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 challenging 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 explained that, there is no specific impurity profiling based analytical method developed to estimate the Cytosines.

Methods: RP-HPLC is developed to quantify and separate Gemcitabine hydrochloride and Cytosine. The Hibar® C18 (250 x 4.6 mm i.d., 5μ), Column choices as stationary phase, tri ethyl ether and Acetonitrile in ratio of 95:5 with 4.3% (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 Cytosines 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 (CYT), RP-HPLC, Genotoxic 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 pharmaceutical, 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’-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-aminoypyrimidin-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 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, the GCH drug also done for radio sensitivity. The linear synthesis been optimized for GCH with modest anomeric selectivity, the developed Synergistic collaboration between cisplatin and GCH in neuroblastoma cell lines and multicellular tumor spheroids, have developed and validated of a UPLCMS/MS assay for the termination of GCH and derivative in oral pharmacokinetics. The GCH drug have developed Y-civatuzumab tetraxetan for pancreatic cancer, another pancreatic cancer study done by because of Ribonucleotide reductase is an operative target to overcome GCH, also thrombosis microangiopathy in an urothelial tumor patient by GCH, Primary Lung Cancer and Metastatic Pulmonary Colorectal Cancer that Responded to GCH/Cisplatin/Bevacizumab Combination Therapy, 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 C8 RP-Column (250mm x 4.6 mm, 5 µ) used for separation. HPLC grade water obtained from Milli QRO 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 orthophosphoric acid. The resulted solution filtered through 0.45µ 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 further 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 pH 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 pH 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 = 13166Xx 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.

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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 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 average retention time (R<sub>t</sub>) 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<sup>2</sup> = 0.8904) and CYS (R<sup>2</sup> = 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 (r<sup>2</sup>) 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 1: Standard chromatogram of GCH and CYS and retention of CYS and GCH is 3.45min and 8.28 min.

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

Figure 3: Standard mixture chromatogram of GCH and CYS.

Figure 4: Typical chromatogram of blank.

Figure 5: Typical chromatogram of marketed product.
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 parameters were 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**


**REFERENCES**