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## A review on gemcitabine hydrochloride

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### ABSTRACT

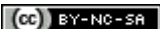
Analytical method development and validation is an integral part during the development of drug substance and drug product in the pharmaceutical industry. It plays important role in the discovery, development, manufacture and quality control of pharmaceuticals. Analytical methods are designed to determine the drug content in formulation, presence of impurities, separation of drug and its related impurities and degraded products. Validation of method proves that it can be suitable for its use in research and development and assures the reliability of proposed method. Now days, need of analytical method development is increasing due to the emergence of new drugs and development of new combinations of various drugs as their standard methods are not available in Pharmacopoeias. An effective method development and its validation prove to be very useful in drug discovery and development. This review is focused on literature findings from 2009-2018 of analytical method development and validation of gemcitabine hydrochloride drug in various dosage forms.

**Keywords:** Gemcitabine, method development, spectrophotometric, RP-HPLC, validation

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## INTRODUCTION

Analytical method development is defined as the process of creating a procedure which is used for identification, quantification and purification of different compounds of interest. Analytical methods are used for the quantitative and qualitative analysis of raw materials, drug substances, drug products, and compounds in biological samples in pharmaceutical industry.[1]

Analytical method could be spectral, chromatographic, electrochemical, or miscellaneous. Analytical method development is the selection of an accurate assay procedure to determine the composition of a formulation. These methods play important roles in monitoring the drugs in their dosage forms and biological samples. Methods for new products are developed, when no official methods are available and alternate method are developed for existing products to reduce the cost and time for better precision and ruggedness.[2]

Then the methods are validated according to ICH guidelines. Validation of an analytical method is the process which proves that the developed method is suitable and can be used for drug development and quality assurance. It provides assurance that the developed method is accurate. The developed method should give reproducible and reliable results when used by different analysts. The method is validated by different parameters like accuracy, precision, linearity, robustness, ruggedness, specificity, detection limit and quantitation limit.[3] The various steps which are involved in method development are following: □ Understanding the Physicochemical properties of drug molecule □ Selection of method conditions □ Developing the approach of analysis □ Sample preparation □ Method optimization □ Method validation In this article various analytical methods developed for drug gemcitabine during last 5 years are reported.

## GEMCITABINE

Gemcitabine is deoxycytidine analogue having antineoplastic activity. It is broad spectrum antimetabolite used in treatment of various forms of cancers such as pancreatic adenocarcinoma, ovarian cancer, small cell lung cancer, bladder cancer, metastatic biliary tract cancer, and breast cancer. It acts by inhibition of thymidilate kinase and DNA synthesis. It is a pro drug and converted to its active metabolites difluorodeoxycytidine diphosphate and difluorodeoxycytidine triphosphate by the action of enzyme deoxycytidine kinase. These incorporate in DNA and block its replication. Gemcitabine

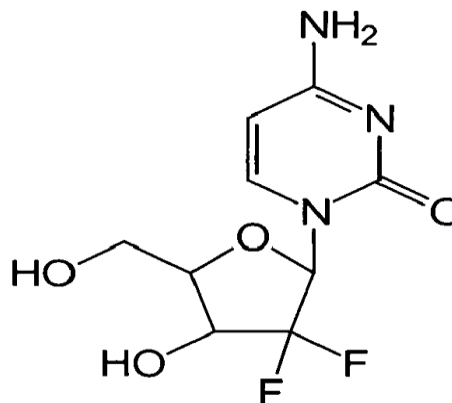


Figure 1: Structure of gemcitabine

Gemcitabine is 2',2'-Difluorodeoxycytidine having molecular formula C<sub>9</sub>H<sub>11</sub>F<sub>2</sub>N<sub>3</sub>O<sub>4</sub> and molecular weight 263.2 g/mol. Its IUPAC name is 4-amino-1[(2R,4R,5R)-3,3-difluoro-4-hydroxy-5(hydroxymethyl)oxolan-2-yl] pyrimidin-2-one. It is white crystalline and odourless powder having melting point 168.64° C. It is soluble in water, slightly soluble in methanol and sparingly soluble in acetone. [4-6]

## LITERATURE REVIEW:

**1. Vinodkumar et al. (2018);** developed a simple and precise method for the simultaneous estimation of gemcitabine and capecitabine in pharmaceutical dosage forms using RP-HPLC. For this method Inertsil C18 (150x4.6 ID) 5µm column and a mixture of 50mM orthophosphoric acid: ACN in ratio of 40:60 was used as mobile phase. The components were detected at 296nm using UV detector. The chromatogram obtained results in symmetric peak and good resolution. Then this method was validated according to ICH guidelines. All the parameters were in acceptable range and this method was applied for determination of these drugs in dosage forms.[1]

**2. Nuland et al. (2017);** developed a LC-MS method for the simultaneous determination of gemcitabine and its metabolite 2',2'-difluorodeoxyuridine in human plasma for a micro dose clinical trial. These are chromatographically separated using Shimadzu LC system. The column temperature was 30°C and gradient elution was applied with mobile phase A was of 10 mM ammonium acetate in water acetonitrile (93:7 v/v) and mobile phase B was of 100% acetonitrile. The flow rate used was 0.2 mL/min. The detector used was triple quadrupole mass spectrometer 6500. Validation of this method was done according to the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA)

guidelines. Calibration curves were found to be linear with regression coefficient  $r^2$  value of 0.9964 for gemcitabine and 0.9962 for dFdU. As compared to sensitivity of previously published methods, this assay showed great improvement and could be used for micro dose trial with gemcitabine.[2]

**3. Patel et al. (2017);** developed a simple, economical, rapid, accurate and precise pH independent method for spectroscopic estimation of gemcitabine hydrochloride in pharmaceutical formulation. To measure the absorbance, double beam UV/Visible spectrophotometer (Shimadzu model 1700) having wavelength accuracy of 0.5 nm and spectral width of 2 nm. Solutions of gemcitabine were prepared in Ammonia buffer pH 10.9 and Phosphate buffer pH 3 and absorbance was determined at 254 nm. The proposed method was found to be linear in concentration range of 1434  $\mu\text{g/ml}$  with correlation coefficient  $r^2$  value of 0.999. The method was found to be accurate with mean percentage recovery of 100.345% and precise with % RSD value of repeatability, interday and intraday precision as 0.344, 0.659 – 1.710 and 0.276 – 0.729 respectively. The limit of quantization (LOQ) and limit of detection (LOD) were found to be 2.964 and 0.978 respectively. The results of validation indicate that the proposed method could be used for routine analysis of gemcitabine in pharmaceutical formulations. [3]

**4. Radha et al. (2017);** developed a rapid, accurate and precise RP-HPLC method for the simultaneous estimation of gemcitabine and capecitabine in bulk and tablet dosage forms. The instrument used for method development was Waters HPLC 2695. The chromatographic conditions include Altima C18 column (4.6 $\times$ 150mm, 5 $\mu$ ) having temperature 35 $^\circ\text{C}$  and mobile phase used was Phosphate Buffer (pH 4.6): Methanol: Acetonitrile (65:25:10 v/v). The separation was carried using isocratic mode with a flow rate of 1mL/min and run time 14 min. Components were detected using UV detector at 234 nm wavelength. The retention time was found to be 6.068, 2.088  $\pm$ 0.02 min for gemcitabine and capecitabine respectively. Calibration curves were plotted, and method was found to be linear within the concentration range of 25-125  $\mu\text{g/mL}$  of capecitabine and 10-50  $\mu\text{g/mL}$  for gemcitabine with  $r^2$  value 0.999 for both drugs. The method was reliable and accurate with percent recovery between 98.22-101.49%. The LOD and LOQ were found to be 4  $\mu\text{g/ml}$  and 8.5  $\mu\text{g/ml}$  for capecitabine and 14.8  $\mu\text{g/ml}$  and 25.7  $\mu\text{g/mL}$  for gemcitabine respectively. The %RSD values for robustness and ruggedness were acceptable. Thus, this method could be used for quality control of bulk and dosage forms containing gemcitabine and capecitabine.[4]

**5. Kaur et al. (2017);** developed a simple, specific, economical, reliable spectroscopic method for the determination of gemcitabine hydrochloride in bulk and polymeric nanoparticles. A double beam systronics UV-visible spectrophotometer (2201) having spectral bandwidth of 1 nm, wavelength accuracy of  $\pm$ 0.5 nm and a pair of 1 cm quartz cells were used to determine the absorbance of the solutions. Solutions were prepared in phosphate buffer pH 6.8 and 7.4 and distilled water and detected at  $\lambda_{\text{max}}$  267.2 nm. The method was validated for different parameters according to ICH guidelines. Linearity was found in concentration range of 5-30  $\mu\text{g/mL}$  with  $r^2$  value of 0.999. Accuracy of method was determined having percent drug recovery in range of 99.32-100.33 %. The % RSD values for interday and intraday precision were in range of 0.089-0.651 %. All other parameters were also in acceptable range. The % drug recovery from polymeric nanoparticles was found to be 97.97%. This method was simple and comply with acceptance criteria and could be used for routine analysis of drug in dosage forms.[5]

**6. Gong et al. (2017);** developed a validated ultrahigh performance liquid chromatography that is coupled with the mass spectrometry method for the determination of gemcitabine, etoposide, vinorelbine and their metabolites in human plasma. Instruments used include Agilent 1290 UHPLC and an Agilent 6460 triple-quadrupole tandem mass spectrometer. The chromatographic conditions used for separation was Waters ACQUITY UPLC HSS T3 column (2.1  $\times$  100 mm, 1.8  $\mu\text{m}$ ) at 50 $^\circ\text{C}$ . Gradient conditions at flow rate 0.3 mL/min were used with binary mobile phase A (0.1% formic acid in acetonitrile, v/v) and B (0.1% formic acid in water, v/v). Run time of sample was 7.5 min and injection volume was 2  $\mu\text{L}$ . For detection mass spectrometer was used having conditions spray voltage (4.0 kV), nebulizer pressure (nitrogen) was 40 psi, source temperature was 105  $^\circ\text{C}$  and desolvation gas include nitrogen at 350  $^\circ\text{C}$  and 10.0 L/min flow rate. The method was validated for different parameters according to US FDA guidelines. Calibration curves were found to be linear with  $r^2$  value 0.995. Accuracy was ranged between 86.35% and 113.44%. LOQ and LOD were found to be in acceptable range. This method had been used for the determination of these drug metabolites in plasma of small lung cancer patients.[6]

**7. Patel et al. (2016);** have developed a rapid, simple and precise RP-HPLC method for the simultaneous determination of Gemcitabine hydrochloride with Letrozole, Bicalutamide and Paclitaxel in nanosponge formulation. Zorbax Eclipse plus C8 (250 $\times$ 4.60 mm 5  $\mu$ ) column with mobile phase consisting of methanol: sodium

dihydrogen phosphate pH 3.5 in ratio of 70:30 v/v was used for chromatographic separation. The run time of each sample was 10 min with flow rate of 1.2 mL/min. The effluents were detected using a photo diode detector at 230 nm for Gemcitabine HCl and Paclitaxel, 257 nm for Gemcitabine HCl and Bicalutamide and 248 nm for Gemcitabine HCl and Letrozole respectively. The retention time for gemcitabine, paclitaxel, bicalutamide and letrozole was found to be 2.6, 7.1, 4.8, and 3.4 minutes respectively. The method was found to be linear with  $r^2$  values more than 0.998 in concentration range of 5-200  $\mu\text{g/mL}$ . The %RSD values for precision, robustness, ruggedness, LOD and LOQ were in acceptable range. The low %RSD value and high % recovery indicate that method was accurate and precise, and method was used for determination of these drugs in pharmaceutical dosage forms.[7]

**8. Rao et al. (2015);** have developed a new simple, rapid, stable RP-HPLC method for the simultaneous estimation of gemcitabine and clarithromycin in combined dosage form. For method development, Nova pack symmetry C18 (150mm x3.9mm, 5 $\mu$ ) column having temperature 30°C at a flow rate of 1.2 mL/min was used. Isocratic conditions were used with mobile phase consisting of buffer (mixture of dipotassium hydrogen orthophosphate and potassium dihydrogen orthophosphate in dilute phosphoric acid having pH of 3.5) and acetonitrile in ratio of 55:45. The components were detected using UV detector at 212 nm wavelength which results in the retention time of 2.373 min for Gemcitabine and 5.995 min for Clarithromycin. Validation parameters were performed according to ICH guidelines. Calibration curves were found to be linear in concentration range of 18.75 -112.50  $\mu\text{g/mL}$  and 12.5 -75  $\mu\text{g/mL}$  for gemcitabine and clarithromycin having  $r^2$  value 0.998 and 0.999 respectively. The % recovery was found to be in the range 99.86 – 99.95 % for gemcitabine and 99.94 – 99.97 % for clarithromycin. Robustness and ruggedness was found to be less than 2 and LOD and LOQ were also in acceptable range. Hence the proposed method was simple and precise, so it can be used for the quality control of these drugs in combined dosage forms.[8]

**9. Thakkar et al. (2015);** have developed sensitive, novel first order derivative method by UV spectrophotometer for simultaneous estimation of gemcitabine and tamoxifen. Instrument used was Shimadzu UV-1800 Spectrophotometer with 10mm matched quartz cells having fast scan speed and slit width of 1.0 nm. Dilutions were made in methanol and to record overlain spectra, absorption maxima of gemcitabine and tamoxifen were detected at 269 nm and 238 nm respectively. According to first

order method, absorbance was measured at 285 nm and 224 nm for gemcitabine and tamoxifen respectively and calibration curves were plotted which show linearity at concentration range of 5-25  $\mu\text{g/mL}$  for both drugs. The correlation coefficient ( $r^2$ ) value was found to be 0.996 for gemcitabine and 0.998 for tamoxifen. The method was accurate with mean % recovery of 99.82% for gemcitabine and 100.14% for tamoxifen and precise with %RSD values of repeatability, and interday precision were 0.0997 and 0.03058 for gemcitabine and 0.127 and 0.105 for tamoxifen respectively. LOD and LOQ values for gemcitabine were found to be 0.02833 and 0.08586 and for tamoxifen were 0.09565 and 0.2898 respectively. As this method was valid according to ICH guidelines and can be used for routine analysis.[9]

**10. Singh et al. (2015);** developed a simple, inexpensive, precise and stable HPLC method for estimation of gemcitabine in injectable dosage forms by using theophylline as internal standard. This method was carried on Phenomenex Luna C18 column (250mm x 4.6 mm; 5 $\mu$ ) maintained at 25°C at a flow rate of 1.0 mL/min. Mobile phase used was water and acetonitrile in ratio 90:10 and pH adjusted to 7.0. Absorbance was detected using PDA detector at 275 nm. Retention time was found to be 3.997. Linearity was found in range of 0.5–50  $\mu\text{g/mL}$  having  $r^2$  value 0.999. The proposed method was accurate with 100.2% to 100.4% recovery. The LOD and LOQ value of this method were found to be 0.1498 and 0.4541  $\mu\text{g/mL}$  respectively. The values of intraday precision, interday precision, robustness and ruggedness were less than 2%. Therefore, this method was successfully used for analysis of gemcitabine in dosage forms.[10]

**11. Chen et al. (2015);** have developed a HPLC method for determination of enzyme degradation studies of gemcitabine and its release from poly lactic-co-glycolic acid nanoparticles. A Grace Smart analytical C18 column (250 x4.6 mm, 5 $\mu\text{m}$ ) fitted with a C18 guard column (10x3.0 mm) with isocratic conditions was used with mobile phase consisting of acetonitrile and 1.38% w/v sodium dihydrogen phosphate buffer (pH 6.5) in ratio of 7:93. The flow rate was maintained at 1 mL/min and injection volume was 20  $\mu\text{L}$ . For detection of sample UV detector was used at 270 nm. Gemcitabine peak was eluted at 4.8 min retention time. Calibration curves plotted between absorbance and concentration results in  $r^2$  value of 0.999 indicating good linearity in concentration range of 1-100  $\mu\text{g/mL}$ . Accuracy and precision of method was found to be excellent in range of 99.91–101.77% and 1.71 respectively and with a 0.014  $\mu\text{g/mL}$  LOD and a 0.043  $\mu\text{g/mL}$  LOQ. This method was found to be effective in evaluating

stability of formulations containing gemcitabine.[11]

**12. Rao et al. (2014);** have developed a new, economical, simple and precise RP-HPLC method for simultaneous determination of gemcitabine hydrochloride and capecitabine hydrochloride in combined tablet dosage forms. The chromatographic conditions used for separation were Inertsil ODS-3 C18 column (250 × 4.6 mm, 5 μm) with mobile phase containing a mixture of 50mM orthophosphoric acid pH 5.5 buffer and acetonitrile in the ratio of 40:60. Analysis was performed using isocratic elution at flow rate of 1.2 mL/min. The components were detected at 298 nm wavelength using UV detector. It results in resolved peaks of both drugs at retention time of 2.943 min for gemcitabine and capecitabine at retention time of 4.797 min. the proposed method was validated for accuracy, precision, robustness, ruggedness, limit of detection and limit of quantification. It was found linear in concentration range of 24-56μg/mL for gemcitabine and 60140μg/mL for capecitabine with r2 value of 0.996. The accuracy of method ranges between 99.1100.4% for capecitabine and 99.3-99.4% for gemcitabine. The precision for both drugs was found to be less than 1 and LOD and LOQ values were 4.0 and 12.2 for capecitabine and 0.6 and 1.9 for Gemcitabine respectively. The values of robustness and ruggedness were also in limit. Thus the method developed was accurate, precise and robust and used for determination of these drugs in combination.[12]

**13. Joselyn et al. (2014);** have developed selective, simple, precise RP-HPLC method for estimation of gemcitabine in pharmaceuticals. Good chromatographic separation was obtained using acetonitrile and water in ratio of 50:50 as mobile phase with a flow rate of 1.2 mL/min and components were detected at 270 nm. Linearity was performed in concentration range of 80μg/mL and 120μg/mL which results in r2 value of 0.999. The % recovery of drug was found to be in range of 101.4 to 99.50% which show accuracy of method. Precision results were less than 0.5 indicate that method was precise. The low %RSD values indicate that method was accurate, precise and robust and successfully applied for analysis in industries and quality control laboratories.[13]

**14. Siddartha et al. (2014);** developed a new, simple, selective and rapid RP-HPLC method which determines gemcitabine hydrochloride in bulk and pharmaceutical formulation. The method was performed on Hypersil BDS C18 column (250×4.6mm, 5 μ) maintained at 30°C and flow rate of 1 mL/min. A mixture of phosphate buffer (pH 3) and acetonitrile in the ratio of 93:7 v/v was

used as mobile phase and drug was detected at 275 nm. By this method gemcitabine was eluted at 3.927 min. It followed linearity in concentration range of 10-60μg/ml with correlation coefficient 0.9997. The method was accurate with % recovery 99.20% and 101.24%. The values of precision and robustness were found to be less than 1%. The limit of detection was 0.296μg/ml and limit of quantification was 0.896μg/ml. Thus, this method was applicable for routine analysis of gemcitabine in bulk drugs and dosage forms.[14]

**15. Xu et al. (2014);** have developed a simple, reliable, reproducible method for simultaneous estimation of hydrophilic gemcitabine, and lipophilic phytochemical curcumin using HPLC. The method used Phenomenex C18 (5 μm, 250 mm×4.60 mm) column for analysis. The gradient elution was used with a mobile phase of water and acetonitrile in ratio of 95:5 for 4.5 min which was followed by 30:70 for next 5 min with flow rate of 1.0 mL/min. For detection of gemcitabine and curcumin 270 nm and 420 nm wavelengths were selected using a diode-array detector. This method was linear in range of 0.125–5 μM and 1.25–50 μM for gemcitabine and curcumin respectively. Values of precision, robustness, specificity were found to be acceptable according to ICH guidelines. The limit of detection and limit of quantification were 0.04 μM, 0.03 μM and 0.12 μM and 0.10 μM for gemcitabine and curcumin respectively. Thus, this method was valid and was employed for analysis of these drugs in dosage forms.[15]

**16. Kudikala et al. (2014);** developed a accurate and stability indicating method for determination of gemcitabine hydrochloride in parenteral and active pharmaceutical ingredients using RP-HPLC. Good resolution and symmetrical peaks were obtained using Enable C18 G (250×4.6mm, 5μm) column at ambient temperature and mobile phase comprised of acetonitrile and methanol in ratio of 55:45 v/v. Flow rate was maintained at 1.0 mL/min. 10μL solution was injected for 10 min run time and detected at 285 nm. Sharp and symmetrical peak of gemcitabine was observed at retention time 2.79 min. The correlation coefficient value was found to be 0.999 in concentration range of 1-45μg/mL. 100.7 to 101.6 % recovery showed that method was accurate. The method was sensitive as its LOD and LOQ was found to be 1.69μg/mL and 5.13μg/mL respectively. Value of %RSD for precision was less than 2% and found to be 0.31 for intraday precision and 1.06 for interday precision which indicate that method was quite precise. Robustness of method was determined by slightly changing the parameters like column temperature, flow rate and wavelength. The value of %RSD for robustness was less than 2% which show that method was

reliable and reproducible and applicable for parenteral dosage forms to determine gemcitabine.[16]

**17. Prakash S. Sarsambi, Kalyan Chakravarthy, (2011) et al** developed spectrophotometric methods have been developed for the estimation of Gemcitabine in bulk drug and pharmaceutical formulation. Method A is based on UV spectrophotometric measurements in which Gemcitabine was dissolved in double distilled water and exhibited absorption maximum at 266 nm and obeyed Beer's law in the concentration range of 5-30 mg/ml. Method B is based on measurements of first order derivative spectroscopy adopted to eliminate spectral interference, in which derivative amplitude was measured at 260.5 nm. Method C is based on calculation of area under curve for analysis of gemcitabine in wavelength range of 260-271 nm. The drug obey's Beer's law in the concentration range of 5-30 µg/ml.

**18. Thakkar Hetal, Das Shilpi, Patel Divyakant (2010) et al** developed an analytical method for the estimation of gemcitabine hydrochloride based on Ultra-violet spectrophotometry. The reported methods of analysis include the chromatographic methods such as HPLC (1) RP-HPLC (2-4), Ion pair HPLC (5), and LC-MSMS (6). Chromatographic methods are tedious, time consuming and are not suitable for routine analysis. However, Ultraviolet spectrophotometric methods which are simple and fast are ideal for routine analysis. Gemcitabine hydrochloride exhibits a sharp peak at 268 nm when scanned in the Ultraviolet region. Thus, it was selected as the analytical wavelength. Linearity was observed in the range of 5µg/ml to 35µg/ml.

**19. V. Rajesh, B. Anupama, V. Jagathi, AND P. Sai Praveen (September 2010) et al** developed A new reverse phase high performance liquid chromatography (RP-HPLC) method has been developed for the simultaneous estimation of gemcitabine hydrochloride and capecitabine hydrochloride in combined tablet dosage form. An inertsil ODS-3 C-18 column having dimensions of 250×4.6 mm and particle size of 5 µm, with mobile phase containing a mixture of acetonitrile: water: triethylamine in the ratio of (70: 28: 2v/v) was used. The pH of mobile phase was adjusted to 4.0 with *ortho*-phosphoric acid. The flow rate was 1 mL/min and the column effluents were monitored at 260 nm. The proposed method was validated in terms of linearity, accuracy, precision, limit of

detection, limit of quantitation and robustness. The method was found to be linear in the range of 10-50 µg/mL and 4-24 µg/mL for gemcitabine hydrochloride and capecitabine hydrochloride, with regression coefficient  $r = 0.999$  and  $r = 0.999$ .

**20. Ling-Zhi Wang,Wei-Peng Yong (2009) et al** developed a novel micro protein precipitation was proposed and employed effectively for bio-sample preparation of only 5 µl of human plasma for rapid liquid chromatography- tandem mass spectrometric (LC-MSMS) determination of gemcitabine (dFdC) and its deaminated metabolite, 2', 2'-difluorodeoxyuridine (dFdU). Gemcitabine-13C, 15N2 Hydrochloride was the internal standard. Baseline chromatographic separation was achieved with an Altima C18 column (2.1×100 mm, 5µm) using isocratic mobile phase [10 mM ammonium acetate (pH = 6.8): methanol, 90:10]. The run time was only 5 min. The mass spectrometer was operated under a positive electrospray ionization condition and a multiple reaction monitoring mode. The linear calibration ranges were 2-2,000 ng/ml for gemcitabine and 20-20,000 ng/ml for dFdU. The recoveries for three analytes were in the same magnitude range from 87.7 to 89.7% with small variation. The intra and inter-day precisions for dFdC and dFdU were  $\leq 5$  and  $\leq 7$  and their accuracy ranged from 98 to 105.3% for dFdC and 93.8 to 104.9% for dFdU, respectively. Ion suppression effect was near negligible. This well validated assay has been applied for quantification of gemcitabine and dFdU in our gemcitabine phase II clinical trial study on Asian non-small cell lung cancer patients treated with different in fusional rates of gemcitabine.

## CONCLUSION

This review provides various published analytical methods and their validation for determination of presence of gemcitabine hydrochloride either single or in combination with other drugs. Analysis proved that these published methods are valid, accurate, reliable and reproducible and can be successfully used for determination of gemcitabine hydrochloride in pharmaceuticals. By understanding the chromatographic separation and changing the experimental conditions, new analytical method could be developed with optimum separation. This review will help the analysts to develop a new method by optimizing the conditions used in these methods which may result in better resolution and separation.

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