Asian Journal of Chemistry; Vol. 23, No. 9 (2011), 3961-3963

ASIAN JOURNAL OF CHEMISTRY

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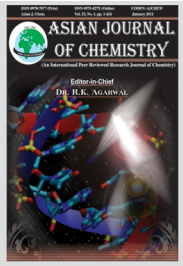

Bioanalytical Method Development and Validation of Gemcitabine Hydrochloride by RP-HPLC Method

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(*Received*: 15 October 2010; *Accepted*: 14 May 2011) AJC-9944

A simple, accurate, precise and sensitive HPLC method with UV detection was developed and validated to separate and detect gemcitabine hydrochloride in human plasma using capecitabine hydrochloride as an internal standard. Gemcitabine hydrochloride and capecitabine hydrochloride (internal standard) were extracted from human plasma using methanol protein precipitation and were chromatographed on a phenomenex C_{18} (250 mm × 4.6 mm. 5µm) column using 20 µl injection volume and detection at 270 nm. An isocratic mobile phase consisting of methanol: water (85:15 % v/v) was used to separate these drugs. The retention times of gemcitabine hydrochloride and internal standard were 4.6 and 6.2 respectively. The method was validated over the range of 406.10-4020.05 ng/mL. The limit of detection was 200 ng/mL and the limit of quantification was 400 ng/mL. Within and between-day precisions are less than 6.5 % for all quality control samples. The absolute recoveries of gemcitabine hydrochloride was greater than 90 % were achieved. The described method can be readily utilized for analysis of pharmaceutical products.

Key Words: Gemcitabine hydrochloride, Capecitabine hydrochloride, HPLC.

INTRODUCTION

Gemcitabine hydrochloride (GTHC) is chemically a 2′-deoxy-2′,2′-difluorocytidine monohydrochloride[1] is a nucleoside metabolic inhibitor that exhibits antitumor[2] activity. Spectroscopic[3,4], HPTLC[5,6] and RP-HPLC method have been reported for the estimation of gemcitabine individually and in combination[7] with other drugs.

Capecitabine hydrochloride (CTHC): Pentyl [1-(3, 4-dihydroxy-5-methyl-oxolan-2-yl)-5-fluoro-2-oxo-pyrimidin-4-yl]aminoformate[8] is cancer treatment for metastatic[9] breast cancer and colorectal[10] cancer, as well as adjuvant therapy for stage III[11]. Various methods such as LC-MS[12], RP-HPLC[13,14] and spectrophotometric method have been reported for the estimation of capecitabine hydrochloride.

Literature survey reveals that no method has been reported so far for the estimation of bio analytical method development and validation of gemcitabine hydrochloride by RP-HPLC method. Hence, in the present study, the bioanalytical[15] method development and validation[16] of gemcitabine hydrochloride dosage forms by RP-HPLC method.

EXPERIMENTAL

Working standards gemcitabine hydrochloride and capecitabine hydrochloride were kindly provided by Swarup Exim (India), Nagpur, India. HPLC Grade solvents (acetonitrile, water and methanol) were obtained from S.D. Fine Chemicals Ltd., India, Qualigens Fine Chemicals Ltd., Mumbai.

Stock standard solutions of gemcitabine hydrochloride and the internal standard were prepared by dissolving appropriate amounts of compounds in a known volume of methanol and water stored at 4 ºC.

Blank human blood was collected with heparin from healthy and drug free volunteers. After centrifugation at 5000 rpm at room temperature, plasma was collected and stored at -30 ºC until analysis.

Analyses were performed on shimadzu scientific instruments composed of LC-20 AT pump and SPD-20 AT variable wavelength detector. The separation of compounds was achieved using a phenomenex C_{18} (250 mm × 4.6 mm, 5µm).

Chromatographic condition: The mobile phase used was methanol and water (85:15 % v/v). Before analyses, the mobile phase was filtered through 0.4 µm filter and then degassed ultrasonically for 15 min. The analyses were conducted at a flow rate of 1.0 mL/min. The eluent was monitored at a wavelength of 270 nm for gemcitabine hydrochloride. The total run time was 10 min.

Protein precipitation: The blank plasma sample was prepared by adding 1 mL of plasma and 1 mL of methanol and vortex for 30 s. Then centrifuge the solution at 4 ºC, 5000

rpm for 5 min. The supernatant liquid is taken and transferred to HPLC vials.

The blank plasma sample was prepared by adding 1 mL of spiked plasma 1 mL of methanol and 0.2 mL of capecitabine hydrochloride standard (250 µg/mL) and vortex for 30 s. then centrifuge the solution at 4 ºC, 5000 rpm for 5 min. the supernatant liquid is taken and transferred to HPLC vials.

Quantification of gemcitabine hydrochloride in plasma: A standard curve was prepared by injecting various concentrations of gemcitabine hydrochloride in plasma. The concentrations of the plasma and quality control samples were calculated by using the regressed equation of the straight line y = mx + c.

The limit of detection (LOD) and limit of quantification (LOQ) were determined as follows: 6 blank samples from six separate subjects for each analyte and matrix were extracted and compared to a low standard of each analyte. Where an obvious peak existed at the same retention time as the analyte, a concentration was calculated for this peak. Where no discreet peak or a series of small noise peaks existed at the same retention time as the analyte, the height of the noise was measured and compared to the height of the low standard. This provided a concentration for the noise. An average of the 6 noise concentrations was calculated and multiply by either 3 (LOD) or 5 (LOQ). Limit of quantification values were subsequently confirmed using six replicates spiked at the target concentration as being within an acceptable variance of 20 %.

Determination of accuracy, precision and recovery: Accuracy, between-day and within-day precision of the method were determined by assaying two replicate samples of plasma at 3 different gemcitabine hydrochloride concentration (3420.06, 2052.04 and 1026.02 ng/mL) in 6 analytical runs. Accuracy was measured as the per cent deviation from the nominal concentration.

The absolute recovery of gemcitabine hydrochloride and internal standard were determined by comparison of the peak areas from non-extracted and extracted samples. The recovery was calculated as the relative standard deviation of the mean (RSD) with RSD (%) = (standard deviation of the mean/mean) × 100.

Specificity and selectivity: Interference from endogenous compounds was investigated by analysis of blank human plasma collected from 6 drug-free volunteers. Chromatograms of plasma from all drug-free volunteers were examined that may co-elute with gemcitabine hydrochloride.

Stability studies: The stability of solutions of gemcitabine hydrochloride was assessed in analytical standard solutions, processed sample extracts and biological matrix by comparison to freshly spiked plasma samples.

Stability of analytical standard solutions: The stability of solutions of gemcitabine hydrochloride and capecitabine hydrochloride in methanol and water (85:15 % v/v) stored at 10 ºC was examined for 6 months. Standard solutions of gemcitabine hydrochloride and capecitabine hydrochloride were prepared throughout the method development phase of the study and stored at 10 ºC. These solutions were compared to freshly prepared standards of gemcitabine hydrochloride and capecitabine hydrochloride. Standard solutions prepared in mobile phase and stored at 10 ºC were stable for at least 6 months.

Short term stock stability: A stock solution of gemcitabine hydrochloride and internal standard was kept at room temperature for 8 h.

Long term stock stability: A stock solution of gemcitabine hydrochloride and internal standard was kept at room temperature for 12 days.

Bench top stability: The replicate concentration of low and high quality control samples were determined by comparing the mean area ratio of freshly thawed samples, which kept at room temperature for 6 h.

Coolant stability: The replicate concentration of low and high quality control samples were determined by comparing the mean area ratio of freshly thawed samples which kept at room temperature for 24 h.

Long term plasma stability: At least three aliquots of each of low and high concentrations at same conditions as study samples. Analyse on three separate occasions. Storage time should exceed the time between the date of first sample collection and the date of last sample analysis.

Ruggedness: This includes different analysts, laboratories, columns, instruments, sources of reagents, chemicals, solvents.

Ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test condition. The ruggedness of the method was studied by changing the experimental condition such as, changing to another column of similar type (phenomenex gemini C_{18}), different operation in the same laboratory.

RESULTS AND DISCUSSION

The method was validated in terms of limit of quantification, recovery, selectivity, precision, accuracy and stability. (Table-1)

TABLE-1

S. No.	Parameters	Result
1	System suitability	Pass
2	Selectivity	Pass
3	LOD	200 ng
4	LOQ	400 ng
5	Accuracy and precision	Pass
6	Linearity	R^2 =0.9999
7	Short term stock stability	12 h
8	Long term stock stability	30 days
9	Coolant stability	7 days
10	Bench top stability	12 h
11	Long term plasma stability	60 days
12	Ruggedness	Pass

Linearity: The method was validated over the range of 406.20-4020.05 ng/mL. The slope, correlation co-efficient were found to be 86.139, 0.9999 respectively.

Sensitivity: In the plasma, the calculated limit of quantification was 400 ng/mL and limit of detection was 200 ng/mL for Gemcitabine hydrochloride.

Recovery: The recovery was determined by comparing the aqueous solution and the spiked drug. The percentage recovery of the drug and the internal standard was calculated and it was 97.55 and 98.11 %, respectively.

Precision and accuracy: The accuracy, precision and intraday precision were carried out by preparing six individual samples and percentage nominal was calculated.

Stability: Stability of the method was carried out by performing short term and long term stock stability. The percentage mean ratio of the drug and internal standard were calculated. Stability of the plasma samples was carried out by performing coolant, bench top stability. The long term plasma stability was carried out by performing from the initial sample to the date of last sample. The percentage % RSD was calculated.

Ruggedness: The ruggedness of the method was carried out by changing by column and by different analyst in the same laboratory. The percentage % RSD was calculated.

Conclusion

The bio analytical method developed is simple and shows good accuracy, specificity and reproducible. It can be used for the estimation of gemcitabine hydrochloride in bio fluids. The separation method developed produce acceptable values of recovery. The chromatogram developed has well resolved peak of gemcitabine hydrochloride without any interference. The developed method could be applied in bioequivalence, pharmacokinetic and toxico kinetic studies.

ACKNOWLEDGEMENTS

The authors are thankful to K.V.S.R. Siddhartha College of Pharmaceutical Sciences for providing the necessary facilities and Swarup Exim (India), Nagpur for providing gift samples of gemcitabine hydrochloride and capecitabine hydrochloride.

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