ABSTRACT

Objective: The objective of the present work was to develop and validate a novel, specific, precise and reliable method for estimation of gemcitabine hydrochloride in bulk and polymeric nanoparticles using UV-visible spectroscopy method.

Methods: The UV-Visible spectrophotometric determination was performed with double beam Systronics UV-visible spectrophotometer; model UV-2201 (India). The proposed methods were validated for various parameters like linearity, precision, accuracy, robustness, ruggedness, detection, quantification limits, and formulation analysis as per international conference on harmonization (ICH) guidelines.

Results: The method was based on measurement of absorbance at wavelength maxima i.e. 267.2 nm, \( \lambda_{max} \) of the drug in distilled water, phosphate buffer pH 6.8 and 7.4. The method obeyed Beer Lambert’s law in the concentration range of 5-30 µg/ml and \( R^2 \)-value was found to be 0.999. Moreover, the % drug recovered from polymeric nanoparticles was found to be 97.97%.

Conclusion: According to results, the currently developed method shows compliance with acceptance criteria with Q2 (R1) and international conference on harmonization (2005) guidelines, because the % RSD was found to be less than 2%. The developed method was simple, accurate and precise.

Keywords: Gemcitabine Hydrochloride, UV-Visible Spectrophotometer, Correlation coefficient, \( \lambda_{max} \)

INTRODUCTION

Method validation is the process which is used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results as well as it is an integral part of any good analytical practice [1].

Gem HCl is 2'-Deoxy-2',2'-difluoro cytidine hydrochloride \( (C_{9}H_{11}F_{2}N_{3}O_{4}.HCl) \) as shown in fig. 1, deoxycytidine analogue with small molecular weight \( (299.66) \), has a short half-life \( (17 \text{ min}) \), appearance (white to off white solid) and quickly decomposed to small molecular weight \( (299.66) \) with its low but still important systemic toxicity [3].

Moreover, the gem HCl is used as an antiviral agent, an enzyme inhibitor, an immuno suppressive agent, radiation-sensitizing agents and also has been successfully used in other tumors such as ovarian cancer, mesothelioma and head and neck cancers in combinations with other cytotoxic drugs [4-5].

By the extensive literature survey, we found that there are numerous methods, such as high-performance liquid chromatography (HPLC) [6-8], UV-spectrophotometric [9], liquid chromatography with mass detector (LC-MS) [10-11], UPLC-MS/MS [12], have been used to measure the gem HCl in formulations and biological samples. However, these methods are involved with sophisticated detectors and costly solvents. Thus, the present investigation was aimed to develop a rapid, simple, sensitive, precise, accurate, robust, reproducible, and economic method for the analysis of gem HCl in the bulk as well as in polymeric nanoparticles and to check the percent drug recovery from a novel delivery system of drug i.e. nanoparticles.

MATERIALS AND METHODS

Chemicals and reagents

Gemcitabine hydrochloride supplied as a gift sample by Neon laboratories, Mumbai. Sodium chloride, potassium dihydrogen orthophosphate, disodium hydrogen phosphate from CDH laboratories. All chemicals and reagents used in the study were of analytical grade.

Instrumentation

A double beam systronics UV-visible spectrophotometer, model UV-2201 (India) with a spectral bandwidth of 1 nm, wavelength accuracy \( \pm 0.5 \text{ nm} \) and a pair of 1 cm quartz cells were used to measure the absorbance of the resulting solutions.

Preparation of solvent system for analysis study

For the spectroscopic analysis of drug, three solvents were selected.
Preparation of standard stock solution and working solution

The 10 mg of gem HCl has weighed accurately and transferred into 10 ml volumetric flask and dissolved. Then, the solution was diluted up to the mark with an appropriate solvent (phosphate buffer pH-7.4, pH 6.8 and distilled water). The clear solution was obtained having the strength of 1000 µg/ml (standard stock solution). From this stock solution, 10 ml was taken into a 100 ml volumetric flask, diluted up to 100 ml to get the solution of 100 µg/ml concentration and filtered through Whatman filter paper before analyzing (working solution) [14].

Procedure for calibration curve

The standard solutions were prepared by the proper dilution of the primary stock solution with phosphate buffer pH 7.4, pH 6.8 and distilled water to obtain working standard. All the measurements were performed at room temperature. The stock solutions scanned in the UV range 200-800 nm by using an appropriate blank. For Linearity study, dilutions were made for the drug in the range of 1-40 µg/ml concentrations were prepared by diluting the stock solution with all the three working solvents.

Assay of gem HCl polymeric nanoparticles

The proposed method was successfully applied for the determination gem HCl in polymeric nanoparticles. An accurately measured volume of polymeric nanoparticles equivalent to 10 mg (7 ml) of the drug was transferred into 10 ml volumetric flask and volume were made up to the mark with distilled water, filtered through whatmann filter paper. The suitable volume of solution was further diluted with water to obtain concentration 10µg/ml. Then, the aliquot was scanned in the UV range (200-400 nm). The amount of drug present in the polymeric nanoparticles was calculated [15].

Validation of methods

Linearity The aliquots of concentration ranging 1-40 µg/ml were analyzed in triplicate. The results obtained were used to calculate the equation of the line by using linear regression by the least-squares regression method [16].

Accuracy The accuracy of the method is the closeness of the measured value to the true value for the sample. For recovery study, the solutions were prepared at levels 75%, 100% and 125% of test solution and taken absorbance of each solution in triplicate [17].

Precision The intra-day and inter-day precisions of the proposed spectrophotometric methods were determined by estimating the corresponding response thrice on the same day and on three different days, over a period of one week and the results were reported in terms of relative standard deviation [18].

Repeatability The repeatability was determined by analyzing six samples of same concentrations of drug (20µg/ml). From the resulting absorbance, the standard deviation and relative standard deviation were calculated [19].

Limit of detection (LOD) and limit of quantification (LOQ)

It is the lowest concentration of analyte in a sample that can be detected but not necessary quantified. The LOD and LOQ were determined by using standard deviation of the response and slope approach as defined in International Conference on Harmonization (ICH) guidelines. LOD and LOQ values were calculated using the relation,

$$\text{LOD} = 3.3 \times \sigma / \text{s}$$

$$\text{LOQ} = 10 \times \sigma / \text{s}$$

Where $\sigma$ is the standard deviation (n=3) of reagent blank determination and $\text{s}$ is the slope of the calibration curve [20-21].

Robustness and ruggedness

The robustness of the proposed method was tested by changing parameters such as wavelength range and slit width. Ruggedness was determined by different analysts, using similar operational and environmental conditions and the results were reported in terms of % RSD [22].

RESULTS AND DISCUSSION

Determination of absorption maxima ($\lambda_{max}$)

The standard stock solution of gem HCl having the concentration 1000 µg/ml was further diluted to 100µg/ml with distilled water, pH 7.4 and 6.8. The absorbance of resulting solution was scanned in the UV spectrophotometer ranging from 200-400 nm. The $\lambda_{max}$ was found to be 267.2 nm as shown in fig. 2. The higher values of correlation coefficient (R) indicate good linearity of calibration curve for drug in bulk as well as in formulation.

![Absorption spectrum of gem HCl showing maximum absorbance at 267.2 nm](image)
Linearity

The linearity studies of the drug were performed by plotting different concentrations of standard solution against their respective absorbance as shown in fig. 3. The drug was found to be linear in the concentration range of 5-30µg/ml. The correlation co-efficient values were found to be 0.999, and the calibration curve shows that the drug obeys beer’s law limit within the concentration range. Furthermore, the overlay spectra of gem HCl in working solutions support the linearity results observed in the standard curve as in fig 4.

Table 1: Comparison of absorbance of gem HCl in different solvent

| Concentration(µg/ml) | PBS pH 7.4±SD | PBS pH 6.8±SD | Distilled water±SD |
|----------------------|---------------|---------------|--------------------|
| 5                    | 0.170±0.003   | 0.185±0.004   | 0.191±0.0033       |
| 10                   | 0.310±0.004   | 0.342±0.003   | 0.356±0.002        |
| 15                   | 0.465±0.002   | 0.476±0.004   | 0.493±0.001        |
| 20                   | 0.618±0.004   | 0.638±0.001   | 0.649±0.004        |
| 25                   | 0.760±0.003   | 0.774±0.002   | 0.777±0.002        |
| 30                   | 0.944±0.002   | 0.946±0.004   | 0.941±0.001        |

*Each value is the average of three determinations (n=3)

Fig. 3: Calibration plot of gemcitabine HCl

Fig. 4: Overlay spectra of drug in phosphate buffer 7.4, 6.8and distilled water
Accuracy

The Precision was determined as:

**Intraday precision**

The intraday precision was determined by analyzing the drug at particular concentration for three times on the same day taking the time intervals of 3 h at 10:00 AM, 1:00 PM and 4:00 PM respectively.

**Interday precision**

The interday precision was determined by analyzing the samples daily for three consecutive days. The values of relative standard deviation (%RSD) were in the range of 0.089-0.651 % respectively. This indicates the reproducibility of the method. The results are shown in table 2. The precision results indicate that the current method was reliable and repeatable [23]. Thus, the methodology can be applied for the determination of drug in different formulations like targeting of pancreatic as well as for bladder cancer.

### Table 2: Inter day and intraday precision data and statistical results

| Solvent          | Absorbance (intraday) (%RSD) | Absorbance (intraday) (%RSD) | Intraday precision (%RSD) | Interday precision (%RSD) | Interday precision (%RSD) |
|------------------|-------------------------------|-------------------------------|---------------------------|---------------------------|---------------------------|
| pH 7.4           | 0.60                          | 0.61                          | 98.3±0.005                | 97.0±0.003                | 100.6±0.003               |
| pH 6.8           | 0.63                          | 0.62                          | 99.9±0.004                | 98.4±0.005                | 101.8±0.004               |
| Distilled Water  | 0.64                          | 0.634                         | 98.3±0.001                | 99.3±0.002                | 100.0±0.004               |

*is the mean of three values (n=3)

### Accuracy

To ensure the accuracy, recovery studies were performed by standard addition method at 75%, 100% and 125% levels of drug concentration, to the pre-analyzed samples and percent recovery values were calculated. Recovery experiment indicated the absence of interferences from the commonly encountered pharmaceutical additives and excipients and values of mean recovery were in the range of 99.32-100.33 %. The results for the recovery studies were found in the desired limits as shown in table 3.

### Table 3: Results of recovery studies at three levels and statistical analysis

| Solvent          | Accuracy (% recovery) | 75% (10+5 µg/ml)±SD | 100% (10+10 µg/ml)±SD | 125% (10+15 µg/ml)±SD |
|------------------|-----------------------|---------------------|-----------------------|-----------------------|
| pH 7.4           | 99.32±0.09            | 100.33±0.48         | 100.69±0.81           |
| pH 6.8           | 99.66±0.1             | 100.66±0.32         | 100.69±0.76           |
| Distilled Water  | 99.77±0.12            | 100.11±0.23         | 100.33±0.98           |

*Each value is the average of three determinations (n=3)

### Repeatability

The repeatability of the instrument was validated by taking the absorbance of six samples of the same concentration (20µg/ml) in different working solvents.

### Table 4: Results of repeatability studies in different working solvents

| Concentration(µg/ml) | Absorbance (pH 7.4)* | Absorbance (pH 6.8)* | Absorbance (distilled water)* |
|----------------------|-----------------------|-----------------------|------------------------------|
| 20                   | 0.616                 | 0.639                 | 0.638                        |
| 20                   | 0.618                 | 0.638                 | 0.638                        |
| 20                   | 0.617                 | 0.638                 | 0.638                        |
| 20                   | 0.618                 | 0.637                 | 0.631                        |
| 20                   | 0.619                 | 0.634                 | 0.638                        |
| Mean                 | 0.617±0.0013          | 0.637±0.00172         | 0.636±0.00285               |
| SD                   | 0.00103               | 0.00172               | 0.00285                     |
| %RSD                 | 0.16                  | 0.27                  | 0.44                         |

*is the mean of three values (n=3)

### Robustness study

Robustness studies assumed that the small variations in any of the variables did not significantly affect the results (table 5). The robustness study provided the liability of the proposed method during routine analysis and proved that change in instrument, show no significant effect on results.

### Ruggedness study

Ruggedness of the method was determined by carrying out the analysis by different analyst and the respective absorbance of 20µg/ml was noted. The result was indicated as %RSD, which should be less than 2 as shown in table 6. The ruggedness analysis, indicates that there was no significant change in reliability and repeatability of methodology with the change of analyst [23].

### Assay of polymeric nanoparticles (PNP’s)

The optimized spectrophotometric method was applied to the direct determination of gem HCl in polymeric nanoparticles without any sample extraction or filtration. From the absorbance value, the drug content was calculated. Furthermore, the results obtained from assay of polymeric nanoparticles showed that the current method was validated because drug content recovered was 97.97% with % RSD 0.44 as shown in table 7.
CONCLUSION

A simple, accurate, precise, robust and rapid UV visible spectrophotometric method has been developed for estimation of gem HCl from its pharmaceutical formulation. Thus it can be extended for routine analysis of gem HCl in pharmaceutical industries and hospitals and research laboratories. Unlike the LC/MS procedure and HPLC procedures, the UV-visible spectrophotometer instrument is simple and not of highly expensive on the other hand in simplicity and user friendly the method could be considered superior in comparison with the previously reported methods. The current method has wide range of accuracy, precision, repeatability, robustness and ruggedness.

CONFLICT OF INTERESTS

The authors declare that they do not have any financial and personal relationships with other people or any other organizations that could inappropriately influence this research work.

ACKNOWLEDGMENT

The authors are grateful to Dr. Chander Mohan, Director-Principal and all staff members of Rayat-Bahra Institute of Pharmacy, Hoshiarpur, Punjab for the providing us the facilities for carrying out this research work. The authors would also thankful to Neon Hoshiarpur, Punjab for the providing us the facilities for carrying out this research work. The authors would also thankful to Neon Hoshiarpur, Punjab for the providing us the facilities for carrying out this research work.

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Table 5: Results of robustness studies and statistical analysis

| Concentration (µg/ml) | Absorbance(pH 7.4)* | Absorbance(pH 6.8)* | Absorbance(distilled water)* |
|----------------------|---------------------|---------------------|-----------------------------|
|                      | 665.9               | 669.2               | 665.9                       |
| 20                   | 0.618               | 0.570               | 0.635                       |
| 20                   | 0.618               | 0.571               | 0.631                       |
| 20                   | 0.619               | 0.571               | 0.634                       |
| 20                   | 0.619               | 0.571               | 0.638                       |
| 20                   | 0.617               | 0.571               | 0.630                       |
| Mean                 | 0.618               | 0.571               | 0.634                       |
| SD                   | 0.000735            | 0.000400            | 0.000408                    |
| %RSD                 | 0.12                | 0.071               | 0.43                        |

* is the mean of three values (n=3)

Table 6: Results of ruggedness studies (by two analysts) in different working solvents

| Concentration (µg/ml) | Absorbance(pH 7.4)* | Absorbance(pH 6.8)* | Absorbance(distilled water)* |
|----------------------|---------------------|---------------------|-----------------------------|
|                      | 667.2               | 669.2               | 665.9                       |
| Analyst I            | 0.619               | 0.617               | 0.637                       |
| Analyst II           | 0.619               | 0.619               | 0.637                       |
| Analyst I            | 0.618               | 0.618               | 0.637                       |
| Analyst II           | 0.618               | 0.619               | 0.637                       |
| Analyst I            | 0.618               | 0.618               | 0.636                       |
| Analyst II           | 0.618               | 0.618               | 0.636                       |
| Analyst I            | 0.617               | 0.617               | 0.636                       |
| Analyst II           | 0.617               | 0.617               | 0.636                       |
| Mean                 | 0.618               | 0.618               | 0.636                       |
| SD                   | 0.000051            | 0.00011             | 0.00013                     |
| %RSD                 | 0.083               | 0.109               | 0.064                       |

* is the mean of three values (n=3)

Table 7: Results of the assay of pharmaceutical formulation

| Formulation | Amount taken (µg/ml) | UV spectrophotometric method | Amount recovered (µg/ml)*±SD | % Drug recovered*±SD | % RSD* |
|-------------|----------------------|-----------------------------|-----------------------------|----------------------|-------|
| PNP’s       | 10                   | 9.797±0.09                  | 97.97±0.9                   | 0.44                 |

* is the mean of three values (n=3)

Table 8: Summary of all the validation parameters

| Validation parameter | Phosphate buffer (pH 7.4) | Phosphate buffer (pH 6.8) | Distilled water |
|----------------------|---------------------------|---------------------------|-----------------|
| Absorption maxima (λ max) | 267.2 nm                  | 267.2 nm                  | 267.2 nm        |
| Linearity Range | 5–30 µg/ml | 5–30 µg/ml | 5–30 µg/ml |
| Standard Regression Equation | y = 0.0307x+0.0074 | y = 0.0301x+0.0346 | y = 0.0295x+0.0304 |
| Intercept (m) | 0.0074 | 0.0346 | 0.0304 |
| Slope (m) | 0.0307 | 0.0301 | 0.0295 |
| Correlation Co-efficient (R) | 0.998 | 0.999 | 0.999 |
| % RSD for Intra-day (n=3) Precision | 0.406 | 0.181 | 0.180 |
| % RSD for Inter-day (n=3) Precision | 0.522 | 0.651 | 0.089 |
| Repeatability (% RSD) | 0.167 | 0.270 | 0.448 |
| LOD | 0.268 | 0.532 | 0.197 |
| LOQ | 0.815 | 1.61 | 0.679 |
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How to cite this article
• Taranjit Kaur, Sukhjinder Kaur, Parminderjit Kaur. Development and validation of UV-spectrophotometric methods for determination of gemcitabine hydrochloride in bulk and polymeric nanoparticles. Int J Appl Pharm 2017;9(5):60-65.