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# Simultaneous Quantification of Gemcitabine and Oxaliplatin by RP-HPLC in Bulk and Synthetic Mixture

**Chaitanya Sucharitha Kolakaluri [1], Govindarao Kamala [2], Sathish Kumar Konidala [1,*], Ramu Samineni [1]**

[1] Department of Pharmaceutical Sciences, School of Biotechnology and Pharmaceutical Sciences, Vignan's Foundation for Science, Technology and Research, Vadlamudi, Guntur, A.P., India-522213; sucharitha57@gmail.com (C.S.K.), govindarao83@gmail.com (G.K.), sathishkonidala@gmail.com (S.K.K.), samineniramu123@gmail.com (R.S.)
[2] Koringa College of Pharmacy, Kakinada
* Correspondence: sathishkonidala@gmail.com (S.K.K.);

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**Abstract:** Gemcitabine and Oxaliplatin are anticancer drugs for treating advanced pancreatic cancer. The combination of GEMOX is preferred in treating hepatocellular cancer, adenocarcinoma, and testicular cancer, because of their synergistic therapeutic potential. So the simultaneous quantification of these drugs should be required to know the efficacy during the product development and analysis by simple, accurate, efficient, and sensitive analytical methods. The literature review revealed that, to date, no proper method is available for the simultaneous estimation of selected drugs, so there is a wide scope to develop an efficient analytical method. This study aims to develop and validate a simple, robust, and accurate method by high-performance liquid chromatography for simultaneously quantifying selected drugs in bulk and synthetic mixture. The separation was carried out on the Enable C18G (250x4.6 mm, 5µm) column using 0.1% orthophosphoric acid: acetonitrile (20:80 %v/v) as mobile phase with a flow rate of 0.8ml/min. The experimental design was optimized and analyzed the variables' effect on the retention time [RT], number of theoretical plates, and tailing factor of both analytes at 268 nm. The developed method was validated by determining specificity, linearity range, accuracy, sensitivity, robustness, precision, and ruggedness parameters according to ICH guidelines. The peaks of the analyte were well resolved, and the retention time of Gemcitabine and Oxaliplatin was found as 2.5min and 3 min, respectively. Calibration curves were linear for Gemcitabine and Oxaliplatin over a 10-60µm/ml concentration range and 50-300µm/ml, respectively. All method validation criteria were within the acceptance range, indicating that the developed method is suitable for the routine simultaneous estimation of Gemcitabine and Oxaliplatin in bulk and synthetic mixture.

**Keywords:** Quantification; RP-HPLC; Gemcitabine; Oxaliplatin.

## 1. Introduction

Gemcitabine (GEM), chemically known as 4-amino-1-2-deoxy-2,2-difluoro-β-D-erythropento furanosyl pyamidine-2(1H)-on [1], and its molecular structure was shown in Figure 1. It has a molecular formula of $C_9H_{11}F_2N_3O_4$ with a molecular weight of 263.19 g/mol. GEM is a chemotherapy medication that treats cancers, including breast cancer, testicular cancer, ovarian cancer, non-small cell lung cancer, pancreatic cancer, and bladder cancer. It is a synthetic pyrimidine nucleoside prodrug in which the hydrogen atoms on the 2 Carbon of deoxycytidine are replaced by fluorine atoms [2].

GEM inhibits DNA synthesis and cell death. Gemcitabine undergoes phosphorylation by deoxycytidine kinase to gemcitabine diphosphate and gemcitabine triphosphate,

respectively. GEM inhibits ribonucleotide reductase, an enzyme responsible for the catalyzing synthesis of deoxynucleoside triphosphate used for DNA synthesis [3,4]. Finally, it inhibits the incorporation of the gemcitabine triphosphate, also called difluoro deoxycytidine triphosphate, into DNA. Hence Gemcitabine prevents cells from replicating their DNA [5]. This stops tumor cells from dividing, and the cells die.

Oxaloplatin (OXA) is a cancer medication used to treat colorectal cancer and is a platinum-based antineoplastic class drug. OXA is chemically known as [(1R,2R) - cyclohexane- 1,2 -diamine](ethanedioato - o,o) platinum [6], and its molecular structure is shown in Figure 2. It has a molecular formula $C_8H_{14}N_2O_4Pt$ with a molecular mass of 397.28 g/mol. The platinum atom of the OXA complexed with oxalate and diamino cyclohexane (DACH). OXA undergoes non-enzymatic conversion to active derivatives by displacement of the labile oxalate ligand. Several reactive species are formed, which covalently bind with macromolecules. After activation, Oxaliplatin binds to cross-linking of DNA and inhibits DNA synthesis, thereby leading to cell death [7–9].

The combination of GEM and OXA is called GEMOX. GEMOX is a chemotherapy combination used to treat pancreatic cancer and other cancers [10,11]. This regimen is currently being studied in a variety of other cancers. Present phase II trials are going on for this combination with other drugs like dexamethasone for various types of cancers [12–16].

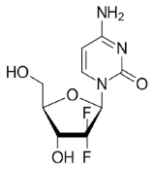


**Figure 1.** Molecular Structure of GEM.

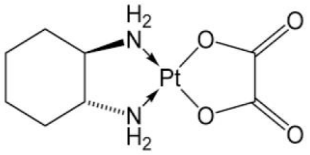


**Figure 2.** Molecular Structure of OXA.

A literature survey revealed that a variety of analytical methods, UV- Visible Spectroscopy [6], HPLC [17–21], UHPLC [22], and LCMS/MS [23–27], has been reported for the estimation of Gemcitabine and Oxaliplatin individually. Since there is no simultaneous estimation of these selected drugs, the present study aims to develop a novel, simple, accurate, and precise method for the simultaneous estimation of GEM and OXA in bulk and synthetic mixture and validate the method per ICH guidelines.

## 2. Materials and Methods

### *2.1. Chemicals and reagents.*

GEM and OXA active pharmaceutical ingredients (API) were acquired as gift samples from Ranbaxy, Hyderabad. HPLC grade methanol and acetonitrile-water was procured from Thermo Fisher Scientific India Pvt.Ltd., Mumbai. Orthophosphoric acid was procured from SD Fine - Chem Limited, Hyderabad, and HPLC-grade chemicals were used in the entire experiment.

### *2.2. Instrumentation.*

SHIMADZU LC - 20AD HPLC system having a binary pump, rheodyne injector with a 20µl loop, Enable C18G (250x4.6mm, i.d 5µ) column connected to SPD-20 detector controlled with LC solution software, LC-GC sensitive balance, etc. were used.

*2.3. Methods.*

2.3.1. Mobile phase preparation.

The mobile phase was prepared by mixing 0.1 %v/v orthophosphoric acid in water with acetonitrile in a 20:80 %v/v ratio, and the same was used as a diluent.

2.3.2. Preparation of standard and working standard solutions.

The standard solution of both analytes has been separately prepared with methanol in a 10ml volumetric flask to obtain 1000µg/ml concentration and considered standard solution A1 for GEM and A2 for OXA. From these solutions, dilute 1ml with diluent to obtain 100µg/ml of concentration solutions B1 and B2 considered as working standard solutions. B1 and B2 solutions were further appropriately diluted with diluent to get solutions having concentrations of 10, 20, 30, 40, 50, and 60µg/ml of GEM and 50, 100, 150, 200, 250, 300µg/ml of OXA respectively.

2.3.3. Preparation of synthetic mixture.

40mg starch, 35 mg hydroxy cellulose, 35mg gum acacia, 20mg lactose, 35mg sodium citrate, 40mg talc, 35 mg sodium alginate, and 35mg magnesium stearate was taken, and all the excipients were mixed in mortar and pestle.

10 mg of GEM and 50mg of OXA were taken in a 100 ml volumetric flask and 50mg of the excipient mixture was added. The contents were dissolved in methanol and made up to 100ml, then sonicated and filtered. 1 ml of the above filtrate was taken to a 10 ml volumetric flask and made up to the mark with diluent.

2.3.4. Method validation.

The developed RP-HPLC method has been validated as stated in ICH Q2(R1A) guidelines [28,29]. The validation parameters are linearity, precision, accuracy, robustness, ruggedness, LOD, and LOQ.

*2.3.4.1. Linearity.*

The linearity was determined by preparing six different concentrations of a standard solution in the range of 10 to 60µg/ml of GEM and 50 to 300µg/ml of OXA, respectively. The linearity was achieved based on the correlation coefficient by plotting a graph with a concentration on X-axis and a peak area on Y-axis.

*2.3.4.2. Precision.*

Precision expresses the closeness of the series of measurements from multiple sampling of the same mixture. Six multiple injections of three known non-identical concentrations of 20,30, 40 µg/ml (GEM) and 100, 150, and 200µg/ml (OXA) were used to study the precision method. % RSD was found to be within range for interday and intraday precision.

*2.3.4.3. Accuracy.*

It is a method to measure the nearness of experimental value to the true value, expressed in terms of % recovery. By comparing the area before and after the addition of the standard drug % recovery was calculated. The standard addition method is performed at 80%, 100%, and 120% levels. The prepared solutions were analyzed in triplicates at each level.

*2.3.4.4. Limit of Quantification (LOQ) and Limit of Detection (LOD).*

The limit of detection is the smallest concentration of the analyte that can be detected by the developed method, which involves an estimable response (signal-to-noise ratio 3), whereas the limit of quantification is the smallest concentration of the analyte which produces a response that can be promptly quantified (signal to noise ratio 10). LOD and LOQ are determined by the calibration curve method. Solutions of GEM and OXA were prepared in the linearity range and injected in triplicate. The average peak area of three analyses was plotted against concentration.

LOQ and LOD were calculated by using the following equations

$$LOQ = 10 \times N/B$$
$$LOD = 3.3 \times N/B$$

Where N is residual variance due to regression

B is the slope.

*2.3.4.5. Robustness.*

The standard and samples of GEM and OXA were injected by changing mobile phase composition and wavelength change. The results obtained were within range.

*2.3.4.6. Ruggedness.*

Ruggedness studies were carried out with two different analysts and showed results within the acceptable range.

## 3. Results and Discussion

The method was developed by performing trials using different columns, mobile phase compositions, wavelengths, runtimes, etc., based on the knowledge of physicochemical parameters of selected drugs and chromatography theoretical aspects. The observations and outcomes of trials were given in Table 1 and the resulting chromatogram was directed in Figure 3.

**Table 1.** Chromatographic parameters used in method development trails.

| S. No | Chromatographic Parameter | Trail 1 | Trail 2 | Trail 3 | Trail 4 | Trail 5 |
|---|---|---|---|---|---|---|
| 1 | Column | Enable $C_{18}$G (250×4.6 mm i.d.,5 µ) | Enable $C_{18}$G (250×4.6 mm i.d.,5 µ) | Enable $C_{18}$G (250×4.6 mm i.d.,5 µ) | Enable $C_{18}$G (250×4.6 mm i.d.,5 µ) | Enable $C_{18}$G (250×4.6 mm i.d.,5 µ) |
| 2 | Mobile Phase | Methanol: acetonitrile (30: 70 %v/v) | Acetonitrile: water (75:25 %v/v) | Acetonitrile: water (85:15 %v/v) | 0.2% orthophosphoric acid: acetonitrile (30:70 %v/v) | 0.2% orthophosphoric acid: acetonitrile (20:80 %v/v) |
| 3 | Wavelength | 268 nm | 268 nm | 268 nm | 268 nm | 268 nm |
| 4 | Flow rate | 1.0 ml/min | 1.0 ml/min | 1.0 ml/min | 0.9 ml/min | 1 ml/min |

| S. No | Chromatographic Parameter | Trail 1 | Trail 2 | Trail 3 | Trail 4 | Trail 5 |
|---|---|---|---|---|---|---|
| 5 | Runtime | 20 min | 20 min | 20 min | 20 min | 20 min |
| 6 | Temperature | Ambient | Ambient | Ambient | Ambient | Ambient |
| | Observation/ result | No proper peaks were observed | Broad peaks were observed | Broad peaks are observed | Peaks not resolved properly | No proper peak shapes were observed |

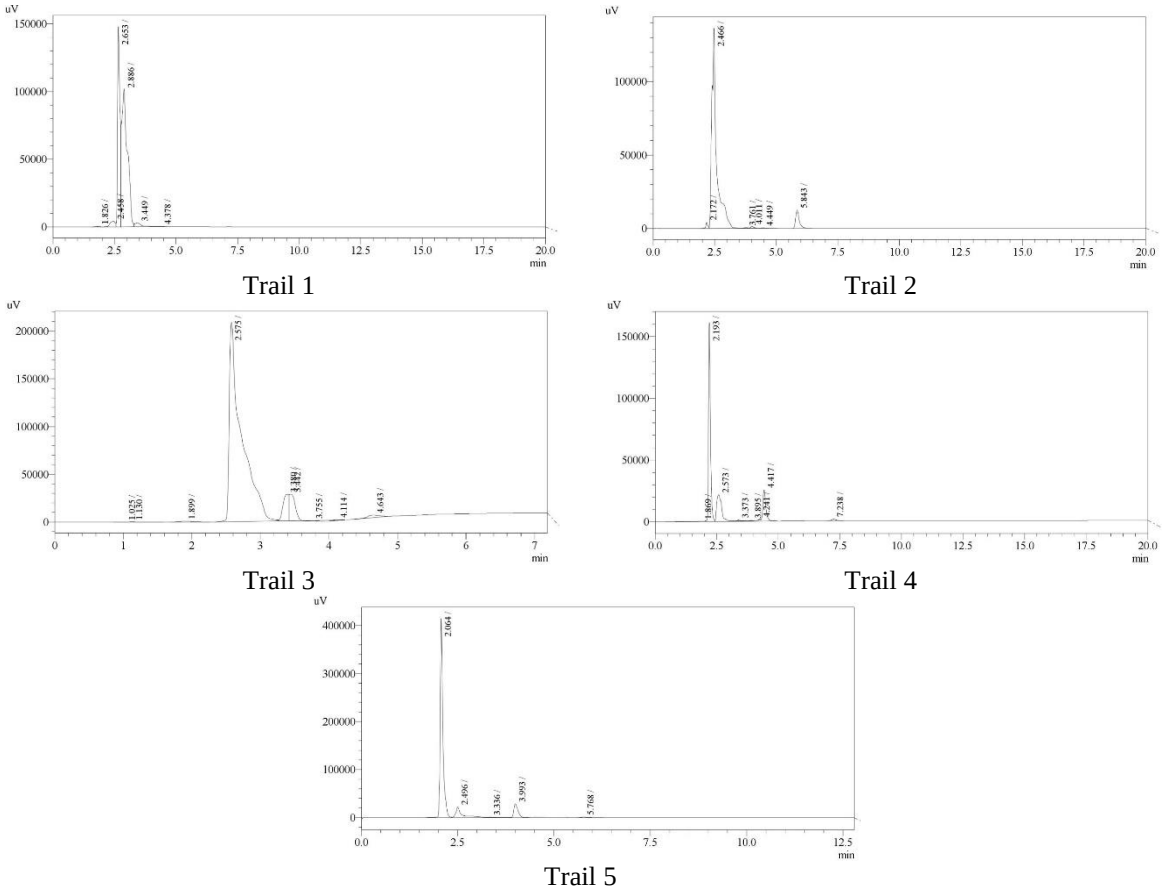


**Figure 3.** Chromatograms of method development trails.

After extensive trial and error, a method was developed to separate the two drugs with proper resolution of peaks with a symmetrical shape. The results of optimized HPLC conditions are shown in Table 2.

**Table 2.** Optimized chromatographic conditions of Gemcitabine and Oxaliplatin.

| Parameter | Condition |
|---|---|
| Mobile Phase | 0.1% Orthophosphoric acid - Acetonitrile (20:80 %v/v) |
| Column | Enable $C_{18}$ G(250X4.6mm, id 5µ) |
| Injection Volume | 10µL |
| Column temperature | Ambient |
| Wavelength | 268nm |
| Flow rate | 0.8ml/min |
| Runtime | 20 min |
| Retention time | 2.5 min (GEM), 3.0 min (OXA) |

*3.1. System suitability studies.*

The system suitability studies were performed by injecting the standard solution mixture of GEM and OXA having 20 µg/ml and 100 µg/ml, respectively, into the optimized HPLC system to record the chromatographic parameter like HETP, Tailing Factor, Resolution, and the number of theoretical plates; the developed method showed that the drugs were effectively resolved with the resolution of more than 2, The retention times of GEM and OXA

were 2.56 min and 3.064 min. The number of theoretical plates calculated was 3729 (GEM) and 4768 for (OXA), and the tailing factor was 1.151 (GEM) and 1.782 (OXA), indicating the efficient column performance; the number of theoretical plates and tailing factors also within the accepted limit and was given in table 3 and the typical chromatogram of standard drugs was depicted in Figure 4.

**Table 3.** System suitability parameters of GEM and OXA.

| Peak number | Retention* | Area* | HETP* | Tailing Factor * | Resolution* | Theoretical plate* |
|---|---|---|---|---|---|---|
| 1 (GEM) | 2.562 | 1114985 | 40.215 | 1.151 | 2.907 | 3729.937 |
| 2 (OXA) | 3.064 | 707510 | 31.460 | 1.782 | | 4768.001 |

* Mean of three replications

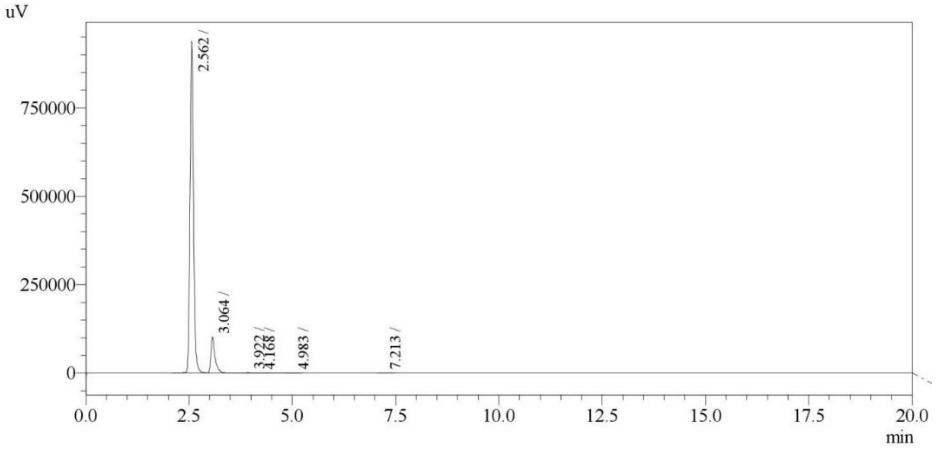


**Figure 4.** Typical chromatogram of GEM and OXA standards.

*3.2. Assay.*

The prepared synthetic mixture was triturated into a fine powder. Then powder equivalent to 10mg of GEM and 50mg of OXA was taken into a 100 ml volumetric flask and dissolved in methanol. The above solution was sonicated for about 10 min. The supernatant liquid was transferred into a 100ml volumetric flask through a Whatman filter paper No.41. The residue was washed twice with solvent, and the combined filtrate was made up to the 100 ml mark. After that 2 ml of the above solution was taken in a 10ml volumetric flask and was diluted with a mixture of 8ml of ACN and 2ml of OPA. The assay results for GEM and OXA are shown in Table 4.

**Table 4.** Assay result for GEM and OXA.

| S. No. | Synthetic Mixture | Amount Taken (mg) | Amount Found | %purity* | % RSD |
|---|---|---|---|---|---|
| 1 | Gemcitabine | 20 | 19.66 | 98.30 | 0.95 |
| 2 | Oxaliplatin | 100 | 99.10 | 99.10 | 1.24 |

* Mean of three replications

*3.3. Validation parameters.*

3.3.1. Linearity.

The method shows linear responses ranging from 10 to 60µg/ml for GEM and 50 to 300µg/ml for OXA. The regression coefficient was found as 1 for both GEM and OXA, which indicates that the method can be used to estimate these drugs simultaneously with the concentrations of 10 to 60µg/ml and 50 to 300µg/ml, respectively. Linear regression data for GEM and OXA were given in Table 5; the linearity curves were shown in Figure 5 (GEM), and Figure 6 (OXA).

**Table 5.** Linearity results of Gemcitabine and Oxaliplatin.

| S.No. | GEM | | OXA | |
|---|---|---|---|---|
| | Conc (µg/ml) | Area (µV) | Conc (µg/ml) | Area (µV) |
| 1 | 10 | 557522 | 50 | 353407 |
| 2 | 20 | 1115034 | 100 | 707409 |
| 3 | 30 | 1672554 | 150 | 1060122 |
| 4 | 40 | 2230083 | 200 | 1414618 |
| 5 | 50 | 2787608 | 250 | 1767145 |
| 6 | 60 | 3345103 | 300 | 2122147 |

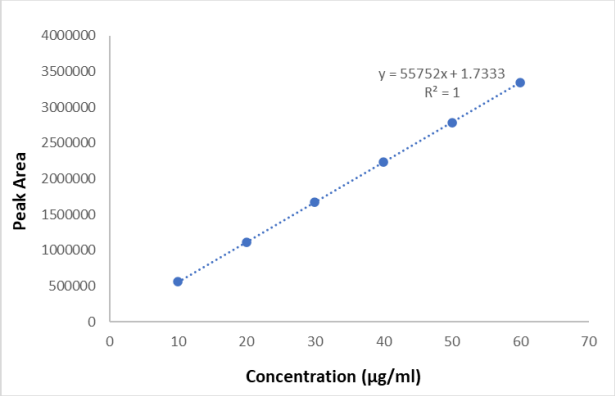


**Figure 5.** Linearity graph of GEM.

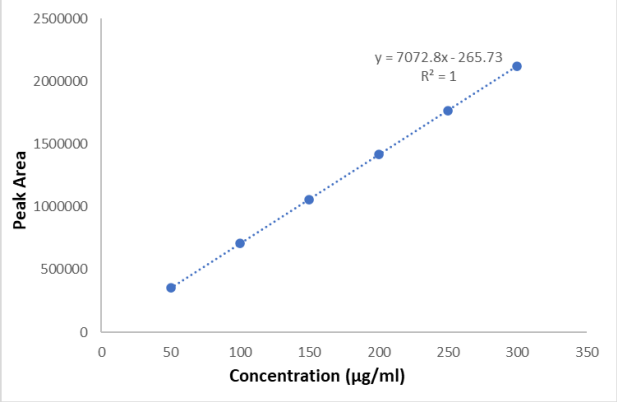


**Figure 6.** Linearity graph of OXA.

3.3.2. Accuracy.

The mean % recoveries of GEM and OXA were found to be 99.2% for Gemcitabine and 98.9% for Oxaliplatin, at different levels of accuracy studies using standard addition method, and the % RSD of these replicates at 80%, 100%, and 120% were found below 2 % which indicated that the method was accurate. The accuracy results are shown in Table 6.

**Table 6.** Accuracy results of GEM and OXA.

| | GEM | | | | OXA | | | |
|---|---|---|---|---|---|---|---|---|
| Spike level | Amount added(µg/ml) | Amount found(µg/ml) | % Recovery | Mean % Recovery | Amount added(µg/ml) | Amount found(µg/ml) | % Recovery | Mean % Recovery |
| 80 | 160 | 158.1 | 98.8 | 99.2 | 32 | 31.6 | 98.7 | 99 |
| 80 | 160 | 158.9 | 99.3 | | 32 | 32.1 | 100.3 | |
| 80 | 160 | 159.3 | 99.5 | | 32 | 31.4 | 98.1 | |
| 100 | 200 | 199.2 | 99.6 | 99.3 | 40 | 39.9 | 99.7 | 98.7 |
| 100 | 200 | 198.6 | 99.3 | | 40 | 39.2 | 98 | |
| 100 | 200 | 198.3 | 99.1 | | 40 | 39.4 | 98.5 | |
| 120 | 240 | 237.4 | 98.9 | 99.3 | 48 | 48.6 | 99.1 | 99 |
| 120 | 240 | 239.3 | 99.7 | | 48 | 48.9 | 99.7 | |
| 120 | 240 | 238.4 | 99.3 | | 48 | 47.1 | 99.0 | |

3.3.3. Precision.

Method Precision: The method precision studies were performed by loading standard solutions of drugs having an intermediate concentration of linearity range of both the drugs in to optimized chromatographic system in six replicates; the peak area was recorded and calculated the % RSD, found as below 2%, (0.02% and 0.17% for GEM and OXA respectively) which indicates that the precision of method results was given in Table 7.

**Table 7.** Method Precision Results of GEM and OXA.

| S.NO | Gemcitabine (40 µg/ml) | Oxaliplatin (150 µg/ml) |
|---|---|---|
| | Peak Area | Peak Area |
| 1 | 2230156 | 1419651 |
| 2 | 2230569 | 1413552 |
| 3 | 2230383 | 1413723 |
| 4 | 2231454 | 1417656 |
| 5 | 2230985 | 1418651 |
| 6 | 2230571 | 1416592 |
| Mean | 2230686 | 1416638 |
| Std Dev | 464.5 | 2537.29 |
| % RSD | 0.02 | 0.17 |

System Precision: The system precision studies were performed by loading a synthetic mixture of Gem and OXA having an intermediate concentration of linearity range of both the drugs in to optimized chromatographic system in six replicates; the peak area was recorded and calculated the % RSD, found as below 2%, (0.01 and 0.23 for GEM and OXA respectively) which indicates that the precision of the method, the results was given in Table 8.

**Table 8.** System Precision results of Gemcitabine and Oxaliplatin.

| S.NO | Gemcitabine (40 µg/ml) | Oxaliplatin (150 µg/ml) |
|---|---|---|
| | Peak Area | Peak Area |
| 1 | 2231083 | 1413555 |
| 2 | 2230965 | 1419832 |
| 3 | 2230849 | 1418893 |
| 4 | 2231643 | 1416542 |
| 5 | 2230654 | 1414253 |
| 6 | 2231153 | 1411352 |
| Mean | 2231058 | 1415738 |
| Std Dev | 336.8 | 3273.19 |
| % RSD | 0.01 | 0.23 |

3.3.4. Robustness.

The method was evaluated for its accuracy though the deliberate changes in method parameters even happened during the analysis of these drugs by injecting the intermediated concentration of these drugs into an HPLC system operated at the slightly higher and lower composition of mobile phase and absorption maxima values, the results (given in Table 9) indicates that the method producing the results within the acceptable level, The %RSD values of ruggedness study were found to be <2%.

**Table 9.** Robustness study of GEM and OXA.

| Drug | Mobile Phase Ratio | Peak Area | | | wavelength | Peak Area | | |
|---|---|---|---|---|---|---|---|---|
| | | Avg. | SD | %RSD | | Avg. | SD | %RSD |
| GEM | 70:30 | 706216 | 11305.27 | 1.60 | 267 | 2231548 | 1794.30 | 0.08 |
| | 80:20 | 2230686 | 464.5 | 0.02 | 268 | 2230686 | 464.5 | 0.02 |
| | 90:10 | 2231790 | 1153.47 | 0.05 | 269 | 2234602 | 2468.51 | 0.11 |

| Drug | Mobile Phase Ratio | Peak Area | | | wavelength | Peak Area | | |
|---|---|---|---|---|---|---|---|---|
| | | Avg. | SD | %RSD | | Avg. | SD | %RSD |
| OXA | 70:30 | 1413666 | 2145.0576 | 0.15 | 267 | 1417445 | 1895.86 | 0.13 |
| | 80:20 | 1416638 | 2537.29 | 0.17 | 268 | 1416638 | 2537.29 | 0.17 |
| | 90:10 | 1418478 | 1704.92 | 0.12 | 269 | 1415231 | 3149.16 | 0.22 |

3.3.5. Ruggedness.

The Method was determined for its flexibility while deliberate changes in analyst, environment, system, etc., through injecting solution having the intermediate concentration into an optimized HPLC system in replication by different analysts. The %RSD of the peak area results (shown in Table 10) is within the accepted limit indicating the ruggedness of the developed method.

**Table 10.** Ruggedness study of GEM and OXA.

| S.No. | GEM | | OXA | |
|---|---|---|---|---|
| | Change of analyst | | Change of analyst | |
| | Analyst 1 | Analyst 2 | Analyst 1 | Analyst 2 |
| 1 | 1672556 | 1675234 | 1060326 | 1067435 |
| 2 | 1674883 | 1670875 | 1063679 | 1062546 |
| 3 | 1678387 | 1673769 | 1062343 | 1064768 |
| 4 | 1675434 | 1676789 | 1065326 | 1068655 |
| 5 | 1670542 | 1669635 | 1061347 | 1060763 |
| 6 | 1673872 | 1674767 | 1069654 | 1062468 |
| **Mean** | 1674279 | 1673512 | 1063779 | 1064439 |
| **SD** | 2671 | 2732 | 3371 | 3093 |
| **%RSD** | 0.15 | 0.16 | 0.31 | 0.29 |

3.3.6. Limit of Detection (LOD) and Limit of Quantification (LOQ).

The LOD for GEM and OXA was found to be 0.125µg/ml and 0.750µg/ml, respectively. The LOQ for GEM and OXA was found to be 0.450µg/ml and 2.35µg/ml, respectively, (shown in Table 11), indicating the method's sensitivity.

**Table 11.** LOD and LOQ data for GEM and OXA.

| Drug | LOD (µg/ml) | LOQ (µg/ml) |
|---|---|---|
| GEM | 0.125 | 0.450 |
| OXA | 0.750 | 2.35 |

## 4. Conclusions

From the present study, it can be concluded that the developed HPLC method for simultaneous determination of GEM and OXA was found as simple, accurate, precise, linear, and robust by performing validation according to the ICH guidelines. Hence this method may be suitable for routine qualitative and quantitative analysis performed for simultaneous estimation of GEM and OXA in bulk and pharmaceutical formulations by RP-HPLC technique.

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## Conflicts of Interest

The authors declare no conflict of interest.

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