FORMULATION OF RELATED SUBSTANCES FOR GEMCITABINE AS INJECTION – A NEW VALIDATED HPLC METHOD

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Abstract

A validated HPLC method was developed for the determination of Gemcitabine (GCB) in pharmaceutical formulation. Isocratic elution at a flow rate of 0.5 ml/min was employed on Reliant C-18; 250mmx4.6mm, 5μm or Equivalent. A mixture buffer : Acetonitrile (60:40 v/v) were prepared and used as mobile phase. The UV detection wavelength was 275nm and 10μl sample was injected. The run time is 5min and the flow rate was found to be 0.8 ml/min. The injection volume is 5μL identified The Approximate retention time was founded for GCB is 38min. The% R.S.D Gemcitabine was identified. The mean Percentage recovery for Gemcitabine is found within the specification limit. The method was validated as per the ICH guidelines. Thus, the proposed HPLC method can be successfully applied for the routine quality control analysis of formulations. The method developed is simple and is better than the methods reported in the literature.

Key words: RP-HPLC Refractive index detector, GCB, flow rate, column, ICH Guidelines, USP reference.

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1.Introduction

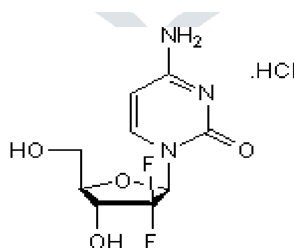

Fig: 1 Molecular structure of GCB

The Molecular formulae for Gemcitabine (GCB)is $C_9H_{11}F_2N_3O_4$. Gemcitabine (GCB), sold under the brand name Gemzar, among others,[1] is a chemotherapy medication used to treat a number of types of cancer.[2] These cancers include breast cancer, ovarian cancer, non-small cell lung cancer, pancreatic cancer, and bladder cancer.[2][3] It is given by slow injection into a vein.[2]GCB was patented in 1983 and was approved for medical use in 1995.[4]Generic versions were introduced in Europe in 2009 and in the US in 2010.[5][6] It is on the World Health Organization's List of Essential Medicines, the most effective and safe medicines needed in a health system.[7] It is used as a second-line treatment in combination with carboplatin

for ovarian cancer.[8-10] GCB is a synthetic pyrimidine nucleoside nucleoside prodrug.[2][11][12] It was intended as an antiviral drug, but preclinical testing showed that it killed leukemia cells in vitro.[13] This can be given by mouth have been a subject of research.[14-16] Women taking GCB should not become pregnant, and pregnant and breastfeeding women should not take it.[17] It was approved in the UK in 1995[8] and approved by the FDA in 1996 for pancreatic cancers.[3]In 1998, GCBreceived FDA approval for treating breast cancer.[3] Rahul Singh et.al.,[18] Proposed GCB and chromatographic separation was achieved on a Phenomenex Luna C-18 column (250 mm × 4.6 mm; 5µ) with a mobile phase consisting of 90% water and 10% acetonitrile. The wave length was recorded at 275 nm. In the range of 0.5–50 µg/mL calibration curves are linear. 0.999 is the correlation coefficient. Kirstein MN et.al., [19] reported on a Waters Spherisorb 4.6 mm x 250 mm, 5 microm C18 column at 40 degrees C. For human plasma 98.7 to 106.2% is the total accuracy and 96.9 - 99.2% for tissue culture media and 96.5 - 99.6% is for dFdU. J.V.L.N. Seshagiri Rao et.al.,[20] performed by using ODS column (250 × 4.6 mm ID), 5 µ particle size with a mobile phase comprising of water and acetonitrile in the ratio 30:70 (v/v) in isocratic mode at a flow rate of 1.0 mL/ min. The eluent was monitored at 234 nm. The retention time was 2.20 min. Ashok K et.al.[21] observed on a Phenomenex Luna C-18 column (250 mm x 4.6 mm; 5µ). 90:10v/v of water and acetonitrile is used a mobile phase. 275mm is the wave length for the both GCB and theophyline. The LOD is 0.1498 µg/mL and LOQ is 0.4541 µg/mL. The inter- and intra-day precision was less than 2%. 100.2% to 100.4% is the accuracy.. GangWang et.al.,[22] developed UPLC–MS/MS method for GCB. The recovery is identified at >81%., this method is applied to know the JDR and GCBafter oral administration to rats. A.V.D.Nagendra kumar et.al.,[23] proposed Loperamide Hydrochloride. Isocratic elution at a flow rate of 2.0ml/min was employed on symmetry Luna C8 5 µm (150 x 4.6mm) Column at ambient temperature. The mobile phase consisted of Acetonitrile : Buffer in the ratio of 45:55 v/v. The UV detection wavelength was 214nm and 50µl sample was injected. The retention time for Loperamide Hydrochloride was 2.59 min. The percentage RSD for precision and accuracy of the method was calculated.

2.Experimental

2.1 Instrumentation:

PeakHPLC containing LC 20AT pump and variable wavelength programmable UV-Visible detector and Rheodyne injector are employed for this present investigation. The chromatographic analysis was performed on a Reliant C18; 250mmx4.6mm, 5µm or EquivalentDegassing of the mobile phase was done using a Loba ultrasonic bath sonicator. A sartorius Analytical balance is used for weighing all drugs and materials.

2.2 Chemicals and Solvents:

The reference sample of Gemcitabine was obtained from the local market. Trifluoro acetic acid, methanol, orthophosphoric, potassium Hydroxide, water Buffer and Acetonitrile were used are HPLC grade and purchased from Fisher scientific, Merck and Mill q India.

2.3 The buffer solution and mobile phase

Transfer 1 mL of Trifluoroacetic acid in 1000 mL of water. Mix well, filter through 0.45 µ membrane filter and degas. As a mobile phase mixture of Buffer 60 v/v and Acetonitrile 40 v/v were prepared.

2.4 Preparation of Solutions:

For this method of analysis various solutions like Diluent, System suitability solution, GCB Standard Stock solution, Cytosine Standard Stock solution, Sensitivity solution (1ppm), Diluted standardSolution, Sample solution(200mg/5.26mL,1g/26.3mL & 2g/52.6mL) and placebo by using standard preparation methods and used in the proposed analysis.

3. METHOD DEVELOPMENT

Various parameters were studied and considered for this analytical method validation of related substances in the drug product GCB injection concentrate 20.0mg/5.26ml.

Choice of stationary phase and Mobile Phase:

By using Reliant C18; 250mmx4.6mm, 5μ or Equivalent the expected separation and peak shapes are obtained. Buffer 60 v/v Acetonitrile 40 v/v are used as a mobile phase which proves to be the most suitable of all the combinations since the chromatographic peak obtained. This shows that better defined and resolved and almost free from tailing.

Flow rate:

In order to measure flow rate the mobiles phase was changed in between 0.1 – 1.0 mL/min for optimum separation. At last it is observed from the experiment that 0.8 mL/min flow rate was ideal for the successful elution of the analyte.

4. Validation or proposed method

4.1 System Suitability

To verify that the analytical system is working properly and can give accurate and precise results, the system suitability parameters are to be set. Injected Blank (Diluent) (1 injection), Standard solution(6 injections) into HPLC and recorded the chromatograms and checked the system suitability parameters into chromatograph and record the chromatograms. From the below results, it is concluded that the system is suitable for analytical method validation. Results are tabulated in table 1.

Injection No.	Gemcitabine	
	Retention Time (In minute)	Area response
1	33.535	162435.561
2	33.520	166210.419
3	33.538	161296.614
4	33.554	163332.787
5	33.539	164607.772
6	33.511	161980.882
Mean	**33.533**	**163310.673**
% RSD	**0.0**	**1.1**

Table:1 System suitability results

4.2 Specificity:

Performed the specificity parameter of the method by injecting Blank (diluent), Placebo preparation, System suitability solution, Diluted standardsolution, Cytosine, GCBα-Anomer,β-Uridine,Sample solution and Spiked sample solution (Sample and Impurity) into the chromatographic system and recorded the retention times. From the results it can be concluded that there is no interference due to the Blank(Diluent),

Placebo, Cytosine, GCBα-Anomer,β-Uridine, diluted standard at the retention time of GCB peak. Results are tabulated in table :2.

Solution		Retention Time
Blank(Diluent)		--
Sensitivity solution	Gemcitabine	38.199
System suitability solution	Gemcitabine	37.954
	GCBα anomer	16.574
Diluted standard solution	Cytosine	5.195
	Gemcitabine	39.075
Placebo preparation		-
Sample prepartion	Cytosine	5.182
	Gemcitabine	37.669
	β-uridine	55.356
GCBα anomer		16.825
Cytosine		5.183
β uridine		55.433
Spked sample preparation	Gemcitabine	37.876
	GCBα anomer	16.783
	Cytosine	5.170
	β uridine	55.390

Table:2 Specificity results

FORCED DEGRADATION STUDIES were studied and finally found that the sample is degrading in thermal (48 Hr) condition. In Peroxide, Neutral, Alkali and acidic condition GCB peak is slightly degraded. However unknown impurities, knonw impurities and degradation impurity peaks are well separated from GCB peak. GCB peak is pure, which was confirmed by chremoleon software. Hence the related substance method is considered specific & stability indicating.

4.3 Precision:

The precision of analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of series of measurement.

4.3.1 System Precision

The %RSD of the Retention time for Gemcitabine peak obtained from 6 injections of Standard solution. The %RSD of the Area response for Gemcitabine peak obtained from 6 injections of Standard solution.From the results, it can be concluded that the retention time and area responses are consistent as evidenced byRelative standard deviation. (Less than 1.0% and less than5.0% respectively).Hence, it is concluded that the system precision parameter meets the requirement of method validation. The results are tabulated in Table 3.

Injection No.	Gemcitabine	
	Retention Time (In minute)	Area response
1	33.535	162435.561
2	33.520	166210.419
3	33.538	161296.614
4	33.554	163332.787
5	33.539	164607.772
6	33.511	161980.882
Mean	**33.533**	**163310.673**
% RSD	**0.0**	**1.1**

Table 3: System precision results

4.3.2 METHOD PRECISION:

Analysed the Sample of Gemcitabine Injection Six times of a same batch as per analytical procedure.Calculated the % of related substance of Gemcitabine injection. The %RSD of the impurities 0.05 and above for 6 determinations is identified. From the above results, it can be concluded that the method is precise. The results are tabulated in Table 4.

	Sample set No	Cytosine impurity	GCBα anomer impurity	β Uridine impurity	Total Impurities
Method Precision	1	0.13	0.13	3.0	3.26
	2	0.13	0.13	3.0	3.26
	3	0.14	0.13	2.9	3.17
	4	0.14	0.13	2.9	3.17
	5	0.13	0.13	3.0	3.26
	6	0.14	0.14	3.0	3.28

Table 4: Method precision results

4.3.3 Comparison for Precision:

The intermediate precision has been carried out in Quality control department Jadcherla to ensure that the analytical results will remain unaffected with change in analyst and day. Repeated the method precision set by different analyst using different instrument, different column and different day. From the obtained results, it can be concluded that method is rugged. The results are tabulated in table 5.

	Sample set No	Cytosine impurity	GCB α anomer impurity	β Uridine impurity	Total Impurities
Method Precision	1	0.13	0.13	3.0	3.26
	2	0.13	0.13	3.0	3.26
	3	0.14	0.13	2.9	3.17
	4	0.14	0.13	2.9	3.17
	5	0.13	0.13	3.0	3.26
	6	0.14	0.14	3.0	3.28
Intermediate Precision	7	0.17	0.17	2.9	3.24
	8	0.17	0.17	2.9	3.24
	9	0.18	0.17	2.9	3.25
	10	0.18	0.17	2.9	3.25
	11	0.17	0.17	2.9	3.24
	12	0.18	0.17	2.9	3.25
	Mean	**0.16**	**0.15**	**2.93**	**3.24**
	%RSD	**13.5**	**13.5**	**1.7**	**1.1**

Table5: Comparison between the method precision and intermediate precision

Stability in analytical solutions

Solution stability at 5°C

The Diluted standard solution is stable for 48 hours at 5°C (% difference of cytosine, GCB is 0.3,-0.5).The spiked sample solution is stable for 36 hours at 5°C (%difference of Cytosine, Gemcitabine, α anomer and β Uridine is 0.1,-.1.1 &0.4). The % difference of the spiked sample preparation from total Impurity is 0.2.

Solution stability at 25°C :

The Diluted standard solution is stable for 57 hours at room temperature (25°C) (% difference of cytosine, GCB is 1.3, 2.4). The spiked sample solution is stable for 41 hours at room temperature(25°C) (%difference of Cytosine,Gemcitabine, α anomer and β Uridine is -1.6,-3.2 &-1.1).The % difference of the spiked sample preparation from total Impurity is -1.4.

4.4 Linearity:

Performed the linearity with using Gemcitabine, Cytosine, GCBα-anomer, β-uridine standard in the range of LOQ to 300% of impurity specification limit. Tested the intercept for statistical equivalence to zero. Also performed precision at higher level by injecting 6 times into the chromatograph. Plotted a graph of Gemcitabine,Cytosine, GCBα anomer,β uridine, as concentration (PPM) on X-axis and area response on Y-axis. For this linearity different solutions are prepared which includes GCB Linearity stock solution, Cytosine Linearity stock solution, GCBα anomer Linearity stock solution and β Uridine Linearity stock solution these are prepared by using standard method of preparation. And graphs are shown in Fig: 2 which indicate the linearity curves.

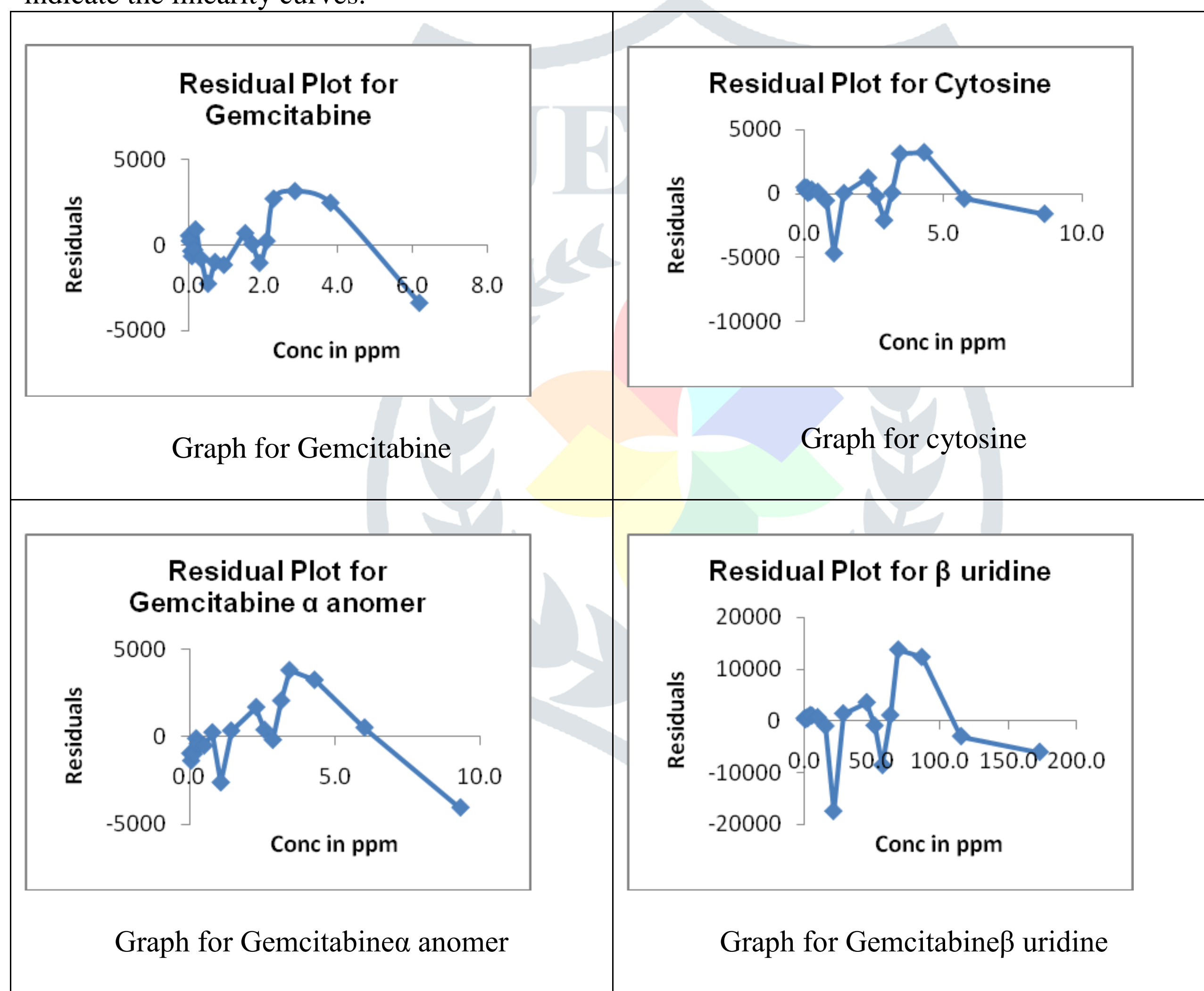

Fig:2 Graphs for Gemcitabine,Cytosine, GCBα anomer and Gemcitabine β uridine

4.5 Precision

From the Statistical treatment of the linearity data of Gemcitabine, cytosine, GCBα anomer and β Uridine it is clear that the responses of Gemcitabine, Cytosine, Gemcitabine, α anomer and β Uridine is linear between LOQ level to 300% level of specification limit. The correlation and regression coefficient are greater than 0.995. In addition, the analysis of residual show that the values are randomly scattered around zero, the P-value was determined. The P value is >0.05 then the intercept is statistically equal to zero for Gemcitabine,

Cytosine, GCBα anomer and β Uridine value is 0.14, 0.50,0.38 and 0.79. Hence it is statistically equal to zero. In addition, the origin is with in the lower and the upper limit of the 95% confidence level that gives high degree of confidence to the value obtained for intercept. Moreover, the value of the intercept is with in ± 5% of the area response at 100% level. Results are tabulated in Table 6.

S. No	Gemcitabine	Cytosine	GCBα anomer	β uridine
1	92658.377	264601.019	130696.553	994238.889
2	95177.823	265131.985	132345.799	998018.482
3	92677.736	263780.853	131263.372	995852.306
4	90894.306	264554.384	132071.877	999205.559
5	95212.129	265181.443	132552.812	997140.348
6	92596.109	265368.435	132735.313	998642.245
Mean	**93202.747**	**264769.687**	**131944.000**	**997182.972**
RSD	**1.8**	**0.2**	**0.6**	**0.2**

Table 6: Precision results

4.6 Limit Of Detection And Quantitation:

Limit of detection is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Limit of quantitation is the lowest amount of analyte in a sample that can bequantitated with acceptable accuracy and precision, under the stated experimental conditions. Calculatedslope,Intercept & correlatation coefficient and the residual standard deviation from the linearity curve. From the intercept, slope and residual standard deviation calculate limit of detectionand Quantitation. Distinct visible peak should be observed at LOD level concentration. The %RSD of the area response obtained from 6 injections (LOQ level) should be NMT10.0. LOQ concentration should be less than or equal to 0.1% of nominal concentration. From the data presented,it is observed that Gemcitabine,Cytosine,GCBα anomer and β uridine are précises at the LOQ Level. The results are tabulated in Table 7.

Confirmed LOD & LOQ Level:

	Gemcitabine	Cytosine	GCBα anomer	β Uridine
LOD(PPM)	0.24	0.16	0.32	0.33
LOQ(PPM)	0.73	0.50	0.97	0.99
LOD (%)	0.013	0.008	0.017	0.017
LOQ (%)	0.038	0.027	0.051	0.052

Table 7: Results for LOD and LOQ

4.7 ACCURACY:

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of an analytical method should be established across its range. Spiked known quantity of Cytosine,GCBα anomer and β Uridine standard at specification level of impurity into the sample. Spiked the known quantity of GCBinto placebo at specification level of unknown impurity.Analyzed these samples in triplicate for each level. From the results, calculate accuracy and range parameters. Cytosine standard Recovery Stock GCBαanomer standard Recovery stock, β Uridine standard Recovery stock, GCB standard stock preparation by takening these solutions for Recovery at LOQ is also performed in the form of

Cytosine standard LOQ Recovery Stock, GCB α anomer standard LOQ Recovery stock, β Uridine standard LOQ Recovery stock, GCBstandard LOQ recovery stock. Finally it is concluded that The Individual and mean recovery at each level should be between 85% at 115% for Known impurity. The Individual and mean recovery at each level should be between 80.0% to 120.0% for known& Unknown impurity.

4.8 Range:

The range of analytical method is the interval between the upper and lower levels of analyte that has been demonstrated to be determined with a suitable accuracy and linearity. Derived the specified range from linearity and accuracy studies. %RSD obtained for all the accuracy level determinations are within the limits. The Correlation and regression coefficient should be NLT 0.995 for Linearity and accuracy level parameter.

4.9 Robustness:

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Change in column oven temperature ±5°C. The System Suitability parameters should pass for all the conditions. All Known impurities should be separate from each other and from GCB peak in sample spiked with impurities. From the results, it can be concluded that the method is robust towards small variations in method parameters.

5. Results and Discussion

A satisfactory separation and good peak symmetry was found in a mixture of buffer and Acetonitrile, 60:40 v/v ratio. Flow rate 0.8ml/minute and wavelength detected at 270nm. The parameters considered for the analytical method validation of GCB injection is 38.02mg/ml. For system suitability the tailing factor is 1.5. The %RSD for 6 injection of standard preparation is 5.0. For Method precision the results are within specification limits. The %RSD calculated for related substance value of GCB from 12 determinations is 0.2, %RSD obtained for all the accuracy levels determinations is 4.2. The Proposed HPLC Method for GCB in the drug product GCB non-Aqueous injection was validated as per analytical method validation. The Method was found Precise, Specific, accurate and robust.

6. Acknowledgement

The Analytical Formulation carried out for related substance of GCB in drug product by using non-Aqueous injection at Shilpa Medicare limited.

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