METHOD DEVELOPMENT AND VALIDATION FOR THE ASSAY OF GEMCITABINE HYDROCHLORIDE IN PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC

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ABSTRACT

A simple and precise, rapid and accurate RP-HPLC method has been developed and validated for the determination of gemcitabine hydrochloride in pharmaceutical dosage forms. The chromatographic separation was achieved on kromasil stainless column (150 X 4.6 mm, 5.0 µ particle size) using acetonitrile : water (40 : 60), flow rate 1.0 mL/min. The analyte was monitored using UV-Visible detector at 270 nm. The retention time of the drug was 4.093 min for gemcitabine hydrochloride. The proposed method was found to have linearity in the concentration range 80-120 µg/mL with correlation coefficient of \( r^2 = 0.999 \). The method was validated for linearity, precision, LOD, LOQ and robustness. The proposed method was optimized and validated as per the ICH guidelines.

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INTRODUCTION

High performance liquid chromatography (HPLC) is the fastest growing analytical technique for the analysis of drugs. The technique of HPLC is developed from advances made in column chromatography. The technique is based on the same modes of separation mentioned above. It differs from conventional column chromatography in the sense that the mobile phase is pumped through the packed column under high pressure. Because of the relatively high pressure necessary to perform this type of chromatography, a more elaborate experimental set up is required.1-6. Gemcitabine hydrochloride is a anti neoplastic. Chemically it is 1-(2-oxo-4-amino-1,2-dihydropyrimidin-1-yl)2-deoxy-2,2-difluororibose hydrochloride, a white powder soluble in water. The hydrochloride salt of an analogue of the antimetabolite nucleoside deoxycytidine with anti neoplastic activity. Gemcitabine is converted intracellularly to the active metabolites difluorodeoxycytidine di-and triphosphate (dFdCDP, dFdCTP). dFdCDP inhibits ribonucleotide reductase, thereby decreasing the deoxynucleotide pool available for DNA synthesis; dFdCTP is incorporated into DNA, resulting in DNA strand termination and apoptosis.7-9. Only very few HPLC methods have been reported in the literature for the estimation of Gemcitabine hydrochloride in biological fluids10-13 and pharmaceutical dosage forms. Hence, the authors have made an attempt to develop new simple and rapid analytical method to estimate the Gemcitabine hydrochloride in its formulation.

Structure of Gemcitabine hydrochloride

![Structure of Gemcitabine hydrochloride](image)

**EXPERIMENTAL**

**Materials and Methods**

**Instrumentation**

Quantitative HPLC was performed on an isocratic LC – 20AT VP SHIMADZU High-Pressure Liquid Chromatographic instrument for the analysis. The instrument is provided with solvent delivery module with UV-visible detector SHIMADZU SPD-20A, ODS Reverse phase column (250 X 4.6mm). A 20 µL Hamilton injecting syringe and window based spinchrome software was used for its semi automatic operation, recording and analysis. A Sartorius electronic balance was used for weighing the materials.

**Chemical and reagents**

Pure drug sample of gemcitabine hydrochloride was kindly supplied as a gift sample by Sun pharma. Pvt. Led. Mumbai. Water (HPLC), and acetonitrile (HPLC) were used.

**Chromatographic conditions**

Mobile phase consists of acetonitrile : water (40 : 60). The mobile phase was pumped from the solvent reservoir to the column at a flow rate 1.0 mL/min. The column was maintained at 27°C and the volume of each injection was 20 µL and run time was kept 8 min. Prior to injection of the solutions, column was equilibrated for at least 10 min with mobile phase flowing through the system. The wavelength was selected by scanning standard solution of the drug over 200 nm to 400 nm and found that the component show reasonably good response at 270 nm so the eluents were monitored at 270 nm.

**Standard preparation**

Weigh accurately 100 mg of gemcitabine hydrochloride was dissolved into 100 ml of mobile phase to get 1 mg/ml solution; sonicated for 5 min and mix. Pipette out 1 ml of the above solution to 100 ml volumetric flask and make up volume with mobile phase (0.1mg/ml).
Sample preparation
Weigh accurately powder equivalent to 100 mg of gemcitabine hydrochloride and transfer in to 100 ml volumetric flask, add 40 ml of mobile phase, keep on rotary shaker for 30 minutes. Sonicated for 10 min with occasional shaking in between. Make up the volume with mobile phase and mix well. Pipette 1 ml of the clear solution in to 100 ml volumetric flask and make up volume with mobile phase (0.1 mg/ml).

Calibration curve
Appropriate aliquot were pipetted out from standard stock solution into a series of 5 mL volumetric flasks. The volume was made up to the mark with mobile phase to get solutions having concentration range 80-120 µg/mL for gemcitabine hydrochloride. Triplicate dilution of each concentration were injected into RP-HPLC system and chromatographed under conditions the above mentioned conditions and elution was monitored at 270 nm. \( Y = 12.2x - 3.027, \quad R^2 = 0.999 \)

![Fig. 2: Calibration curve of gemcitabine hydrochloride](image)

Estimation of Gemcitabine hydrochloride in formulation by proposed HPLC method
Weigh accurately powder equivalent to 100 mg of gemcitabine hydrochloride and transfer into 100 ml volumetric flask, add 40 ml of mobile phase, keep on rotary shaker for 30 minutes. Sonicated for 10 min with occasional shaking in between. This solution was filtered through 0.45 µm membrane filter and filtrate was collected. From the filtrate, different aliquots were taken in separate 5 mL volumetric flask. The contents of the flask were made up to the volume with the mobile phase and mixed well. The solutions were sonicated for 10 min and 20 µL of each sample solution was injected into the column under above mentioned chromatographic conditions. The amount of gemcitabine hydrochloride was calculated by comparing the peak area values of sample and standard solutions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Label claim (mg)</th>
<th>Amount found (mg)</th>
<th>%Labeled claim ± SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>200</td>
<td>198.54</td>
<td>99.27 ± 1.52</td>
</tr>
<tr>
<td>Sample 2</td>
<td>200</td>
<td>200.40</td>
<td>100.18 ± 1.86</td>
</tr>
</tbody>
</table>

* Average of six determinations

Validation of method
System suitability tests
System suitability tests were carried at on freshly prepared stock solution of gemcitabine Hydrochloride for five times. 20 µL of each solution was injected under optimized chromatographic conditions (Table 2). System suitably parameters for the method listed in Table 3.

Linearity and range
The linearity of the method is its ability to elicit test results that is directly proportional to the concentration of analyte in sample. The stock solution were further diluted with mobile phase to get a concentration of 80-120 µg/mL of gemcitabine hydrochloride. Each concentration was injected six times into the column and the corresponding chromatograms were obtained. Evaluation of the drug was performed with UV-detector at 270 nm and a calibration graph was obtained by plotting peak area vs. the respective concentration of drug. The plots of area versus the
respective concentration were found to be linear with coefficient of correlation ($R^2 = 0.999$) for drug.

**Table 2: Optimized chromatographic conditions**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instrument</td>
<td>Shimadzu pump LC - 20AT VP</td>
</tr>
<tr>
<td>Detector</td>
<td>SPD-20AT VP U.V – visible detector</td>
</tr>
<tr>
<td>Column</td>
<td>Kromasil Stainless steel</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>Acetonitrile : Water (40 : 60)</td>
</tr>
<tr>
<td>Temperature</td>
<td>27°C</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 ml/min</td>
</tr>
<tr>
<td>Wave length</td>
<td>270 nm</td>
</tr>
<tr>
<td>Runtime</td>
<td>8 min</td>
</tr>
<tr>
<td>Sample size</td>
<td>20 µL</td>
</tr>
</tbody>
</table>

**Recovery studies**

Accuracy of the method was determined by recovery studies of gemcitabine hydrochloride. Known amount of standard was added to the pre-analyzed sample and subjected to the proposed HPLC method. The study was done with three different concentration levels. Each determination was performed in triplicate. The results of recovery analysis are presented in Table 4. The mean recovery was found to be reasonably accurate.

**Table 4: Results of recovery studies**

<table>
<thead>
<tr>
<th>Amount of drug taken from sample (mg)</th>
<th>Amount of standard drug added (mg)</th>
<th>% Recovery ± SD*</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>10</td>
<td>80</td>
<td>100.00±1.18</td>
</tr>
<tr>
<td>80</td>
<td>20</td>
<td>100.08</td>
<td>100.20±1.73</td>
</tr>
<tr>
<td>90</td>
<td>30</td>
<td>120.05</td>
<td>100.80±2.91</td>
</tr>
</tbody>
</table>

*Average of three determinations

**Limit of detection (LOD) and Limit of quantification (LOQ)**

The limit of detection and limit of quantification of the method were determined by injecting progressively low concentration of the standard solution of gemcitabine hydrochloride with the optimized chromatographic conditions. The limit of detection and limit of quantification was found to be 0.00164 µg/mL and 0.004592 µg/mL respectively.

**Robustness**

Robustness of the method was checked by deliberately change in ratio of the mobile phase and temperature of the column.

**Ruggedness**

Ruggedness as the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions.
conditions such as different labs, different analysis, different lots of reagents etc. Ruggedness is a measure of reproducibility of test results under normal expected operational conditions from laboratory to laboratory and from analyst to analyst.

Table 5: Results of ruggedness study (Analyst to Analyst)

<table>
<thead>
<tr>
<th>S.N0</th>
<th>% of Labelled claim Analyst-1</th>
<th>Analyst-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99.07</td>
<td>99.25</td>
</tr>
<tr>
<td>2</td>
<td>99.92</td>
<td>100.50</td>
</tr>
<tr>
<td>3</td>
<td>100.16</td>
<td>98.92</td>
</tr>
<tr>
<td>Mean</td>
<td>99.71</td>
<td>99.55</td>
</tr>
<tr>
<td>S.D</td>
<td>0.5843</td>
<td>0.8344</td>
</tr>
<tr>
<td>%RSD</td>
<td>0.5862</td>
<td>0.5457</td>
</tr>
</tbody>
</table>

Fig.3: Chromatogram of blank, Fig.4: chromatogram of standard, Fig.5: Chromatogram of sample

RESULTS AND DISCUSSION

The official methods for determination of Gemcitabine Hydrochloride in pharmaceutical dosage forms are prescribed in the United States Pharmacopoeia (USP). USP 27 prescribes HPLC method for determination of Gemcitabine Hydrochloride in powder form, using a reversed phase column with particle size 5 µm, length 4.2 mm x 25 cm, L7, sodium phosphate and phosphoric acid as mobile phase. Gemcitabine Hydrochloride RS as internal standard and UV detection at 275 nm and flow rate 1.2 mL/min. Different liquid chromatographic methods have been reported for determination of Gemcitabine Hydrochloride especially in human plasma, serum. But no method has been developed on the validated liquid chromatographic method for estimation of Gemcitabine Hydrochloride lyophilized powder dosage form. Typical bio analytical sample preparation techniques such as extraction into an organic solvent one not effective because of the extremely hydrophilic nature of the compound. Analysis of the urinary excretion in one study was only to be accomplished with radio labelled drug was already reported. A number of different options were explored in the development of the separation. The highly polar nature of the analysts causes them to elute quite rapidly from reversed phase columns even very low organic content mobile phases. The variability was controlled by adding small quantities of triethylamine and acetic acid to the mobile phase. A small quantity of water was also added to achieve a constant level of hydration of the chromatographic system. Five replicates from each pool were assayed on each two hours so that within-day precision and accuracy could be determined. The linearity of the response for the assay was established over the concentration range from 80 µg/ml to 120 µg/ml. Typical correlation co-efficient were greater than 0.999. Specificity is the ability of the method to determine accurately and specifically the analyte of interest in the presence of other components in a sample matrix under the stated conditions of the test. An isocratic reversed phase HPLC assay with UV detection used for the proposed HPLC method. The separation was performed on kromasil stainless steel column (150 x 4.6 mm; 5 µm particle sizes.) packed with ODS chemically bonded porous silica particles column at 27 °C. The mobile phase acetonitrile and water (40:60), was pumped at a flow rate 1 ml/min.
The run time was 8 min. Detection was performed with a UV-Visible detector at 270 nm. System suitability tests were performed and chromatographic parameters such as asymmetry factor, resolution, retention time, no. of theoretical plates and area were calculated. The validity of the liquid chromatographic assay was established through a study of linearity, system precision, method precision, intra-day precision, accuracy, robustness, ruggedness, limit of detection and limit of quantification. The linearity was established with a series of working solution prepared by diluting the stock solution with dilution to the final concentration. Each concentration was injected in to liquid chromatography and the value of peak area was taken for the calibration curve. The calibration curve was plotted using concentration against peak area. The correlation co-efficient value was found to be 0.999 indicates that the concentration of Gemcitabine Hydrochloride had good linearity. In method precision study, the % RSD for Gemcitabine Hydrochloride was found to be 0.4863. The result indicates that method is validated for method precision. In intra-day precision study, the % RSD for Gemcitabine Hydrochloride was found to be 0.4260. There is no significant difference by same analyst by different time intervals on the day. Therefore the intra-day precision of the method can be considered to be acceptable. In accuracy or recovery studies, the overall % of recovery and % RSD for Gemcitabine Hydrochloride in marketed formulation indicated that there is no significant difference in percentage of recovery. Therefore, accuracy of the method considered acceptable as it was well within 98 to 102 %. In robustness or system suitability study, there was no significant impact on the asymmetry factor, retention time and no. of theoretical plates. The results of the robustness study also indicated that the method is robust and is unaffected by small variation in chromatographic conditions. The detection limit and quantification limit for Gemcitabine Hydrochloride was found to be 0.00164 µg/mL and 0.004592 µg/mL respectively.

**CONCLUSION**

Method validation was carried out as per ICH guidelines. The evaluation of obtained values suggests that the proposed HPLC methods provide simple, precise, rapid and robust quantitative analytical method for determination of Gemcitabine Hydrochloride in dosage form. The mobile phase is simple to prepare and economical. After validating proposed method as per ICH guidelines and correlating obtained values with the standard values, satisfactory results were obtained. Hence, the method can be easily and conveniently adopted for routine estimation of Gemcitabine Hydrochloride in dosage form.

**REFERENCES**


