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Original Article

Simultaneous Method for Quantification of Genotoxic Impurity in the Gemcitabine Hydrochloride by RP-HPLC

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ABSTRACT

Objective: In the pharmaceutical analytical field, effort to analyzing the degradation or related and impurity substances from the complex matrix in the ultra-trace levels is a really a challengeable task. Impurity is any component of the new drug substance that is not the chemical entity defined as the new drug substance. Gemcitabine hydrochloride is a new anticancer bio molecule. According to Indian Pharmacopoeia there is two impurities present in this drug. The literature review exploded that, there is no specific impurity profiling based analytical method developed to estimate the Cytosine. **Methods:** RP HPLC is developed to quantify and separate Gemcitabine hydrochloride and Cytosine. The Hibar® C^{18} (250 x 4.6 mm i.d., 5µ), Column choices as stationary phase, tri ethyl ether and Acetonitrile in ratio of 95:5 with 4.35 (with Orthophosphoric acid) as mobile phase, the flow rate fixed as 1ml/ min. at 279 nm. **Results:** A retention time found to be 3.44min and 8.28min for gemcitabine and cytosine respectively. The developed method was validated as per ICH guidelines and the method was proved as selective, specific and linear. The assay and recovery studies were carried out the assay limit was 99.19 and 99.32 for bulk drug and formulations respectively. The Cytosine were present 0.7821 µg/ml and 0.6531 µg/ml in the bulk drug and formulation respectively. **Conclusion:** It can be concluded that the developed RP-HPLC method was found to be suitable for the appraisal of cytosine impurities in Gemcitabine HCl. The developed RP-HPLC is economic and suitable for the use.

Key words: Gemcitabine HCl (GCH), Cytosine (CYS), RP-HPLC, Genotoxin, Impurity, Anticancer.

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INTRODUCTION

Impurity profiling is an integral part of the drug development program. Impurity is any component of the new drug substance that is not the chemical entity defined as the new drug substance.[1] In present circumstance various regulatory authorities like International Conference on Harmonization (ICH), the United States Food and Drug Administration (USFDA) and the Canadian Drug and Health Agency (CDHA), Therapeutic Good Administration (TGA) etc., are emphasizing on the transparency requirements in the identification of impurities in Active Pharmaceutical Ingredients (APIs) by means of its effects on pharmacological, toxicological and genetically related issues, which drives the drug related monitoring bodies to developed special attention on the impurity regulation. Impurities generally divided into three major categories which covers organic, inorganic and related substances. Out of which organic impurities attain maximum attention among the controlling body and researcher due to their serious side effects in the different levels of biological systems. To predict the known / unknown impurities and to assure the selectivity of the developed method ICH installed the concept of force degradation studies with different stress condition like acid, base, hydrolysis, photolytic cleavage, oxidative degradation, decarboxylation, enantiomeric impurity and so on. The different pharmacopoeias such as the British Pharmacopoeia (BP), United State Pharmacopoeia (USP) and Indian Pharmacopoeia (IP) are slowly incorporating the daily intake limit for the known and unknown impurities in the finished formulation and APIs. The impurities present / developed in the product and APIs may also occur due to in proper handling, storage, by contact with packaging materials etc., in this challenge the simultaneous estimation of impurities in presence of APIs and in presence of excipients place a vital role in regulatory as well in analytical field to separate the various impurities from their complex matrix. In the present study the gemcitabine hydrochloride (GCH) nominated as a chemical entity. GCH chemically known as 2'-deoxy-2', 2'-difluorocytidine monohydrochloride (β-isomer). Pharmacologically used in the treatment of breast cancer, pancreatic cancer and lungs cancer at the different differ dose like 1000mg /vial, 1250mg /vial, 750mg /vial at different interval like weekly once. The mechanism of action of GCH is basically works on the pro drug concept, once it transports in to the cell then it will be phosphorylated by a deoxycytidine kinase and it convert in to an active form.it also inhibit the DNA synthesis. Incorporation of GCH is causes cell death. The GCH entered in to commercial market at May 19, 2004. The selected drug molecule recommended to have major impurities Cytosine (CYT). (Gemcitabine impurity A1), Gemcitabine alpha anomer. (Gemcitabine impurity B2)[2] respectively. Cytosine (CYS) present in the GCH as a reactant impurity, CYS common IUPAC name is 4-aminopyrimidin-2(1H)-one and the molecular weight is 111.1 g/mol. It is a derivative of pyrimidine. It has been added to the synthesis of GCH on the 4th stage. The mechanism of exploit of GCH is basically ace drug and once it will conveyance in to the cell then it must be phosphorylated by a deoxycytidine kinase to a dynamic form. In prevent of DNA synthesis equally of two gemcitabine diphosphate and gemcitabine triphosphate is vital. Assimilation of gemcitabine diphosphate is major role in GCH causes cell death. After adding of GCH in to the DNA analog there is no additional position for added to other substance, so the DNA

polymerase are incompetent to proceed. The previous track of literature review explored that GCH Lyophilized product cycle[3] studied by using Differential scanning calorimeter as a tool. The stability indicating by RP HPLC method was done to evaluate stability of GCH in with different stress condition,[4] the GCH drug also done for radio sensitivity.[5] The linear synthesis been optimized for GCH with modest anomeric selectivity,[6] the developed Synergistic collaboration between cisplatin and GCH in neuroblastoma cell lines and multicellular tumor spheroids,[7] have developed and validated of a UPLCMS/MS assay for the termination of GCH and derivative in oral pharmacokinetics.[8] The GCH drug have developed Y-clivatuzumab tetraxetan for pancreatic cancer, another pancreatic cancer study done by because of Ribonucleotide reductase is an operative target to overcome GCH, also thrombotic microangiopathy in an urothelial tumor patient by GCH, Primary Lung Cancer and Metastatic Pulmonary Colorectal Cancer that Responded to GCH/Cisplatin/Bevacizumab Combination Therapy[9-12] were studied. As go through the updated literature assessment there was no specific method found for the simultaneous estimation of genotoxic impurity in the GCH.

MATERILAS AND METHODS

Methods

Acetonitrile and tri ethyl amine were procured from Loba Chemi Pvt. Ltd, Mumbai. GCH obtained as a gift sample from the Industry, GCH dry powder injection purchased from local commercial market manufacture from Eli Lilly Company. Shimadzu HPLC LC 2010A auto injector model with UV detector with Class- VP software used for method development. Hibar C_{18} RP-Column (250mm × 4.6 mm, 5 µ) used for separation. HPLC grade water obtained from Milli Q RO system.

Preparation of standard solution

Preparation of buffer

Measured volume of 1 ml trimethylamine mixed with 300ml of milli Q water the pH of water adjusted to 4.35 using orthophosporic acid. The resulted solution filtered through 0.45u cellulose membrane filter.

Preparation of standard solution of GCH and CYS

The standard 10 mg of GCH and CYS weighed and transferred in to individual 10 ml volumetric flask few ml of methanol used to dissolve both the substance and the remaining volume makeup with methanol. These standard solutions were stored at 50 until farther analysis.

Preparation of working standard solution of GCH and CYS

The 100 µg/ ml working standard solutions of GCH and CYS were prepared by diluting 1 ml respective solution to 10 ml methanol.

Preparation of assay solution

The assay performed for both bulk and injectable dosage forms. Amount equivalent to 100mg of GCH have been weighed and transfer to the 100ml volumetric flask, the weighed substance has been dissolved and the marked volume made up with mobile phase as a solvent. The solution was involved for the membrane filtration to avoid the fiber and other external interferences. The Assay of CYS was performed for marketed accessible formulation by adopting RP- HPLC as analytical tool.

Optimization of separation condition

Optimization of separation condition was performed based on trial. The different mobile phase composition was taken in to consideration to develop ideal separation condition. In the first trial methanol and water used as a solvent with 50: 50 % v/v ratio later the methanol was replaced with acetonitrile and the separation condition was screened. The composition methanol, water, acetonitrile have been also tried to get good resolution.

Optimization of pH

To obtain the sharp and symmetric peak the *p*H optimization stage is essential in the method development. During method development to reduce the tailing affect, to improve the theatrical plates. The tri ethyl amine buffer *p*H was adjusted up to 4.35 by using ortho phosphoric acid and the mobile phase composition was fixed to 5:95% v/v for acetonitrile and tri ethyl respectively.

Method validation

Accuracy and recovery

Accuracy is a measure of the closeness of the investigational value to the genuine amount of the substance in the background. The closeness of the agreement between the value is mainly described the accuracy. Which is primarily designate by the acceptance either as conventional true and the accepted reference value found. Standard addition and recovery trials were conducted to regulate accuracy.

Precision study

Precision measurement provides an idea to assess the closeness of results within the day and between days. It also delivers information about the variations while different analysts handle the same procedure. This above parameter scrutinized with the different concentrations label like 0.3, 0.5, 0.7µg/ ml for GCH and CYS. The average relative standard deviation for GCH and CYS was 0.360 and 0.480 respectively.

Linearity and range

Linearity is a basically evaluation tool to find the degree of range of concentration in which the intended method procedure able to convey result as linear line. The linearity was examined by linear relapse analysis, which was calculated by the least square lapse method.

System suitability

System suitability is a scale to cheek the system performance towards the designed protocol. It also governs the adaptability of column, resolution, Theoretical plates, peak symmetry and tailing factor etc., the obtained system suitability in present protocol where reported in Table 1. The linearity and range for GCH and CYS was 0.1-1.0µg/ml both. Correlation co-efficient (R2) for GCH and CYS was 0.9904 and 0.9936 respectively. Slope equations y = 131665x and y=99907x +46884 for GCH and CYS.

Validation and system suitability parameters

The developed method was validated for different parameters like accuracy, linearity, precision, specificity, repeatability, Limit of detection (LOD), Limit of quantification (LOQ) and robustness. Suitability of the chromatographic system was tested before each stage of validation. Five replicate injections of standard preparation were injected and Retention time, Tailing factor, number of theoretical plates and relative standard deviation of peak area were determined.

Table 1: Report for system suitability.

S. No	Validation Parameters	Observations	
		GCH	CYS
1.	Linearity and Range*	0.1-1.0µg/ml	0.1-1.0µg/ml
2.	Correlation co-efficient (R^2)	0.9904	0.9936
3.	Slope equations	y = 128513x	y=99907x +46884
4.	Limit of Quantification (LOQ)	2.621ng/ml	2.282ng/ml
5.	Asymmetric factor	1.0	0.99
6.	Tailing factor	1.0	1.0

RESULTS

Impurity analysis become mandatory from the drug regulatory bodies aspect for any kind of drug applications that newly introduced into market. In this aspect the transparency in drug efficacy and safety plays a vital role from the consumer side. To predict the known and unknown effects of other chemical substances present in the lead molecule, the first stage is separation and quantification of those substances from the lead in this context the analytical method development plays a major role. In present state genotoxic substances assessment equally produce a challenge to the analyst. The selected drug entity, Gemcitabine HCl is an important drug for treatment of different cancer. However, the presence of CYS has become mandatory because it considered as a reactant in the synthesis of GCH. Though several analytical methods have been reported in the GCH as per the previous track of research there was no specific method to quantify CYS. The present study aim was to develop simple accurate, precise and sensitive method to estimate the amount of CYS and GCH present in the selected bulk drugs and pharmaceutical dosage forms. The fixed aim obtained RP-HPLC technique. Initially selection of mobile phase methanol: water and acetonitrile: water has been experimented in different proportion, but the poor peak shape and poor system suitability parameters diverted to select the method and acetonitrile as a mobile phase with different ratio. The second experiment peak shape was not perfect enough to get symmetry. Finally, acetonitrile and tri methyl ether mixture was tried at *p*H 4.35 adjusted with ortho-phosphoric acid (5:95 v/v) at a flow rate of 1 ml/min was found to be satisfactory and good system suitability parameters. The average retention time (R_t) for CYS and GCH was found to be 3.45 min and 8.28 min, the standard chromatogram were given in the Figure 1- 5. The developed HPLC method was validated as per the ICH guidelines. The linearity and range was developed for the GCH (R^2= 0.8904) and CYS (R^2= 0.9936) (Table 2). The correlation coefficient were for CYS and GCH were 0.9936 and 0.8904 respectively, the solve equation were Y = 99907X+46884 and Y = 131665X as respectively, where Y is the peak area and X is the concentration. The linearity over the concentration range of 0.1-1.2 µg/ml for both respectively and the correlation coefficient (r2) were calculated. The developed method assay was produced 99.19 and 99.32 for bulk drug and formulations respectively in presence of CYS as a related substance is given in the Table 3. The Cytosine was present 0.7821 µg/ml and 0.6531 µg/ ml in the bulk drug and formulation respectively. The experiment was carried by 3 times and the percentage relative standard deviation (%RSD) for the GCH was found to be 99.12% and 99.32%

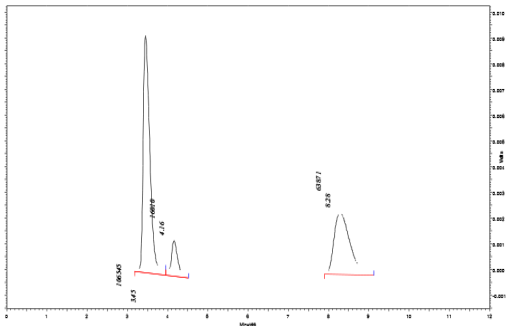

Figure 3: Standard mixture chromatogram of GCH and CYS.

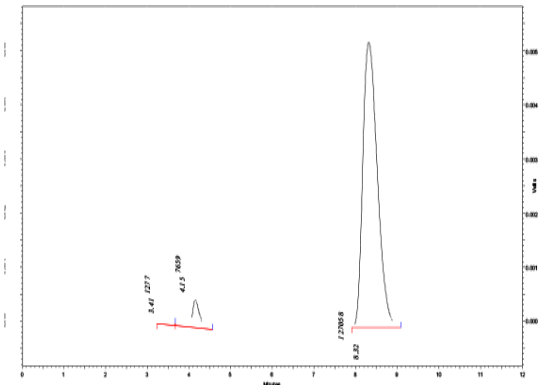

Figure 1: Standard chromatogram of GCH and CYS and retention of CYS and GCH is 3.45min and 8.28 min.

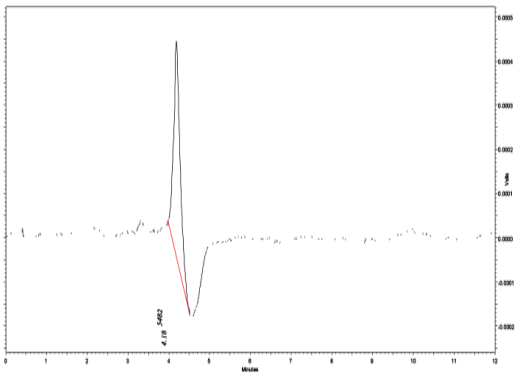

Figure 4: Typical chromatogram of blank.

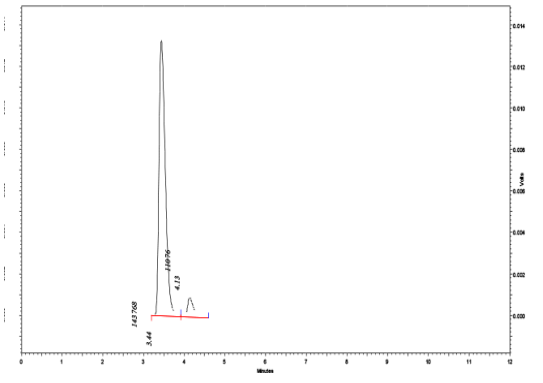

Figure 2: Standard chromatogram of CYS retention time, of 3.44 min.

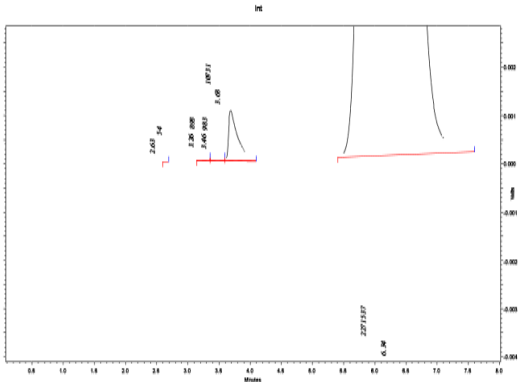

Figure 5: Typical chromatogram of marketed product.

Table 2: Linearity and range for GCH and CYS.

Concentration (µg/ml)	Peak area	
	GCH	CYS
0	0	0
0.1	19405	54049
0.3	41331	77418
0.5	67263	99655
0.7	89193	119288
1.0	126058	143768

Table 3: Results of accuracy and assay.

Brand Name	Labelled Amount	Amount Found	%Recovery	Assay %W/W	Amount of CYS
Bulk drug	1000mg	991.90 mg	96.10± 1.02	99.19	0.7821 µg/gm
Formulation	1000mg	993.20 mg	97.27± 1.5	99.32	0.6531 µg/gm

Table 4: Report for precision study.

Co ncentration (µg/ml)		Retention time analysis for GCH	Retention time analysis for CYS	Amount found
0.3(µg/ml)	AVG	8.33	3.45	0.307 µg/ml
	SD	0.033714	0.0228	0.005
	%RSD	0.404331	0.6609	1.6609
0.5(µg/ml)	AVG	8.32	3.43	0.507 µg/ml
	SD	0.0450	0.0208	0.0039
	%RSD	0.5417	0.6063	0.7746
0.7 (µg/ml)	AVG	8.35	3.46	0.707 µg/ml
	SD	0.0115	0.0057	0.005
	%RSD	0.1382	0.1665	0.7078

for bulk drug and commercial marketed formulation respectively. And also, for CYS was found to be 0.7821 µg/g and 0.6531 µg/g in the bulk drug and commercial marketed formulation respectively. The impurity quantity was found to be with in the limit. The developed method precision and recovery were studied and the limits of standard deviation were within acceptable limit the results were discussed in Table 3. The methodology was evaluated for specificity and system suitability, in order to establish suitability of the analytical method. Stability of analytical solution, filter compatibility, LOD and LOQ were also observed. Limit of Quantification (LOQ) 2.621ng/ml and 2.282ng/ml. Asymmetric factor 1.0 and 0.99 and also tailing factor was 1 for both. The System suitability results reflected that the obtained value reasonably satisfied as per the limits. Robustness of HPLC analysis method was evaluated the system suitability report were presented in the Table 4. The system suitability were analyzed, the obtained results explored in the Table 1.

DISCUSSION

According to the various regulatory bodies present worldwide, there are regulations set by them for limiting the impurities so that genotoxicity is not experienced. The selected drug Gemcitabine HCl, is known to have to have two major impurities CYS and GCH. No method was available for simultaneous estimation according to the literature.

For the research described above, the method have been developed using HPLC LC 2010A auto injector model with UV detector with Class- VP software used for method development. Hiber C_{18} RP-Column (250mm × 4.6 mm, 5 µ) used for separation, triethyl ether buffer with pH 4.35 and acetonitrile is used as mobile phase. CYS and GCH was freely soluble in methanol. The wavelength was found to be 279 nm. The assay reports of the bulk and dry powder injectables were found within the limits.

The developed method was checked for different studies which gave results within the limits and reported in the tables and chromatograms. Accuracy and recovery was within the stipulated limits. Precision studies are performed at 3 concentration levels for intra and inter day. Linearity and range with the intended method procedure can be conveyed as linear line. System suitability parameters are performed and observations were within the limitations. Acetonitrile and tri methyl ether mixture was tried at pH 4.35 adjusted with orthophosphoric acid (5:95 v/v) at a flow rate of 1 ml/min was found to be satisfactory and good system suitability parameters. The retention time (Rt) for CYS and GCH was found to be 3.45 and 8.28 min. respectively. The slope liner diagram were given 1 for GCH and CYS. The correlation coefficient for CYS and GCH were 0.9936 and 0.8904 with equations Y = 99907X+46884 and Y = 131665X as respectively. The developed method produced an assay value of 99.19 and 99.32 for bulk drug and formulations respectively in presence of CYS as related substances is given. The developed method precision and recovery were studied and the limit of standard deviation was within acceptable limit the results were discussed. Robustness of HPLC analysis method was evaluated the system suitability report were presented. The system suitability was analyzed and results were obtained.

The developed method is expected to be applied in routine analysis for quantifying the limit of CYS and GCH in the future and also for testing the stability of the drug.

The method developed can be used for reducing the impurities that are a major part of the selected drug and create a hindrance in the therapy, as it is one of the major diseases like cancer and we could not afford to have any lacuna in the drug.

CONCLUSION

The developed method was having a total run time of 12 mins. Within the stipulated retention time the drug and impurity were eventually separated. Method validation results have proved the method to be specific, precise accurate and robust. This method can be successfully applied for routine analysis as well as stability study for the separation of GCH in presence of CYS.

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CONFLICT OF INTEREST

The paper authors have no conflict of interest.

ABBREVIATIONS

GCH (HCl): Gemcitabine; **CYS:** Cytosine; **RP HPLC:** Reverse phase liquid Chromatography; **UPLC:** Ultra performance liquid chromatography; **MS:** Mass Spectrometry; **LOD:** limit of detection; **LOQ:** Limit of Quantification; **µg/ml:** Microgram/ mille litter; **IUPAC:** International Union of Pure and Applied Chemistry; **AVG:** Average; **SD:** Standard Deviation; **RSD:** Related Standard Deviation.

REFERENCES

1. ICH, Impurities in New Drug Substances. Q3A;(R2):1.
2. Indian Pharmacopoeia. 2018;2:2158-60.
3. Sreedhar B, Seshasai M, Yeluri R, Chandra R. Optimization of Lyophilization

cycles for gemcitabine. Int J Pharm Pharm Sci. 2013;5(2):216-21.
4. Mastanamma S, Ramkumar G, Anantha KD, Seshagiri RJVLN. A Stability Indicating RP-HPLC Method for the Estimation of Gemcitabine HCl in Injectable Dosage forms. E J Chem. 2010:7(S1):S239-44.
5. Heshmati E, Mozdarani H, Abdolmaleki P, Khoshaman K. Radiosensitizing effects of gemcitabine on aerobic and chronically hypoxic HeLa and MRC5 cells *in-vitro*. Int J Radiat Res. 2012;10(1):11-8.
6. Brown K, Weymouth-Wilson A, Linclau B. A linear synthesis of gemcitabine. Carbohydr Res. 2015;405:71-5.
7. Besançon OG, Tytgat GA, Meinsma R, Leen R, Hoebink J, Kalayda GV, *et al.* Synergistic interaction between cisplatin and gemcitabine in neuroblastoma cell lines and multicellular tumor spheroids. Cancer Lett. 2012;319(1):23-30.
8. Picozzi VJ, Ramanathan RK, Lowery MA, Ocean AJ, Mitchel EP, O'Neil BH, *et al.* (90)Y-clivatuzumab tetraxetan with or without low-dose gemcitabine: A phase Ib study in patients with metastatic pancreatic cancer after two or more prior therapies. Eur J Cancer. 2015;51(14):1857-64.
9. Minami K, Shinsato Y, Yamamoto M, Takahashi H, Zhang S, Nishizawa Y. Ribonucleotide reductase is an effective target to overcome gemcitabine resistance in gemcitabine-resistant pancreatic cancer cells with dual resistant factors. J Pharmacol Sci. 2015;127(3):319-25.
10. Wang G, Zhao D, Chen H, Ding D, Kou L, Kan Q. Development and validation of a UPLC-MS/MS assay for the determination of gemcitabine and its L-carnitine ester derivative in rat plasma and its application in oral pharmacokinetics. Asian J Pharm. 2016;30104:S1818-76.
11. Ryu H, Kang E, Park S, Park S, Lee K, Joo KW, *et al.* A case of gemcitabine-induced thrombotic microangiopathy in an urothelial tumor patient with a single kidney. Am J Kidney Dis. 2015;34(4):237-40.
12. Chen HM, Pan CC, Tsai CM, Hsu WH, Yang SH, Yen CC. Concomitant primary lung cancer and metastatic pulmonary colorectal cancer that responded to gemcitabine/cisplatin/bevacizumab combination therapy. J cancer Res Ther. 2015;2(1):76-82.

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