RESEARCH PAPER

Analytical Quality By Design Approach In A Validated Rp-Hplc Method Development For Estimation Of Gemcitabine And Methotrexate In Their Dosage Forms.

SachinS.Shinde[1*], Dr. Preeti Khulbe[2],

[1*]School of Pharmacy, Suresh Gyan Vihar University, Jaipur ORCID ID: 0000-0002-4111-0439
Email: sachinshinde18@gmail.com
[2]Associate Professor, School of Pharmacy Suresh Gyan Vihar University, Jaipur
Email:preeti.khulbe@mygyanvihar.com ORCID: 0009-0000-3412-6961

***Corresponding Author:** SachinS.Shinde
School of Pharmacy, Suresh Gyan Vihar University, Jaipur ORCID ID: 0000-0002-4111-0439
*Email: sachinshinde18@gmail.com

ABSTRACT
Analytical control of anticancer drugs requires reliable quantification methods because of their therapeutic sensitivities and clinical significance. This study applied an Analytical Quality by Design (AQbD) framework to develop and validate RP-HPLC methods for gemcitabine and methotrexate. An Analytical Target Profile was defined, and potential chromatographic factors were screened using risk assessment tools to identify the critical method parameters for each drug. These parameters were optimized using a Box–Behnken response surface design to establish a statistically supported Method Operable Design Region (MODR), ensuring robust performance under variable analytical conditions.
Gemcitabine was analyzed on an L-7 column (250 × 4.6 mm, 5 µm) with phosphate buffer (pH 6.5): methanol (97:3, v/v) at 1.0 mL·min^{-1} in 10 min, and methotrexate on a C18 column (100 × 6 mm, 5 µm) using phosphate buffer (pH 6.0): acetonitrile (92:8, v/v) at 1.4 mL·min^{-1} in 10 min.
The obtained MODR demonstrated wide operational flexibility and enhanced reliability for routine quality control. Validation according to ICH Q2 confirmed linearity ($r^2 = 0.999$), accuracy within 98–102%, precision with %RSD < 2%, robustness to deliberate variations, and 48-h solution stability. The developed AQbD-based methods are suitable for the routine pharmaceutical analysis of the selected anticancer drugs.
Keywords: Analytical Quality by Design, RP-HPLC, Anticancer, Method Developmen

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1 INTRODUCTION

Cancer remains one of the most serious global health challenges, responsible for millions of deaths annually worldwide. The development of effective anticancer drugs is essential for controlling disease progression and improving patient outcomes. Among the various therapeutic agents available, small-molecule chemotherapeutics and targeted drugs play critical roles in inhibiting malignant cell growth through diverse molecular mechanisms.
Gemcitabine ($C_9H_{11}F_2N_3O_4$) is a nucleoside analog indicated for pancreatic, lung, ovarian, and breast cancers that inhibits DNA polymerization and cell replication[1]. Methotrexate ($C_{20}H_{22}N_8O_5$) is an antifolate antimetabolite that inhibits dihydrofolate reductase, blocking nucleotide synthesis[2].
Given the clinical relevance of these drugs and the increasing expectations for reliable analytical control, a structured, science-based approach to method development is essential. Analytical Quality by Design (AQbD) enables a systematic understanding of method variables, ensuring robustness, regulatory confidence, and consistent assessment of drug quality throughout its lifecycle.
Several HPLC methods have been developed for these individual anticancer drugs and their combinations with other drugs[3][4][5][6]. However, the analytical quality-by-design approach has not yet been explored for these drugs.
In this study, a risk-based approach and analytical quality by design approach were used for the HPLC method development for the determination of gemcitabine and methotrexate. The developed HPLC method was validated in accordance with the ICH Q2 guidelines.

2 MATERIALS AND METHODS

2.1 Instrumentation and Software

Chromatographic analysis was performed using a Jasco LC-4600 RP-HPLC system equipped with a UV detector and Fine-pack Jasco C18 column (250 mm × 4.6 mm, 5 µm). A Jasco V-630 UV spectrophotometer was used for wavelength selection. The supporting instruments included an analytical balance (Mettler Toledo), a pH meter (Mettler Toledo), and an ultrasonic sonicator (Lab India).

*Author for Correspondence: sachinshinde18@gmail.com

2.2 Materials and Reagents

The study utilized Gemcitabine HCl (WS-005, 99.95% purity, 0.05% LOD), and Methotrexate (WS-005, 99.98% purity, 0.10% LOD). Gemlive 1000 injection and Folitrax-5 tablets, along with analytical-grade reagents such as HPLC-grade water, methanol, acetonitrile, phosphoric acid, orthophosphoric acid, buffers, and sodium hydroxide, were procured from Merck Life Science and RANKEM (India).

2.3 Chromatographic conditions

The following chromatographic conditions were used for the analytical method validation.

2.3.1 Gemcitabine

The chromatographic conditions for gemcitabine were based on an L-7 column (250 × 4.6 mm, 5 µm) and a mobile phase containing phosphate buffer (pH 6.5) and methanol in a ratio 97:3 v/v under isocratic elution. The flow rate was maintained at 1.0 mL/min with UV detection at 266 nm, an injection volume of 20 µL, and a 10-minute run time. The mobile phase was used as both the blank and diluent.

2.3.2 Methotrexate

The chromatographic conditions for Methotrexate used a C18 column (100 × 6 mm, 5 µm) with an isocratic mobile phase of phosphate buffer (pH 6.0) and acetonitrile in the ratio 92:8 v/v. The flow rate was set at 1.4 mL/min, detection wavelength at 303 nm, injection volume at 20 µL, and run time at 10 min. The mobile phase was used as the blank.

2.4 Preparation of standard solutions

2.4.1 Gemcitabine

Approximately 40 mg of Gemcitabine HCl working standard was weighed and transferred to a 100 ml volumetric flask. Then, 70 ml of diluent was added and sonicated to dissolve the sample. The solution was diluted to the required volume with a diluent and mixed. Then, 1.0 ml of the solution was transferred into a 100 ml of volumetric flask, diluted with the diluent, and mixed. The solution was filtered through a 0.2µm nylon membrane filter.

2.4.2 Methotrexate

Approximately 25 mg of the methotrexate working standard was accurately weighed and transferred to a 25 ml volumetric flask. Then, 10 ml of diluent was added and sonicated to dissolve the sample. The solution was diluted to the required volume with a diluent and mixed. Then, 1.0 ml of the solution was transferred into a 10 ml of volumetric flask, diluted to volume with the diluent, and mixed.

2.5 Preparation of sample solutions

2.5.1 Gemcitabine

A 1 ml of sample solution was pipetted into a 100 ml volumetric flask. Approximately 70 ml of diluent was then added and shaken for 5 min mechanically or manually, followed by sonication for 5 min. Then, 1.0 ml of the solution was transferred into a 100 ml of volumetric flask, diluted with the diluent, and mixed. The solution was filtered through a 0.2µm nylon membrane filter.

2.5.2 Methotrexate

The sample powder (547 mg) was weighed and transferred to a 25 ml volumetric flask. Then, approximately 10 ml of diluent was added and shaken for 20 min mechanically or manually, followed by sonication for 30 min. The solution was diluted to the mark with the diluent. The solution was centrifuged at 8000 rpm for 10 min. The supernatant solution was decanted into another test tube, and 1.0 ml of the supernatant solution was transferred into another 10 ml volumetric flask, and the volume was made up with diluent. Further, 1.0 ml of the solution was transferred into another 10 ml volumetric flask, and the volume was made up with diluent. The solution was filtered through a 0.2µm nylon membrane filter.

2.6 Analytical target profile (ATP), critical method parameters (CMPs), critical method attributes (CMAs) and risk assessment

The analytical target profile is an important element of analytical QBD. This helps establish the goals of method development and provides strict criteria for method development. ATP was prepared to develop an analytical method. The ATP is presented in Table 1 .

To identify the Parameters the Ishikawa Fishbone diagram (***Figure 2***) was used, and the cause-effect relationship between various risk factors was established.

3 RESULTS AND DISCUSSION

3.1 Screening and verification of the critical method parameters (CMPs)

The potential risk factors in risk assessment were related to chromatographic conditions as well as the drug. The drug specific parameters were solubility, potency (which depend on the drug's properties) and pH of buffer (which depends on drug pKa and ionization state). Other risk factors were scored identically in both drugs, as they are not specifically drug related, instead they are HPLC system and chromatographic condition related.

3.1.1 Gemcitabine

A focused Ishikawa and RPN risk assessment was performed to identify chromatographic factors most likely to affect the predefined CMAs: Number of Theoretical Plates and Asymmetry Factor. The risk assessment results (

Table ***2***, Figure 3) identified the percentage of buffer in the mobile phase, buffer pH, and flow rate as high-risk parameters; thus, CMPs were considered for these parameters. Low-risk factors were also fixed.

Preliminary trials were conducted for gemcitabine using a highly aqueous phosphate buffer system owing to the hydrophilic nature of the molecule. Screening of mobile phase compositions from Buffers with 96–98% methanol as the organic component showed stable peak shapes and acceptable run times at mid-range compositions, whereas lower aqueous proportions led to early elution and reduced efficiencies. A pH range of 6.4–6.6 was evaluated because of limited ionization in this region (due to its low pKa 3.6), which is required for considerable rentention. Here small adjustments in pH produced noticeable shifts in the theoretical plate counts. Flow rate screening between 0.8 and 1.2 mL/min demonstrated a minor influence on plate counts while maintaining an acceptable backpressure. Thus, the CMP working ranges were set as 96–98% buffer, pH 6.4–6.6, and 0.8–1.2 mL/min,

3.1.2 Methotrexate

A focused Ishikawa analysis followed by RPN scoring was performed to identify chromatographic variables that most likely affect the predefined CMAs, Number of Theoretical Plates and Asymmetry Factor. All relevant factors were screened and ranked by RPN, and the results are summarized in

Table 3. From the assessment, the percentage of buffer in the mobile phase, buffer pH, and flow rate showed the highest risk and were therefore selected as CMPs for further study, whereas the lower-risk factors (column temperature, injection volume, wavelength, and diluent) were fixed.

For Methotrexate, initial chromatographic screening was performed using phosphate buffer and acetonitrile systems on a C18 stationary phase. High aqueous content was required for adequate retention, and screening demonstrated that compositions below 90% buffer produced insufficient retention, whereas compositions above 94% excessively extended the run times. The molecule is a weak dicarboxylic acid and possesses pKa values of 4.7–5.5 with poor aqueous solubility (0.01 mg/mL). The solubility of methotrexate increases as we move from pH 5.0 to 7.0. Thus to have optimum solubility and ionization, the pH range was explored from 5.0 to 6.0. pH trials between 5.0 and 6.0 revealed measurable effects on peak symmetry and theoretical plates owing to ionization changes. Flow rates between 1.2 and 1.4 mL/min were assessed, and although the impact on CMAs was moderate, the operational backpressure and run-time optimization favored this range. Based on empirical outcomes, the CMP working limits were finalized as 90–94% buffer, pH 5.0–6.0, and flow 1.2–1.4 mL/min.

3.2 Optimization of the critical method parameters (CMPs) using response surface methodology (RSM)

The parameters obtained from the screening experiments were extensively studied using response surface methodology. Response surface methodology is one of the best statistical tools for optimization, which helps in determining the individual and combination effects of the experimental variables. Box Behnken Design (BBD), an RSM-based design, was employed to optimize and finalize the CMPs. BBD was useful for identifying the method operable design space region (MODR) using numerical and graphical optimization.

3.2.1 Gemcitabine

Response surface methodology (RSM) using a Box–Behnken design was employed to evaluate and optimize the chromatographic performance of gemcitabine. A total of 17 experimental runs were generated based on three control method parameters: percentage of buffer in the mobile phase (A), buffer pH (B), and flow rate (C), and the obtained responses for the number of theoretical plates (TP) and asymmetry factor (AF) were incorporated into the model. The experimental data were analyzed using a quadratic model through ANOVA, which was found to be statistically significant for both responses, while the lack of fit remained non-significant, confirming the model's suitability.

For TP, the squared terms A^2, B^2, and C^2 exhibited significant effects, whereas for AF, the quadratic terms A^2 and C^2 were significant contributors.

Number of Theoretical Plates= $5857.8 + 6.625A + 0.375B + 1.25C + -0.75AB + 12.5AC + -215.025A^2 + -212.525B^2 + -206.775C^2$

Asymmetry Factor= $1.636 - 0.01875A + 0.00375C + 0.0025AB - 0.01AC - 0.0125BC + 0.0445A^2 + 0.022B^2 + 0.0295C^2$

The 3D plots were analyzed to evaluate the relationships between the CMPs and the responses to gemcitabine. R1 (number of theoretical plates) reached a maximum of 5244.22 at a higher buffer (% A = 98%), lower pH (B = 6.4), and higher flow rate (C = 1.2 $mL \cdot min^{-1}$), and a minimum of 5204.48 at an intermediate buffer level (% A = 97%), lower pH (B = 6.4), and higher flow rate (C = 1.2 $mL \cdot min^{-1}$).

R2 (asymmetry factor) showed a maximum of 1.7795 at an intermediate buffer level (% A = 97%), lower pH (B = 6.4), and higher flow rate (C = 1.2 $mL \cdot min^{-1}$), and a minimum of 1.697 at a higher buffer (% A = 98%), higher pH (B = 6.6), and higher flow rate (C = 1.2 $mL \cdot min^{-1}$).

The analysis of the 3D plots demonstrates that a high-aqueous environment (buffer in mobile phase) maximizes chromatographic efficiency by facilitating the enhanced retention of the polar Gemcitabine HCl. Because the drug is highly soluble in the aqueous buffer and possesses low affinity for organic phase, increasing the aqueous percentage reduces the elution strength of the mobile phase, causing the analyte to interact more extensively with the stationary phase. It also allows for a more uniform partitioning between the mobile and

stationary phases. This increased retention time coupled with an pH of 6.4 and flow rate of 1.2 mL/min, results in a narrower peak width relative to retention, thereby significantly increasing the number of theoretical plates to a maximum of 5244.22 However the the asymmetry factor achieved a minimum value of 1.697 under higher buffer and pH conditions (%A = 98%, pH 6.6), suggesting that the increased aqueous fraction not only improves efficiency but also enhances peak symmetry. This improvement in peak symmetry is attributed to the higher pH level, which effectively minimizes secondary interactions between the basic drug molecules and the acidic residual silanol groups on the stationary phase that typically cause tailing. Additionally, the 1.2 mL/min flow rate facilitates efficient mass transfer kinetics, preventing the analyte from spreading excessively.For gemcitabine, numerical optimization was performed to determine the most favorable combination of CMPs for a robust chromatographic method. The buffer percentage (CMP-A), buffer pH (CMP-B), and flow rate (CMP-C) were optimized within the defined experimental ranges to maximize the number of theoretical plates and minimize the asymmetry. The proposed solution was a Buffer: Methanol (97:3%V/V), pH 6.5, and a flow rate of 1.0 mL·min^{-1}. The global desirability value observed from the optimization figure was 0.874, indicating approximately 87% achievement of the optimization criteria and confirming the suitability of the selected conditions for the analysis of gemcitabine.

Based on numerical optimization, optimized chromatographic conditions were selected, and the method operable design was identified. The optimized conditions fell within the operable design region of the method.

3.2.2 Methotrexate

Response surface methodology (RSM) using a Box–Behnken design was employed to evaluate and optimize the chromatographic performance of methotrexate. A total of 17 experimental runs were generated based on three control method parameters: percentage of buffer in the mobile phase (A), buffer pH (B), and flow rate (C), and the obtained responses for the number of theoretical plates (TP) and asymmetry factor (AF) were incorporated into the model. The experimental data were analyzed using a quadratic model through ANOVA, which was found to be statistically significant for both responses, while the lack of fit remained non-significant, confirming the model's suitability.

For TP, the squared terms A^2, B^2, and C^2 exhibited significant effects, whereas for AF, the quadratic term A^2 and other squared contributions were identified as significant contributors to the model.

Number of Theoretical Plates= 6604.8 + 55.625A + 96.875B + 9.5C - 39AB + 68.75AC + 27.25BC - 316.65A^2 + 17.35B^2 + 4.6C^2

Asymmetry Factor= 1.534 - 0.03625A - 0.02875B - 0.01C + 0.0175AB - 0.025AC -0.025BC + 0.16425A^2 - 0.01575B^2 + 0.02175C^2

The 3D plots were analyzed to evaluate the relationships between CMPs and MTX responses. R1 (number of theoretical plates) reached a maximum of 6529.10 at higher buffer (% A = 94%), higher pH (B = 6), and higher flow rate (C = 1.4 mL·min^{-1}), and a minimum of 6032.10 at the central/lower region of the design (approximate conditions near % A = 92%, B = 5.5, and C = 1.3 mL·min^{-1}). R2 (asymmetry factor) showed a maximum of 1.82675 in the central region (approx. % A = 92%, B = 5.5, C = 1.3 mL·min^{-1}) and a minimum of 1.59675 at higher buffer (% A = 94%), higher pH (B = 6) and higher flow rate (C = 1.4 mL·min^{-1}) as indicated on the asymmetry cube.

The 3D response surface analysis reveals that maximum chromatographic efficiency and optimal peak symmetry are achieved at a high-aqueous mobile phase (94% buffer) and pH 6.0. This outcome is primarily governed by the pH-dependent ionization of Methotrexate. While MTX has low solubility in pure water, at pH 6.0 the molecule exists in its anionic state. This increased polarity enhances solute-solvent compatibility within the high-aqueous environment, which reduces the elution strength and leads to significant peak compression, thereby maximizing theoretical plates.

The improvement in the asymmetry factor at pH 6.0, compared to the central design region (pH 5.5), is attributed to the suppression of secondary interactions. At pH 6.0, the drug is fully deprotonated, which minimizes its affinity for residual silanol groups on the C18 stationary phase—a common cause of tailing in acidic or amphoteric analytes. Furthermore, the 1.4 mL•min^{-1} flow rate improves column efficiency by optimizing the mobile phase velocity. Under these conditions, the higher velocity effectively limits longitudinal diffusion, ensuring the analyte is eluted as a sharp, symmetrical peak rather than spreading within the column's interstitial spaces."

For methotrexate, numerical optimization was performed to determine the most favorable combination of CMPs for a robust chromatographic method. The buffer percentage (CMP-A), buffer pH (CMP-B), and flow rate (CMP-C) were optimized within the defined experimental ranges to maximize the number of theoretical plates and minimize the asymmetry. The proposed solution was a Buffer: ACN (92:8% v/v), pH 6, and a flow rate of 1.4 mL·min^{-1}. The global desirability value observed from the optimization figure was 0.995, indicating approximately 99% achievement of the optimization criteria and confirming the suitability of the selected conditions for the analysis of methotrexate.

3.3 Identification of method operable design region (MODR)

The operable design region of the method was determined using graphical optimization. The MODR for gemcitabine and methotrexate are shown in Figure 9. The operable design methods are highlighted in yellow. The optimum chromatographic condition results are flagged and are well within the method-operable design region.

3.4 Method validation

Method validation was performed according to the ICH Q2 guideline.

3.4.1 System Suitability

The system suitability was evaluated by analyzing five replicate injections of standard solutions of Gemcitabine, and Methotrexate under the optimized chromatographic conditions. The parameter assessed was the peak area repeatability, and the %RSD was calculated to determine the system precision. All analytes exhibited %RSD values below 2%, with gemcitabine (0.76%) and methotrexate (0.60%), indicating excellent repeatability and confirming that the system met the predefined suitability criteria.

3.4.2 LOD LOQ

The Quantitation and Detection Limits were calculated using the standard deviation of the response and slope. The LOD and LOQ values for gemcitabine and methotrexate are presented in Table 8.

3.4.3 Linearity

Linearity was verified by injecting five different drug concentrations between 50% and 150% of the target concentration. The results of the linearity are presented in

Table *9*.

3.4.4 Specificity/Selectivity

Selectivity was assessed by injecting the diluent blank solution, excipient blend, system suitability solution, and test solution. The diluent blank and excipient blend solutions did not show any peaks at the retention times of Gemcitabine and Methotrexate.

3.4.5 Precision

The method precision was evaluated by performing six replicate injections of the test solution at the same concentration under identical operating conditions, and the precision was expressed in terms of the%RSD of peak areas.

Intermediate precision (ruggedness) was assessed by analyzing a total of 12 injections, where six replicates were performed by Analyst 1 using Column 1 on System 1 on Day 1, and the remaining six replicates were carried out by a different analyst on a different day using an independent system and column. This design enabled the evaluation of the method's reproducibility across analysts, instruments, columns, and days of analysis. The results are presented in

Table *9* below.

3.4.6 Accuracy

The accuracy of the method was established through recovery studies conducted using a standard addition technique. Known quantities of the analyte were spiked into the pre-analyzed sample matrix at five concentration levels corresponding to 50%, 75%, 100%, 125%, and 150% of the target concentration, with each level analyzed in triplicate. The percentage recovery and %RSD at each level were calculated to determine the closeness of the measured values to the true added concentrations, thereby demonstrating the accuracy and reliability of the analytical method over a wide concentration range. The results for the accuracy are presented in

Table *9*.

3.4.7 Robustness

The robustness of the method was evaluated by intentionally introducing deliberate variations in the key chromatographic parameters and assessing their effects on the system suitability results. The parameters subjected to variation included changes in the column lot, flow rate (±0.2 mL/min), detection wavelength (±2 nm), and mobile phase composition (±2%). The results were compared with those obtained under optimized conditions to determine the method's capacity to remain unaffected by small but purposeful changes, thereby confirming its reliability for routine analysis (

Table *9*).

3.4.8 ***Solution stability***

Solution stability was assessed to determine the integrity of the sample and standard solutions over a defined period under normal laboratory storage conditions. Both the sample and standard solutions were stored at room temperature and analyzed at predetermined time intervals (initial, 12, 24, and 48 h) using the optimized chromatographic conditions. The results were evaluated by comparing the measured peak areas and system suitability parameters with their initial values. The percentage assay difference and %RSD were calculated to ensure that no significant degradation or variability occurred, thereby confirming the stability of the solutions over the specified durations. The results are presented in

Table *9* below.

4 CONCLUSION

The Analytical QbD approach enabled the systematic development and optimization of RP-HPLC methods for gemcitabine and methotrexate. Individual risk assessments identified distinct critical method parameters for each drug, which were optimized using Box–Behnken RSM to establish robust method conditions and a well-defined MODR for each drug. The final methods complied with the predefined ATP and fulfilled the ICH Q2 validation criteria, showing excellent linearity, precision, accuracy, robustness, and solution stability. These results confirm the suitability of the optimized methods for the routine quality control of the respective dosage forms.

Authors' Contributions statement

Mr. Sachin Shinde performed the research and was responsible for data generation, interpretation and manuscript preparation. Dr. Preeti Khulbe provided overall guidance, critically reviewed the work, and involved in drafting and revising the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest

AUTHORS DO NOT HAVE ANY CONFLICT OF INTEREST.

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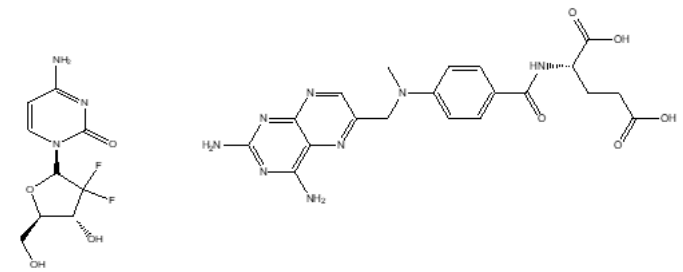

Figure 1 Chemical Structure of Gemcitabine and Methotrexate

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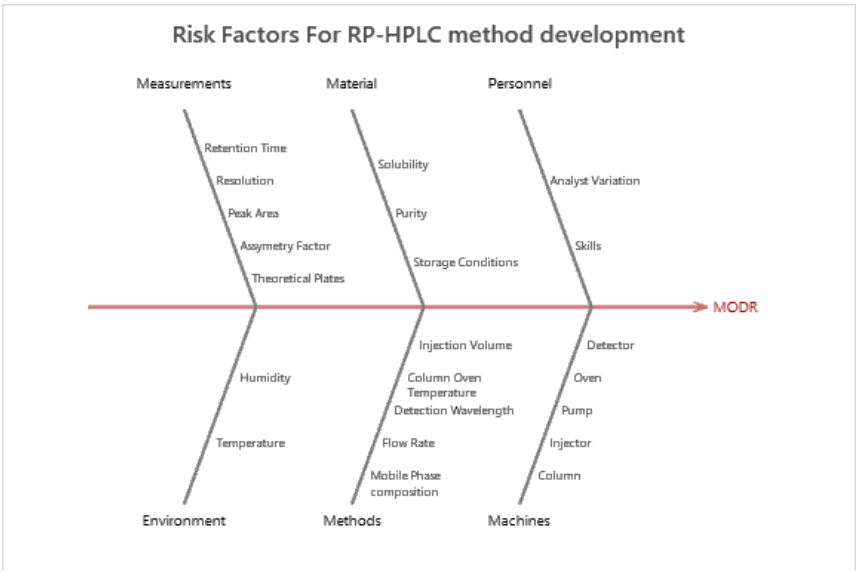

Figure 2 Cause effect analysis of the risk factors for HPLC method development using the Ishikawa Fishbone Diagram

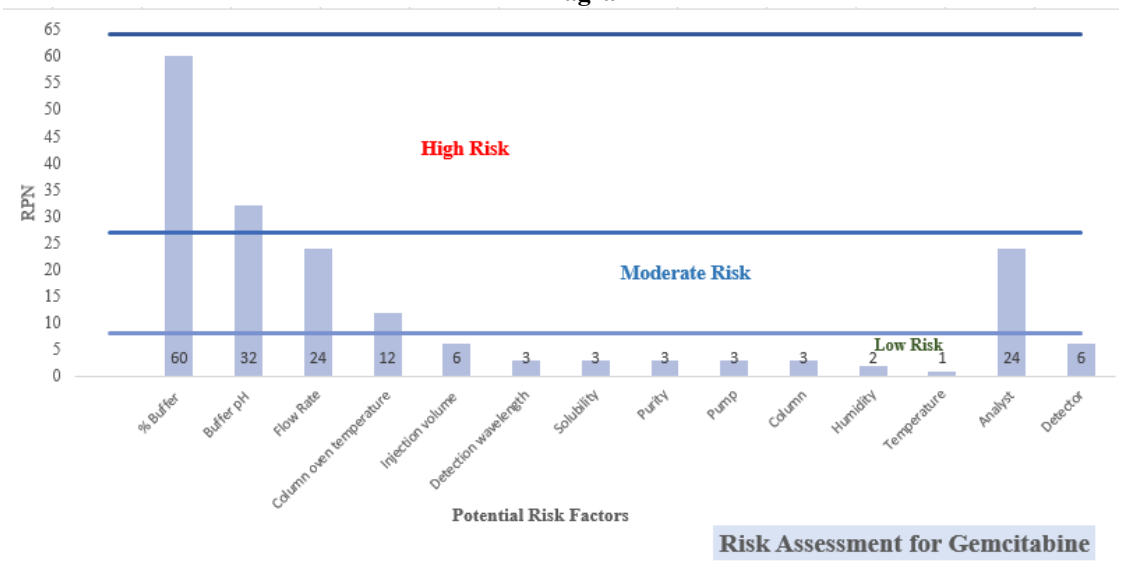

Figure 3 Risk Assessment for Gemcitabine

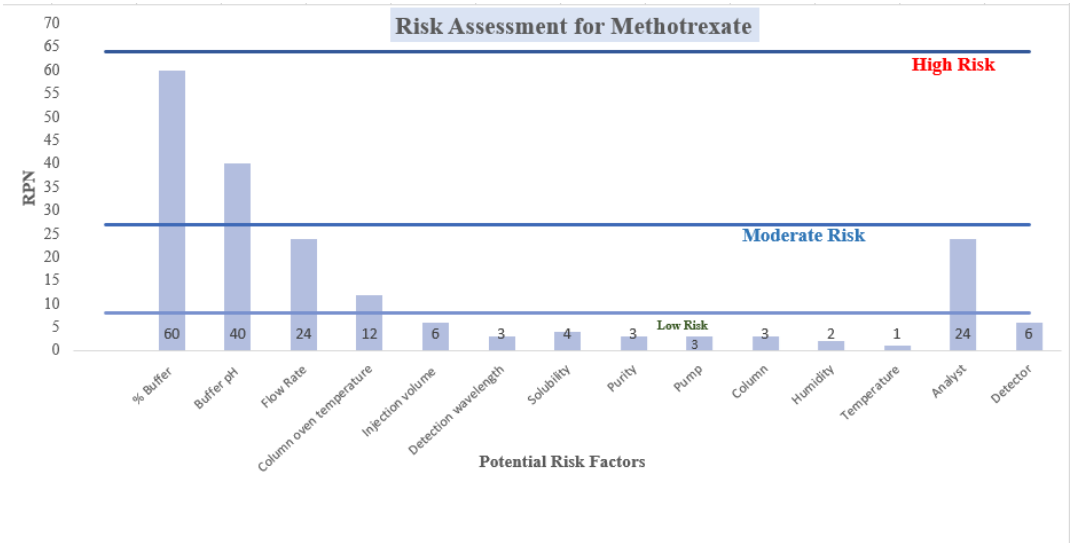

Figure 4 Risk Assessment for Methotrexate

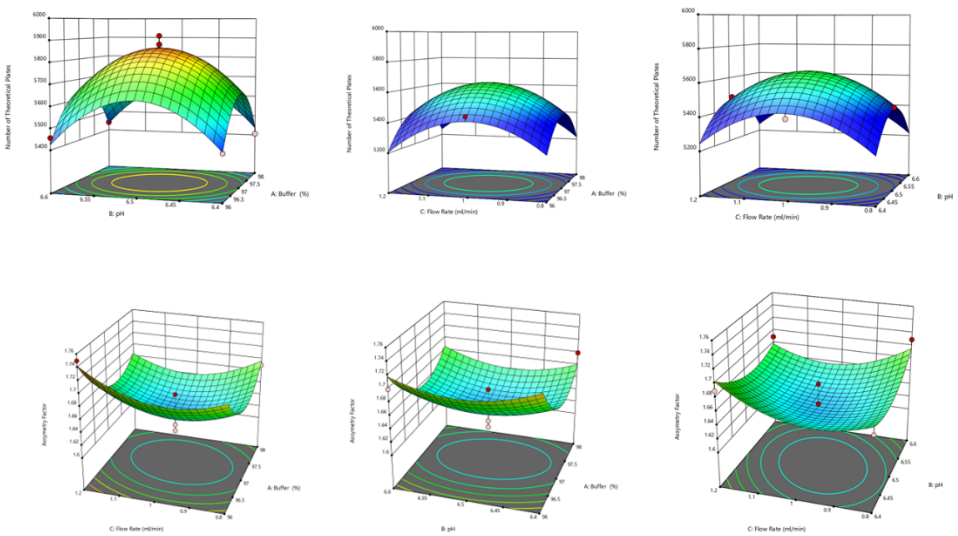

Figure 5 3D plots for effect of CMPs on CMAs for the Gemcitabine

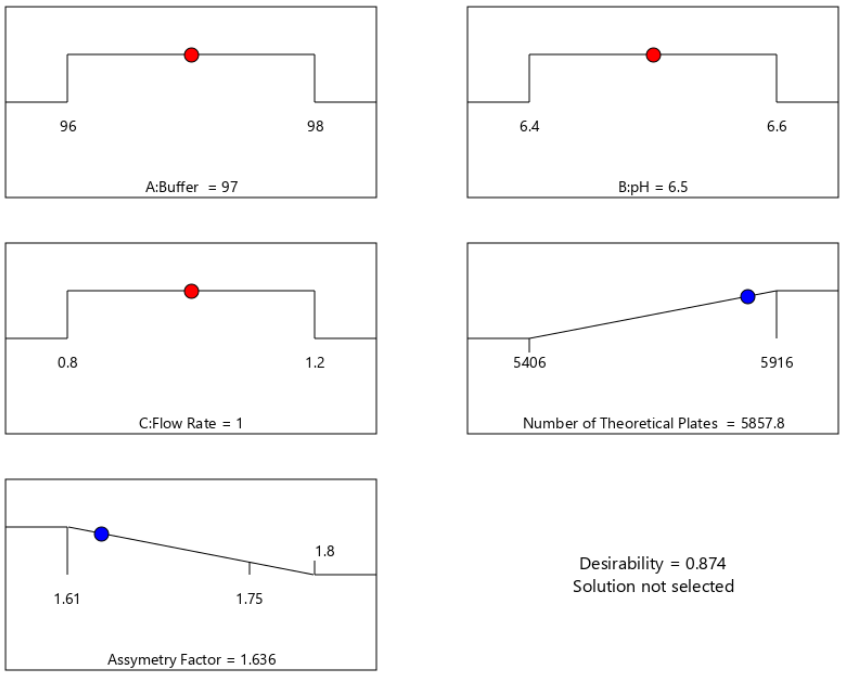

Figure 6 Numerical optimization desirability ramps for the Gemcitabine

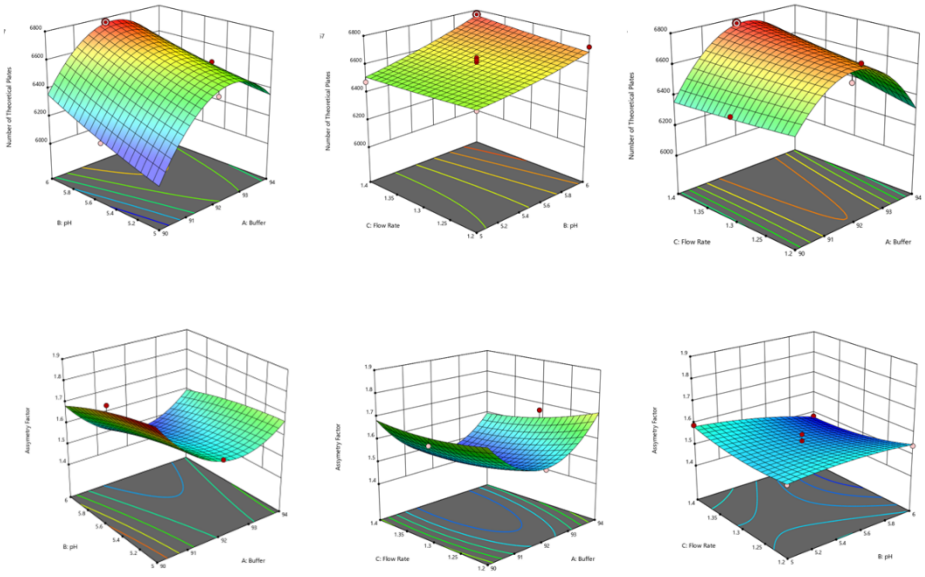

Figure 7 3D plots for effect of CMPs on CMAs for the Methotrexate

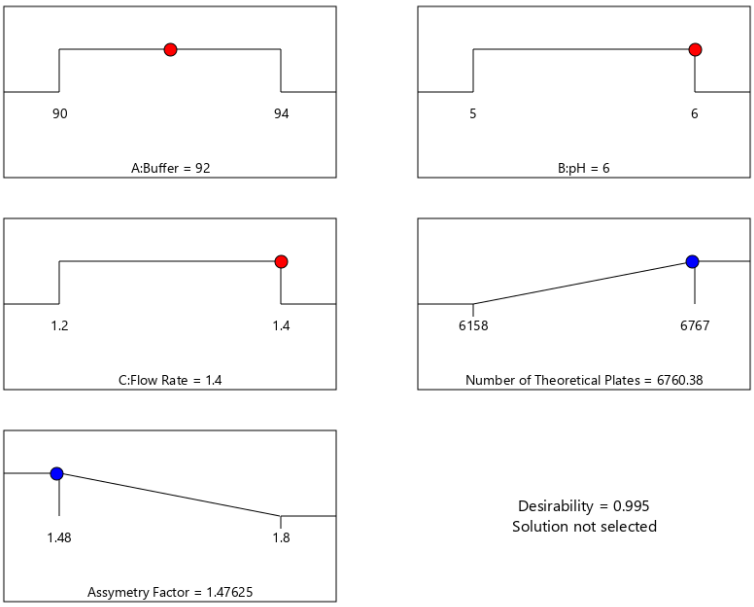

Figure 8 Numerical optimization desirability ramps for methotrexate

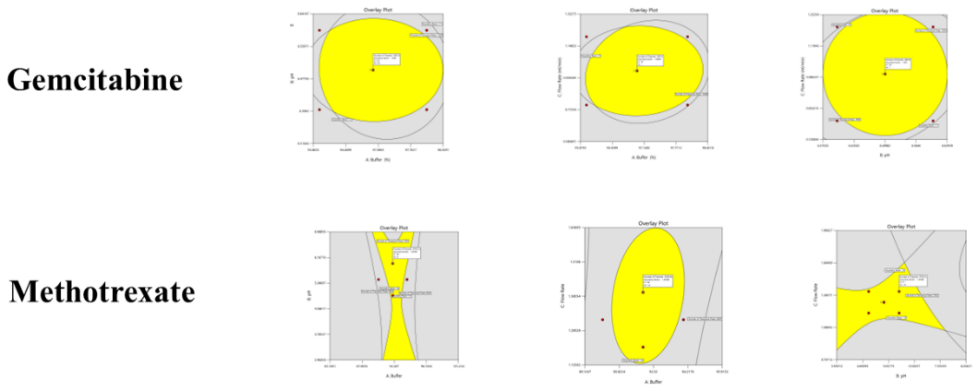

Figure 9 Method Operable Design Region for Gemcitabine and Methotrexate

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Table 1 Analytical Target Profile for Gemcitabine and Methotrexate

Parameters	**DetailsandAcceptance Limits**
Objective	Estimation of selected anticancer agents
SelectionofTargetAnalyte	Gemcitabine, Methotrexate (Separate dosage forms)
Selection of Analytical Method	RP-HPLC
Specificity	There should be no interference of placebo in determination of Gemcitabine and Methotrexate.
Accuracy	98-102%
Precision	% RSD <2%
Resolution	>2
Number of Theoretical Plates	>2000
Tailing Factor	<2

Table 2 Risk Assessment for CMAs and CMPs for HPLC method development for Gemcitabine

Potential Risk Factor	S	O	D	RPN	Risk Ranking	Remarks
% Buffer	5	4	3	60	High	Affects retention time, peak area, peak shape. Mobile phase composition is critical to the peak separation, it needs to be optimized based on various trials.
Buffer pH	4	4	2	32	High	Based on the pKa of the drug, the pH range is selected. It needs to be optimized for proper peak retention. Affects retention time, peak area.
Flow Rate	4	3	2	24	Moderate	Flow rate need to be optimized for the proper peak separation. Affects retention time, peak shape.
Column oven temperature	2	2	3	12	Moderate	Direct controls present, and variation can be measured easily. Does not affect the responses significantly.
Injection volume	2	1	3	6	Low	Injector calibration is performed periodically.
Detection wavelength	3	1	1	3	Low	The detection wavelength is selected based on the spectrum of the drug. It can be set accordingly in HPLC system. Periodic verification of detector is performed.
Solubility	3	1	1	3	Low	Solubility can be checked visually and also the direct detection methods are present. The drug is easily soluble in the given mobile phase.
Purity	3	1	1	3	Low	The COA from the supplier is present.
Pump	3	1	1	3	Low	Pump calibration can be performed.

Column	3	1	1	3	Low	Column calibration can be performed.
Humidity	2	1	1	2	Low	Temperature can be measured and recorded using thermometer.
Temperature	1	1	1	1	Low	Humidity can be measured and recorded using hygrometer.
Analyst	4	3	2	24	Moderate	Covered in robustness
Detector	3	1	2	6	Low	[i]

Potential Risk Factor	S	O	D	RPN
% Buffer	5	4	3	60
Buffer pH	4	4	2	32
Flow Rate	4	3	2	24
Column oven temperature	2	2	3	12
Injection volume	2	1	3	6
Detection wavelength	3	1	1	3
Solubility	3	1	1	3
Purity	3	1	1	3
Pump	3	1	1	3
Column	3	1	1	3
Humidity	2	1	1	2
Temperature	1	1	1	1
Analyst	4	3	2	24
Detector	3	1	2	6

Table 3 Risk Assessment for CMAs and CMPs for HPLC method development for Methotrexate[1]

Potential Risk Factor	**S**	**O**	**D**	**RPN**	**Risk Ranking**	**Remarks**
% Buffer	5	4	3	60	High	Affects retention time, peak area, peak shape. Mobile phase composition is critical to the peak separation, it needs to be optimized based on various trials.
Buffer pH	5	4	2	40	High	Based on the pKa of the drug, the pH range is selected. The pKa of the drug is critical here, because the drug is soluble in mobile phase at

[1]RPN = Severity X Occurrence X Detectability

Scale for RPN

0 to 8: Low risk
8 to 27: Medium risk
28 to 64: High risk
65 to 125: Critical risk

RPN Scoring Criteria:

1: Not severe; unlikely occurrence; immediately detectable (e.g., pH meter/paper).
2: Slightly severe; 0 to 20% occurrence; highly detectable (detection methods present).
3: Severe; likely, 20% to 60% occurrence; detectable (e.g., direct/indirect methods with multiple steps).
4: Highly severe; highly likely, 60% to 80% occurrence; hard to detect.
5: Critical; certain (>80%) occurrence; not detectable.

						particular pH. It needs to be optimized for proper peak retention. Affects retention time, peak area.
Flow Rate	4	3	2	24	Moderate	Flow rate need to be optimized for the proper peak separation. Affects retention time, peak shape.
Column oven temperature	2	2	3	12	Moderate	Direct controls present, and variation can be measured easily. Does not affect the responses significantly.
Injection volume	2	1	3	6	Low	Injector calibration is performed periodically.
Detection wavelength	3	1	1	3	Low	Covered in robustness also. The detection wavelength is selected based on the spectrum of the drug. It can be set accordingly in HPLC system. Periodic verification of detector is performed.
Solubility	4	1	1	4	Low	The solubility is severe risk factor, but chances of occurrence of failure to achieve solubility are less. The mobile phase pH is maintained based on drug solubility. Solubility can be checked visually and also the direct detection methods are present.
Purity	3	1	1	3	Low	The COA from the supplier is present.
Pump	3	1	1	3	Low	Pump calibration can be performed.
Column	3	1	1	3	Low	Column calibration can be performed.
Humidity	2	1	1	2	Low	Temperature can be measured and recorded using thermometer.
Temperature	1	1	1	1	Low	Humidity can be measured and recorded using hygrometer.
Analyst	4	3	2	24	Moderate	Covered in robustness.
Detector	3	1	2	6	Low	Detector can be calibrated and validated periodically. Direct controls are present. If detector is not proper, the HPLC system cannot be used.

Table 4 Box Behnken Design for the Gemcitabine

	Factor 1	Factor 2	Factor 3	Response 1	Response 2
Run	A: Buffer	B: pH	C: Flow Rate	Number of Theoretical Plates of Gemcitabine peak	Asymmetry Factor of Gemcitabine peak
	%		ml/min		
1	96	6.4	1	5418	1.73
2	98	6.5	1.2	5476	1.68
3	97	6.4	1.2	5451	1.69
4	97	6.5	1	5875	1.67
5	97	6.5	1	5849	1.61
6	97	6.4	0.8	5461	1.66
7	97	6.5	1	5775	1.64
8	96	6.5	0.8	5421	1.72
9	96	6.6	1	5456	1.70
10	97	6.5	1	5916	1.64
11	97	6.5	1	5874	1.62
12	98	6.5	0.8	5436	1.69
13	96	6.5	1.2	5411	1.75
14	97	6.6	1.2	5416	1.69
15	98	6.6	1	5441	1.68
16	97	6.6	0.8	5426	1.71
17	98	6.4	1	5406	1.70

Table 5 P-Values for the Individual, Squared and combined effects of CMPs on CMAs for Gemcitabine

	Intercept	**A**	**B**	**C**	**AB**	**AC**	**BC**	**A^2**	**B^2**	**C^2**
TP	5857.8	6.625	0.375	1.25	-0.75	12.5	3.04436E-14	**-215.025**	**-212.525**	**-206.775**
p-values		0.6994	0.9825	0.9416	0.9752	0.6080	1.0000	**< 0.0001**	**< 0.0001**	**< 0.0001**
AF	1.636	-0.01875	1.83613E-16	0.00375	0.0025	-0.01	-0.0125	**0.0445**	0.022	**0.0295**
p-values		0.0543	1.0000	0.6583	0.8339	0.4129	0.3126	**0.0054**	0.0902	**0.0337**

Table 6 Box Behnken Design for the Methotrexate

	Factor 1	Factor 2	Factor 3	Response 1	Response 2
Run	A: Buffer	B: pH	C: Flow Rate	Number of Theoretical Plates of Methotrexate peak	Asymmetry Factor of Methotrexate peak
1	90	6	1.3	6399	1.64
2	94	5.5	1.2	6290	1.69
3	92	5.5	1.3	6632	1.53
4	92	6	1.2	6723	1.54
5	92	6	1.4	6767	1.48
6	92	5	1.4	6476	1.59
7	92	5	1.2	6541	1.55
8	90	5	1.3	6170	1.73
9	94	5	1.3	6290	1.69
10	94	5.5	1.4	6476	1.61
11	92	5.5	1.3	6618	1.52
12	94	6	1.3	6363	1.67
13	92	5.5	1.3	6600	1.54
14	92	5.5	1.3	6526	1.57
15	92	5.5	1.3	6648	1.51
16	90	5.5	1.4	6158	1.8
17	90	5.5	1.2	6247	1.78

Table 7 P-Values for the Individual, Squared and combined effects of CMPs on CMAs for Methotrexate

	Intercept	**A**	**B**	**C**	**AB**	**AC**	**BC**	**A^2**	**B^2**	**C^2**
Number of Theoretical Plates	6604.8	**55.625**	**96.875**	9.5	-39	68.75	27.25	**-316.65**	17.35	4.6
p-values		**0.0312**	**0.0023**	0.6602	0.2246	0.0512	0.3829	**< 0.0001**	0.5624	0.8765
Asymmetry Factor	1.534	**-0.03625**	-0.02875	-0.01	0.0175	-0.025	-0.025	**0.16425**	-0.01575	0.02175
p-values		**0.0389**	0.0842	0.5068	0.4154	0.2561	0.2561	**< 0.0001**	0.4504	0.3062

Table 8 Detection and Quantitation Limits for the HPLC method development of Gemcitabine and Methotrexate

Drug	**UV LOD**	**UV LOQ**	**HPLC LOD**	**HPLC LOQ**
Gemcitabine	0.0384	0.1165	2.0523	6.2192
Methotrexate	0.5495	1.6651	3.9160	11.8666

Table 9 Results for analytical method validation of Gemcitabine and Methotrexate

Test	**Details**	**Acceptance Criteria**	**Gemcitabine**		**Methotrexate**		**Remark**
Linearity	Range	-	50-150 ug/ml		50-150 ug/ml		
	Correlation Coefficient (r^2)	>0.99	1.000		0.9996		Pass
Accuracy % Recovery (Mean ± SD)	n=9 (triplicates of std addition 50%, 75% and 150%)	% recovery: 98-102%	100.15±0.74		100.60 ±0.96		Pass
Method Precision	N=6	RSD <2%	0.67		0.51		Pass
Intermediate Precision	N=12	RSD <2%	0.01		0.01		Pass
Robustness (RSD)	Change in Column lot (n-2)	RSD <2%	1.22		0,10		Pass
	Change in flow rate (± 0.2 ml/min)	RSD <2%	1.72		0.67		Pass
	Change in wavelength (± 0.2 nm)	RSD <2%	0.92		0.93		Pass
	Change in mobile phase pH (± 2%)	RSD <2%	1.12		1.59		Pass
Stability of analytical solution		% RSD	0.76		0.32		Pass
			Assay (%)		Assay (%)		Pass
			0 Hr	101.03	0 Hr	99.97	Pass
			12 Hr	101.66	12 Hr	99.15	Pass
			24 Hr	101.7	24 Hr	100.67	Pass
			36 Hr	100.19	36 Hr	100.04	Pass
			48 Hr	100.95	48 Hr	101.08	Pass