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RESEARCH ARTICLE

RP-HPLC Method for Simultaneous Estimation of a Hydrophilic Drug Gemcitabine Hydrochloride in Combination with Paclitaxel, Bicalutamide and Letrozole in Nanosponges

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ABSTRACT

Simple, fast and precise reverse phase high performance liquid chromatographic methods have been developed for the simultaneous determination of Gemcitabine HCl in combination with lipophilic drugs such as Paclitaxel, Bicalutamide and Letrozole. The chromatographic separation was achieved on Eclipse plus C-8 (250 x 4.6 mm) as stationary phase with a mobile phase comprising of methanol and phosphate buffer (pH 3.5) in the ratio of 70:30 v/v. The flow rate was maintained at 1.2 mL/min and eluents were detected at 230, 257 and 248 nm. The retention time of Gemcitabine HCl was found to be 2.6 minutes and that for paclitaxel, bicalutamide and letrozole was found to be 7.1, 4.8 and 3.4 minutes respectively. The proposed methods were validated by determining specificity, precision, accuracy, and robustness. The linearity were found to be in the range of 5 µg/mL and 200 µg/mL with correlation coefficient greater than 0.998. Due to its simplicity, accuracy and high precision the proposed HPLC methods were found to be appropriate for the estimation of Gemcitabine HCl, Paclitaxel, Bicalutamide and Letrozole in pharmaceutical dosage forms.

KEYWORDS

Gemcitabine HCl, Paclitaxel, Bicalutamide, Letrozole, HPLC, Validation

INTRODUCTION

Gemcitabine hydrochloride, 2-deoxy-2'2'diflurocytidine monohydrochloride has antitumour activity. The cytotoxic effect of Gemcitabine is attributed to combination of two actions of the diphosphate and the triphosphate nucleosides, which leads to inhibition of DNA synthesis. After the Gemcitabine nucleotide is incorporated into DNA, only one additional nucleotide is added to the growing DNA strands. After this addition, there is inhibition of further DNA synthesis.

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DNA polymerase epsilon is unable to remove the Gemcitabine nucleotide and repair the growing DNA strands (masked chain termination). In lymphoblastoid cells, Gemcitabine induces internucleosomal DNA fragmentation, one of the characteristics of programmed cell death[1].

Combination chemotherapy has shown significant promise in cancer treatment. Combined therapy of two or more drugs promotes synergism among the different drugs against cancer cells and suppresses drug resistance through distinct mechanisms of action[2]. Gemcitabine is useful in combination with paclitaxel as first-line therapy for metastatic breast cancer. It is useful for initial treatment in patients with inoperable, locally advanced (stage

IIIA or IIIB) or metastatic (stage IV) non-small cell lung cancer in combination with cisplatin. It is useful as first-line drug for the treatment of adrenocarcinoma of pancreas, also used as second-line therapy in patients previously treated with fluorouracil. It is used alone or in combination with cisplatin for the treatment of advanced or metastatic bladder cancer. It is currently being investigated for use in the treatment of advanced epithelial cell cancer[1].

Literature review indicated that there are LC-MS[3,4], HPLC[5-16] methods for the determination of Gemcitabine individually and in combination with other drugs in dosage forms and biological fluids. The present investigation by the authors describes rapid, accurate and precise RP-HPLC methods for the determination of Gemcitabine HCl (GEM) in combination with other lipophilic drugs paclitaxel (PAC), Bicalutamide (BIC) and Letrozole (LET) in pharmaceutical dosage forms.

MATERIAL AND METHODS

Instrumentation

The HPLC system consisted of a LC Waters (Waters, Milford, MA, USA) using a quaternary gradient system (600 Controller), in line degasser (Waters, model AF). The system was equipped with a photodiode array detector (Water, 2998 model) and auto sampler (Waters, model 717 plus). The separation of drugs was carried out on a 250X4.60 mm 5 µ C8 Zorbax Eclipse plus Column obtained from Agilent. The mobile phase comprised of methanol:Sodium dihydrogen phosphate pH 3.5 (70:30 v/v). The flow rate was maintained at 1.2 mL/min. The run time for each run was set for 10 minutes. The effluents were monitored at 230 nm, 257 nm and 248 nm for 1) Gemcitabine HCl and Paclitaxel, 2) Gemcitabine HCl and Bicalutamide and 3) Gemcitabine HCl and Letrozole respectively.

Chemicals and Reagents

Gemcitabine HCl, Bicalutamide, and Letrozole for these studies were provided by Cadila Healthcare Ltd., India and Paclitaxel was provided by Sun Pharmaceuticals Ltd., India. βCD (MW 1134) was purchased from Roquette. Diphenyl Carbonate was obtained from EMD Millipore. All other reagents used were of reagent grade and all solvents were of HPLC grade. Milli-Q water was used to prepare buffer solutions and other aqueous solutions.

Preparation of Stock and Standard Solutions

The standard stock solutions containing 1 mg mL^{-1} each of GEM, PAC, BIC and LET were prepared separately by dissolving reference standards in methanol and diluting with the same diluent. 10 mL aliquots from the standard stock solutions of drugs were transferred to 100 mL calibrated volumetric flask and the volume was made up to the mark with the same solvent mixture to prepare a mixed standard preparation having a concentration of 100 µg mL^{-1} for all drugs. Calibration curve solutions containing 5-200 µg mL^{-1} each of GEM, PAC, BIC and LET were prepared by diluting the standard stock solution to the appropriate volume with the same diluent.

Preparation of Test Solution

Nanosponge preparations equivalent to 7.5 mg each of GEM and PAC, GEM and BIC and GEM and LET were accurately weighed and transferred to a 100 mL calibrated volumetric flask. Around 50 mL of methanol was added, and the solution sonicated for 10 min. Volume was made up to the mark with the same solvent mixture. The solution was filtered through 0.45 mm membrane filter. This solution contains 75 µg mL^{-1}, each of GEM and PAC, GEM and BIC and GEM and LET.

Monobasic Phosphate Buffer (pH 3.5)

13.8g of monobasic sodium phosphate was accurately weighed and transferred in to 1000 ml volumetric flask, 2.0ml of phosphoric acid was added and 300 ml water was added. The solute was made to dissolve. Then the volume was made up to 1000 ml with water. pH was adjusted to 3.5 with Triethylamine. The solution was filtered through a 0.45 µm membrane filter.

Mobile Phase

Methanol and Phosphate buffer (pH 3.5) was mixed in the ratio of 70:30 v/v. Mobile phase was filtered and degassed prior to HPLC use.

Method Validation

The developed methods were validated for specificity, precision, accuracy and robustness. System precision was determined on six replicate injections of standard preparations. Accuracy and robustness was determined on three replicate injections.

Linearity

Calibration graphs were constructed by plotting peak area vs concentration of GEM and PAC, GEM and BIC and GEM and LET and the regression equations were calculated. The calibration graphs were plotted over 8 different linear concentrations in the range of 5-200 µg/mL for both drugs. Aliquots (20 µl) of each solution were injected under the operating chromatographic condition described above [Number of replicates (n = 6)].

Precision

The day to day and within day precision of the proposed method was determined by analyzing mixed standard solution of GEM and PAC, GEM and BIC and GEM and LET at concentration 75, 100 and 150 µg/mL 3 times on the same day and on 3 different days. The results are reported in terms of relative standard deviation in table 2 and 3.

Accuracy

The accuracy of the method was established by recovery studies i.e external standard addition method. The known amount of standard was added at two different levels to preanalyzed sample. Each determination was performed in triplicate, the results of the study are reported in table 5.

Robustness

Robustness is the measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters (e.g. pH, mobile phase composition, temperature, instrument settings, etc.) and provides an indication of its reliability during normal usage. The robustness of the method was evaluated by assaying the test solutions after slight but deliberate changes in the analytical conditions like flow rate (± 2%), and mobile phase ratio (±5%). Each determination was performed in triplicate, the results of the study are reported in table 6.

Analysis of Drugs in Nanosponge Formulations

The responses of sample solutions were measured for quantitation of GEM and PAC, GEM and BIC and GEM and LET by the method described above. The amount of GEM and PAC, GEM and BIC and GEM and LET present in the sample solution were determined by applying values of peak area to regression equation of the calibration graph. Each determination was performed on six replicate injections; the results of the study are reported in table 7.

RESULTS AND DISCUSSION

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 this HPLC method was determined by comparing the chromatograms obtained followed by the injection of just the mobile phase and the chromatogram of the mobile phase containing the drugs. Drugs (15µg/ml) were spiked in the mobile phase and injected onto HPLC. The retention time was found to be 2.6 minutes for Gemcitabine and 7.1, 4.8 and 3.4 minutes for paclitaxel, bicalutamide and letrozole respectively. The absence of any overlapping or extraneous peaks in both chromatograms indicates the specificity of the HPLC method. The representative chromatogram of the mobile phase alone (without drug) and the representative chromatogram of the mobile phase containing 15 µg/mL of Gemcitabine in combination with Paclitaxel, Bicalutamide and Letrozole is shown in figure 1. Since drug peaks could be clearly distinguished from the other peaks therefore, this method was said to be specific for the analysis of gemcitabine in combination with paclitaxel, bicalutamide and letrozole.

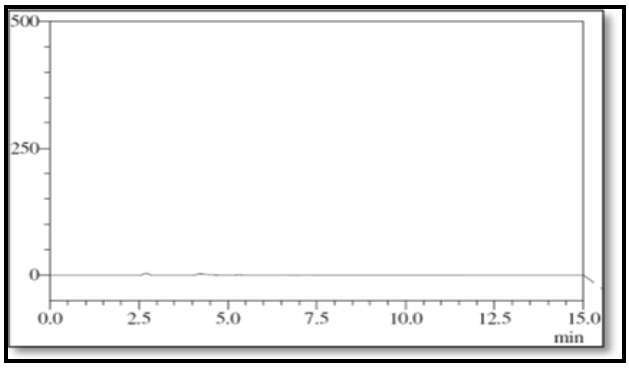

Figure 1A: Sample chromatogram mobile phase, and 15 µg/mL injection

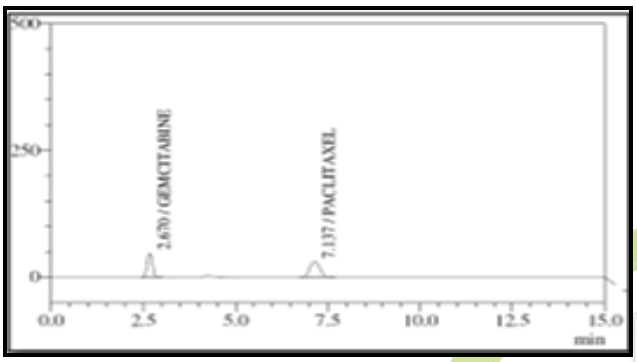

Figure 1B: Sample chromatogram Gemcitabine and Paclitaxel injection

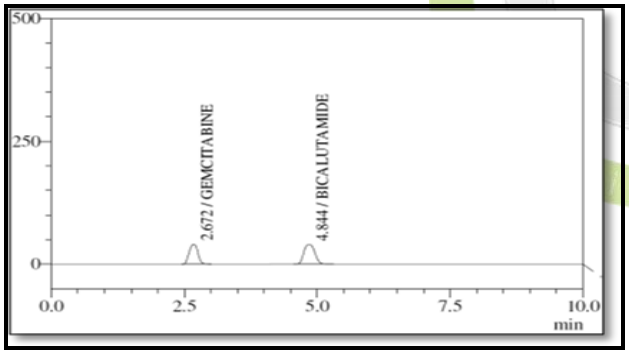

Figure 1C: Sample chromatogram Gemcitabine and Bicalutamide injection

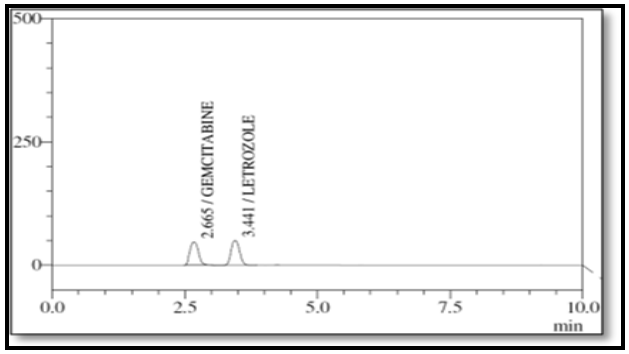

Figure 1D: Sample chromatogram Gemcitabine and Letrozole injection

Linearity

A linear study identifies a specified concentration range where analyte's response is linearly proportional to the concentration. The standard curve was found to be linear over the concentration range of 5 - 200 µg/mL. The equation of the standard curve relating the peak area to the drug concentration (C in µg/ml) in this range, along with the correlation coefficient are provided in table 1 and figure 2 below. It is a statistical measure of the extent to which variations in one variable are related to variations in another. The stronger the relationship, the more the data points on the scatter plot will align them in a straight line. When $r^2 = 1$, then all data points fall on the regression line.

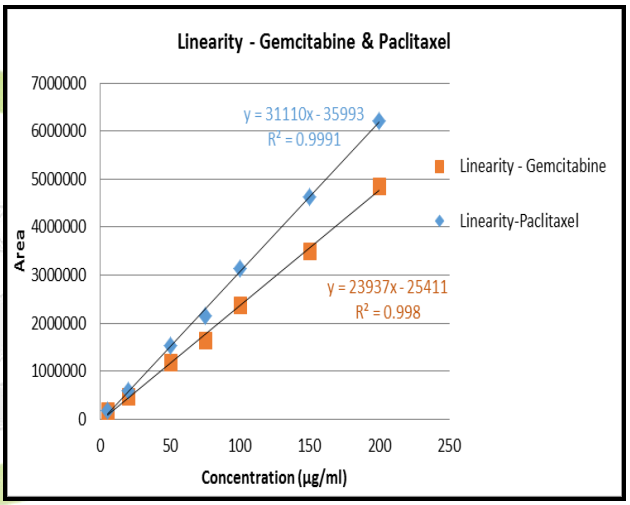

Figure 2A: Standard Curve for Gemcitabine and Paclitaxel

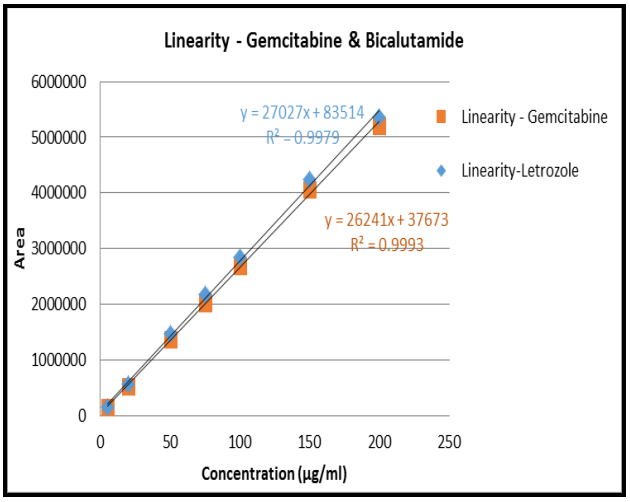

Figure 2B: Standard Curve for Gemcitabine and Bicalutamide

Table 1: Linear equation and correlation coefficient of analytical methods

Parameters	Gemcitabine and Paclitaxel		Gemcitabine and Bicalutamide		Gemcitabine and Letrozole	
	Gemcitabine	Paclitaxel	Gemcitabine	Bicalutamide	Gemcitabine	Letrozole
Linear Equation	y = 23937x – 25411	y = 31110x – 35993	y = 26053x – 3586.5	y = 33244x - 16140	y = 26241x + 37673	y = 27027x + 83514
Correlation Coefficient	R^2 = 0.998	R^2 = 0.9991	R^2 = 0.9995	R^2 = 0.9996	R^2 = 0.9993	R^2 = 0.9979
Linearity Range (µg/ml)	5-200					

Table 2: Within day precision for HPLC methods

Conc^n (µg/ml)	Interday											
	Gemcitabine and Paclitaxel				Gemcitabine and Bicalutamide				Gemcitabine and Letrozole			
	Gemcitabine		Paclitaxel		Gemcitabine		Bicalutamide		Gemcitabine		Letrozole	
	Mean Peak Area	RSD (%)	Mean Peak Area	RSD (%)	Mean Peak Area	RSD (%)	Mean Peak Area	RSD (%)	Mean Peak Area	RSD (%)	Mean Peak Area	RSD (%)
75	1649716	0.32	1649716	0.24	1976007	0.77	1976007	0.95	2011700	0.83	2162162	0.82
100	2361974	3.96	2361974	4.13	2575833	2.68	2575833	2.70	2760298	2.20	2930584	2.02
150	3483166	0.50	3483166	0.50	3950069	4.28	3950069	4.19	4117875	1.39	4295148	1.23

Table 3: Day to day precision for HPLC methods

Conc^n (µg/ml)	Intraday											
	Gemcitabine and Paclitaxel				Gemcitabine and Bicalutamide				Gemcitabine and Letrozole			
	Gemcitabine		Paclitaxel		Gemcitabine		Bicalutamide		Gemcitabine		Letrozole	
	Mean Peak Area	RSD (%)	Mean Peak Area	RSD (%)	Mean Peak Area	RSD (%)	Mean Peak Area	RSD (%)	Mean Peak Area	RSD (%)	Mean Peak Area	RSD (%)
75	1655012	0.56	2170534	0.61	1974206	0.33	2515410	0.41	2019197	0.82	2172050	0.81
100	2330128	0.31	3077092	0.30	2578304	0.92	3287575	1.16	2754862	1.77	2926606	1.66
150	3486755	0.37	4615039	0.38	3878147	0.78	4937042	0.73	4079406	1.18	4262686	1.05

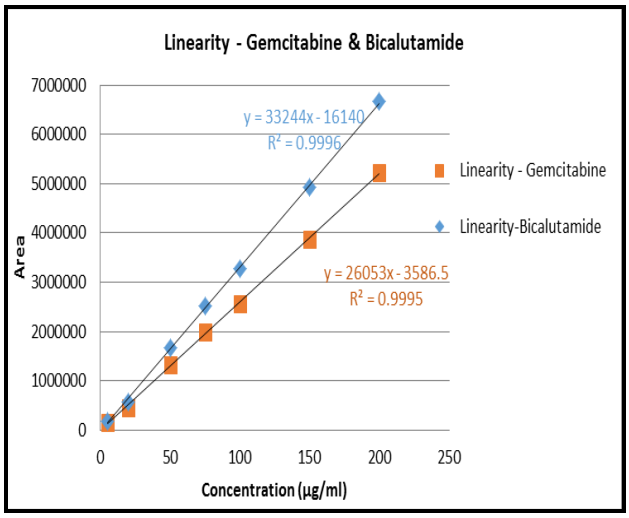

Figure 2C: Standard Curve for Gemcitabine and Letrozole

Precision

Precision is a measure of the consistency and reproducibility of a method. A precise method gives very close values for repeated measurements of same sample under identical experimental conditions. For current assay validation, within day precision and day to day precision were used. The within day precision was determined by repeated injection of standard solutions at three different levels for three times on the same day. Day to day precision was determined by injecting three sets of standard solutions on three different days over a period of 30 days. The relative standard deviation (RSD) values were calculated for both within day Precision and day to day precision and were found to fall within the acceptable limits. The precision results for the HPLC method are listed in table 2 and 3.

Accuracy

Accuracy, sometimes also referred to as recovery is an indicator of the trueness of test measurements. To determine the accuracy of the method quality control samples, 75 and 150 µg/mL concentrations were used. Samples were spiked and analyzed three times. The accuracy of the assay was evaluated by comparing the estimated concentration with the known concentration of drugs. The results of this study are depicted in table 5.

Robustness

The robustness data for GEM, PAC, BIC and LET are presented in Table 6. The average % RSD for robustness were found to be 0.64 and 1.3 for GEM and PAC, 1.15 and 1.35 for GEM and BIC, 1.17 and 1.11 for GEM and LET respectively.

Analysis of Drugs in Nanosponge Formulations

Nanosponge formulations were analyzed and amount of drugs was determined by proposed method; it was in good agreement with the label claim. Results of estimation in different formulations are shown in table 7.

Table 5: Accuracy results of HPLC methods

Actual Concentration (µg/ml)	Measured Concentrations (µg/ml) ± RSD, Accuracy (n = 3)					
	Gemcitabine and Paclitaxel		Gemcitabine and Bicalutamide		Gemcitabine and Letrozole	
	Gemcitabine	Paclitaxel	Gemcitabine	Bicalutamide	Gemcitabine	Letrozole
75	74.46 ± 1.72, 99.28%	73.69 ± 0.51, 98.26%	73.80 ± 1.40, 98.40%	74.93 ± 1.49, 99.91%	75.30 ± 1.48, 100.40%	76.36 ± 1.06, 101.81%
150	147.61 ± 0.58, 98.40%	148.60 ± 0.96, 99.06%	147.23 ± 0.78, 98.15%	150.15 ± 0.99, 100.10%	151.10 ± 0.79, 100.73%	148.96 ± 1.53, 99.31%

Table 6: Robustness results of HPLC methods

Parameters	Robustness (%), RSD (n=3)					
	Gemcitabine and Paclitaxel		Gemcitabine and Bicalutamide		Gemcitabine and Letrozole	
	Gemcitabine	Paclitaxel	Gemcitabine	Bicalutamide	Gemcitabine	Letrozole
Flow Rate (±2%)	99.33% ± 0.62	98.32% ± 0.25	98.08% ± 1.28	100.01% ± 1.62	101.07% ± 1.54	101.04% ± 0.98
Ratio of Mobile Phase (±5%)	98.55% ± 0.66	98.94% ± 1.05	97.85% ± 1.02	99.09% ± 1.08	100.82% ± 0.80	99.52% ± 1.24

Table 7: Analysis of GEM and PAC, GEM and BIC and GEM and LET in nanosponge formulations by HPLC method

Actual Concentration (µg/ml)	Measured Concentrations (µg/ml) ± RSD (n=6)					
	Gemcitabine and Paclitaxel		Gemcitabine and Bicalutamide		Gemcitabine and Letrozole	
	Gemcitabine	Paclitaxel	Gemcitabine	Bicalutamide	Gemcitabine	Letrozole
100	99.03 ± 0.12	100.64 ± 0.14	98.92 ± 0.03	98.97 ± 0.07	100.95 ± 0.34	102.68 ± 0.30

CONCLUSION

A simple, specific, accurate and precise RP-HPLC method has been developed and validated for simultaneous estimation of gemcitabine hydrochloride in combination with Paclitaxel, Bicalutamide and Letrozole. The chromatographic separation was carried out on a C8 Zorbax Eclipse plus column (250 x 4.60 mm, 5 µ) obtained from Agilent using a mobile comprised of methanol and sodium dihydrogen phosphate pH 3.5 in a ratio of 70:30 v/v.

The flow rate was maintained at 1.2 mL/min. The effluents were read at 230 nm, 257 nm and 248 nm for 1) Gemcitabine HCl and Paclitaxel, 2) Gemcitabine HCl and Bicalutamide and 3) Gemcitabine HCl and Letrozole respectively using a photo diode detector.

The developed HPLC method was found to be specific for gemcitabine hydrochloride, Paclitaxel, Bicalutamide and Letrozole. A strong linear correlation with an r2 > 0.9979 was observed between the concentration of the drug and peak area obtained upon chromatographic extraction over a concentration range of 5 to 200 µg/mL. Precision and accuracy of the developed method are expressed in %RSD and % of recovery of the active pharmaceutical ingredient respectively. Low %RSD value and high percent of recovery indicate that the method is highly precise and accurate.

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