Mobilephase-B at a flow rate of 1.2 mL/min. Gradient profile at Initial: 97-3, 8 minutes: 97-3, 13 minutes: 50-50, 20 minutes: 50-50, 25minutes: 97-3, 35minutes: 97-3 and with UV detection at 275 nm. Linearity was observed in the concentration range of Cytosin 0.04-3.0 µg/mL (R2 = 0.999), Alpha Anomer impurity 0.10–2.91 µg/mL (R2 = 0.999), the concentration range of Gemcitabine 0.15-5.5 µg/mL (R2 = 0.999), and the concentration range of Beta Uridine Impurity 0.05–72.5µg/mL (R2 = 0.999),). The limit of detection (LOD) AND limit of Quantitation (LOQ) were found to be Cytosin impurity 0.02&0.04 µg/mL, Alpha Anomer impurity 0.06&0.15µg/mL, Gemcitabine 0.06&0.17 µg/mL and Beta Uridine Impurity 0.02&0.06 µg/mL, respectively. The method was validated as per ICH guidelines. The RSD for intra-day (0.1-1.3) and inter-day (0.5-1.1) precision were found to be less than 10.0 %. The percentage recovery was in good agreement with the labelled amount in the pharmaceutical formulations and the method is simple, specific, precise and accurate for the determination of related substances Gemcitabine in pharmaceutical formulations.

Article History
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Keywords:*Gemcitabine, Estimation of Related Substances of Gemcitabine, HPLC, validation.*

I. INTRODUCTION

Gemcitabine hydrochloride, 4-amino-1-[(2R, 4R, 5R)-3, 3-difluoro-4-hydroxy-5-(hydroxymethyl) oxolan-2-yl] pyrimidin-2-one) is a β-difluoronucleoside, purine antimetabolite. The drug is an antitumor agent, employed extensively against several human malignancies like ovarian, lung, pancreatic, bladder, urothelial, and breast cancer. It is currently marketed as a lyophilized

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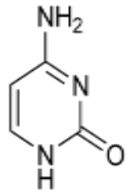
powder. The drug is also extensively employed as antiviral agent, enzyme inhibitor, immunosuppressive agent, and radiation-sensitizing agents. Gemcitabine is a prodrug that enters the cell by means of nucleoside transporters and becomes active through an intracellular transformation catalyzed by deoxycytidine kinase to its diphosphate and triphosphate derivatives. The triphosphate derivative is incorporated into the DNA strand, inhibiting thymidylatesynthetase which inhibits DNA synthesis and chain elongation, contributing to the antineoplastic activity of the drug. The diphosphate derivative inhibits ribonucleotide reductase, the enzyme responsible for catalyzing synthesis of deoxynucleoside-triphosphate required for DNA synthesis. Gemcitabine triphosphate competes with endogenous nucleoside triphosphate for incorporation into DNA [1–3]

A literature survey reveals that only a few methods based on ultraviolet spectroscopy [4], HPTLC [5], and HPLC [6–13] are available for determination of drug in formulation. Although several HPLC [14–22] methods have been reported for estimation of drug and its metabolites in biological fluids. A few stability indicating that HPLC methods [3, 11, 12] have been reported, which provides variable level of degradation of Gemcitabine. Jansen et al. [3] reported the separation and identification of degraded product of Gemcitabine in acidic stress condition. Mastanamma et al. [11] and Kudikala et al. [12] have reported the validated stability indicating method which can separate the hydrolytic degraded product of Gemcitabine. However, to the best of our knowledge none of the HPLC method reported the oxidative degraded product of Gemcitabine. Previously published methods for formulation are less robust and need more investigations for method development and validation. Stability-indicating methods have to demonstrate that they are specific, which involves evaluating the drug in the presence of its degradation products [23]. The present investigation describes a simple, rapid, accurate, precise, robust stability indicating RP-HPLC method for the determination of Gemcitabine for dosage forms.

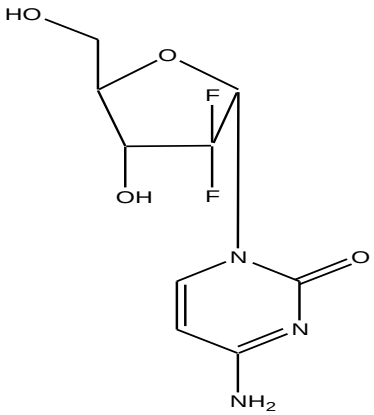
i. Cytosine



Molecular Formula: $C_4H_5N_3O$

Molecular Wt.: 111.10

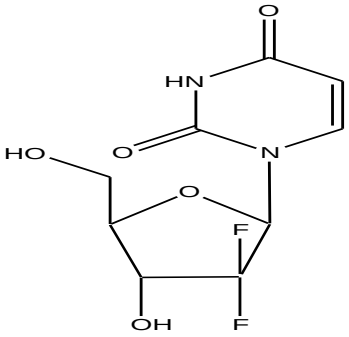
ii. α-anomer



Molecular Formula: $C_9H_{11}F_2N_3O_4$

Molecular Wt.: 263.20

iii. β-uridine



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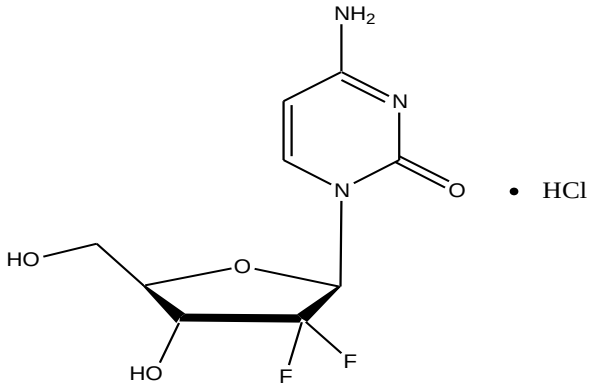
Molecular Formula: $C_9H_{10}F_2N_2O_5$

Molecular Wt.: 264.18

iv. Gemcitabine Hydrochloride



Molecular Formula: $C_9H_{12}ClF_2N_3O_4$

Molecular Wt.: 299.66

II. MATERIALS, EQUIPEMENTS DETAILS, CHEMICAL NAME FOR GEMCITABINE AND IT'S IMPURITIES, METHOD PROCEDURE

1. Reagents and Solvents:

Monobasic sodium phosphate (AR grade), Ortho-phosphoric acid (AR grade), Potassium hydroxide (AR grade), Methanol (HPLC grade), Milli-Q water purification system from Millipore.

2. Equipment Details:

Two LC systems were used for method development and validation. LC 1 was a Agilent 1100 series of variable wavelength absorbance detector) with empower software. LC 2 was a Agilent [1100 separation module and a 1100 series of photodiode array (PDA) detector] with Empower-2 software.

3. Chemical Name for Gemcitabine and it's Impurities:

The chemical names are as described in the following

Gemcitabine HCL: 4-Amino-1-(2-deoxy-2,2-difluoropentofuranosyl)pyrimidin-2(1H)-one.

α-Anomer: 3',5'-Di-O-benzoyl-2'-deoxy-2',2'-difluorocytidine.

β-Uridine :1-(2-deoxy-2,2-difluoropentofuranosyl)pyrimidine-2,4(1H,3H)-dione

4. Method Procedure:

A new gradient method was developed for separating process impurities of Gencitabine from its degradation peaks, thus proving the method to be stability indicating. The chromatographic method employed mobile phase A, consisting of *pH 3.0 (adjusted with Phosphoric acid) of monobasic sodium phosphate buffer* and mobile phase B, consisting of *Methanol*. The method employed the gradient programs listed in Table I for the analysis of impurities. The method was developed by using anZorbax Rx C8, (250mm x 4.6mm) 5µ (Agilent). The flow rate of the mobile phase was 1.2 mL/min. The column temperature was maintained at 25°C, the sample cooling rack temperature was maintained at 25°C and the detection wavelength was monitored at 275 nm. The injection volume was 20 µL. Water used as diluent.

Table I

HPLC Gradient Program for Analysis of Impurities

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow rate (mL/min)
0.00	97.0	3.0	1.0
8.0	97.0	3.0	1.0
13.0	50.0	50.0	1.0
20.0	50.0	50.0	1.0
25.0	97.0	3.0	1.0
35.0	97.0	3.0	1.0

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III. ANLYTICAL METHOD VALIDATION

Solution stability

The stability of Gemcitabine and its impurities in solution for the related substance method was determined by injecting the spiked sample solution at 25°C temperature for 64 hours and measuring the amounts of the four impurities in particular intervals and cumulative RSD of Cytosine Impurity was found 0.16%, ß Uridine Impurity was found 0.45%, α Anomer was found 0.38%,. Injected the standard solution at 25°C temperature for about 64 hours and measuring the cumulative RSD of Gemcitabine was found 0.52% at about 46 hours (stability hours).

Specificity

Specificity is the ability of the method to measure the response of the analyte in the presence of its potential impurities and degradation products. The specificity of the developed LC method for Gemcitabine HCL was tested in the presence of its impurities. The sample was subjected to acid hydrolysis, alkaline hydrolysis and oxidation conditions. The sample was also subjected to thermal and photo degradation in a dry state. Different stress conditions were used to achieve degradation.

Linearity

Linearity solutions for the method of impurities were prepared by diluting impurity stock solutions to the required concentrations. Linearity was established over a specified range of the LOQ to the 150% of the specification limit of known impurities and Gemcitabine.

Limits of detection and quantification

The Limit of Quantitation (LOQ) and Limit of Detection (LOD) values of known impurities and that of Gemcitabine were determined based on calibration curve plotted between concentration of impurity and their respective responses. The respective LOD and LOQ of impurities were calculated from the residual standard deviation obtained from calibration curve. Precision at limit of Quantitation and verification of limit of detection value were performed. A precision study was also conducted at the LOQ level by injecting six individual preparations of all four impurities and Gemcitabine and calculating the relative standard deviation (RSD) of the area.

Accuracy

The accuracy was performed by spiking respective impurity standards with Gemcitabine HCl sample at 50%, 100%, and 150% of specification level and by spiking respective impurity standards with Gemcitabine HCl API + Placebo at LOQ level. The solution was prepared in triplicate at each level.

Precision

System precision was performed by injecting six replicate injections of standard solution of Gemcitabine HCl. Method precision was performed by analysing six sample preparations, as per method. Intermediate precision was performed by analysing six sample preparations, as per method by a different analyst, on a different day, on a different instrument, using a column of different serial no.

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution between Gemcitabine and its α Anomer and the tailing factor, plate count for Gemcitabine and Cytosine were recorded. The effect of flow rate was studied at

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1.0& 1.4 mL/min and compared with the flow rate of the method at 1.2 mL/min. The effect of Mobile phase Buffer pH was studied at 2.8&3.2 and compared with the Mobile phase Buffer pH of the method at 3.0.The effect of column temperature was studied at 23 & 27°C and compared with the column temperature of the method at 25°C. The effect of wavelength was studied at 273 & 278 and compared with the wavelength of the method at 275.

IV. RESULTS OF PARAMETERS

Selectivity

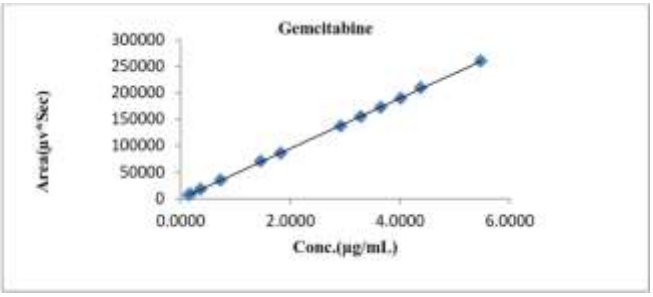
Name of the component	Retention time in Individual solutions	Retention time in Spiked sample solution
Cytosine	2.7	2.8
α Anomer	5.2	5.3
ß Uridine	13.5	13.6
Gemcitabine	7.9	7.9

Specificity

Stress condition	Degradation in Control Sample in %w/w
Control (Unstressed)	-
Thermal Sample(24 Hrs at 105°C)	0.80
Acid Sample 5mL of 5N HCl(23 Hours, RT)	0.07
Peroxide Sample 5mL of 30%H2O2 (22 Hours, RT)	3.73
Alkali Sample 5mL of 5N NaOH (23 Hours, RT)	7.58
Photolytic stress (1.2 million lux hours)	0.01

Linearity and Range

Gemcitabine		
Level(%)	**Level(%)**	**Level(%)**
150%	5.48	260644
120%	4.38	209931
110%	4.02	189612
100%	3.65	172718
90%	3.29	155201
80%	2.92	137221
50%	1.83	85829
40%	1.46	70692
20%	0.73	34784
10%	0.37	17550
5%	0.18	8650
LOQ	0.15	6891
Correlation coefficient		0.99993
Slope		47495.98369
Intercept		-81.61320
Residual sum of squares		10940084.8



Cytosine Impurity		
Level(%)	**Concentration (µg/mL)**	**Area**
150%	2.95	278446
120%	2.36	222955
110%	2.17	205883
100%	1.97	186129
90%	1.77	168657
80%	1.57	149227
50%	0.98	96824
40%	0.79	76092
20%	0.39	37995
10%	0.20	18755
5%	0.10	9801
4%	0.08	7928

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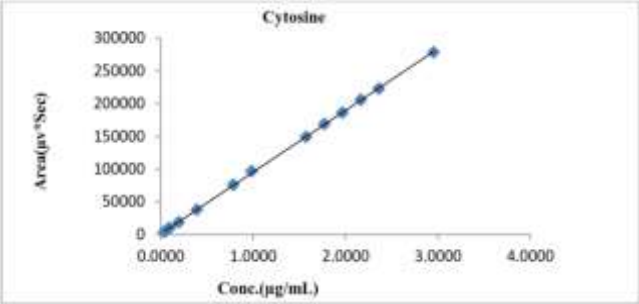
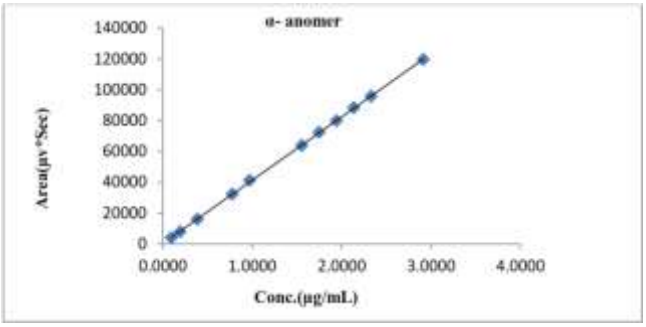
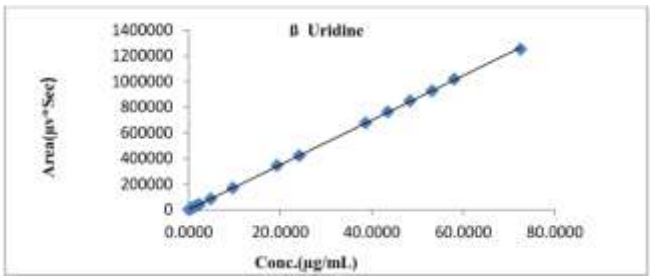
3%	0.06	5994
2%	0.04	4140
Correlation coefficient		0.99994
Slope		94307.46028
Intercept		954.15842
Residual sum of squares		14337754.4



α Anomer Impurity		
Level (%)	**Level (%)**	**Level (%)**
150%	2.91	119591
118%	2.33	95758
109%	2.14	88317
99%	1.94	79911
89%	1.75	72414
79%	1.55	63837
49%	0.97	41172
39%	0.78	32294
20%	0.39	16015
10%	0.19	7828
5%	0.10	3942
Correlation coefficient		0.99994
Slope		41068.12676
Intercept		244.92557
Residual sum of squares		1814868



Uridine		
Level(%)	**Level(%)**	**Level(%)**
150%	72.57	1252171
120%	58.06	1018639
110%	53.22	926608
100%	48.38	848681
90%	43.54	764142
80%	38.71	677474
50%	24.19	421684
40%	19.35	344294
20%	9.68	170373
10%	4.84	86214
5.0%	2.41	42546
4.0%	1.93	33793
3.0%	1.44	25220
2.0%	0.96	16851
1.0%	0.48	8713
0.5%	0.24	4231
0.2%	0.10	1677
0.1%	0.05	941
Correlation coefficient		0.99995
Slope		17408.14204
Intercept		1469.21087
Residual sum of squares		305063015.8



Limits of detection and quantification

Component	Concentration LOD (%) w/w	Concentration LOQ (%) w/w
Cytosine	0.001	0.002
α-Anomer	0.003	0.008
ß-Uridine	0.001	0.003
Gemcitabine	0.003	0.009

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Verification of Limit of detection

Injection	Area of Gemcitabine	Area of Cytosine	Area of α Anomer	Area of ß Uridine
1	3.08838	1.32054	2.84856	0.552254
2	3.53243	1.48724	2.71426	0.434875
3	3.50368	1.28064	2.60229	0.596428
4	3.53403	1.13230	2.22032	0.443062
5	2.72437	1.29435	2.92800	0.361061
6	3.60646	1.51925	2.36686	0.416946
Mean	3.33156	1.33905	2.61338	0.46744
%RSD	10.52	10.71	10.55	18.98

Precision at Limit of Quantitation

Injection	Area of Gemcitabine	Area of Cytosine	Area of α Anomer	Area of ß Uridine
1	7.56077	3.13509	6.71355	1.25950
2	8.24548	3.39840	6.22124	1.22450
3	8.18768	3.04704	6.41992	1.05919
4	8.08732	3.10509	6.57392	1.35173
5	7.29372	3.13627	6.53449	1.18133
6	8.31150	3.01494	7.16601	1.34811
Mean	7.94775	3.13947	6.60486	1.23739
%RSD	5.27	4.33	4.85	8.92

Accuracy

Cytosine Impurity:

Level	Sample	Amount added (mg)	Amount recovered (mg)	% Recovery	Average %Recovery at each level
LOQ	1	0.0042	0.0037	88.5	86.9
	2	0.0042	0.0036	86.8	
	3	0.0042	0.0036	85.6	
50%	1	0.0956	0.0829	86.7	86.9
	2	0.0956	0.0829	86.7	
	3	0.0956	0.0834	87.2	
100%	1	0.1912	0.1665	87.1	87.2

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	2	0.1912	0.1665	87.1	
	3	0.1912	0.1672	87.4	
150%	1	0.2868	0.2493	86.9	86.9
	2	0.2868	0.2498	87.1	
	3	0.2868	0.2489	86.8	
			Overall mean		86.99
			Overall SD		0.650
			Overall % RSD		0.75

αAnomer

Level	Sample	Amount added(mg)	Amount recovered (mg)	% Recovery	Average %Recovery at each level
LOQ	1	0.015	0.015	99.9	99.8
	2	0.015	0.015	100.2	
	3	0.015	0.015	99.3	
50%	1	0.099	0.100	100.9	99.7
	2	0.099	0.097	98.6	
	3	0.099	0.098	99.6	
100%	1	0.197	0.200	101.4	101.3
	2	0.197	0.200	101.2	
	3	0.197	0.200	101.3	
150%	1	0.296	0.297	100.4	100.5
	2	0.296	0.298	100.6	
	3	0.296	0.298	100.7	
			Overall mean		**100.3**
			Overall SD		**0.87**
			Overall % RSD		**0.9**

ß-Uridine

Level	Sample	Amount added (mg)	Amount recovered (mg)	% Recovery	Average % Recovery at each level
LOQ	1	0.0061	0.0063	102.5	101.3
	2	0.0061	0.0061	100.3	
	3	0.0061	0.0062	101.0	
50%	1	0.9733	1.0955	112.6	112.5
	2	0.9733	1.0943	112.4	
	3	0.9733	1.0962	112.6	
100%	1	1.9465	2.1890	112.5	112.6
	2	1.9465	2.1908	112.6	
	3	1.9465	2.1967	112.9	
150%	1	2.9198	3.2730	112.1	112.1

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	2	2.9198	3.2787	112.3	
	3	2.9198	3.2702	112.0	
			Overall mean		**109.6**
			Overall SD		**5.1**
			Overall % RSD		**4.6**

Precision

System Precision

Injection	Peak area of Gemcitabine	Peak area of Cytosine
1	196154	90041
2	196241	90247
3	196168	90018
4	195916	90227
5	196036	90294
6	195917	90074
Mean	196072	90150
%RSD	0.1	0.1

Method Precision (in % w/w)

Sample Preparation	% Cytosine	%α Anomer	%ß Uridine	% Total impurity (Excluding ß Uridine)
1	0.09	0.10	0.11	0.24
2	0.09	0.10	0.11	0.24
3	0.09	0.10	0.11	0.24
4	0.09	0.10	0.11	0.24
5	0.09	0.10	0.11	0.24
6	0.09	0.10	0.11	0.23
Mean	**0.09**	**0.10**	**0.11**	**0.24**
%RSD	**0.0**	**1.0**	**0.0**	**1.3**

Intermediate Precision (in % w/w)

Sample Preparation	% Cytosine	%α Anomer	%ß Uridine	% Total impurity (Excluding ß Uridine)
1	0.09	0.10	0.10	0.22
2	0.09	0.10	0.10	0.22
3	0.09	0.10	0.10	0.22
4	0.09	0.10	0.10	0.22

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5	0.09	0.10	0.10	0.22
6	0.09	0.10	0.10	0.22
Mean	**0.09**	**0.10**	**0.10**	**0.22**
%RSD	**1.1**	**1**	**1**	**0.5**

Relative Retention Time and Response Factor

β-uridine	1.70	2.70

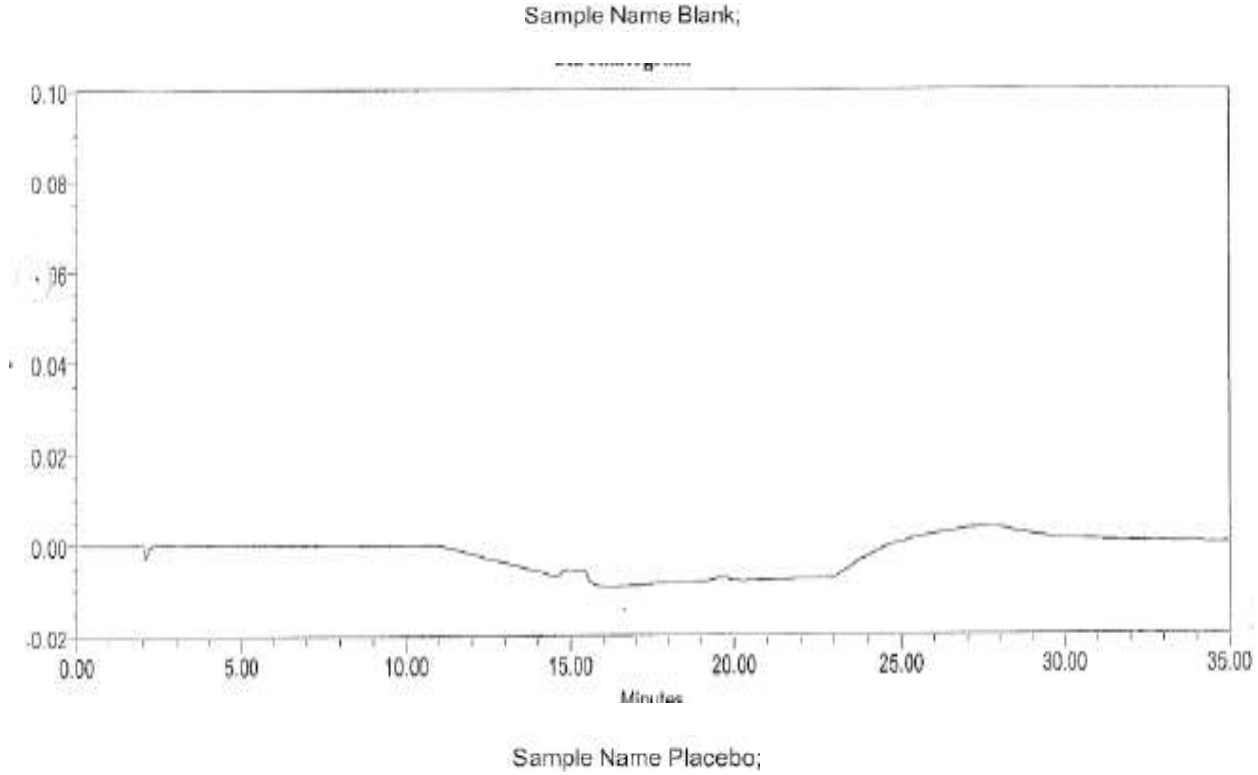
Impurity Name	RRT (approx.)	RF
Gemcitabine	--	1.00
Cytosine	0.34	0.50
α-anomer	0.63	1.15

Robustness

The system suitability parameters complied in every robustness condition ((flow rate, mobile phase buffer pH, column temperature and gradient programme). The method was found to be robust.

Chromatograms

Fig.1: Blank



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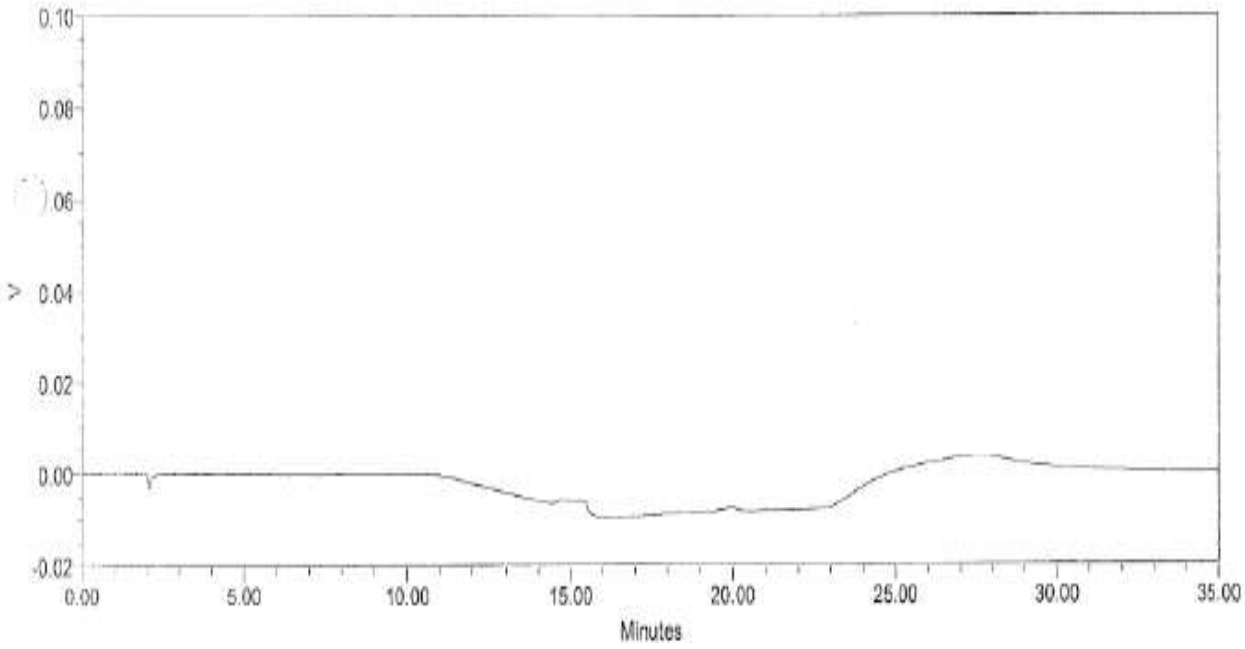

Fig.2: Diluted Spiked Sample solution

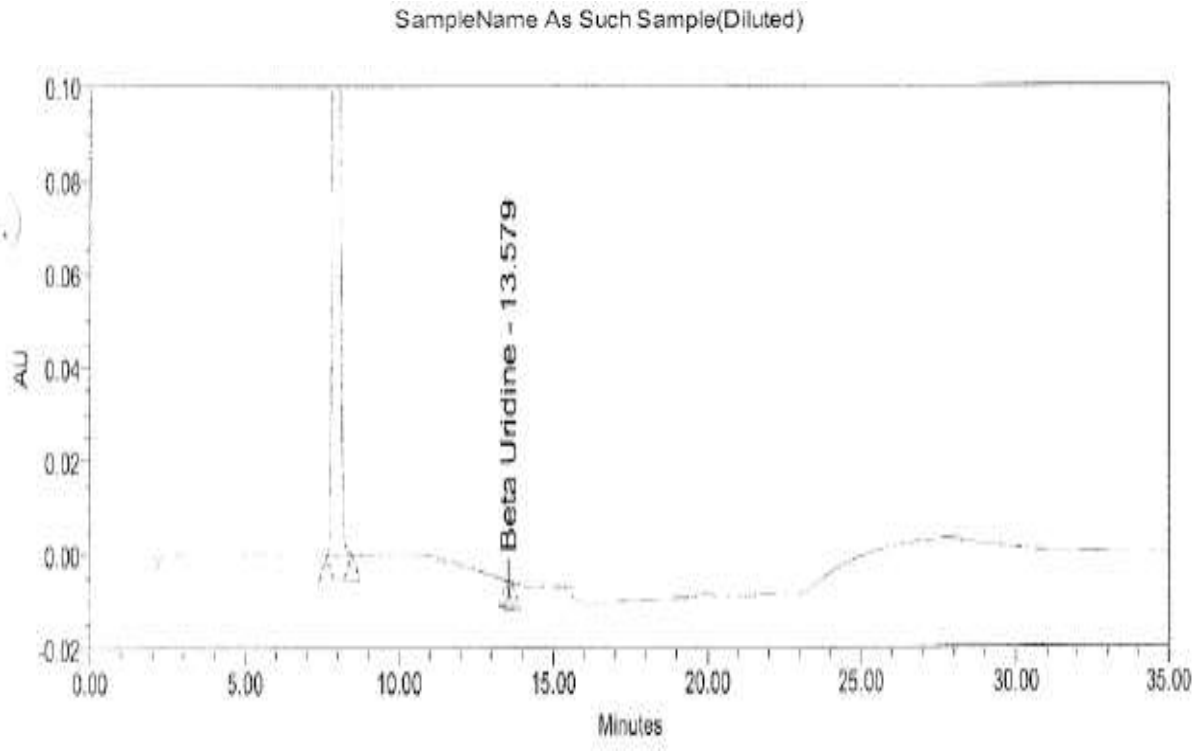

Fig.3: Sample solution

Sample Name System Suitability Solution;

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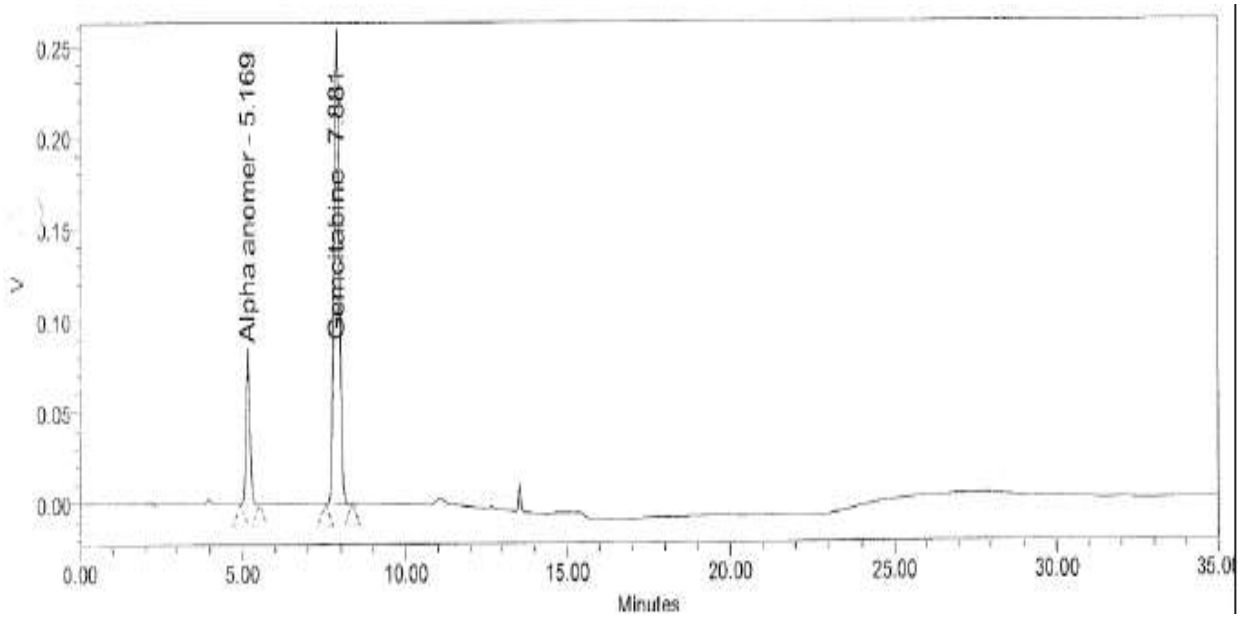
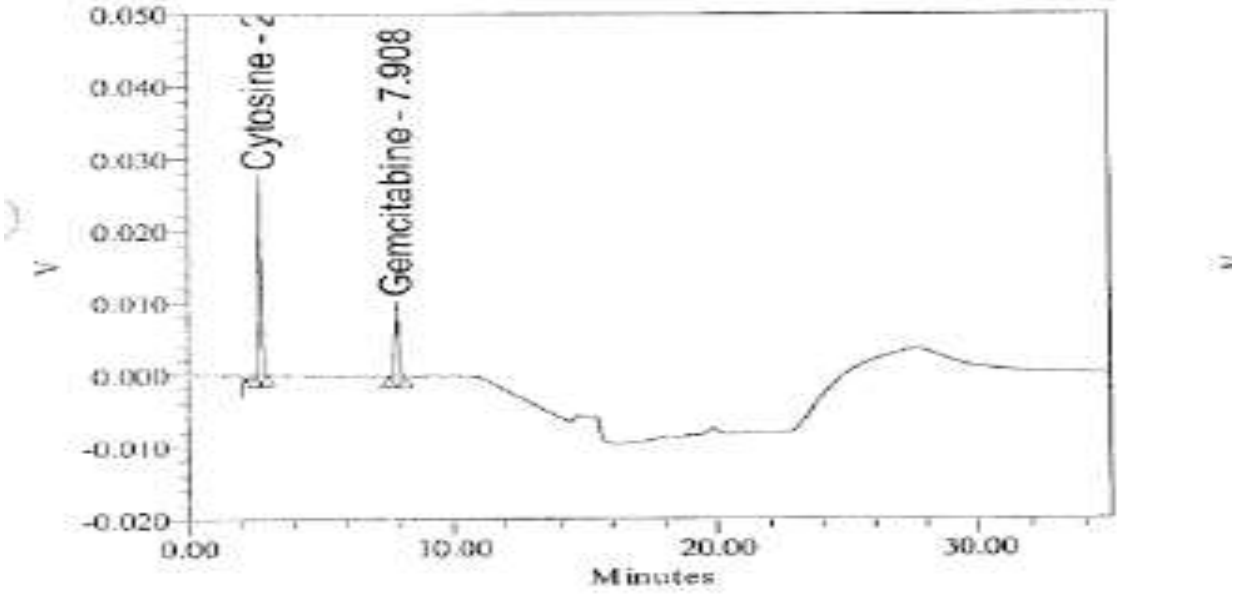


SampleName Standard Solution;



SampleName As Such Sample

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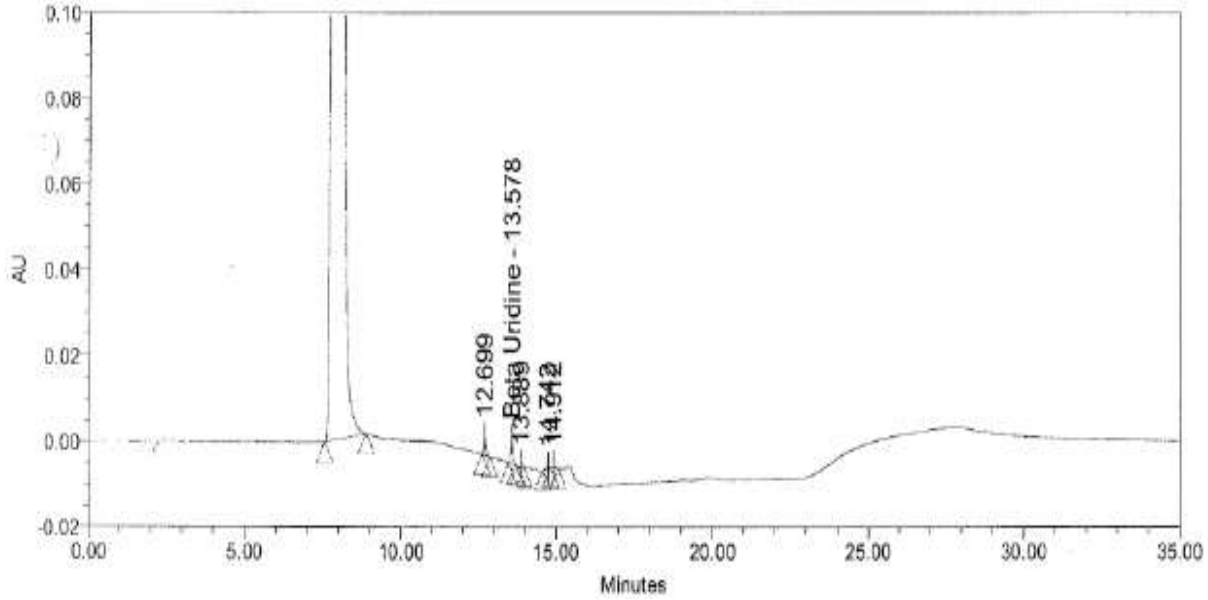
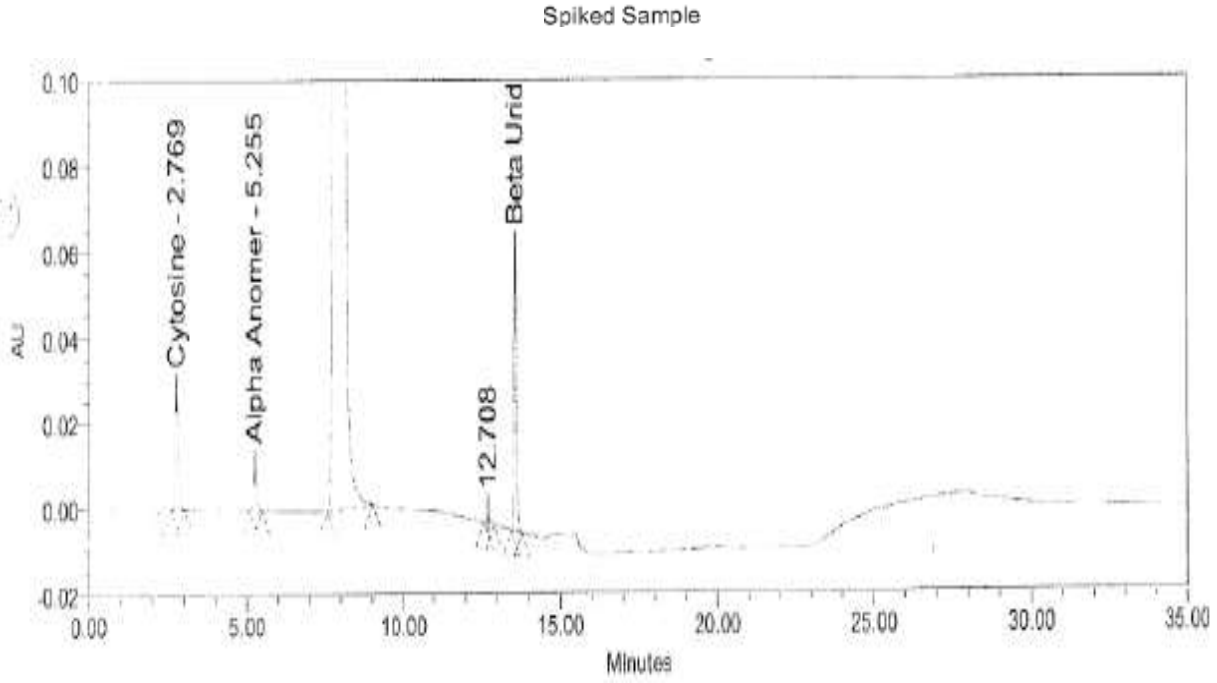


Fig.4: Spiked sample solution



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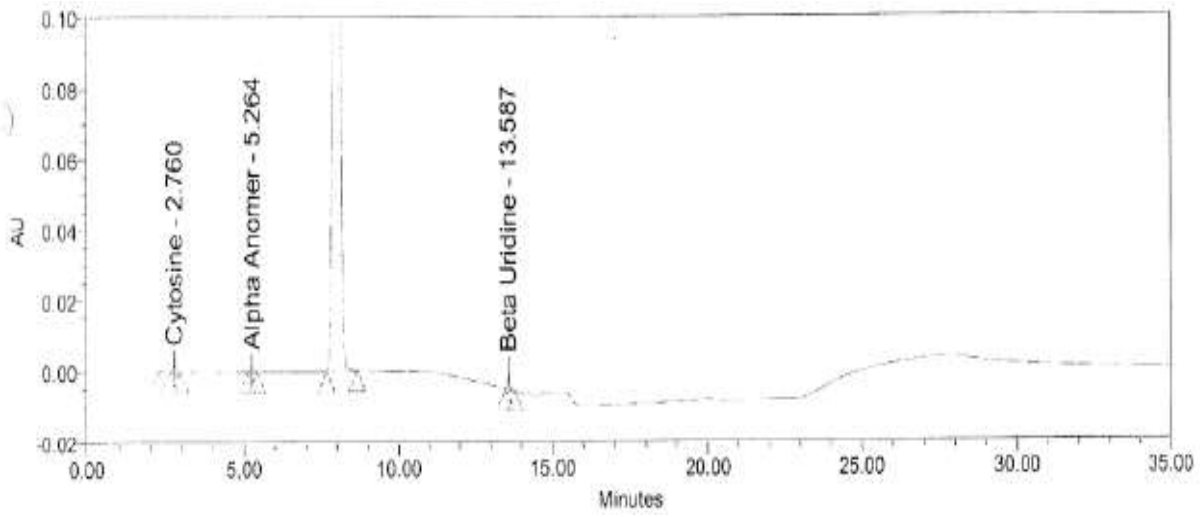

V. CONCLUSION

The present study emerged with a suitable method for impurities for evaluation of the pharmaceutical quality of Gemcitabine. The impurities method was designed by taking adequate care to separate process-related impurities and degradation products from each other and from Gemcitabine. The method also identified the retention times of known impurities and accurately ensured their quantification by employing RRF's. A simple and accurate method for the determination of related substances for Gemcitabine was established. The developed method is stability indicating and can be used for the routine analysis of production samples and to check the stability of Gemcitabine injection samples.

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