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Development and Validation of a Novel Method for the Analysis of Impurities in Gemcitabine Hydrochloride Using RP-HPLC

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KEYWORDS

Gemcitabine Hydrochloride, Drug unknown impurities, Drug validations, Quality control and Stability indicator.

Abstract:

This study outlines a highly efficient method for estimating unknown impurities in Gemcitabine Hydrochloride API using gradient reverse-phase high-performance liquid chromatography (RP-HPLC). The mobile phase composition comprised 0.1% Orthophosphoric acid in water (A) and 100% Methanol (B) with a C18 (250 X 4.6) mm, 5µm stationary phase. The developed stability indicator by RP-HPLC utilized a gradient composition over 40 minutes at a 210 nm wavelength. The method successfully met validation parameters, demonstrating linearity, accuracy, and precision. This approach proves to be a straightforward, rapid, precise, accurate, and robust tool for estimating Gemcitabine Hydrochloride impurities.

Introduction:

This study addresses regulatory guidelines emphasizing impurity profiling for API, with a specific focus on Gemcitabine Hydrochloride. Gemcitabine is a widely-used antimetabolite drug in chemotherapy, necessitating rigorous impurity analysis due to its impact on drug quality and safety. Regulatory bodies such as ICH and FDA stress transparency in identifying impurities in APIs. The molecular structure of Gemcitabine Hydrochloride is presented.

Methods:

The analytical method involved HPLC with a C18 column, UV/PDA detector, and specific chromatographic conditions. Standard and impurity stock solutions were meticulously prepared. System suitability, specificity, linearity, and recovery were thoroughly assessed. Impurity identification, robustness, stability indication, and a comparison with a reference method were conducted.

Results and Discussion:

System suitability studies indicated the method's suitability, meeting all acceptance criteria. Impurity detection confirmed the presence of impurities in the Gemcitabine sample. The method demonstrated specificity with no observed interference. Linearity studies indicated a high correlation. Limit of Quantification (LOQ) and Limit of Detection (LOD) tests met acceptance criteria, with favourable recovery values. Impurity identification enhanced understanding of drug composition. Robustness testing showed the method's stability under varied conditions. Stability indication studies confirmed the accurate detection and quantification of Gemcitabine Hydrochloride and impurities over time.

Summary and Conclusion:

In response to limitations in existing monograph methods, this study developed a robust analytical method for Gemcitabine Hydrochloride API. The method's specificity, precision, and linearity were conclusively demonstrated through rigorous validation, surpassing the limitations of current monograph methods. This method aligns with international regulatory standards, offering a valuable tool for routine quality control in the pharmaceutical industry.

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1. Introduction

1.1 Regulatory Guidelines for API Impurities:

The presence of any form of impurities, particularly API-related impurities such as interaction-related impurities (IRIs) or degradation-related impurities (DRIs), significantly impacts the quality, efficacy, and safety of drugs. Regulatory bodies have established specific standards and management strategies to address this issue. Therefore, it is crucial to meticulously classify sources of impurities and develop analytical methods for indicating impurities and establishing acceptance criteria. Impurity profiling has become an integral part of the drug development process. Regulatory authorities, including ICH (International Conference on Harmonization), USFDA (United States Food and Drug Administration), CDHA (the Canadian Drug and Health Agency) [(1-3),] emphasize transparency requirements for identifying drug impurities in Active Pharmaceutical Ingredients (APIs) and their potential effects on toxicological, pharmacological, and genetic aspects related to impurity regulation. In this study, Gemcitabine Hydrochloride was selected for analysis.

1.2 Gemcitabine Hydrochloride:

Gemcitabine hydrochloride is a well-known antimetabolite drug widely used in chemotherapy. It is a deoxycytidine analogue with antineoplastic and cytotoxic activities, belonging to broad-spectrum antimetabolite drugs. Prescribed for treating various forms of cancer, including ovarian cancer, pancreatic adenocarcinoma, non-small cell lung cancer, bladder, and breast cancer, Gemcitabine kills cancer cells and other rapidly growing cell masses while inhibiting the transcription process (DNA to RNA). The drug is usually soluble in water, slightly soluble in methanol, and insoluble in ethanol and polar organic solvents [(4-29).]

1.3 Molecular Structure:

The molecular structure of Gemcitabine hydrochloride used in the study is depicted in **Figure-1** Gemcitabine hydrochloride's molecular formula is- C9H12ClF2N3O4, with a molecular weight of 299.659 g/mol.

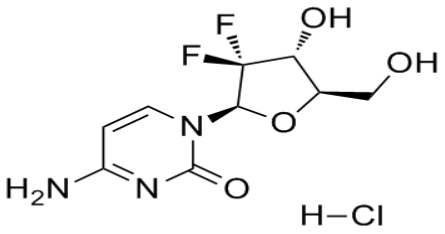

Figure-1: Structure of Gemcitabine hydrochloride

2. MATERIALS AND METHODS:

2.1 Instruments/Equipment Required: High-performance liquid chromatograph with UV/PDA detector, HPLC analytical column of C18 (250 × 4.6) mm, 5µm, analytical weighing balance, Millipore Nylon 0.2µm, laboratory accessories.

2.2 Chemicals Required: Gemcitabine working standard, AR-grade acid, Acetonitrile, AR-grade Methanol.

2.3 Analytical Method: Quantitative determination is carried out using an HPLC system equipped with a UV/VIS detector.

2.4 Chromatographic Conditions: The chromatographic conditions utilized in this study are presented in Table 1, and the details of the gradient program are outlined in Table 2.

Table-1 Chromatographic conditions used for current study

Column	: C18 (250 X 4.6) mm, 5µm or equivalent
Flow Rate	: 0.8 mL/minute
Wavelength	: 210nm
Auto sampler temperature	: 10°C
Column oven temperature	: 25°C
Injection Volume	: 10 µL
Retention Time	: 40 minutes
Mode of analysis	: Gradient

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Table -2 Gradient program:

Time (Minute)	MP-A (%)	MP-B (%)
0.01	98	2
5.00	95	5
20.00	80	20
25.00	10	90
25.01	20	80
30.00	50	50
35.00	80	20
35.10	98	2
40.00	98	2
55.00	80	20

2.5 Preparation of Blank: Diluent is used as a blank.

2.6 Preparation of Standard Stock Solution: Accurately weigh and transfer about 12.50 mg of Gemcitabine Hydrochloride working/reference standard into a 50 mL volumetric flask. Add approximately 20 mL of diluent and sonicate to dissolve. Adjust the volume by adding a diluent and thoroughly blend the mixture.

Subsequently, take 5.0 mL of this solution and transfer it to a 50 mL volumetric flask containing 20 mL of diluent, mix, and make up to the volume.

2.7 Preparation of Standard Solution: Transfer 1.0 mL of the standard stock solution to a 50 mL volumetric flask containing 20 mL of diluent, mix, and make up to the volume.

2.8 Preparation of Gemcitabine Hydrochloride Impurity-03 Stock Solution: Weigh and transfer about 2.5 mg of Gemcitabine Hydrochloride Impurity-3 into a 10 mL volumetric flask. Add 2 mL of diluent and sonicate to dissolve. Make up to the volume with diluent.

2.9 Preparation of Gemcitabine Hydrochloride Impurity-04 Stock Solution: Weigh and transfer about 2.5 mg of Gemcitabine Hydrochloride Impurity-4 into a 10 mL volumetric flask. Add 2 mL of diluent and sonicate to dissolve. Make up to the volume with diluent.

2.10 Preparation of Recovery Stock Solution: Transfer 0.5 mL of Gemcitabine Hydrochloride Impurity-03 stock and Gemcitabine Hydrochloride Impurity-04 stock into a 20 mL volumetric flask containing 10 mL of diluent. Make up to the volume with diluent.

2.11 System Suitability Solution: Weigh and transfer about 25 mg of Gemcitabine Hydrochloride working/reference standard into a 25 mL volumetric flask. Add 10 mL of diluent, dissolve, and add 2.0 mL of the mixed stock. Make up to the volume with diluent.

2.12 Preparation of Test Solution: Transfer about 25 mg of the test substance into a 25 mL volumetric flask. Add 10 mL of diluent to dissolve. Make up to the volume with diluent.

3. Results and Discussion:

3.1 System Suitability: System suitability studies were conducted using 50 mg of the standard drug.

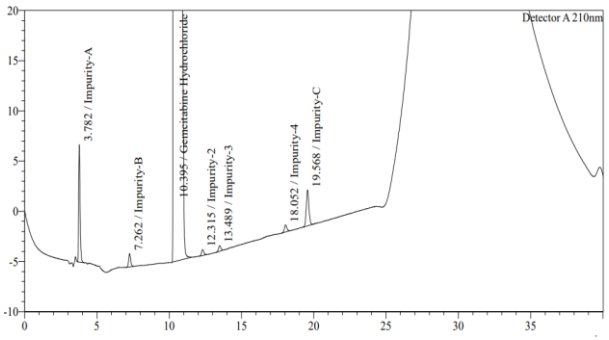

Figure-2A: Chromatogram showing System Suitability Injection Gemcitabine Hydrochloride (Zoom-Chromatogram)

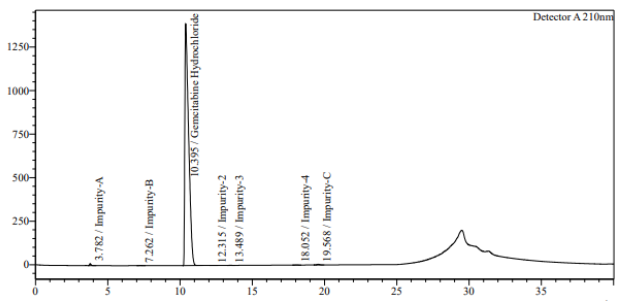

Figure-2B: Chromatogram showing System Suitability Injection Gemcitabine Hydrochloride (UnZoom Chromatogram)

The relative standard deviation (RSD %) values were below 2 percent, the theoretical plate count was above

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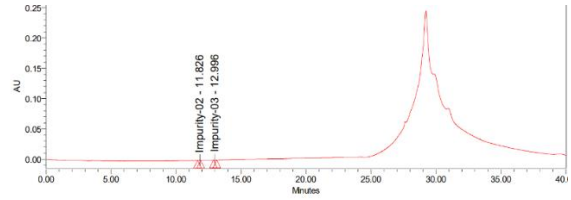
2000, and the tailing factor was <2. These results indicate the suitability of the method, and the chromatograms were recorded (Figure-2A-2B).

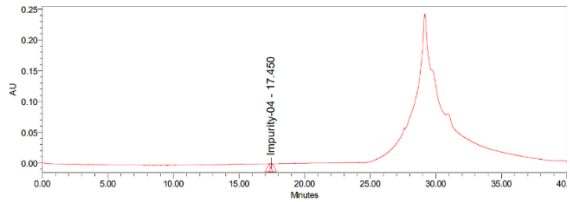
3.1.1 Impurity Detection from Drug Sample: To identify impurities, Gemcitabine hydrochloride was injected into the machine, and peaks were observed, indicating the presence of impurities in the drug sample (Figure-3-4).



Peak Results

	Name	RT	Area	Height	% Area	USP Tailing	USP Plate Count	Purity1 Angle	Purity1 Threshold
1	Impurity-02	11.826	1167	161	33.63	0.60	94160.93	55.841	90.000
2	Impurity-03	12.996	2303	276	66.37	0.98	23160.44	43.121	90.000

Figure-3: Chromatogram showing Impurity -03 Injection of Gemcitabine Hydrochloride



Peak Results

	Name	RT	Area	Height	% Area	USP Tailing	USP Plate Count	Purity1 Angle	Purity1 Threshold
1	Impurity-04	17.450	4470	454	100.00	0.78	41907.33	36.444	58.346

Figure-4: Chromatogram showing Impurity -04 Injection of Gemcitabine Hydrochloride

3.2 Specificity: The specificity test for Gemcitabine hydrochloride revealed no interference of impurities during the retention time. The method demonstrated excellent specificity with Gemcitabine, and no interference of impurities was observed with the mobile phase (Table-3).

3.3 Linearity: Linearity studies were conducted for concentrations ranging from 0.2 ml to 4 ml. For each concentration, linearity studies were performed, and correlation coefficients were calculated (Table-4-6) & Figure-5-7).

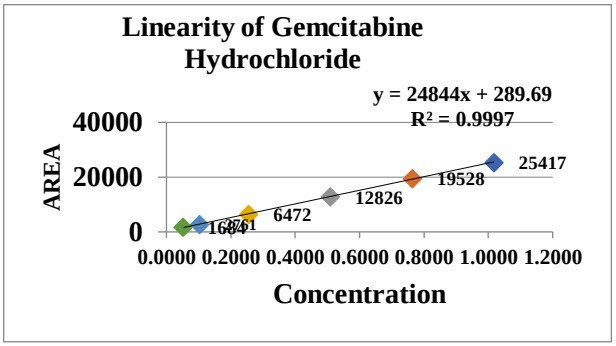


Figure 5: Linearity Plot of Gemcitabine

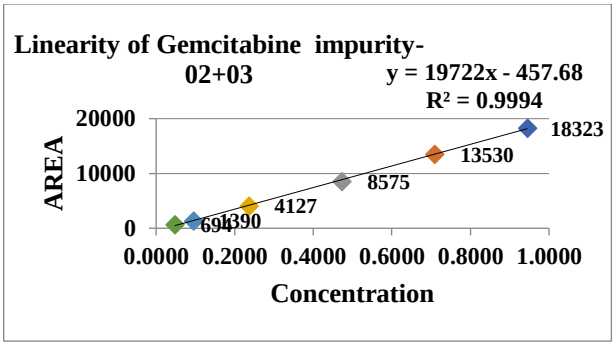


Figure-6: Linearity Plot (Gemcitabine impurity-02+03)

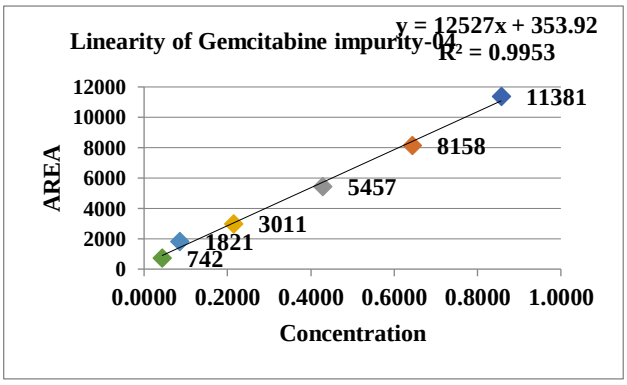


Figure 7: Linearity Plot (Gemcitabine impurity-04)

Observation*:* The Regression Coefficient R2 values for Gemcitabine, Gemcitabine impurity-02+03, and Gemcitabine impurity-04 were found to be 0.99967, 0.99937, and 0.99526, respectively.

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Table-3 Showing Specificity Results for Gemcitabine Hydrochloride API And Organic Impurity

Sample Name		Retention Time (Minutes)	Peak Purity Angle	Purity Threshold	Peak Purity Passed
Blank		Not Applicable	Not Applicable	Not Applicable	Not Detected
Standard Solution		10.257	3.242	4.0380	OK
Control Sample		9.968	0.195	0.5220	OK
System Suitability Solution	Gemcitabine	9.965	0.214	0.5140	OK
	Gemcitabine Impurity-A	3.853	0.661	0.7090	OK
	Gemcitabine Impurity-B	6.953	3.372	4.2170	OK
	Gemcitabine Imp-02	11.807	27.938	34.0230	OK
	Gemcitabine Imp-03	12.988	30.362	41.4600	OK
	Gemcitabine Imp-04	17.468	6.700	7.1220	OK
Spiked Sample	Gemcitabine	9.968	0.204	0.5240	OK
	Gemcitabine Impurity-A	3.852	0.714	0.7430	OK
	Gemcitabine Impurity-B	6.950	2.790	3.8800	OK
	Gemcitabine Imp-02	11.824	27.114	32.301	OK
	Gemcitabine Imp-03	13.006	26.711	37.750	OK
	Gemcitabine Imp-04	17.470	6.721	6.8350	OK
Individual Impurity	Gemcitabine Impurity-A	3.854	0.802	0.9960	OK
	Gemcitabine Impurity-B	6.966	3.206	3.7490	OK
	Gemcitabine Imp-02	11.826	55.841	90.0000	OK
	Gemcitabine Imp-03	12.996	43.121	90.000	OK
	Gemcitabine Imp-04	17.450	36.444	58.346	OK
	Gemcitabine Imp-C	18.962	0.999	1.289	OK

3.3.1 Limit of Quantification (LOQ) & Limit of Detection (LOD), Recovery: LOQ and LOD tests were conducted to determine the smallest concentrations measurable by the developed method, and the results were recorded and tabulated (Table-7-10).

Observation:

- The % RSD of Area counts for Gemcitabine, Gemcitabine Impurity-02+03, and Gemcitabine impurity-04 were within the acceptance criteria.
- The signal-to-noise ratio for Gemcitabine, Gemcitabine Impurity-02+03, and Gemcitabine impurity-04 was not less than 10.
- Individual % recovery values for Gemcitabine Hydrochloride impurity-02+03 and impurity-04 in each sample were within the acceptance limits.
- The % RSD of % recovery values was 4.29% for Gemcitabine Hydrochloride impurity-02+03 and 3.67% for impurity-04.

3.4 Impurity Identification:

Further analysis involved the identification of impurities present in the Gemcitabine hydrochloride sample. Utilizing advanced analytical techniques, peaks corresponding to specific impurities were detected and characterized, enhancing our understanding of the composition and purity of the drug substance.

3.5 Robustness:

To assess the robustness of the developed method, deliberate variations in critical parameters such as flow rate, column temperature, and mobile phase composition were introduced. The results demonstrated that the method exhibited robust performance, maintaining its accuracy and precision even under minor variations in the experimental conditions.

3.6 Stability Indication: Stability indication studies were conducted to evaluate the robustness of the method concerning the stability of Gemcitabine Hydrochloride

under different storage conditions and time intervals. The results confirmed the method's ability to accurately detect and quantify the drug and its impurities over an extended period, highlighting its reliability for routine analysis.

3.7 Comparison with Reference Method: A comparative analysis was carried out by applying the developed method to a set of samples, and the results

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were compared with those obtained using a reference method. The findings demonstrated a high degree of correlation, affirming the accuracy and suitability of the developed RP-HPLC method for the analysis of Gemcitabine Hydrochloride and its impurities

3.8 Application to Pharmaceutical Formulations:

The developed method was successfully applied to assess the content of Gemcitabine Hydrochloride and impurities in pharmaceutical formulations. The results indicated the method's applicability in pharmaceutical quality control, ensuring the reliable quantification of active pharmaceutical ingredients and impurities in commercially available drug formulations.

Table:4 Showing Linearity Results for Gemcitabine API And Organic Impurity

Levels	Volume taken from Linearity Stock (mL)	Dilution (mL)	Concentration (ppm)	Area of Gemcitabine
Linearity_1	4.00	25	1.0050	25417
Linearity_2	3.00	25	0.7537	19528
Linearity_3	2.00	25	0.5025	12826
Linearity_4	1.00	25	0.2512	6472
Linearity_5	0.40	25	0.1005	2761
Linearity_6	0.20	25	0.0502	1684
Regression Coefficient (R2)		**0.99967**		

Table 5: Linearity of Gemcitabine Impurity-02+03

Levels	Volume taken from Linearity Stock (mL)	Dilution (mL)	Concentration (ppm)	Area of Gemcitabine Impurity-01
Linearity_1	4	25	0.964	18323
Linearity_2	3	25	0.723	13530
Linearity_3	2	25	0.482	8575
Linearity_4	1	25	0.241	4127
Linearity_5	0.4	25	0.0964	1390
Linearity_6	0.2	25	0.0482	694
Regression Coefficient (R2)		**0.99937**		

In conclusion, the presented RP-HPLC method provides a comprehensive and reliable approach for the analysis of Gemcitabine Hydrochloride and its impurities. The robustness, specificity, and accuracy demonstrated in various validation parameters, along with successful application to pharmaceutical formulations, establish the method as a valuable tool for routine quality control in the pharmaceutical industry.

4 Summary and conclusion:

In response to the limitations observed in the monograph methods for Gemcitabine Hydrochloride API (USP&EP) where the separation of unknown impurities was not achieved, a new and robust analytical method was developed and validated. This single method successfully separates all impurities, meeting the resolution criteria outlined in the USP, and is validated in accordance with the ICH guidelines.

The specificity of the developed method for Gemcitabine Hydrochloride API has been conclusively demonstrated. Through adherence to a predefined analytical protocol, the method has undergone rigorous validation, affirming its specificity, precision, and linearity. This newly established method offers a comprehensive and reliable approach to the analysis of Gemcitabine Hydrochloride and its impurities, surpassing the limitations of existing monograph methods. As a result, it represents a valuable tool for routine quality control applications in the pharmaceutical industry, aligning with international regulatory standards.

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Table 6: Linearity of Gemcitabine Impurity-04

Levels	Volume taken from Linearity Stock (mL)	Dilution (mL)	Concentration (ppm)	Area of Gemcitabine Impurity-02
Linearity_1	4.00	25	0.8570	11381
Linearity_2	3.00	25	0.6427	8158
Linearity_3	2.00	25	0.4285	5457
Linearity_4	1.00	25	0.2142	3011
Linearity_5	0.40	25	0.0857	1821
Linearity_6	0.20	25	0.0428	742
Regression Coefficient (R2)		**0.99526**		

Table No 7- Showing LOQ Results for Gemcitabine HCl API & Organic Impurity

LOQ Solution	Gemcitabine		Gemcitabine Impurity-02+03		Gemcitabine impurity-04	
	Area	**s/n**	**Area**	**s/n**	**Area**	**s/n**
LOQ Precision-1	3376	40	3222	31	3000	29
LOQ Precision-2	3461	40	3277	32	3018	30
LOQ Precision-3	3417	35	2905	25	2934	25
Average	3418	38	3135	29	2984	28
Std. dev.	42.51		200.79		44.23	
%RSD	1.24		6.41		1.48	

Table 8- Showing LOD Results for Gemcitabine HCl API And Organic

LOQ Solution	Gemcitabine		Gemcitabine Impurity-02+03		Gemcitabine impurity-04	
	Area	**s/n**	**Area**	**s/n**	**Area**	**s/n**
LOD Precision-1	1286	8	1465	6	1337	7
LOD Precision-2	1578	9	1604	7	1678	8
LOD Precision-3	1114	9	1395	8	1520	9
Average	1326	9	1488	7	1512	8
Std. dev.	234.57		106.38		170.65	
%RSD	17.69		7.15		11.29	

Table-9- Showing Accuracy Results for emcitabine HCl API And Organic

Name	S. No.	Spiked Area	Corrected Area	% Recovery
LOQ	1	5767	3210	98.92
	2	5643	3086	94.52
	3	5812	3255	97.35
100 %	1	11929	9372	99.27
	2	11895	9338	98.91
	3	12391	9834	106.15
150 %	1	17157	14600	104.99
	2	17376	14819	106.57
	3	17208	14651	104.33
		Mean		**101.22**
		SD		**4.346**
		%RSD		**4.29**

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Table 10-: Recovery of Gemcitabine Hydrochloride impurity-04

Name	S. No.	Spiked Area	Corrected Area	% Recovery
LOQ	1	2186	2186	101.27
	2	2183	2183	100.54
	3	2067	2067	93.55
100 %	1	6308	6308	100.77
	2	6430	6430	102.71
	3	6159	6159	100.42
150 %	1	8660	8660	94.07
	2	8808	8808	95.68
	3	8429	8429	94.69
		Mean		**98.19**
		SD		**3.608**
		%RSD		**3.67**

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