World Journal of Pharmaceutical research

Volume 3, Issue 2, 3017-3025. Research Article ISSN 2277 – 7105

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR GEMICITABINE HCl IN BULK AND PHARMACEUTICAL DOSAGE FORM BY RP-HPLC METHOD

B. Siddartha[1*], Dr. I. Sudheer Babu[2], Ch. Ravichandra Gupta[3], C. Parthiban[1]

[1]Department of Pharmaceutical Analysis, Malla Reddy College of Pharmacy, Secunderabad.

[2]Sir C.R.Reddy College of Pharmaceutical Sciences, Eluru.

[3]Suven Life Sciences Limited, Patancheru (Mandal), Medak (Dist).

Article Received on
05 January 2014,

Revised on 25 January 2014,
Accepted on 27 February 2014

***Correspondence for Author**
B. Siddartha
Department of Pharmaceutical Analysis, Malla Reddy College of Pharmacy, Secunderabad. India.

ABSTRACT

A simple, sensitive and rapid reverse phase high performance liquid chromatographic method was developed for the estimation of gemicitabine hydrochloride in pure and pharmaceutical dosage forms. A Hypersil BDS C_{18} column (250 x 4.6mm x 5 µ) was used as a stationary phase with a mobile phase containing a mixture of buffer and acetonitrile in the ratio of 93:7v/v. The flow rate was 1.0ml/min, effluent was monitored at 275nm and eluted at 3.927min. Calibration curve was plotted with a range from 10-60µg/ml for gemicitabine hydrochloride and the correlation was found to be 0.9997. The accuracy range was found between 99.20% and 101.24%. The % RSD values for both intraday and interday precision were less than 1%. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.296µg/ml and 0.896µg/ml respectively. The assay was validated for the parameters like specificity, system suitability, precision, accuracy, robustness and ruggedness parameters. The proposed method will be useful for the routine determination of gemicitabine hydrochloride in pharmaceutical dosage form.

Keywords: Gemicitabine HCl, Calibration curve, RP-HPLC, Validation.

INTRODUCTION

Gemcitabine hydrochloride is 2´-deoxy-2´, 2´-difluorocytidine monohydrochloride (β-isomer). It is a nucleoside metabolic inhibitor that exhibits antitumor activity. The empirical

formula for gemcitabine hydrochloride is $C_9H_{11}F_2N_3O_4$•HCl. It has a molecular weight of 299.66. 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. Gemcitabine kills cells undergoing DNA synthesis and blocks the progression of cells through the G1/S-phase boundary. Gemcitabine diphosphate inhibits ribonucleotide reductase, an enzyme responsible for catalyzing the reactions that generate deoxynucleoside triphosphates for DNA synthesis, resulting in reductions in deoxynucleotide concentrations, including dCTP. The reduction in the intracellular concentration of dCTP by the action of the diphosphate enhances the incorporation of gemcitabine triphosphate into DNA (self-potentiation). After the gemcitabine nucleotide is incorporated into DNA, only one additional nucleotide is added to the growing DNA strands, which eventually results in the initiation of apoptotic cell death[1]. A literature survey reveals the report of a few analytical methods for determination of gemcitabine in pharmaceutical dosage forms and in biological fluids by HPLC and LC-MS/MS[2-11]. But, no stability indicating method is being developed. The present work describes the development of a validated RP-HPLC method in pharmaceutical dosage form. The present study was validated following the ICH guidelines[12].

EXPERIMENTAL

Reagents

Gemicitabine hydrochloride was kindly supplied by Dr. Reddy Laboratory (Hyderabad, A.P., and India). Acetonitrile, water (HPLC grade, Merck) and all the other reagents of AR grade were purchased from M R Enterprisers. An injection CYTOGEM (Dr. Reddy's Labs) contains 200mg of gemicitabine hydrochloride.

Instrumentation

The LC system consisted of a Waters model 515, PDA detector 2998 with 20 µL sample loop. The output signals were monitored and integrated using Empower 2 software.

Chromatographic conditions

The elution was isocratic and the mobile phase consisted of a mixture of buffer (accurately weighed and transferred 2.72gm of potassium dihydrogen orthophosphate in a 1000ml of volumetric flask and add about 900ml of milli-Q water. Add 0.5ml of triethylamine and degas to sonicate and finally make up the volume with water, then pH adjusted to 3.0 with dil. Ortho phosphoric acid solution) and acetonitrile (93 : 7 v/v). The mobile phase was filtered through a 0.45-µm (HVLP, Germany) membrane filter prior to use. A Hypersil BDS C_{18}

column (250 x 4.6mm x 5 µ) was used for determination. The flow rate was 1.0 ml/min and the column was operated at ambient temperature (~30 ^{o}C). The volume of sample injected was 10 µL. Prior to injection of the solutions, column was equilibrated for at least 30min with mobile phase flowing through the system. The UV detector was set at wavelength of 275nm. A typical chromatogram of gemicitabine hydrochloride is shown in (Fig. 2).

Diluent: Mobile phase.

Standard Preparation

Stock solution was prepared by dissolving 40mg of Gemicitabine HCl working standard and transfer to 100 ml volumetric flask. Add 70ml of diluents and sonicate to dissolve. Transfer 1ml from the above solution into 10ml volumetric flask to get concentration of 40µg/ml.

Sample Preparation

Five injection ampoules of gemicitabine hydrochloride (CYTOGEM) were taken and their volumes were pooled up in a volumetric flask. The powders in all the vials were collected and the average weight of powder in one vial was calculated. About 1 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 1 mg/ml solution. From this, the working solution containing 40 µg/ml was prepared by suitable dilution and injected into the column (n=6) for the estimation of the drug in the injection.

METHOD VALIDATION

The developed method was validated as per ICH guidelines [13-14] for its accuracy, linearity, precision, specificity, robustness, ruggedness, limit of detection and limit of quantification by using the following procedures. The parameters are validated as shown in table 9.

System suitability

System suitability and chromatographic parameters were validated such as asymmetry factor, tailing factor and number of theoretical plates were calculated (Figure: 1).

Linearity

Linearity of this method was evaluated by linear regression analysis and calculated by least square method and studied by preparing standard solutions of gemicitabine hydrochloride at

different concentration levels. Absorbance of resulting solutions was measured and the calibration curve was plotted between absorbance vs concentration of the drug (Figure: 2). The response was found to be linear in the range 10-60 µg/ml for gemicitabine hydrochloride. The data was given in table 1.

Accuracy

Accuracy was performed in triplicate for various concentrations of gemicitabine hydrochloride equivalent to 50%, 100% and 150% of the standard amount were injected into the HPLC system per the test procedure. The average % recovery was calculated. The data was given in table 2.

Precision

A) Method Repeatability

Six sample solutions of the same concentration (100%) were prepared and injected into the HPLC system as per test procedure. The results were given in table 3.

B) Intermediate Precision (Day to Day variability)

Two days as per test method conducted the study. For Day-1 and Day-2, six sample solutions of the same concentration (100%) were prepared and injected into the HPLC system as per test procedure. The results were given in table 6.

Limit of detection and Limit of Quantification

LOD and LOQ were calculated from the average slope and standard deviation from the calibration curve as per ICH guidelines. LOD and LOQ were found to be 0.296µg/ml and 0.896µg/ml respectively.

Robustness and Ruggedness

Robustness was done by small deliberate changes in the chromatographic conditions and retention time of gemicitabine hydrochloride was noted. The factors selected were flow rate and variation in the mobile phase composition. The results remained unaffected by small variations in these parameters as shown in table 4 and 5. Ruggedness of the method was checked by using different days and instruments. The relative standard deviation of the results obtained from different days and instruments was <2.0%. The results were given in table 6 and 7.

Assay

The assay & % purity was performed by taking CYTOGEM with label claim 200mg. The observed value was compared with that of standard value without interference from the excipients used in the tablet dosage form. The results were given in table 8.

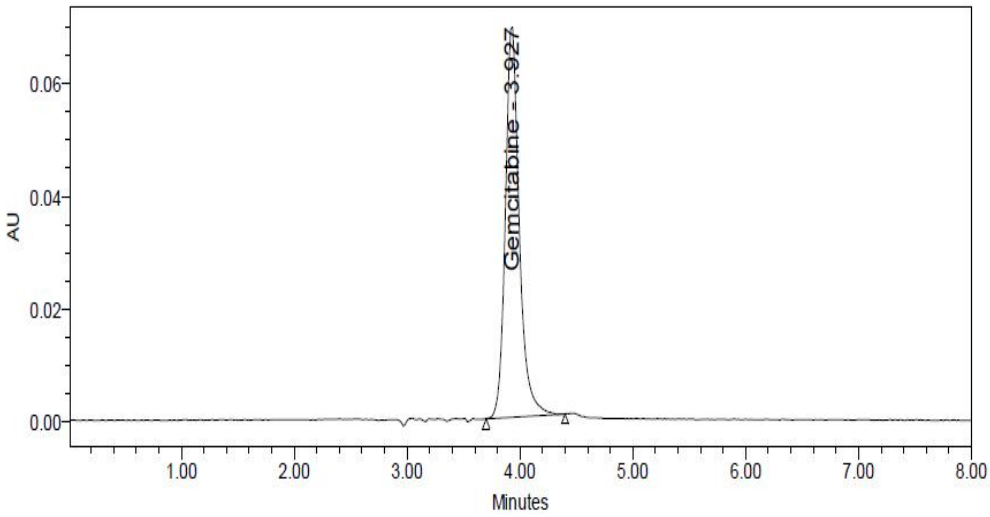

Fig – 1 HPLC Chromatogram of Gemicitabine HCl

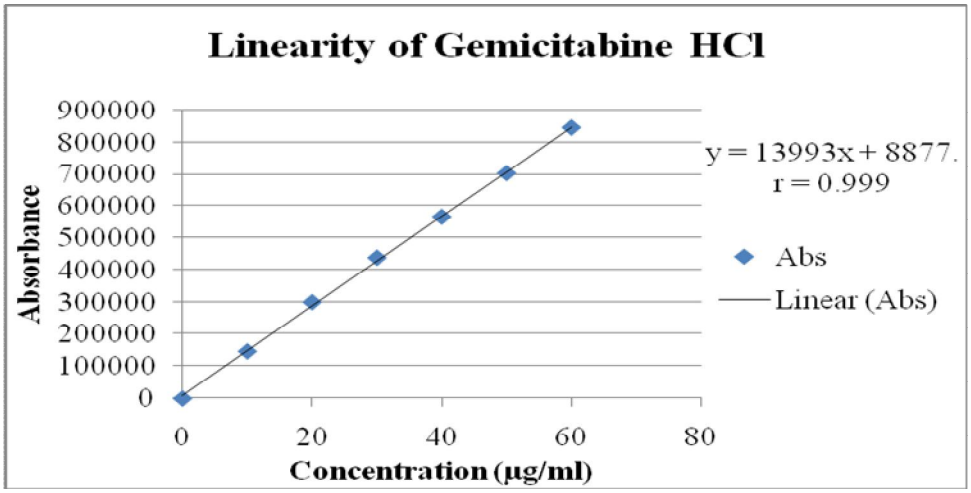

Fig – 2 Linearity of Gemicitabine HCl in the range 10 to 60µg/ml.

Table 1 Linearity data of Gemicitabine HCl

S.No	Concentration (µg/ml)	Injection	Retention time (mins)	Area
1	10	1	3.939	146827
2	20	1	3.942	299939
3	30	1	3.943	438236
4	40	1	3.927	565702
5	50	1	3.932	703940
6	60	1	3.941	846006
r = 0.9997				
y = 13993x + 8877				

Table 2 Accuracy data

S.No.	Spiked level	Amount Added(µg/ml)	Amount Present(µg/ml)	Mean %Recovery*	%RSD
1(n=6)	50%	20.16	20.34	100.91	0.33
2(n=6)	100%	40.03	39.81	99.46	0.18
3(n=6)	150%	59.97	59.60	99.38	0.22

*n=3 (Average of 3 determinations)

Table 3 Precision data of 40µg/ml

S.No.	Concentration(µg/ml)	Injection	Retention time (mins)	Area
1	40	1	3.959	567830
2	40	1	3.961	565559
3	40	1	3.968	568025
4	40	1	3.962	565515
5	40	1	3.949	565125
6	40	1	3.943	566142
Mean				566366
Std.Dev				1254
%RSD				0.22

Table 4 Robustness data relating to change in flow rate (1.0ml/min)

S.No	Flow rate (ml/min)	Average Peak Area*	SD	%RSD
1	0.9ml/min	553514	1427	0.26
2	1.0ml/min	565616	755	0.13
3	1.1ml/min	552768	1182	0.21

*n=3 (Average of 3 determinations)

Table 5 Robustness data relating to change in mobile phase composition

S.No	Mobile Phase Variation (%)	Average Peak Area*	SD	%RSD
1	M.P-1-(BUFFER:ACN::91:9)	555557	2045	0.37
2	M.P-2-(BUFFER:ACN::93:7)	564477	778	0.14
3	M.P-3-(BUFFER:ACN::95:5)	553922	1510	0.27

*n=3 (Average of 3 determinations)

Table 6 Ruggedness data relating to change of day

S.No	Inter-day Precision		
	Peak Area		
	Concentration (µg/ml)	Day – 1	Day – 2
1	40	565765	565366
2	40	567354	567432
3	40	567955	567025
4	40	565515	565642
5	40	564906	563906
6	40	563142	567238
Mean		565773	566102
SD		1732	1377
%RSD		0.31	0.24

Table 7 Ruggedness data relating to change of instrument

S.No	Instrument to Instrument		
	Peak Area		
	Concentration (µg/ml)	Instrument – 1	Instrument – 2
1	40	565343	564787
2	40	567354	567432
3	40	567955	567025
4	40	565515	565323
5	40	565906	564906
6	40	563323	563135
Mean		565899	565435
SD		1639	1582
%RSD		0.29	0.28

Table-8 Results of analysis of laboratory samples (Assay)

Sample	Label	Amount found	% Purity ± RSD*
Brand-1 (CYTOGEM)	200mg	199.40mg	99.50 ± 0.31

*n=3 (Average of 3 determinations)

Table 9 System suitability parameters

Validation parameters	Results
Linearity range (µg/ml)	10 – 60
Regression equation	y = 13993x + 8877
Correlation Coefficient(r)	0.9997
Accuracy	99.20% to 101.24%
Precision (%RSD)	0.22
Robustness (%RSD)	
Flow rate (0.9ml/min & 1.1ml/min)	NMT 0.26
Mobile phase – Buffer : ACN(91:7 & 95:5)	NMT 0.37
Ruggedness (%RSD)	
Interday – (Day 1 & Day 2)	NMT 0.31
Instrument to Instrument (Inst-1 & Inst-2)	NMT 0.29

RESULTS

A reverse-phase column procedure was proposed as a suitable method for the determination of gemicitabine hcl dosage form. The chromatographic conditions were optimized by changing the mobile phase composition. Different ratios were experimented to optimize the mobile phase. Finally, buffer and acetonitrile in the ratio 93:7v/v was used as mobile phase, which showed good resolution of gemicitabine hydrochloridel peak. The wavelength of detection selected was 275nm, as the drug showed optimized absorbance at this wavelength. By our proposed method the retention time of gemicitabine hydrochloride was about 3.927 minute and none of the impurities were interfering in its assay.

DISCUSSIONS

The statistical analysis of data and the drug recovery data showed that the method was simple, rapid, economical, sensitive, precise and accurate. It can thereby easily adopt for routine quality control analysis. The results of this analysis confirmed that the proposed method was suitable for determination of drug in pharmaceutical formulation with virtually no interference of additives. Hence the proposed method can be successfully applied in estimation of gemicitabine hydrochloride in marketed formulation.

CONCLUSION

The proposed method is rapid, accurate and sensitive. It makes use of fewer amounts of solvents and change of set of conditions requires a short time. This method can be suitably analyzed for the routine analysis of gemicitabine hydrochloride in bulk and its tablet dosage forms. It does not suffer from any interference due to common excipients present in pharmaceutical preparation and can be conveniently adopted for quality control analysis.

ACKNOWLEDGEMENTS

The authors are thankful to Malla Reddy College of pharmacy, Secunderabad for providing all facilities to complete the work and Dr. Reddy Laboratory for providing the gemicitabine hydrochloride as gift sample.

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