Chapter 2: Analytical Method Development

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2.1 Introduction

Analytical methods are important tools to estimate the entrapment efficiencyof the formulations and assess the stabilityof the drug in terms of percent drug retained (PDR) in the formulations over the period of time. Instrumental analysis are very sensitivity and accurate measure of estimation. Hence, UV spectrophotometric, a simplest instrumentation method capable of drug estimation in micrograms was used. In the presence of interfering components, derivative spectroscopy is used for drug estimation. HPLC method is more sophisticated method used for the estimation of samples with very low quantity of the drug.The analytical methods employed in these investigations are described below [1].

A literature survey reveals the report of a few analytical methods for the determination of gemcitabine in pharmaceutical dosage forms and in biological fluids by HPLC [2, 3].

2.2 Materials and Instruments

2.2.1 Instrument and Software for UV Spectrophotometric Measurement

Spectrophotometric measurements were carried out on a Shimadzu 1700 double beam UV Visible spectrophotometer with a fix slit width of 1nm coupled with UVPROB V2.34 software. The spectral bandwidth was 1 nm and the wavelength scanning speed was 2800 nm/min. Matched quartz cuvettes (1cm) were used for all the spectral measurements.

2.2.2. Instrument and Software for HPLC Measurement

The chromatographic system (Shimadzu,Kyoto, Japan) was Shimadzu LC-20 AT Prominence solvent delivery module, a manual Rheodyne injector with a 20µl fixed loop and SPD-20A ProminenceUV-Visible detector. The separation was performed on a Phenomenex C8 column (particle size 5µm, length 250mm X 4.6mm; Phenomenex Torrance, USA). Chromatographic data were recorded and processed using Spinchrome Chromatographic Station® CFR Version 2.4.0.193 (Spinchrome Pvt.Ltd., Chennai, India).

2.3 Materials and Reagents

Gemcitabine hydrochloride (Gifted by Sun Pharma Advanced Research Center (SPARC), Vadodara, Gujarat, India), Methanol (HPLC grade), Water (HPLC grade), Tetrazolimu Blue, Tetramethyl ammonium hydroxide in MeOH/Toluene, Glacial acetic acid, Acetontrile (HPLC Grade). All the reagents were AR Grade.

2.4 Methods

2.4.1 Method Validation Parameters for UV Spectroscopic Method

2.4.1.1. Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value [4]. The accuracy of the method was determined by calculating the recoveries of the analyte by the method of standard additionsdifferent levels of drug concentrations. Known amounts of standard drug (80%, 100% and 120%) were added to the pre-analyzed samples and the absorbance was measured. Accuracy is assessed as the percentage relative error and mean % recovery. The % recovery of the added pure drug was calculated as the % recovery = [(Ct–Cs)/Ca)]*100, where Ct is the total drug concentration measured after standard addition; Cs drug concentration in solution sample; Ca drug concentration added to solution.

2.4.1.2 Precision/ Repeatability/Stability

Precision is a measure of the consistency and reproducibility of a method. The precise analytical method is the degree of agreement among the individual test results gives very close values for repeated measurement of same samplewhen the procedure is applied repeatedly to multiple sampling of homogeneous sample. The precision of an analytical method is usually expressed as the SD (Standard Deviation) or RSD (% Relative Standard Deviation)

2.4.1.3 Linearity and Range

Linearity of an analytical method is the ability to elicit the test results that are directly or by well-defined transformation proportional to the concentration of the analyte in the samples within the given range.

2.4.1.4 Specificity

Analytical specificity of a method may be defined as its ability to accurately identify, measure and resolve an analyte in the presence of other closely related compounds that is, how well an assay detects only a specific substance and not the other closely related substances during an analysis. The Analytical specificity of analytical method was determined by comparing the graph obtained followed by the analysis of just the solvent and the graph of the solvent containing the drug.

2.4.2 Method Validation Parameters for HPLC Method

The parameters mentioned under UV spectroscopic method (Linearity and Range, Accuracy, Precision/Repeatability, Specificity were performed for HPLC method.

2.4.3 Direct Estimation of Gemcitabine HCl by UV Spectrometry

Most of the drugs being organic in nature having chromophoric group and absorb in the UV range. Hence, can be estimated by a direct UV spectroscopic method.

2.4.3.1 Preparation of Stock Solution of Drug

Stock solution containing 0.1mg/ml was prepared by dissolving drug in different solvents such as Distilled water, Phosphate Buffer (pH 7.4) and Methanol.

2.4.3.2 Preparation of Standard Solution of Drug

Standard solutions were prepared by pipetting out required volume of stock solution in 10 ml volumetric flasks and making the volumeup to the mark with different solvents such as Distilled water, Phosphate Buffer (pH 7.4) and Methanol to obtain known final concentrations in µg/ml. The different spectra of the standard solutions were recorded using UV Visible spectrophotometer for 200nm to 400nm range against respective solvent as blank. The observations were recorded in triplicate. The experimental conditions for Gemcitabine HCl by UV method were as below **(Table 2.1)**.

TABLE 2 1 Experimental conditions for Gemcitabine HCl by UV method were as below.

Solvents	**Distilled water, Phosphate Buffer[#] (pH 7.4) and Methanol**
Stock Solution Concentration	0.1mg/ml
Serial Concentration Range	2-30 µg/ml
Spectrum Range	200nm to 400nm
Spectrum Blank	Distilled water, Phosphate Buffer (pH 7.4)and Methanol

[#] **Preparation of phosphate buffer pH 7.4:** Dissolved 2.35gm of Disodium Hydrogen Phosphate, 0.19gm of Potassium Dihydrogen Phosphate and 8gm of Sodium Chloride in sufficient distilled water to produced 1000mL in volumetric flask and adjust the pH if necessary with 0.1N HCl or 0.1N NaOH. (As per Indian Pharmacopoeia 1996).

2.4.3.3. Estimation of Drug forIn VitroRelease Study Samples, Excipients and Formulations

A definite volume of sample to be estimated like supernatant of the saturated excipients, *in vitro* drug release study samples and formulations like solution andliposomes was taken in a 10mlvolumetric flask and diluted up to the mark with methanol. The resultant solution was then sonicated for 2 min at ambient temperature, filtered through 0.22 µm membrane filter. Further dilutions were made up with methanol and the samples were analyzed in triplicate.

2.4.4 Stability study

Stability of the solution of Gemcitabine HCl in distilled water, PBS pH 7.4 and methanol was ascertained by observing the changes in the absorbance of the solution at the analytical wavelength over a period of 24hr at room temperature. The readings were recorded in triplicate.

2.4.5 Interference study of the formulation component

The interference of different lipids (DPPC, DSPG, Cholesterol, DSPE-mPEG$_{2000}$) in the estimation of the Gemcitabine HCl was determine by measuring the individual absorbance maxima at 268.8 nm against methanol. The spectrum of blank liposomal formulation and drug loaded liposomal formulation were compared for interference study.

2.4.6 Estimation of Gemcitabine HClby HPLC

Estimation of Gemcitabine HCl by HPLC has been reported USP NF 30[5]. The reported method was used for estimation of Gemcitabine HCl in formulations, *in vitro* release medium and exscipients.

2.4.6.1 Preparation of Stock Solution of Drug

Stock solution containing 0.1mg/ml was prepared by dissolving drug in methanol.

2.4.6.2 Preparation of Standard Solutions of Drug

Appropriate and accurate aliquots of the stock solutions were transferred to 10ml calibrated flasks anddiluted up to the volume with methanol in order to get a series of known final concentrations in µg/ml.

2.4.6.3 Estimation of Drug forIn VitroRelease Study Samples, Excipients and Formulations

A definite volume of sample to be estimated like supernatant of the saturated excipients, *in vitro* drug release study samples and formulations like solution andliposomes was taken in a 10mlvolumetric flask and diluted up to the mark with methanol. The resultant solution was then sonicated for 2 min at ambient temperature, filtered through 0.22 µm membrane filter. Further dilutions were made up with methanol and the samples were injected in triplicate.

2.5 Analytical Conditions

Analysis was isocratic with fixed flow rate of the mobile phase. The mobile phase was prepared freshly every day. The mobile phase was filtered through a 0.22 µm membrane filter to remove any particulate matter, mixed and degassed by sonication before use. The absorbance of drugs at the required wavelength was checked for any interference. Prior to injecting solutions, the column was equilibrated for 60 minutes with the mobile phase

flowing through the system. Each solution was injected in triplicate and relative standard deviation was required to be below 2% on peak area basis (USP). The experimental conditions for Gemcitabine HCl by UV method were as below.

2.5.1 Chemicals and Reagents

Methanol was of HPLC grade and purchased from Merck chemicals, India. All the other solvents and reagents used were of analytical grade were filtered through a 0.2µm Ultipor ® Nylon 66 membrane filter (Pall Life Sciences, USA) prior to use **(Table 2.2)**.

TABLE 2 2 Experimental conditions for Gemcitabine HCl by HPLC method were as below.

Column	**C8, 0.5 µm size, 4.6 mm X 250mm**
Mobile Phase	Sodium dihydrogen phosphate (pH 2.5±0.1 adjusted with phosphoric acid)
Stock Solution Concentration	100 µg/ml
Serial Concentration Range	10-100 µg/ml
Flow Rate	1.2mL/min
Injection Volume	20 µl
UV Detection	275nm
Retention Time	≅ 6.5 min

2.6 Result & Discussion

2.6.1 Direct Estimation of Gemcitabine HCl by UV Spectrometry

The UV-Visible spectrum obtain by scanning the 30µg/mL of Gemcitabine HCl in distilled water, phosphate buffer pH 7.4, methanol recorded between 200-400 nm was shown in **Figure 2-1, 2-2 and 2-3** respectively. It was observed that Gemcitabine HCl showed the characteristic peak at 268.4 nm in distilled water and phosphate buffer pH 7.4 and 268.8 nm in methanol respectively and hence, the respective wavelength was selected as the analytical wavelength throughout the further analysis.

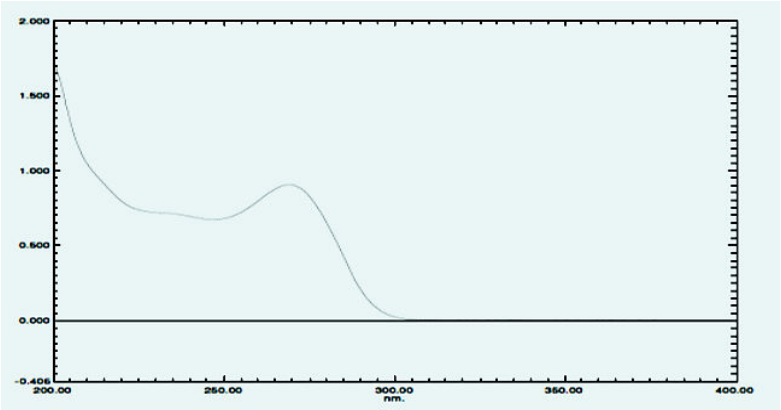

FIGURE 2 1. UV Absorption Spectrum of Gemcitabine HCl in Distilled Water

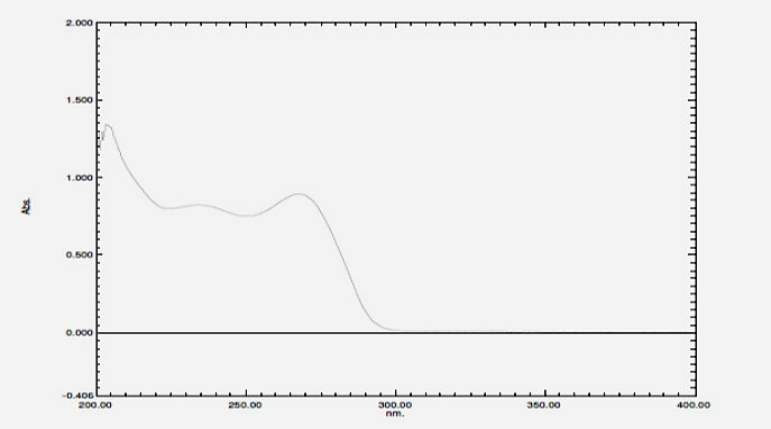

FIGURE 2 2. UV Absorption Spectrum of Gemcitabine HCl in Phosphate Buffer pH 7.4

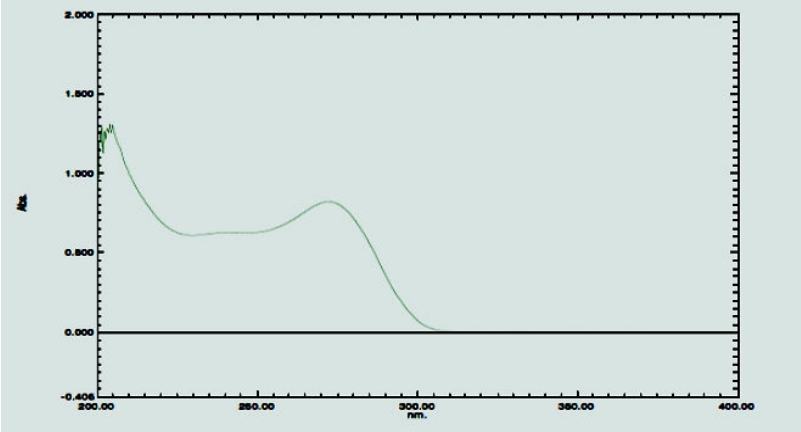

FIGURE 2 3. UV Absorption Spectrum of Gemcitabine HCl in Methanol

2.6.2 Validation Parameters for UV Spectroscopic Method

Linearity

The mean absorbance value and calibration data along with the standard deviation for Gemcitabine HCl in distilled water, phosphate buffer pH 7.4 and methanol were shown in **Table 2-3** and **Figure 2-4**. The value of correlation coefficients in the respective solvents indicated that absorbance and concentration of the drug was in significant linear correlation. Beer's law was found to be obeyed in the range of 2 to 30µg/mL in all the three solvents. The parameters indicating the linearity of the method was shown in **Table 2-3.**

TABLE 2 3. Absorbance and Calibration Data of Gemcitabine HCl for Linearity

Concentration (ppm)	Absorbance Mean ± SD (n =3)		
	Distilled water	**PBS (pH 7.4)**	**Methanol**
2	0.065 ±0.007	0.048 ± 0.015	0.051 ±0.011
4	0.118 ±0.008	0.097 ± 0.011	0.104 ±0.009
6	0.170 ±0.009	0.151 ± 0.041	0.162 ±0.010
8	0.231 ±0.007	0.208 ± 0.096	0.230 ±0.011
10	0.291 ±0.008	0.240 ± 0.007	0.293 ±0.010
15	0.423 ±0.015	0.381 ± 0.019	0.475 ±0.025
20	0.561 ±0.019	0.480 ± 0.009	0.648 ±0.066
25	0.705 ±0.025	0.631 ± 0.066	0.820 ±0.072
30	0.855 ±0.041	0.720 ± 0.025	0.996 ±0.096
Regression equation	Y=0.0243x+0.0041	Y=0.0281x + 0.0053	Y=0.0342x - 0.0362
Regression Coefficient (R^2)	0.9978	0.9998	0.9993

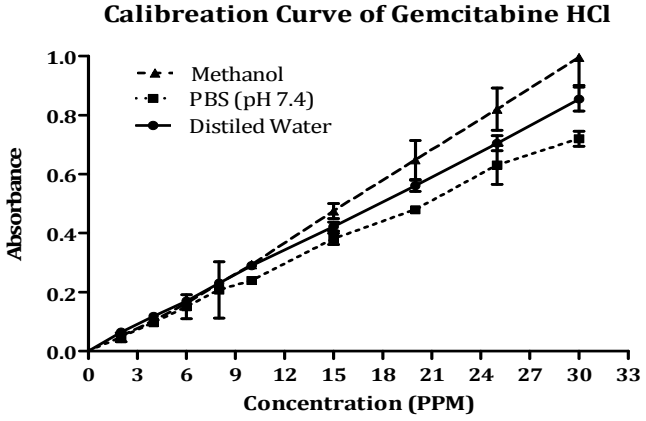

FIGURE 2 4. Calibration Curve of Gemcitabine HCl in Different Solvents

Stability

The stability of the drug in all the three solvents was ascertained over the period of 24hr by measuring absorbance of the solution at time interval of 24 hrs and the results (**Table 2-4**) revealed that there was no significant difference between the readings. Thus, it can be concluded that Gemcitabine HCl was stable in all three solvents over the period of analysis.

TABLE 2 4. Absorbance and Calibration Data of Gemcitabine HCl (after 24hr) for Stability

Concentration (ppm)	Absorbance Mean ± SD (n =3)		
	Distilled water	**PBS (pH 7.4)**	**Methanol**
2	0.061 ± 0.006	0.066 ± 0.007	0.051 ± 0.004
4	0.119 ± 0.004	0.134 ± 0.009	0.100 ± 0.006
6	0.161 ± 0.005	0.189 ± 0.003	0.144 ± 0.014
8	0.222 ± 0.005	0.241 ± 0.004	0.194 ± 0.009
10	0.281 ± 0.008	0.312 ± 0.006	0.240 ± 0.003
15	0.413 ± 0.009	0.470 ± 0.007	0.355 ± 0.008
20	0.570 ± 0.004	0.616 ± 0.008	0.509 ± 0.018
25	0.689 ± 0.007	0.731 ± 0.011	0.639 ± 0.009
30	0.831 ± 0.003	0.880 ± 0.008	0.773 ± 0.011
Regression equation	Y=0.0293X+ 0.0111	Y = 0.0293X + 0.0126	Y = 0.0257X – 0.0082
Regression Coefficient (R^2)	0.9989	0.9985	0.9986

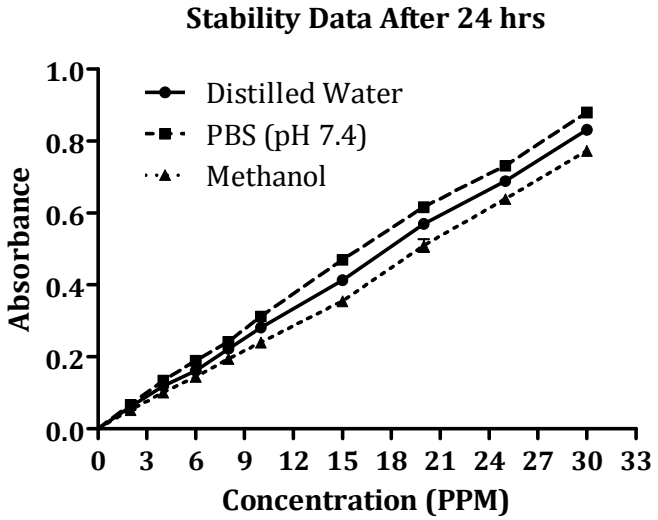

FIGURE 2 5. Calibration curve of Gemcitabine HCl in Different Solvents after 24hr for stability study

Precision

Precision was determined by studying the repeatability and intermediate precision. Intraday and inter-day precision results (**Table2-5**) indicated the precision under the same operating conditions over the interval of time was precise. In addition, the RSD values obtained for the analytical methods were within the acceptable range indicating that these methods have excellent precision.

Intraday and Interday precision analysis

TABLE 2 5. Intraday and Interday Precision Analysis of UV Method

Concentration (ppm)	Intraday Precision					Interday Precision				
	Absorbance			Mean	RSD	Absorbance			Mean	RSD
	Set 1	Set 2	Set 3			Day 1	Day 2	Day 3		
2	0.065	0.059	0.062	0.062	4.83	0.066	0.062	0.063	0.064	3.25
4	0.111	0.116	0.124	0.117	5.60	0.114	0.116	0.121	0.117	3.08
6	0.169	0.161	0.159	0.163	3.24	0.166	0.158	0.153	0.159	4.12
8	0.220	0.226	0.226	0.224	1.54	0.221	0.231	0.235	0.229	3.14
10	0.290	0.285	0.277	0.284	2.30	0.285	0.299	0.289	0.291	2.47
15	0.415	0.403	0.415	0.411	1.68	0.422	0.416	0.428	0.422	1.42
20	0.580	0.575	0.567	0.574	1.14	0.555	0.563	0.565	0.561	0.94
25	0.700	0.685	0.694	0.693	1.09	0.699	0.704	0.712	0.705	0.93
30	0.856	0.839	0.831	0.842	1.51	0.829	0.836	0.837	0.834	0.52

Accuracy

The excellent mean % recovery value, close to 100% with less standard deviation (SD < 0.1) represent high accuracy of the analytical method. The validity and reliability of the proposed method was further assessed by recovery studies by standard addition or spiking method.The mean % recovery for lower, intermediate and higher concentrationwere found to be 98.46, 101.97 and 98.36respectively **(Table 2-6)**. These results revealed that any small change in the drug concentration in the solution could accurately be determined by the proposed analytical method.

TABLE 2 6. Accuracy of the UV Method

Spiking	Drug in solution (ppm)	Pure drug (ppm)	Total drug found (ppm) (mean ± SD)	% Analytical Recovery
80%	10	8	17.88 ± 0.015	98.46 ± 0.191
100%	10	10	20.20 ± 0.042	101.97±0.416
120%	10	12	21.80 ± 0.068	98.36 ± 0.567

Interference and Specificity Study

Analytical specificity of a method may be defined as its ability to accurately identify, measure and resolve an analyte in the presence of other closely related compounds that is, how well an assay detects only a specific substance and not the other closely related substances during an analysis. The Analytical specificity of this UV spectroscopy method was determined by comparing the graph obtained followed by the analysis of just the drug solution in selected solvent and the graph of the selected solventcontaining the drug and other excipients. The representative graph of the drug solution alone (without drug) and the representative graph of the selected solvent containing drug and other excipientswere shown in was shown in **Figure 2-6**. The absorbance maxima of gemcitabine was found to be 268.8 nm. The absence of any overlapping or extraneous peaks in graph indicates the specificity of theUV method. Since gemcitabine peak could be clearly distinguished from the other peaks therefore, this method was said to be specific for the analysis of gemcitabine **(Table 2.7)**.

TABLE 2 7. Specificity and Interference Study of Formulation Component

Sample for interference study	Absorbance at 268.8 nm
Loaded liposome formulation	0.499
Plain DPPC	0.0041
Plain DSPG	0.0012
Plain cholesterol	0.0002
Plain DSPE MPEG 2000	0.0031
Blank formulation	0.031

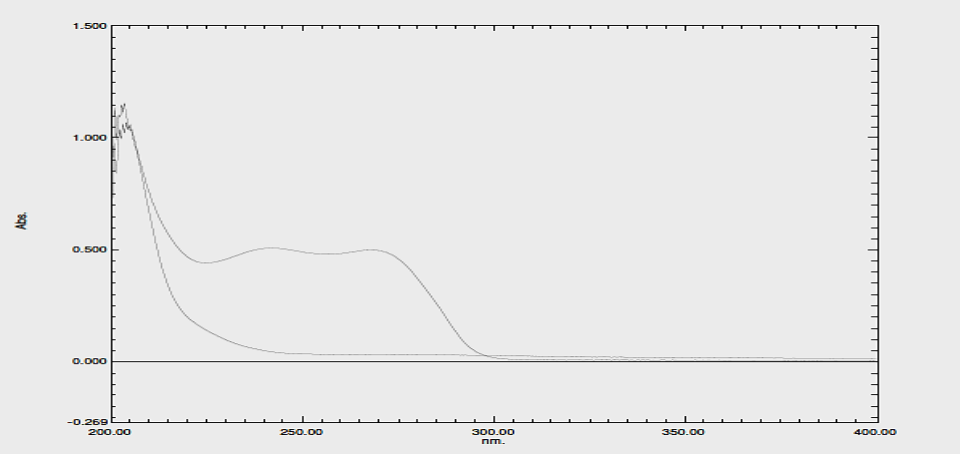

FIGURE 2 6. Specificity and Interference Study Graph between Gemcitabine HCl and Excipients

2.6.3 Direct Estimation of Gemcitabine HCl by HPLC Method

HPLC method was based on reverse phase chromatography on a C8 column. The elution was done with theflow rate of 1.2 ml/min of mobile phase and the retention time of gemcitabine HClwas found to be ≅ 6.5 min at 275 nm of detection (**Figure 2-7)**.

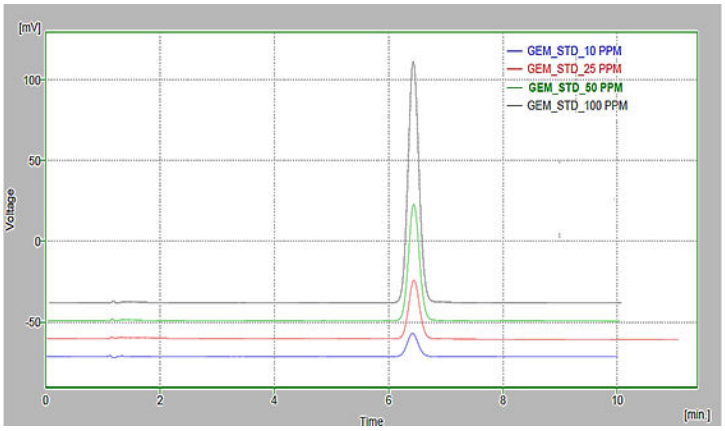

FIGURE 2 7. Typical **Chromatogram of Gemcitabine HCl by HPLC**

2.6.4 Validation Parameters for HPLC Method

Linearity

The mean area value and calibration data along with the standard deviation for gemcitabine HCl for HPLC method were shown in **Table 2-8** and **Figure 2-8**. The value of correlation coefficients indicated that area and concentration of the drug was in significant linear

correlation. Beer's law was found to be obeyed in the range of 10 to 100µg/mL. The parameters indicating the linearity of the method was shown in **Table 2-8.**

TABLE 2 8. Peak Area and Calibration Data of Gemcitabine HCl for Linearity

Concentration (ppm)	Peak Area (mV) (Mean ± SD, n=3)
10	195.768± 4.456
25	491.234 ± 9.387
50	972.115 ± 13.643
100	2007.095 ± 20.001
Regression equation	Y=20.048X-8.5202
Regression Coefficient (R^2)	0.9997

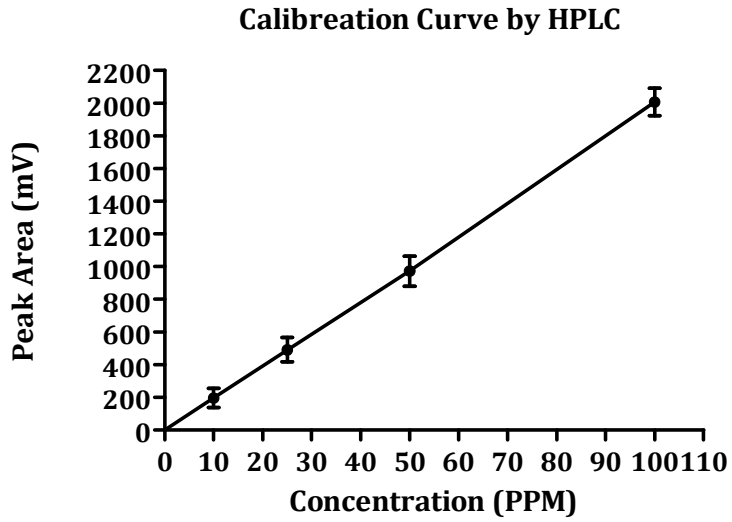

FIGURE 2 8. Calibration Curve of Gemcitabine HCl by HPLC

Precision

Precision was determined by studying the repeatability and intermediate precision. Intraday and inter-day precision (**Table2-9**) results indicated the precision under the same operating conditions over the interval of time was precise. In addition, the RSD values obtained for the analytical methods were within the acceptable range indicating that these methods have excellent precision.

2.6.5 Intraday and Interday precision analysis

TABLE 2 9. Intraday and Interday Precision Analysis of HPLC Method

Concentration (ppm)	Intraday Precision					Interday Precision				
	Peak Area			Mean	RSD	Peak Area			Mean	RSD
	Set 1	Set 2	Set 3			Day 1	Day 2	Day 3		
10	195.768	189.332	197.889	194.330	2.29	197.217	190.890	193.520	193.876	1.64
25	491.234	504.231	485.998	493.821	1.90	495.378	478.923	500.450	491.584	2.29
50	972.115	955.987	983.113	970.405	1.41	983.548	970.963	992.131	982.214	1.08
100	2007.095	1975.963	2013.321	1998.793	1.00	2020.973	1973.223	1995.371	1996.522	1.20

Accuracy

The excellent mean % recovery value, close to 100% with less standard deviation (SD < 0.1) represent high accuracy of the analytical method. The validity and reliability of the proposed method was further assessed by recovery studies by standard addition or spiking method.The mean % recovery for lower, intermediate and higher concentration were found to be 99.04, 98.68 and 101.56 respectively**(Table 2-10)**. These results revealed that any small change in the drug concentration in the solution could accurately be determined by the proposed analytical method.

TABLE 2 10. Accuracy of the HPLC Method

Spiking	Drug in solution (ppm)	Pure drug (ppm)	Total drug found (ppm) (mean ± SD)	% Analytical Recovery
80%	25	20	44.56 ± 0.802	99.04 ± 0.792
100%	25	25	49.34 ± 0.740	98.68 ±0.977
120%	25	30	55.86 0.726	101.56± 0.508

2.7 Conclusion

A rapid analytical method for the quantitative estimation of gemcitabine hydrochloride was successfully developed and validated using reverse UV spectrometry. The spectrometric analysis was carried out between 200nm - 400nm using a three different solvents such as distilled water, PBS pH 7.4 and methanol. The UV absorption by the drug was read at 268.4 nm in distilled water and phosphate buffer pH 7.4 and 268.8 nm in methanol respectively. The developed UV method was found to be specific for gemcitabine hydrochloride. A strong linear correlation with an r^2>0.9978, r^2>0.9998 and r^2>0.9993 in distilled water, PBS pH 7.4 and methanol respectivelywas observed between the concentration of the drug and absorbance obtained fora concentration range of 2 to 30 µg/mL. The precision of the analytical method was tested using intraday and inter-day validation. Also, the method depicted an accuracy of ~ 98.46, 101.97and 98.36 % for concentrations 8, 10 and 12µg/mL respectively.

In addition, to UV method of analysis another analytical method for the quantitative estimation of gemcitabine hydrochloride was successfully developed and validated using high performance liquid chromatography. The chromatographic separation was carried out on a C8 column (250 x 4.60 mm, 5µm) obtained from phenomenex using a reported mobile phase. The flow rate was maintained at 1.2 mL/min. The UV absorption by the effluents was read at 275nm. The developed HPLC method was found to be specific for gemcitabine hydrochloride. A strong linear correlation with an r^2>0.9997was observed between the concentration of the drug and peak area obtained upon chromatographic extraction over a concentration range of 10 to 100 µg/mL. The precision of the analytical method was tested using intraday and inter-day validation. Also, the method depicted an accuracy of 99.04, 98.68 and 101.56 for concentrations 20, 25 and 30µg/mL respectively.

2.8 References

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