A Stability Indicating RP-HPLC Method for the Estimation of Gemcitabine HCl in Injectable Dosage forms

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Abstract: A stability indicating RP HPLC method has been developed for the determination of gemcitabine hydrochloride. Chromatography was carried out on an ODS C₁₈ column (250×4.6 mm; 5μ) using a mixture of methanol and phosphate buffer (40: 60 v/v) as the mobile phase at a flow rate of 1.0 mL/min. The detection of the drug was monitored at 270 nm. The retention time of the drug was found to be 2.31 min. The method produced linear responses in the concentration range of 10 to 60 μg/mL of gemcitabine HCl. The method was found to be reproducible for analysis of the drug in injectable dosage forms. The stability of the drug was assessed by forced degradation studies.

Keywords: Gemcitabine HCl, Lyophilized injection, Forced degradation study, HPLC.

Introduction

Gemcitabine¹ (2'-deoxy-2', 2''-difluorocytidine, 2', 2''-difluorodeoxycytidine) is a pyrimidine analog that is proven to be active against a variety of solid tumors. It is widely used in the treatment of cancers of pancreas, lung, breast, bladder, kidney and biliary tract either singly or in combination with other cytotoxic agents. Promising response rates with gemcitabine have been observed in other tumor types such as ovarian and testicular tumors and lymphomas. Gemcitabine is a prodrug that is converted to its active metabolite gemcitabine triphosphate (dFd CTP) after the uptake of the parent compound into the cells *via* nucleoside transporters. Several enzymes are involved in the conversion of gemcitabine to gemcitabine monophosphate (rate limiting step), gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP) *via* successive phosphorylation. Gemcitabine triphosphate is

incorporated into DNA, blocking DNA polymerase. In addition, dFdCDP inhibits the ribonucleotide reductase resulting in a decrease of the deoxy ribonucleotide pool necessary for DNA synthesis, thereby contributing to the antitumor effect.

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 HPLC²⁻¹² and LC-MS/MS¹³⁻¹⁴. So far, no stability indicating method for the determination of gemcitabine HCl is available. Hence, the authors have attempted to develop a validated stability indicating RP-HPLC method for the determination of gemcitabine HCl in bulk samples and in pharmaceutical formulations. The forced degradation studies were performed by subjecting the drug to acidic, basic and oxidation conditions and heat and UV light treatment.

Experimental

The reference sample of gemcitabine HCl was procured from Dr. Reddy's Laboratories, Hyderabad. HPLC grade methanol and GR grade potassium dihydrogen phosphate and phosphoric acid were purchased from Merck Schuchardt, Germany. High purity water was prepared by using Millipore Milli Q plus water purification system. Commercial samples of gemcitabine HCl lyophilized injection (Cytogem of Dr. Reddy's Laboratories) were used in the study.

Instrumentation

A Shimadzu Prominence HPLC instrument equipped with a Luna C₁₈ (250 mm x 4.6 mm; 5 μ) analytical column, a LC-20AT pump, a CTO-20A column oven, a Rheodyne 7725 sample injector with a 20 μ L loop and an SPD-20A UV-VIS detector was employed for this analysis. LC Solutions software was used for the data acquisition and quantification of peaks.

A freshly prepared 40:60 v/v mixture of methanol and phosphate buffer was used as the mobile phase. The mixture was filtered through a 0.45 μ membrane filter and sonicated before use. A DGU-20A3 degasser was used to enhance the solubility of the drug and to remove entrapped air in the solution. The flow rate of the mobile phase was maintained at 1 mL/min. The column temperature was maintained at 25 $\pm$ 1 $^{\circ}$ C. The detection was carried out at 270 nm.

Preparation of standard solution

About 20 mg of gemcitabine HCl was weighed accurately and transferred into a 20 mL volumetric flask and dissolved in 10 mL of the mobile phase. The solution was sonicated for 20 min and then the volume made up with a further quantity of the mobile phase to get 1 mg/mL solution. Subsequent dilutions of this solution ranging from 10-60 μ g/mL were made in 10mL volumetric flasks. A 20 μ L volume of the solution was injected each time into the column at a flow rate of 1 mL/min. Each dilution was injected five times into the column and the corresponding chromatograms were obtained. From these chromatograms, the area under the peak of the drug to that of the reference standard for each dilution was calculated. The regression of the drug concentrations over the ratios was computed from the relevant plot. This regression equation was used to estimate the amount of gemcitabine HCl in pharmaceutical dosage forms.

Estimation of gemcitabine HCl

Five injection ampoules of cytogem were taken and their volumes were pooled up in a volumetric flask. Their aluminium closures were removed. The powders in all the vials were pooled up and the average weight of powder in one vial was calculated. About one ml of

injection sample containing 100 mg of gemcitabine HCl was accurately measured and dissolved in 50 mL of the mobile phase in a 100 mL volumetric flask with sonication for about 5 min. The volume was made up with the mobile phase to get a 1 mg/mL solution. From this, the working solution containing 30 µg/mL was prepared by suitable dilution and injected into the column (n=6) for the estimation of the drug in the injection.

Results and Discussion

The present study was carried out to develop a sensitive, precise, accurate, stability-indicating HPLC method for the analysis of gemcitabine HCl in pharmaceutical dosage forms. In order to achieve efficient separation of the component peaks under isocratic conditions, mixtures of methanol and phosphate buffer (pH 3.5) in different proportions were tested as the mobile phase on a C₁₈ stationary phase. A binary mixture of methanol and phosphate buffer in a 40:60 v/v proportion was proved to be the most suitable since the chromatographic peaks were better defined and resolved and almost free from tailing. The retention time obtained for gemcitabine HCl was 2.31 min. A model chromatogram showing the separation of the drug is shown in Figure 1.

Figure 1. Typical chromatogram showing the separation of gemcitabine HCl

For the linearity study, each of the samples was injected five times and almost the same retention times were observed in all the cases. The peak areas of gemcitabine HCl for different concentrations setup as above were calculated and the average value for 5 such determinations are shown in Table 1.

Table 1. Linearity range

Concentration, µg/mL	Average area
10	176911.8
20	353823.5
30	520735.3
40	707647.0
50	884558.8
60	1061471

Regression equation $y=17691.18x+1428.57$ ($r=0.9999$)

The peak areas of both the standard and the pharmaceutical formulation were reproducible as indicated by low coefficient of variation (0.98). A good linear relationship ($r=0.9990$) was observed between the concentration of gemcitabine HCl and the respective peak area ratios. The regression of the curve was computed by linear regression fitting and its mathematical expression was $y=17691.18x+1428.57$ (where y is the ratio of peak areas of the drug to that of the reference standard and x is the concentration of gemcitabine HCl). When gemcitabine HCl solutions containing 5 to 15 $\mu\text{g/mL}$ was analyzed by the proposed method for finding out intra and inter-day variations as low coefficient of variation was observed (Table 2). This shows that the present HPLC method is highly precise. The amounts of gemcitabine HCl obtained from the pre analyzed samples containing known amounts of the added drug are shown in Table 3. About 99.61% of gemcitabine HCl could be recovered from the pre-analyzed samples indicating high accuracy of the proposed method.

The drug content in the injection sample was quantified by using the proposed analytical method. The sample was found to contain an average of 99.60% of the labeled amount of the drug. The low coefficient of variation indicates the reproducibility of the assay of gemcitabine HCl in pharmaceutical dosage forms.

Precision

Intra-day precision was conducted at 50, 100 and 150 percent levels of the drug ($n=3$). The percent recovery at each level and the mean amount recovered are presented in Table 2.

Table 2. Precision of the method

Concentration	Amount added, μg	Amount Found, μg	% Recovery	Mean $\pm$ S.D (amount recovered) ($n=3$)	% RSD
50% Sample (1)	100	99.94	99.4	99.8 $\pm$ 0.92	0.90
50% Sample (2)	100	100.0	100.0		
50% Sample (3)	100	100.05	100.02		
100% Sample (1)	200	199.80	99.9	99.9 $\pm$ 0.37	0.37
100% Sample (2)	200	200.03	100.01		
100% Sample (3)	200	199.97	99.85		
150% Sample (1)	300	299.89	99.8	99.5 $\pm$ 0.17	0.17
150 %Sample (2)	300	298.6	99.6		
150% Sample (3)	300	297.5	99.4		

Accuracy

Accuracy was determined by recovery study of gemcitabine HCl. Known amount of standard gemcitabine HCl was added to pre-analyzed sample which was then subjected to the proposed HPLC method. Results of recovery studies are shown in Table 3. The study was carried out at three different concentration levels.

Table 3. Accuracy studies

S.No.	Labeled amount, mg	Amount added, mg	Amount recovered, mg	% Recovery
1	200	50	249.2	99.68
2	200	100	299.4	99.80
3	200	150	349.4	99.82

Robustness

To determine the robustness of the method the experimental conditions were deliberately altered slightly. The method conditions such as flow rate ($\pm 10\%$), organic content in mobile phase ($\pm 2\%$) and pH of buffer in mobile phase (± 0.2) were altered and the influence of these changes on the assay, peak tailing, number of theoretical plates and peak area were evaluated. The method was found robust enough that the selected factors were affected within the allowed limits.

Limit of detection and limit of quantification

Limit of detection and limit of quantification were calculated based on the signal to noise ratio. The LOD and LOQ are found to be 0.187 and 0.617 $\mu\text{g/mL}$ respectively.

System suitability

System suitability parameters were evaluated by using a 100 $\mu\text{g/mL}$ of the standard drug solution and the results are presented in Table 4.

Table 4. System suitability parameters

Parameter	Value
Retention time in minutes (t)	2.31
Column length in cm. (L)	25
Theoretical plates (n)	7350
Theoretical plates per meter (N)	51450
Ht. equivalent to theoretical plates (HETP)	0.034
Tailing Factor	0.98
Peak asymmetry (T)	0.93

Results of forced degradation studies

Forced degradation studies were conducted to evaluate the stability and specificity of the method. No significant degradation of gemcitabine HCl was observed when the drug was subjected to acidic and basic treatment, exposure to UV light and to oxidation conditions. The assay values of the drug after subjecting to the above conditions were 99.3, 99.7 and 99.5 respectively. The small peaks found at the retention times of 1.62, 1.54, 1.95 at various stress conditions were well resolved from the drug peaks. The drug peaks obtained from all the stressed samples were found to be homogenous and pure. Hence the method is found to be specific. The results are given in Table 5, 6 and 7 respectively.

Table 5. Acid stress study (0.1 N HCl)

Conc.	Time, h	Gemcitabine		Rt. of degraded product
		Assay	Rt.	
200 $\mu\text{g/mL}$	0		2.315	-
	8		2.32	-
	24	99.3	2.325	1.62

Table 6. Base stress study (0.1 N NaOH)

Conc.	Time, h	Gemcitabine		Rt. of degraded product
		Assay	Rt.	
200 $\mu\text{g/mL}$	0		2.30	-
	8		2.32	-
	24	99.7	2.302	1.54 & 1.95

Table 7. Peroxide stress study (5% H₂O₂)

Conc.	Time, h	gemcitabine		R _t of degraded product
		Assay	R _t	
200 µg/mL	0		2.305	-
	8		2.322	-
	24	99.5	2.301	-

Stability of the standard solutions

The stability of the standard and the formulation sample solutions were studied over a period of 48 h and the assay results were compared with those of the freshly prepared solutions. Only a small reduction in percent recovery of the drug was noticed, which indicates that the solutions are stable for about 48 h.

Conclusion

The proposed RP-HPLC method developed for quantitative determination of gemcitabine HCl is precise, accurate and selective. The method was completely validated and satisfactory results were obtained. This method can also be used for assessing the stability of gemcitabine HCl from bulk drug samples and in its pharmaceutical formulations.

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