RESEARCH PAPER

Analytical Qbd Approach For Development And Validation Of Stability-Indicating Rp-Hplc Method For Related Substances In Leuprolide Acetate Depot Formulations

Vaibhav R. Mandale [1]*, Dr. Sampada D. Dalvi [1], Dr. Avinash R. Tekade [2]

[1]*Department of Pharmaceutical Chemistry, Marathwada Mitra Mandal's College of Pharmacy, S.No.4/17, Sector No.34, Off Kalewadi Phata-Pimpri Road, Thergaon, Pune, Maharashtra, India, 411033.*
[2] *Department of Pharmaceutics, Marathwada Mitra Mandal's College of Pharmacy, S.No.4/17, Sector No.34, Off Kalewadi Phata-Pimpri Road, Thergaon, Pune, Maharashtra, India, 411033*

ABSTRACT
Objective:
The study aimed to develop and validate a stability-indicating RP-HPLC method for the estimation of related substances in Leuprolide acetate depot formulations using an Analytical Quality by Design (AQbD) approach.
Materials and Methods: Critical method parameters such as flow rate, column oven temperature, and mobile phase pH were screened and optimized using a Central Composite Design. Preliminary method development included risk assessment to identify critical analytical attributes. The optimized method was validated as per ICH guidelines for specificity, linearity, accuracy, precision, robustness, and stability.
Results and Discussion: Optimization studies revealed that flow rate, temperature, and pH significantly influenced resolution and peak tailing. The method demonstrated acceptable resolution and peak symmetry, with satisfactory ANOVA values confirming the suitability of the design model. Validation results established the method as specific, precise, linear, accurate, robust, and stable, fulfilling ICH acceptance criteria.
Conclusion: The developed RP-HPLC method, guided by AQbD principles, proved to be reliable and stability-indicating for quantification of related substances in Leuprolide acetate depot formulations. This method offers a robust tool for routine quality control and stability studies in pharmaceutical analysis..
Keywords: Leuprolide acetate; Analytical Quality by Design (AQbD); RP-HPLC; Related substances; Stability-indicating method; Method validation
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INTRODUCTION

Leuprolide acetate, a synthetic nonapeptide analog of gonadotropin-releasing hormone (GnRH), is widely prescribed for the management of hormone-dependent conditions such as prostate cancer, endometriosis, uterine fibroids, and central precocious puberty. It exerts its therapeutic effect by initially stimulating and subsequently downregulating the pituitary release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), leading to suppression of gonadal steroidogenesis. For long-term therapy, Leuprolide acetate is formulated as depot injections with extended-release profiles, typically administered over one, three, or six months. Owing to its peptide nature and parenteral delivery, the stability and purity of Leuprolide acetate are critical for therapeutic safety and efficacy. Even trace levels of impurities or degradation products may compromise clinical outcomes and increase toxicity risks, highlighting the necessity for highly sensitive and selective analytical methods.

The determination of related substances in peptide-based depot formulations presents unique challenges due to their complex physicochemical properties, susceptibility to degradation, and formulation variability. High-performance liquid chromatography (HPLC), particularly reverse-phase (RP-HPLC), is the most widely applied technique for impurity profiling and stability assessment in peptides, owing to its high resolution, reproducibility, and adaptability. However, conventional method development often involves trial-and-error experimentation, which can be labor-intensive, less systematic, and unable to consistently address variability in analytical outcomes.

To overcome these limitations, the concept of Analytical Quality by Design (AQbD) has emerged as a systematic and science-based approach for developing robust analytical methods. AQbD emphasizes risk assessment, identification of critical method parameters (CMPs), and establishing design space to ensure method performance remains reliable under varying conditions. This approach not only enhances method robustness and efficiency but also complies with regulatory expectations set by ICH Q8(R2), Q9, Q10, and Q14 guidelines. In peptide analysis, where method sensitivity and robustness are paramount, AQbD provides a structured framework to achieve consistent performance.

Previous studies on peptide formulations have shown the importance of employing design of experiments (DoE) and

**Author for Correspondence: mandalevaibhav0@gmail.com*

statistical modeling, such as Central Composite Design (CCD), to systematically evaluate the influence of chromatographic variables on resolution, peak symmetry, and sensitivity. These tools enable optimization of method conditions while minimizing experimental runs, thereby improving efficiency and reducing development costs. Despite the significance of Leuprolide acetate in clinical use, limited literature exists on the application of AQbD principles for stability-indicating RP-HPLC method development specifically targeting its related substances in depot formulations.

Therefore, the present study was undertaken with the objective of developing and validating a stability-indicating RP-HPLC method for the determination of related substances in Leuprolide acetate depot formulations using an AQbD framework. The method was optimized through risk assessment, DoE-based modeling, and statistical analysis to identify critical variables influencing chromatographic performance. The validated method was further assessed in line with ICH guidelines to ensure specificity, precision, accuracy, linearity, robustness, and stability. This systematic approach not only assures regulatory compliance but also enhances the reliability of impurity profiling in long-acting peptide depot systems.

MATERIAL AND METHODS:

Materials:

Analytical and HPLC grade reagents were used, including sodium hydroxide and orthophosphoric acid (Merck), triethylamine and n-propyl alcohol (Spectrochem), dichloromethane (Qualigens), acetonitrile (Merck, Rankem), and Milli-Q water. Hydranal solution AG-oven (Honeywell) was used for Karl Fischer analysis. Leuprolide acetate USP WS (assay 100.4%) and impurity standards, D-Ser leuprolide, D-His leuprolide, L-Leu6–leuprolide and O-acetyl-L-ser leuprolide were used within their validity periods. All materials complied with ICH guidelines for the development and validation of a stability-indicating RP-HPLC method for related substances in leuprolide acetate depot formulations (11.25 mg, 22.5 mg, and 30 mg).

Chromatographic Conditions:

The related substances of leuprolide acetate depot formulations were analyzed using an isocratic RP-HPLC method with UV detection. Separation was performed on a YMC ODS-A (100 × 4.6 mm, 3 µm) column, with ACE Excel 3 C18 (100 × 4.6 mm, 3 µm) validated as an equivalent column. The mobile phase was delivered at a flow rate of 1.0 mL/min, with the column maintained at 30 °C and the sample cooler at 5 °C. The injection volume was 25 µL, and detection was carried out at 220 nm. Needle wash (acetonitrile:water, 50:50 v/v) and seal wash (acetonitrile:water, 10:90 v/v) were applied to ensure system cleanliness. The total run time was 120 minutes. Based on column equivalency studies, YMC ODS-A was preferred for routine analysis, while ACE Excel C18 was employed as an alternative in cases of peak shape variability.

Table 1: Chromatographic conditions for HPLC analysis of related substances in leuprolide acetate depot formulations

Parameter	Condition
Mode	Isocratic
Column	YMC ODS-A (100 × 4.6 mm, 3 µm, Part No. AA12S03-1046WT) Equivalent: ACE Excel 3, C18 (100 × 4.6 mm, 3 µm, Part No. EXL-111-1046U)
Wavelength	220 nm
Column oven temperature	30 °C
Sample cooler temperature	5 °C
Flow rate	1.0 mL/min
Injection volume	25 µL
Run time	120 min
Needle wash	Acetonitrile:Water (50:50 v/v)
Seal wash	Acetonitrile:Water (10:90 v/v)

Preparation of Solutions and Sample Processing:

Preparation of Solution A: Triethylamine (21 mL) was transferred into 800 mL of water, mixed thoroughly, and the pH was adjusted to 3.0 ± 0.05 using orthophosphoric acid. The volume was made up to 1000 mL with water, mixed well, and filtered through a 0.45 µm membrane filter.

Preparation of Solution B: A mixture of acetonitrile and n-propyl alcohol (300:200, v/v) was prepared in a suitable beaker and mixed well.

Mobile Phase: Solution A and Solution B were mixed in a ratio of 850:150 (v/v), sonicated for 5 min to degas, and used as the mobile phase. The mobile phase was stable for up to 168 hours.

Diluents and Blank: Dichloromethane was used as diluent-1, while pH 3.0 buffer (Solution A) was used as diluent-2. The blank was prepared by mixing 10 mL each of diluent-1 and diluent-2 in a 20 mL volumetric flask, followed by separation of the upper aqueous layer for injection.

Auxiliary Solutions: A 1N sodium hydroxide solution was prepared by dissolving 4.0 g of sodium hydroxide in 100 mL of water, while 1M phosphoric acid was prepared by diluting 6.8 mL of orthophosphoric acid to 100 mL with water.

Resolution Solution (100 ppm): Leuprolide acetate WS (50.0 mg) was dissolved in 30 mL water, sonicated, and diluted to 50 mL. One milliliter of this solution was further diluted to 10 mL, followed by treatment of 5 mL with 100 µL of 1N sodium hydroxide, heating at 60 °C for 10 min, cooling, and neutralization with 50 µL of 1M phosphoric acid.

Sample Preparation: For 11.25 mg/unit and 30 mg/unit (pediatric) formulations, 240.0 mg of sample was dispersed in 20 mL of diluent-1, vortexed for 5 min, followed by the addition of 20 mL diluent-2 and vortexing. The biphasic mixture was transferred into a separating funnel, and the aqueous layer (diluent-2) was collected for analysis, while the organic layer (diluent-1) was discarded.

Dichloromethane was used only for extraction and not considered in quantification.

Impurity Standards: Impurity stock solution (100 ppm each) was prepared by dissolving 1.0 mg of D-Ser Leuprolide, D-His Leuprolide, L-Leu Leuprolide, and O-acetyl Leuprolide in 10 mL water. The impurity identification solution was prepared by spiking 10.0 mg of leuprolide acetate WS with 1.0 mL impurity stock solution and diluting to 10 mL with water, yielding 10 ppm of each impurity with 1000 ppm of leuprolide acetate.

Analytical Procedure

The chromatographic system was set up as per the described instrumental conditions. The column was equilibrated with the mobile phase until a stable baseline was achieved. Blank, resolution solution, impurity identification solution, and sample solution were injected sequentially into the HPLC system for analysis.

System Suitability

System suitability was evaluated using blank, resolution, and impurity identification solutions. The system was acceptable when the resolution between D-His leuprolide and leuprolide acetate was ≥1.5 and the USP tailing factor of the leuprolide peak was ≤2.0. Impurity retention times were verified with the identification solution, while placebo peaks (RRT ~0.041 and 0.079) and unknown impurities <0.1% were disregarded.

Acceptance limits included: acetyl leuprolide ≤1.0%; D-Ser, D-His, and L-Leu6 impurities ≤0.5% at release and ≤1.0% at shelf life; any unspecified impurity ≤0.5%; total impurities ≤3.0% (release) and ≤5.0% (shelf life). In this study, the resolution was 2.8 and the tailing factor was 0.9, confirming system performance.

Specificity

Specificity was established by injecting blank, placebo, known impurities, spiked samples, and resolution solutions. No interference was observed at the analyte retention times. All impurities were baseline separated from the drug peak and from each other. Peak purity analysis confirmed homogeneity, and relative retention times of impurities matched the method specifications. Thus, the method was specific for related substance determination in leuprolide acetate depot formulations.

Precision

Repeatability was assessed by analyzing six replicate spiked samples. Acceptance criteria were: %RSD ≤25% (0.05–0.10%), ≤15% (0.11–0.50%), ≤10% (0.51–1.0%), and ≤5% (>1.0%), with total impurities NMT 15%. For the 11.25 mg/unit formulation, impurities detected were D-His leuprolide (0.0731%, BLOQ) and an unspecified impurity (0.1372%), giving total impurities of ~0.1%, well within limits.

Intermediate precision

Intermediate precision was evaluated by a second analyst under varied conditions, with results meeting the same acceptance limits, demonstrating reproducibility of the method.

Limit of Detection (LOD), Limit of Quantification (LOQ), and Precision at LOQ Level

The LOD and LOQ were determined using the signal-to-noise (S/N) ratio approach, by comparing analyte responses at low concentrations against blank injections. The LOD was defined as the concentration yielding an S/N ratio of at least 3, while the LOQ corresponded to an S/N ratio of at least 10. Once established, LOD and LOQ solutions were prepared and injected into the chromatographic system.

Precision at the LOQ was evaluated by injecting the LOQ solution in six replicates and the LOD solution in triplicate. The %RSD of LOQ replicates was required to be ≤15.0, while the S/N ratio for each replicate had to meet the acceptance limit of ≥10:1. LOD and LOQ for known impurities were determined using individual impurity standards, whereas for unknown impurities, the analyte standard was used.

Linearity

Method linearity was assessed across a concentration range from the LOQ up to 150% of the target level for leuprolide and its known impurities. Calibration curves were constructed by plotting peak area against concentration, and regression analysis was used to calculate slope, y-intercept, correlation coefficient, and relative response factors (RRFs). The method was considered linear when the correlation coefficient was ≥0.99 and the y-intercept deviation was within ±10%.

Accuracy

Accuracy was determined by spiking known impurities into the sample matrix at LOQ, 50%, 100%, and 150% of the target concentration. Each level was analyzed in triplicate, and recovery was expressed as a percentage of the expected value. The mean recovery, %RSD at each level, overall %RSD, and 95% confidence intervals were calculated. The acceptance limits were: 70–130% recovery at the LOQ, 80–120% recovery at other levels, and ≤10% RSD across replicates.

Robustness

Robustness was studied by introducing deliberate variations in chromatographic conditions while analyzing blank, placebo, standard, and spiked samples. Parameters tested included: flow rate (±10%), column temperature (30 °c ± 5 °c), detection wavelength (220 nm ± 2 nm), buffer ph (3.0 ± 0.2), and organic content in the mobile phase (±10% relative to the optimized ratio of 850:150, v/v). The method was considered robust when system suitability parameters were met and the %rsd for impurity levels (known and unknown) remained within acceptance criteria.

Stability of analytical solutions

The stability of analytical solutions was assessed at room temperature (approximately 25 °c), and refrigerated conditions (5 ± 3 °c). Solutions including resolution, impurity identification, placebo, sample, and spiked sample preparations were analyzed at initial and subsequent intervals up to 168 hours. The evaluation involved monitoring system suitability, checking for new peaks above the LOQ, and assessing variations in impurity levels under each storage condition. Acceptance criteria required compliance with system suitability parameters, absence of new peaks above the LOQ, and defined limits for

differences in impurity levels compared with the initial value.

Stability of mobile phase

The stability of the mobile phase was studied over time by both physical inspection and chromatographic testing. Physically, the mobile phase was examined for clarity, color, turbidity, and presence of any precipitation or particulate matter. Chromatographic evaluation included monitoring retention times of known impurities from spiked samples at different intervals and comparing them with the initial values. Acceptance criteria specified that the mobile phase should remain physically stable and that retention times of impurities should be consistent throughout the study period.

Range of the analytical method

The analytical range was established as the interval between the lowest and highest concentrations of analyte that could be quantified with acceptable accuracy, precision, and linearity. The range was determined using data generated from linearity, accuracy, and precision experiments. Acceptance criteria defined that the validated range must be supported by compliance with all three validation parameters.

RESULTS & DISCUSSION:

Preliminary method development

Preliminary trials were conducted to establish suitable chromatographic conditions for the determination of related substances in Leuprolide acetate. Various mobile phase compositions, flow rates, and pH levels were evaluated to achieve sharp peak shape, adequate resolution, and acceptable tailing. Based on these trials, critical parameters such as flow rate, column oven temperature, and mobile phase pH were identified for further optimization using a design of experiments approach.

Risk Assessment

Critical method parameters such as flow rate, column oven temperature, and mobile phase pH were identified through risk assessment as the most influential factors that could potentially affect chromatographic performance. These variables were selected for systematic evaluation to ensure robustness and reliability of the HPLC method. By narrowing the focus to these parameters, unnecessary variability was minimized, ensuring that the method consistently met system suitability requirements.

Central Composite Design

To study the impact of these critical factors, a Central Composite Design (CCD) was applied. Seventeen experimental runs were conducted with flow rate (0.9–1.1 ml/min), temperature (25–35 °C), and pH (2.8–3.2) as independent variables, while USP resolution and tailing factor were evaluated as responses. The results indicated stable chromatographic performance across all experimental conditions. Although ANOVA results showed no statistically significant effect of the studied factors, the method demonstrated robustness within the design space, confirming its suitability for routine analysis of related substances in Leuprolide acetate.

Chromatographic Factors Selection

The optimization study employed a Central Composite Design (CCD) with three independent factors: flow rate (0.9–1.1 mL/min), column oven temperature (25–35 °C), and mobile phase pH (2.8–3.2). These variables were chosen as they directly influence peak resolution and symmetry in HPLC analysis of related substances in Leuprolide acetate.

Table 2: Chromatographic factors and level for Central Composite Design

Independent Factors	Low level (-1)	Medium level (0)	High level (+1)
X1: Flow Rate	0.9	1.0	1.1
X2: Column Oven Temperature	25	30	35
X3: Mobile Phase- pH	2.8	3.0	3.2

Table 3: Central Composite Design optimization layout

Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2
	A:Flow	B: Temp.	C:pH	USP Resolution	USP Tailing
	mL/min	°C	pH		
1	1	25	2.8	2.1	1.1
2	1.1	30	3.2	2.3	1.2
3	0.9	30	2.8	2.2	0.9
4	0.9	35	3	2.1	1
5	0.9	30	3.2	2.2	1.2
6	1	30	3	2.1	1.1
7	0.9	25	3	2.4	1.2
8	1	25	3.2	2.1	1.1
9	1	35	3.2	2.1	1.1
10	1	30	3	2.3	1.2

11	1.1	35	3	2.4	0.9
12	1.1	30	2.8	2.1	1
13	1	30	3	2.3	1
14	1.1	25	3	2.4	1.1
15	1	35	2.8	2.3	1.2
16	1	30	3	2.2	1.1
17	1	30	3	2.1	1.2

Experimental Design and Responses

Seventeen experimental runs were generated using CCD to study the effect of factor variations on two responses: USP resolution (Y1) and USP tailing factor (Y2). The observed USP resolution ranged from 2.1 to 2.4, while tailing values varied between 0.9 and 1.2. These results indicate that all experimental conditions maintained acceptable chromatographic performance, with minor differences attributed to factor interactions.

Statistical Analysis (ANOVA):

ANOVA results showed relatively low R^2 values (0.4192 for resolution and 0.2329 for tailing), indicating limited predictive power of the model. Adjusted R^2 values (0.0640 and 0.0327, respectively) further suggest that factor contributions were not statistically significant. P-values (>0.05) confirmed that none of the independent factors had a significant effect on responses within the studied design space. However, coefficients of variation (5.34% for resolution and 9.38% for tailing) were within acceptable limits, confirming method robustness.

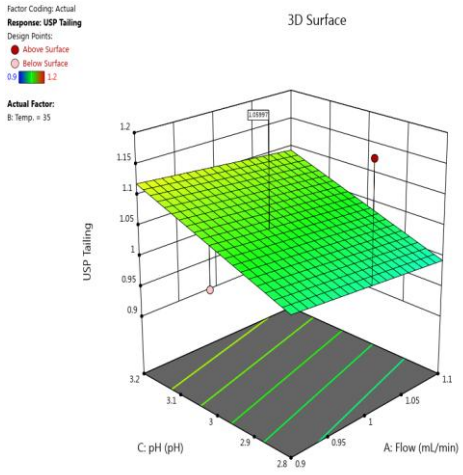
The CCD-based optimization demonstrated that variations in flow rate, temperature, and mobile phase pH within the tested range produced consistent chromatographic performance. Although statistical analysis indicated no significant factor influence, the method remained stable with adequate resolution and tailing, supporting its reliability for routine application.

Table 4: ANOVA results for each CAAs

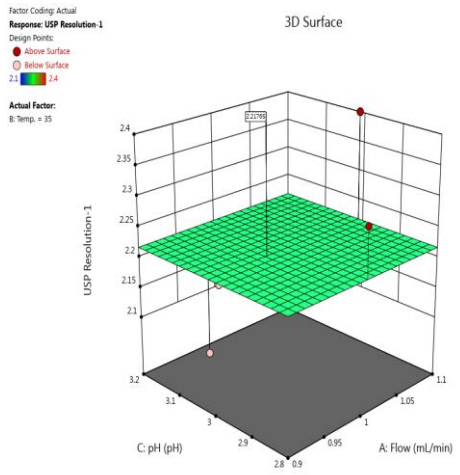
ANOVA Parameters	Y1: USP Resolution	Y2: USP Tailing
R- square	0.4192	0.2329
Adjusted R- square	0.0640	0.0327
F- Value	1.55	1.73
P- Value	0.3621	0.3142
C.V. %	5.34	9.38

Identification of optimum method conditions

The optimization process indicated that the most suitable chromatographic conditions were achieved at a flow rate of 1.0 ml/min, a column oven temperature of 30 °c, and a mobile phase pH of 3.0. these conditions provided consistent peak shape, acceptable USP resolution, and controlled tailing, confirming their suitability for routine analysis of related substances in leuprolide acetate.

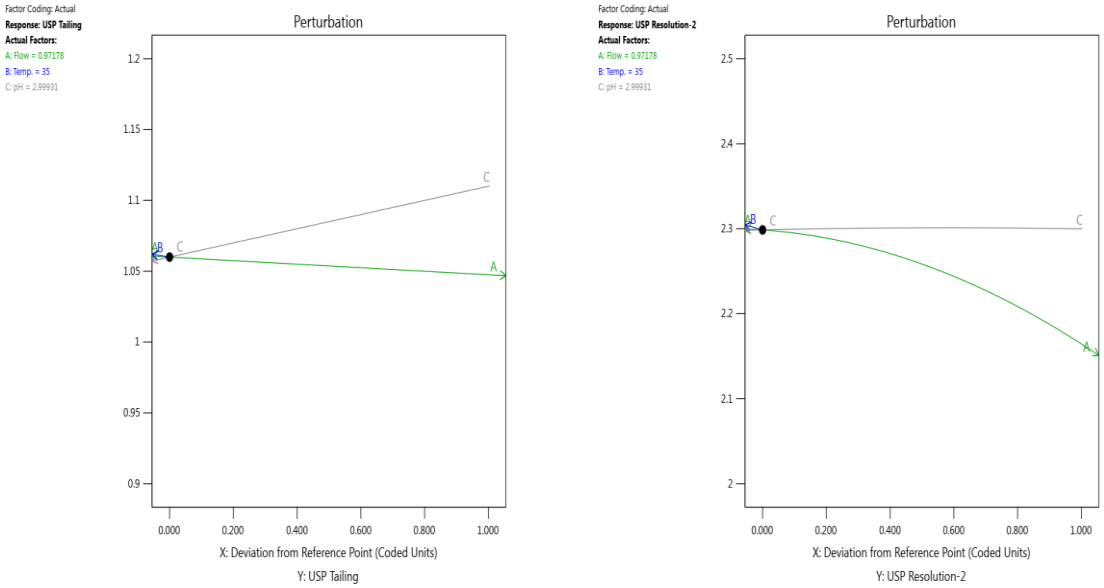


(A)



(B)

Figure 1: 3D plots (A) Effect of PH and Flow (mL/min) of mobile phase on USP Tailing of leuprolide Acetate. (B) Effect of PH and Flow (mL/min) of mobile phase on USP Resolution-1 Count of leuprolide Acetate.



(A) **(B)**

Figure 2: Perturbation plot showing (A) the effect of independent factor on USP Tailing of leuprolide Acetate. (B) the effect of independent factor on USP Resolution-1 Count of leuprolide Acetate.

Analytical method validation:

System Suitability

System suitability was assessed by injecting blank, resolution, and impurity identification solutions into the chromatographic system. The evaluation focused on the resolution between D-His Leuprolide and Leuprolide acetate, as well as the USP tailing factor for the Leuprolide peak. The obtained resolution was 2.8, which exceeds the minimum requirement of 1.5, while the USP tailing factor was 0.9, well below the acceptable limit of 2.0. These results confirm that the chromatographic system is reliable and suitable for the determination of related substances in Leuprolide acetate depot suspension formulations (11.25 mg, 22.5 mg, and 30 mg, pediatric) by HPLC.

Table 5: System Suitability Parameters

Parameter	Acceptance Criteria	Result
Resolution (D-His Leuprolide vs. Leuprolide acetate)	≥ 1.5	2.8
USP Tailing Factor (Leuprolide peak)	≤ 2.0	0.9

Specificity

The specificity of the developed HPLC method was evaluated to confirm its ability to selectively quantify Leuprolide acetate and its related impurities without interference from blank, placebo, or diluents. For this purpose, blank, diluent for identification, resolution solution, impurity identification solution, placebo, identification solutions of individual impurities, sample, and spiked sample solutions were analyzed. The criteria included clear separation of analyte peaks from placebo and blank, absence of interfering peaks, compliance with peak purity requirements, and comparability of relative retention times (RRTs) with the established method values.

Retention Times of Analytes

Table 6a summarizes the retention times of Leuprolide and its impurities under different test conditions. No peaks were detected in blank, diluent, or placebo solutions. Retention times were consistent in both sample and spiked sample injections.

Table 6a: Retention times of analytes under different solutions

Solution Name	Leuprolide	Acetyl-Leuprolide	D-Ser-Leuprolide	D-His-Leuprolide	L-Leu6-Leuprolide
Blank	ND	ND	ND	ND	ND
Diluent (ID)	ND	ND	ND	ND	ND
Resolution solution	45.7	ND	35.8	40.7	ND
Placebo	ND	ND	ND	ND	ND
Impurity ID solution	44.0	64.4	35.6	40.3	50.4
Acetyl-Leuprolide ID	ND	65.4	ND	ND	ND

D-Ser-Leuprolide ID	ND	ND	36.2	ND	ND
D-His-Leuprolide ID	ND	ND	ND	41.2	ND
L-Leu6-Leuprolide ID	ND	ND	ND	ND	53.5
Sample	44.4	ND	ND	40.7	ND
Spiked sample	44.5	65.1	35.9	40.6	52.9

Peak Purity

The peak purity study confirmed that all analyte peaks were spectrally pure, with purity angles lower than the corresponding purity thresholds in both as-such and spiked samples (Table 6b).

Table 6b: Peak purity results

Name	As-such sample (Purity angle / threshold)	Spiked sample (Purity angle / threshold)
Leuprolide	0.108 / 0.261	0.096 / 0.257
Acetyl-Leuprolide	ND	1.519 / 2.113
D-Ser-Leuprolide	ND	0.757 / 1.289
D-His-Leuprolide	24.722 / 32.960	0.902 / 1.320
L-Leu6-Leuprolide	ND	1.219 / 1.691

Retention Time and Relative Retention Time

Retention times and RRTs from spiked samples are provided in table 6c, showing consistency with the method specifications.

Table 6c: Retention time (RT) and relative retention time (RRT)

Name	RT (min)	RRT
Leuprolide	44.5	1.00
Acetyl-Leuprolide	65.2	1.46
D-Ser-Leuprolide	35.9	0.81
D-His-Leuprolide	40.7	0.91
L-Leu6-Leuprolide	52.9	1.19

Impurity Profile in As-Such Samples

Table 6d presents impurity levels detected in as-such samples. Only D-His-Leuprolide and unspecified impurities were observed at trace levels, while other impurities were not detected.

Table 6d: Impurity results from as-such sample solution

Impurity	% Impurity
Acetyl-Leuprolide	ND
D-Ser-Leuprolide	ND
D-His-Leuprolide	0.0601 (≈0.1, BLOQ)
L-Leu6-Leuprolide	ND
Any unspecified impurity	0.1646 (≈0.2)
Total impurity	≈0.2

Impurity Profile in Spiked Samples

In spiked samples, all impurities were detected and quantified at their respective levels, as presented in Table 6e.

Table 6e: Impurity results from spiked sample solution

Impurity	% Impurity
Acetyl-Leuprolide	1.0563 (≈1.1)
D-Ser-Leuprolide	1.0480 (≈1.0)
D-His-Leuprolide	1.1330 (≈1.1)
L-Leu6-Leuprolide	0.9211 (≈0.9)
Any unspecified impurity	0.0786 (≈0.1, BLOQ)
Total impurity	4.1584 (≈4.2)

The blank and placebo solutions showed no interference at the retention times of principal or impurity peaks. All

known impurities were well resolved from the main peak and from each other. Peak purity confirmed that analyte peaks were spectrally pure, while RRT values were consistent with method specifications.

Precision

A. Method precision

Method precision was evaluated by analyzing six replicate preparations of sample solutions spiked with known impurities at specification levels. The precision of the method was assessed by calculating mean, standard deviation (SD), relative standard deviation (%RSD), and 95% confidence intervals for each impurity. As such samples (without spiking) were also analyzed to verify that impurity levels remained within specification limits.

For the 11.25 mg/unit formulation, the as such sample showed no detectable impurities except D-His Leuprolide (0.073%, below quantitation) and an unspecified impurity (0.137%), with a total impurity of ~0.1%. The spiked samples demonstrated consistent recovery of impurities with mean values of ~1.0% for Acetyl Leuprolide, D-Ser Leuprolide, and L-Leu6 Leuprolide, ~1.1% for D-His Leuprolide, and ~0.1% for unspecified impurity. The total impurity was ~4.3% with %RSD values ≤0.5% for all specified impurities, meeting acceptance limits.

For the 30 mg/unit formulation, the as such sample showed low impurity levels, with D-His Leuprolide at 0.052% (below quantitation) and an unspecified impurity at 0.167%, giving a total impurity of ~0.2%. In spiked samples, impurity recoveries were consistent, with mean values similar to the 11.25 mg unit: ~1.0% for Acetyl Leuprolide, D-Ser Leuprolide, and L-Leu6 Leuprolide, ~1.1% for D-His Leuprolide, and ~0.1% for unspecified impurity. The total impurity averaged ~4.3% with %RSD values ≤0.4%, again fulfilling acceptance requirements.

Table 7: Summary of Method Precision Results

Strength	Sample Type	Acetyl Leuprolide (%)	D-Ser Leuprolide (%)	D-His Leuprolide (%)	L-Leu6 Leuprolide (%)	Unspecified Impurity (%)	Total Impurity (%)	%RSD (Range)
11.25 mg	As such	ND	ND	0.073 (BLOQ)	ND	0.137	0.137 (~0.1)	–
11.25 mg	Spiked (n=6)	1.02 (~1.0)	1.04 (~1.0)	1.11 (~1.1)	0.99 (~1.0)	0.11 (~0.1)	4.26 (~4.3)	0.1–0.5
30 mg	As such	ND	ND	0.052 (BLOQ)	ND	0.167	0.167 (~0.2)	–
30 mg	Spiked (n=6)	1.03 (~1.0)	1.05 (~1.0)	1.11 (~1.1)	1.00 (~1.0)	0.14 (~0.1)	4.33 (~4.3)	0.1–0.4

Overall, the method demonstrated excellent repeatability, with %RSD values well within the acceptance criteria for all impurity levels.

The precision study confirms that the HPLC method is precise and reliable for quantifying related substances in Leuprolide acetate depot suspension formulations (11.25 mg, 22.5 mg, and 30 mg, pediatric).

B. Intermediate Precision

Intermediate precision was assessed by preparing and analyzing six replicate spiked samples along with as such samples for the 11.25 mg/unit formulation. Impurities were quantified and evaluated in terms of mean values, SD, %RSD, and 95% confidence intervals. Additionally, combined analysis of method precision and intermediate precision (ruggedness) was performed to assess reproducibility.

For the as such sample, impurities were minimal, with only D-His Leuprolide detected at trace levels (0.059%, below quantitation) and an unspecified impurity at ~0.14%, giving a total impurity content of ~0.1%.

In spiked samples, consistent recoveries of all impurities were observed, with mean values around 1.0% for Acetyl Leuprolide, ~1.1% for D-Ser and D-His Leuprolide, ~1.0% for L-Leu6 Leuprolide, and ~0.1% for unspecified impurity. The total impurity averaged ~4.4%. The %RSD values for all impurities were ≤0.5%, confirming excellent reproducibility.

When combining method precision and intermediate precision data, overall mean impurity levels were consistent with previous results (~1.0% for major impurities and ~0.1% for unspecified impurity), with total impurities averaging ~4.3%. The overall %RSD values remained within acceptance criteria (<2% for specified impurities and <5% for low-level impurities), demonstrating robustness of the method.

Table 8: Summary of Intermediate Precision and Overall Precision Results

Parameter	Acetyl Leuprolide (%)	D-Ser Leuprolide (%)	D-His Leuprolide (%)	L-Leu6 Leuprolide (%)	Unspecified Impurity (%)	Total Impurity (%)	%RSD (Overall)

As such (11.25 mg)	ND	ND	0.059 (BLOQ)	ND	0.141	0.141 (~0.1)	–
Spiked (n=6, 11.25 mg)	1.04 (~1.0)	1.07 (~1.1)	1.15 (~1.1)	1.01 (~1.0)	0.11 (~0.1)	4.38 (~4.4)	0.2–0.5
Overall (Method + Intermediate)	1.03 (~1.0)	1.06 (~1.1)	1.13 (~1.1)	1.00 (~1.0)	0.11 (~0.1)	4.32 (~4.3)	1.2–1.7

Intermediate precision results were within acceptance limits, confirming that random variations do not significantly impact the reproducibility of the HPLC method. The method is therefore rugged and precise for the quantification of related substances in Leuprolide acetate depot suspension (11.25 mg, 22.5 mg, and 30 mg, pediatric).

Limit of Detection and Limit of Quantification

A. LOD/LOQ Establishment by signal-to-noise ratio method:

The sensitivity of the method was determined using the signal-to-noise ratio approach. Analyte responses at low concentrations were compared against blank injections to establish the minimum detectable and quantifiable levels. A signal-to-noise ratio of at least 3 was considered for LOD determination, while values ≥10 confirmed LOQ levels. Once established, corresponding solutions were prepared and injected into the chromatographic system for confirmation.

The method demonstrated high sensitivity, with LOD values ranging between 0.1966–0.2516 ppm and LOQ values between 0.7909–1.0064 ppm. Relative to the sample concentration, the detection limits were approximately 0.0190–0.0242%, while quantitation limits were around 0.0761–0.0969%. Signal-to-noise ratios were well above acceptance limits, confirming reliability of detection and quantification.

Table 9a: Limit of Detection (LOD) Results

Name	Absolute conc. (ppm)	w.r.t. sample conc. (%)	USP S/N ratio
Acetyl Leuprolide	0.1977	0.0190	8
D-Ser Leuprolide	0.1970	0.0189	28
D-His Leuprolide	0.1966	0.0189	16
L-Leu6 Leuprolide	0.2011	0.0193	16
Leuprolide	0.2516	0.0242	17

Table 9b: Limit of Quantitation (LOQ) Results

Name	Absolute conc. (ppm)	w.r.t. sample conc. (%)	USP S/N ratio
Acetyl Leuprolide	0.7909	0.0761	30
D-Ser Leuprolide	0.7883	0.0759	70
D-His Leuprolide	0.7865	0.0757	60
L-Leu6 Leuprolide	0.8045	0.0775	57
Leuprolide	1.0064	0.0969	62

Precision at LOD and LOQ

The method's sensitivity was evaluated by establishing the limit of detection (LOD) and limit of quantification (LOQ) for Leuprolide acetate and its related impurities using the signal-to-noise (S/N) approach. LOD solutions were injected in triplicate and LOQ solutions in six replicates. Precision at the LOQ level was assessed by calculating mean peak area, %RSD, and confirming S/N ratios.

The results demonstrated that all analytes met the acceptance criteria, with LOD values showing adequate S/N ratios (>3) and LOQ replicates yielding %RSD well below the 15% threshold. This confirms that the method is highly sensitive and capable of accurately detecting and quantifying impurities even at very low concentration levels.

Table 10: LOD and LOQ Verification with Precision Results

Analyte	LOD Mean Peak Area	LOD Mean S/N	LOQ Mean Peak Area	LOQ Mean S/N	LOQ %RSD
Acetyl Leuprolide	7337	6	27292	30	9.4
D-Ser Leuprolide	8814	11	32586	61	5.2
D-His Leuprolide	7011	10	31206	54	3.7
L-Leu6 Leuprolide	7513	8	25413	36	7.3
Leuprolide	10108	12	40222	62	3.7

All impurities demonstrated acceptable S/N ratios at LOD levels and consistent %RSD values at LOQ levels, confirming that the HPLC method is precise, sensitive, and reliable for detecting and quantifying related substances in Leuprolide acetate depot suspension (11.25 mg, 22.5 mg, and 30 mg, pediatric).

Linearity

Linearity of the developed HPLC method was evaluated across a concentration range from LOQ to 150% of the sample concentration for Leuprolide acetate and its known impurities. Peak areas were plotted against concentrations to generate calibration curves, and regression parameters such as slope, intercept, correlation coefficient, %Y-intercept, residual sum of squares, and relative response factor (RRF) were determined.

All analytes exhibited strong linear relationships within the tested range. Correlation coefficients were consistently above 0.999, with %Y-intercept values well within ±10%, fulfilling the acceptance criteria. These findings confirm the linearity and reliability of the method for quantitative analysis of related substances in Leuprolide acetate depot suspension (11.25 mg, 22.5 mg, and 30 mg, pediatric).

Table 11: Linearity parameters for Leuprolide acetate and its impurities

Analyte	Conc. Range (µg/mL)	Correlation Coefficient	Intercept	Slope	% Y-Intercept	Residual SS	RRF
Acetyl Leuprolide	0.80 – 15.81	0.9999	-5912.78	36280.65	-1.6	2.02E+07	0.90
D-Ser Leuprolide	0.80 – 14.84	0.9999	-3527.57	41193.79	-0.9	2.35E+07	1.03
D-His Leuprolide	0.80 – 15.43	0.9999	-705.57	40295.42	-0.2	1.95E+07	1.00
L-Leu6 Leuprolide	0.80 – 15.55	0.9999	-9141.04	38387.87	-2.4	3.31E+07	0.96
Leuprolide	1.00 – 7.84	0.9999	-2122.60	40170.02	-1.0	1.52E+07	1.00

The method demonstrated excellent linearity over the tested concentration range for both Leuprolide and its related impurities. High correlation coefficients and acceptable %Y-intercepts confirm the suitability of this HPLC method for quantitative estimation of impurities in Leuprolide acetate depot formulations.

Accuracy

The accuracy of the HPLC method was assessed at LOQ, 50%, 100%, and 150% concentration levels by spiking known impurities into the sample solution. Each level was prepared in triplicate, and recoveries were calculated in terms of individual values, mean recovery, %RSD, and 95% confidence intervals (excluding LOQ).

The as-such sample showed no detectable levels of Acetyl Leuprolide, D-ser Leuprolide, or L-Leu6 Leuprolide. A trace level of D-His Leuprolide was observed below the LOQ, and one unspecified impurity (0.14%) contributed to the total impurity level, which remained well within specification.

For all known impurities, recoveries across LOQ to 150% levels were within the acceptance criteria. At LOQ, recoveries varied between 83.9% and 109.6%, while at higher levels (50–150%) the mean recoveries ranged from 98.5% to 109.9%. The overall %RSD values for replicate determinations at each spike level were below 10%, confirming the consistency of the method.

Table 12: Accuracy results for known impurities in Leuprolide acetate

Impurity	LOQ Mean Recovery (%)	50% Mean Recovery (%)	100% Mean Recovery (%)	150% Mean Recovery (%)	Overall %RSD (Excl. LOQ)	95% CL (Excl. LOQ)
Acetyl Leuprolide	103.9	107.4	106.9	106.3	0.7	0.54
D-ser Leuprolide	107.8	104.2	102.8	107.8	2.6	2.22
D-His Leuprolide	109.6	102.1	102.4	109.9	1.1	0.98

**Author for Correspondence: mandalevaibhav0@gmail.com*

L-Leu6 Leuprolide	83.9	98.5	104.5	98.6	3.0	2.32

Table 13: As-such impurity levels in sample

Impurity Name	**Result (%)**
Acetyl Leuprolide	ND
D-ser Leuprolide	ND
D-His Leuprolide	0.0585 (BLOQ)
L-Leu6 Leuprolide	ND
Any unspecified	0.1408
Total Impurity	0.1408 ≈ 0.1

The recovery results at all concentration levels confirmed that the method meets the acceptance criteria for accuracy. With recoveries between 98.5% and 109.9% at 50–150% levels and consistent precision, the method is demonstrated to be accurate and reliable for quantifying related substances in Leuprolide acetate depot suspension (11.25 mg, 22.5 mg, and 30 mg, pediatric) using HPLC.

Robustness

The robustness of the method was evaluated by deliberately varying key chromatographic parameters including flow rate, column oven temperature, detection wavelength, buffer pH, and organic content in the mobile phase. Blank, placebo, resolution, sample, and spiked sample solutions were analyzed under each altered condition. The system suitability parameters and impurity profiles were assessed to verify the consistency of the method.

Overall, the system suitability criteria were met under most variations, with resolution between D-His Leuprolide and Leuprolide acetate remaining above 2.3, and USP tailing factors consistently ≤1.1. For spiked samples, mean recoveries of individual impurities were stable across conditions, with total impurities consistently around 4.2–4.3%. The %RSD values were within acceptance limits, confirming reproducibility. However, slight sensitivity was observed at reduced column oven temperature and lower organic content, where partial merging or incomplete elution of peaks occurred.

Table 14: Summary of robustness evaluation for spiked sample (11.25 mg/unit)

Condition Varied	**Resolution (D-His vs Leuprolide)**	**USP Tailing (Leuprolide)**	**Mean Recovery (%) – Range of Impurities**	**Total Impurities (%)**	**%RSD (Overall)**	**Observation**
Flow rate +10% (1.1 mL/min)	2.6	0.8	0.98–1.11	4.26	≤0.7	Within criteria
Flow rate -10% (0.9 mL/min)	2.8	0.8	0.98–1.11	4.26	≤0.7	Within criteria
Column temp. +5°C (35°C)	3.4	1.1	0.98–1.12	4.27	≤1.7	Within criteria
Column temp. -2°C (25°C)	2.9	0.9	0.99–1.12	4.28	≤1.3	Within criteria
Wavelength +2 nm (222 nm)	2.5	1.0	0.98–1.12	4.25	≤1.4	Within criteria
Wavelength -2 nm (218 nm)	2.5	1.0	0.98–1.12	4.25	≤1.3	Within criteria
Buffer pH +0.2 (3.2)	2.9	0.9	1.00–1.12	4.27	≤1.6	Within criteria
Buffer pH -0.2 (2.8)	2.9	0.9	0.99–1.12	4.29	≤1.6	Within criteria
Organic +10% (835:165 v/v)	2.3	1.0	0.98–1.12	4.25	≤1.8	Within criteria
Organic -5% (857.5:142.5 v/v)	3.3	1.1	0.99–1.12	4.29	≤1.9	Delayed elution of Acetyl Leuprolide

The method was confirmed robust under variations in flow rate, wavelength, buffer pH, column oven temperature and increased organic content. However, slight sensitivity was observed at reduced organic content (delayed elution of Acetyl Leuprolide and late-eluting impurities). Despite these observations, impurity recoveries and precision remained within acceptance criteria, demonstrating that the method is reliable for routine use in the analysis of related substances in Leuprolide acetate depot suspension.

Solution stability

Stability of analytical solutions

The stability of analytical solutions was assessed at room temperature (~25 °c) and refrigerated conditions. Resolution, impurity identification, placebo, sample, and spiked sample solutions were analyzed at initial preparation and at different time points up to 168 h. Across all conditions, system suitability requirements were consistently met, and no additional peaks above the loq were observed. Impurity levels remained within the specified limits, with differences from initial values well below the acceptance thresholds. This confirmed that all prepared solutions, including the resolution and spiked sample solutions, were stable for up to 168 h under the tested storage conditions.

Stability of mobile phase

The mobile phase was monitored both physically and chromatographically for stability over 168 h. Physically, it remained clear, colorless, and free from turbidity or particulate matter throughout the study. Chromatographic evaluation demonstrated consistent retention times and peak profiles for known impurities when compared with initial injections. These observations confirmed that the mobile phase was stable for up to 168 h without any changes that could affect the analytical performance.

Range

The range of the developed HPLC method was established based on precision, accuracy, and linearity data. It was defined as the concentration interval between the lowest and highest levels of analyte that could be quantified with acceptable reliability. The validated range covered both impurities and the active drug, demonstrating suitability of the method across the required concentration span.

Table 15: Validated Range of Method

Analyte	Concentration (ppm) From–To	Concentration (% w.r.t. sample) From–To
Acetyl Leuprolide	0.8031 – 15.1869	0.0773 – 1.4602
D-ser Leuprolide	0.8022 – 14.8199	0.0772 – 1.4249
D-His Leuprolide	0.8044 – 15.0390	0.0774 – 1.4460
L-Leu6 Leuprolide	0.7967 – 14.9964	0.0767 – 1.4419
Leuprolide	1.0045 – 7.8446	0.0967 – 0.7557

These results confirm that the method is capable of accurately quantifying impurities and the drug substance across a wide concentration interval, ensuring reliability in both routine quality control and stability studies.

The HPLC method for determination of related substances in Leuprolide acetate depot suspension (11.25 mg, 22.5 mg and 30 mg) was successfully validated. The method demonstrated compliance with all validation parameters, including specificity, precision, LOD/LOQ with precision at LOQ, linearity, accuracy, robustness, and solution/mobile phase stability. All results were within the predefined criteria, confirming that the method is reliable, validated, and suitable for assessing the quality of finished products and stability study samples.

CONCLUSION:

The present study successfully applied an Analytical Quality by Design (AQbD) approach for the development and validation of a stability-indicating RP-HPLC method for the determination of related substances in Leuprolide acetate depot formulations. Critical method parameters, including flow rate, column oven temperature, and mobile phase pH, were identified and optimized using Central Composite Design to ensure robust method performance. The validated method demonstrated specificity, precision, sensitivity (LOD and LOQ), linearity, accuracy, robustness, and solution stability, meeting all acceptance criteria. The optimized chromatographic conditions provided reliable separation with acceptable resolution and peak symmetry, confirming the method's suitability for routine quality control and stability testing of Leuprolide acetate depot formulations.

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CONFLICT OF INTREST:

The authors declares that the no conflict of interest.

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*Author for Correspondence: mandalevaibhav0@gmail.com

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