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ANALYTICAL METHOD VALIDATION FOR THE SENSITIVE RESIDUAL DETERMINATION OF POTENT HORMONAL DRUG LEVONORGESTREL IN PHARMACEUTICAL MANUFACTURING EQUIPMENT CLEANED SURFACES BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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1.0 ABSTRACT

This work presents a newly developed RP-HPLC technique designed to identify and quantify trace levels of hormonal drug residues on the surfaces of pharmaceutical production equipment. The importance of cleaning validation in ensuring both product safety and efficacy is well recognized, and this method offers a highly sensitive, accurate, and selective solution for the identification of trace residues. By providing a validated RP-HPLC approach, the study emphasizes its vital role in meeting cleaning standards, preventing cross-contamination, and maintaining the integrity of pharmaceutical production. Additionally, the paper discusses the challenges associated with residual analysis, method optimization, and regulatory compliance, underscoring the significance of robust analytical techniques in upholding quality control and ensuring regulatory adherence in the pharmaceutical sector.

2.0 KEYWORDS

Hormonal, Highly potent, Sensitive Determination, Liquid Chromatography Validation, Pharmaceuticals, Cleaning.

3.0 INTRODUCTION

Cleaning validation is an essential component of pharmaceutical manufacturing, particularly in facilities that produce multiple drug products using shared equipment. Its primary objective is to ensure that all residues from previous production runs, including active pharmaceutical ingredients (APIs), are effectively removed to prevent contamination of subsequent batches. This process becomes critically important when handling potent hormonal drugs, which exert significant biological effects at very low concentrations. Even trace levels of these compounds can compromise product safety and therapeutic efficacy.

Multi-product facilities are especially vulnerable to cross-contamination risks due to equipment reuse. Recognizing these

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challenges, regulatory agencies such as the U.S. Both the FDA and the EMA enforce strict regulatory frameworks to verify the effectiveness of cleaning processes and to reduce the likelihood of contamination in pharmaceutical manufacturing. Compliance with these regulations requires analytical techniques that are highly sensitive, reproducible, and dependable for identifying residues at ultra-trace concentrations. RP HPLC approach tailored for the detection of hormonal drug residues on pharmaceutical production equipment surfaces is introduced and validated in this work in order to satisfy these regulatory requirements.

RP-HPLC was selected for its high specificity, sensitivity, and suitability for low-level quantification. The goal is to support robust cleaning validation practices that align with regulatory standards and ensure the highest levels of product safety and quality.

The aim of this research was to establish and validate a simple, accurate, and sensitive RP-HPLC-UV method for the quantitative estimation of Levonorgestrel residues in cleaning validation swab samples collected from manufacturing surfaces following the production of Levonorgestrel tablets (1.5 mg)**.** Method validation was carried out using 316L stainless steel plates, representing the typical surface material of pharmaceutical manufacturing equipment.

Levonorgestrel drug was evaluated as the worst case, it is a synthetic progestogen hormone and having contraceptive properties and used as emergency contraceptive.

Levonorgestrel–Chemical Overview

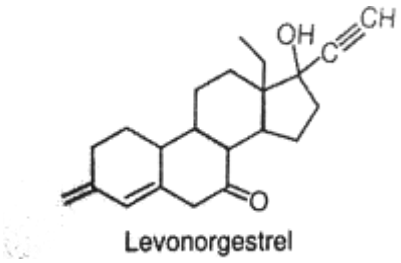

Figure 1. Chemical Structure of Levonorgestrel

IUPAC Name

13-ethyl-17-hydroxy-18,19-dinor-17alpha-pregn-4-en-20-yn-3-one or 13-beta-ethyl-17alpha- ethynyl-17beta-hydroxygon-4-en-3-one

Molecular Formula $C_{21}H_{28}O_2$

Molecular Weight

312.45g/mo

Solubility Profile

Levonorgestrel appears as a white or nearly white crystalline solid. It shows limited solubility in solvents such as chloroform and dichloromethane, demonstrates slight solubility in 95% ethanol and water, and is considered practically insoluble in water.

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4.0 MATERIALS AND METHODS

(A) Reagents and Standards

Hormonal Drugs: A selection of potent hormonal drugs Levonorgestrel Tablets were selected due to their potent therapeutic roles and stringent residue limits in cleaning validation.

Reagents: HPLC-grade methanol, acetonitrile, water were used to prepare the mobile phases and sample, solvents.

Reference Standards: Purity-tested working standards of the Levonorgestrel hormonal drugs were obtained from reputable suppliers and used to prepare standard solution to validate the method.

Test Sample: Levonorgestrel tablets 1.5 mg were obtained from a local pharmacy for the preparation of test solution to validate the method.

(B) Instrumentation & Material Characterization

Chromatographic System: For accurate analyte detection, a HPLC system with a UV-visible detector, like the Agilent 1200 series, was used for the analysis.

Column & Mobile Phase: It was separated using a C18 reversed-phase column (150 mm × 4.6 mm, 5 µm particle size) with a flow rate of 1.5 mL/min. The sensitivity and reproducibility were guaranteed by setting the injection volume at 20 µL. The mobile phase, which was designed for gradient elution based on the polarity and chemical processes of the target hormonal molecule, was made up of a 50:50 v/v mixture of water and acetonitrile.

Chromatographic Optimization: To attain optimal sensitivity and peak resolution, critical chromatographic parameters such as injection volume, column temperature, flow rate, and mobile phase composition were methodically tuned. The finalized conditions provided sharp, symmetric peaks with minimal baseline noise, ensuring reliable quantification.

Detection: UV detection was carried out within the range of 210–280 nm**,** corresponding to the absorption maxima of Levonorgestrel. The λ max values were identified at 210 nm and 280 nm**,** with an Isobestic point at 225 nm**,** indicating consistent absorption behavior across different concentrations. For this method, 210 nm was selected as the primary detection wavelength due to its highest absorbance response for Levonorgestrel. Additionally, three different mobile phase ratios were evaluated during method development to ensure optimal separation and compliance with system suitability criteria.

Method Validation: Following the guidelines set forth by the International Council for Harmonization (ICH), the newly established RP HPLC technique was validated. The following important validation characteristics were meticulously assessed: robustness, linearity, specificity, precision, and precision, limit of detection (LOD), limit of measurement (LOQ), and suitability. The method's sensitivity, dependability, and suitability for quantitative examination of the cholesterol-lowering drug residues in cleaning validation trials were confirmed by evaluating all parameters under carefully monitored experimental settings.

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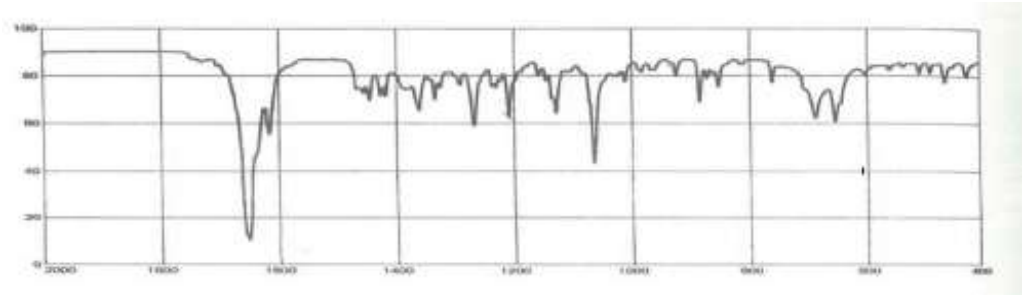
Sample Characterization: A standard reference of Levonorgestrel was obtained from the in-house laboratory, while Levonorgestrel tablets (1.5 mg) were sourced from a local pharmacy. The identity and purity of the compound were confirmed through Fourier-Transform Infrared (FT-IR) Spectroscopy using a Shimadzu FT-IR spectrophotometer**.** The spectral scans were recorded in the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}**.** The resulting FT-IR spectrum was analyzed and compared with standard reference data, which confirmed the characteristic peaks corresponding to Levonorgestrel, thereby validating the authenticity of the sample.



Levonorgestrel infrared spectrum

5.0 WORST CASE PRODUCT SELECTION FOR CLEANING VALIDATION

The worst-case scenario for the product will be the one that can be used to represent any other items produced using the same cleaning process in the same pieces of equipment. The solubility of the product's active ingredients in water with the cleaning chemical used to clean the equipment indicates how difficult it is to remove an active ingredient that is more insoluble. Consequently, the product that contains an insoluble active ingredient will be the worst-case scenario.

6.0 VALIDATION OF THE RP-HPLC METHOD

Preparation of Standard Solution: 10 mg of levonorgestrel were precisely weighed and then transferred into a 20 mL volumetric flask to create a stock solution. The sample was dissolved by adding a tiny amount of acetonitrile (ACN), and to guarantee full solubilization, the mixture was sonicated for 15 minutes. After then, ACN was used to make up the final volume. The mobile phase (ACN: water, 50:50 v/v) was used to dilute 2 mL of this stock solution to volume in a 20 mL volumetric flask, yielding a working standard of 50 µg/mL.

Preparation of Test Solution: After weighing and coarsely powdering twenty Levonorgestrel tablets, 10 mg of the drug was added to a 20 mL volumetric flask. The mixture was subjected to 45 minutes of sonication after a minor amount of ACN was introduced. After using ACN to regulate the volume, the solution was filtered via Whatman filter paper. A test solution containing 50 µg/mL of the cholesterol-lowering drug was obtained by transferring 2 mL of this filtrate into a second 20 mL volumetric flask and diluting it with the mobile phase (ACN: water, 50:50 v/v). Under the specified chromatographic

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conditions, this solution was subsequently injected into the HPLC system, and the peak area was noted.

System Suitability: System suitability testing was carried out to verify the performance of the HPLC system and method**.** The evaluation was conducted using five replicate injections of the standard solution. The assessed parameters included:

- **%RSD of peak area** (acceptance limit: < 5.0%)
- **Tailing factor** (acceptable range: 0.8 – 2.0)
- **Number of theoretical plates** (minimum requirement: > 2000)

All system suitability results complied with the acceptance criteria, confirming that the method is robust and reliable for routine analysis.

Linearity & range: The RP-HPLC method exhibited excellent linearity across a wide concentration range of Levonorgestrel (80%,90%,100%,110%,and120%).Inject the sample and note the area response plot the graph between concentration and area response. The correlation coefficients is 0.99 and linearity graph found liner indicating strong calibration curve in different concentration.

Levonorgestrel					
Concentration	**80%**	**90%**	**100%**	**110%**	**120%**
Sample	**Area**				
Rep. I	605528	682268	757094	831118	907575
Rep. II	604617	682773	757272	831626	908283
Average	**605073**	**682521**	**757183**	**831372**	**907929**
Co-relation Coefficient =1.0000					

Linearity graph of Levonorgestrel

Specificity: The RP-HPLC method was highly specific, with no interference from common excipients or matrix components present on pharmaceutical manufacturing surfaces, confirming the reliability of the method in real-world cleaning validation.

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The specificity was checked by the three replicate injection of Levonorgestrel standard solution & Retention Time of active ingredient in sample is same that of standard.

Levonorgestrel				
Blank	**Placebo Prep.**	**Placebo+ Std.**	**Std. Prep.**	**Test Perp.**
Mobile Phase	648.4mg Placebo in 50 ml volumetric flask add 25ml ACN and sonicate. Make up to 50ml with water dilute 5ml to10ml with mobile Phase.	647.8mg Placebo+ 25.1mg Levonorgestrel std in 100ml volumetric flask. add 50ml ACN and Sonicate to make up to 100ml with water dilute 5ml to 25ml with Mobile Phase.	25.3mg Levonorgestrel std in 100ml V.F. add 50ml CAN and sonicate Make up to 100ml with water. Dilute 5ml to 25ml with Mobile Phase.	653.8mg of sample in 50ml Volumetric flask add 25ml ACN and sonicate to make up 50ml with water dilute5ml to 10ml with mobile Phase.

Observation	**Interference**	**Area**	**Assay (%)**
Blank	No Interference	Not Detected	NA
Placebo	No Interference	Not Detected	NA
Placebo+LevonorgestrelStd.(RT)	7.495	754229	NA
LevonorgestrelStd.(RT)	7.491	757598	NA
TestSolution(RT)	7.487	759163	99.96

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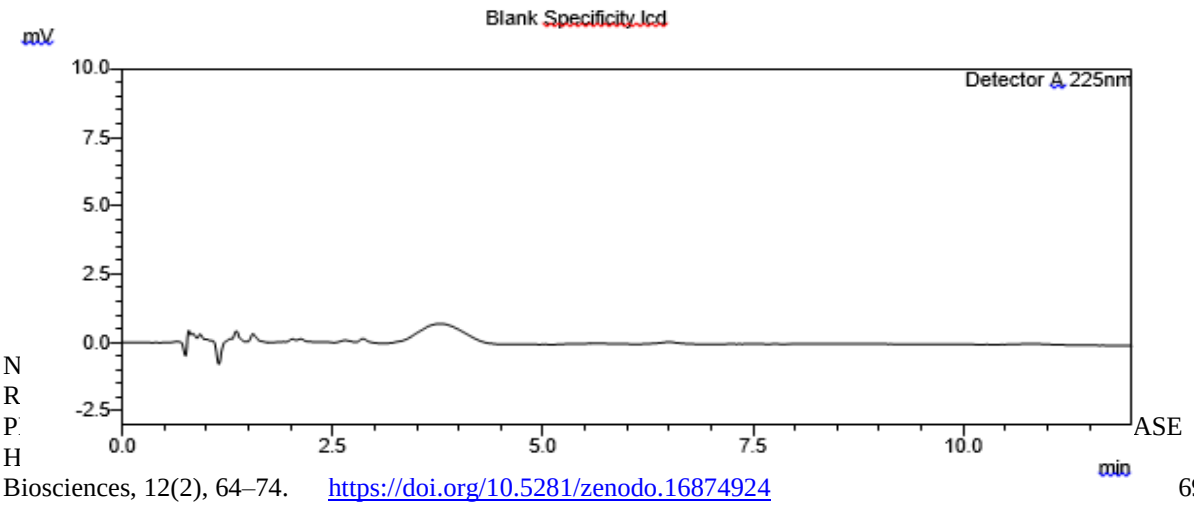

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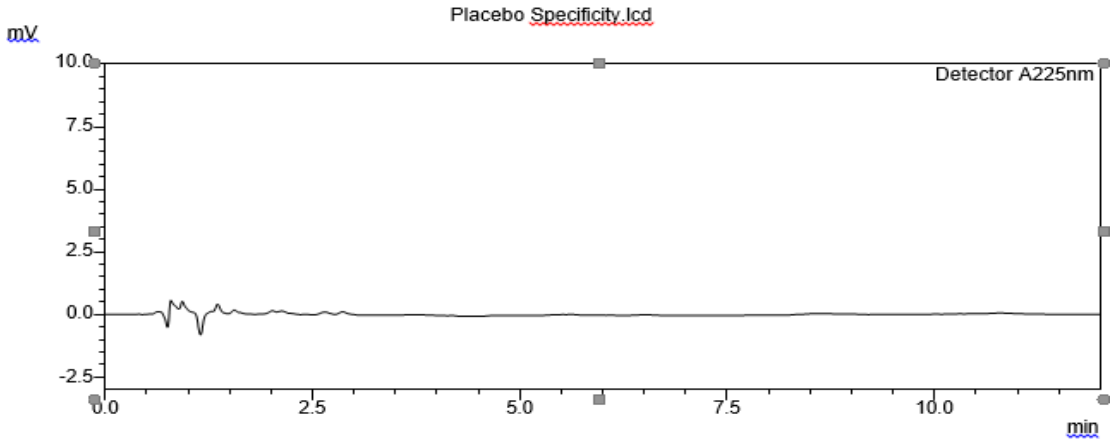

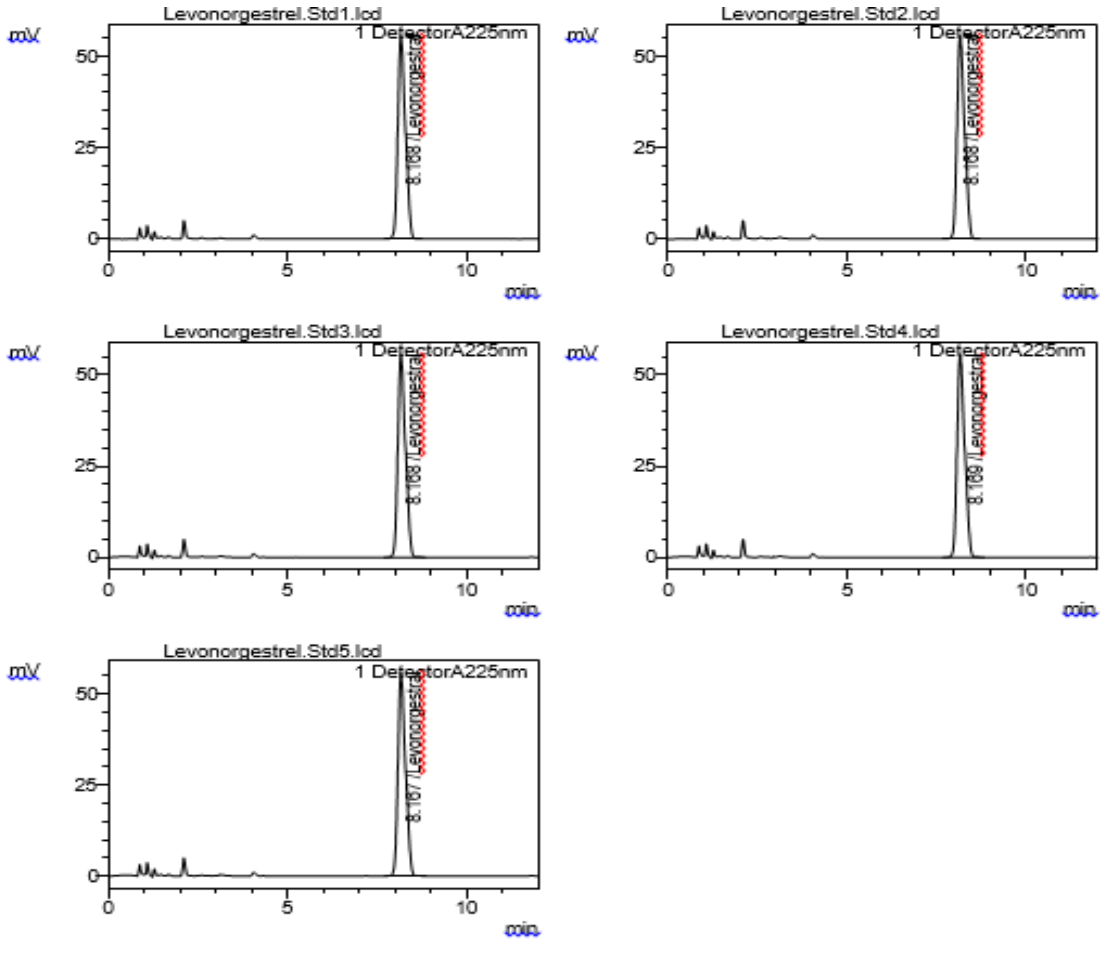

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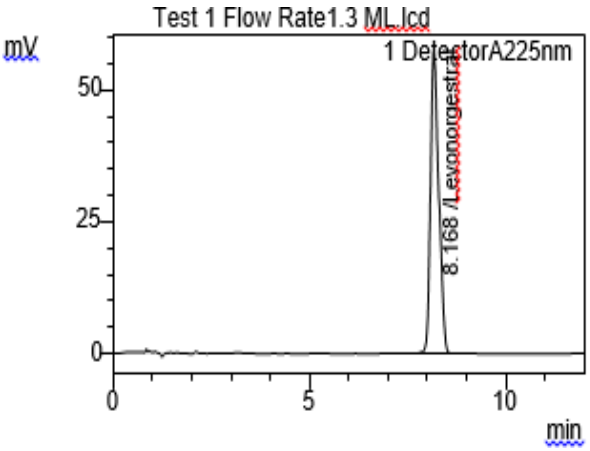

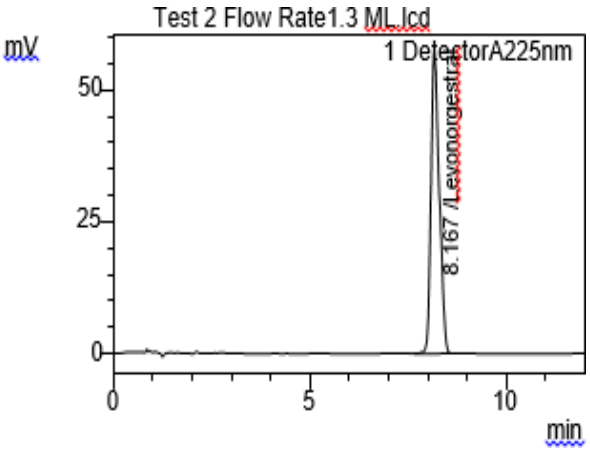

Accuracy and Precision: Three concentration levels—low, medium, and high—were used in recovery tests to assess the accuracy of the suggested approach. The method's accuracy was confirmed when it was discovered that the mean recovery values fell between 90 and 110 percent. The proportion of the acquired result that differed from the associated reference measurement was used to express accuracy.

The precision of the approach was evaluated using both inter-day (intermediate precision) and intra-day (repeatability) tests. The Levonorgestrel standard solution was injected three times in a row on the same day to assess repeatability, and the same process was repeated three days in close succession to assess intermediate precision.

In both evaluations, the relative standard deviation (RSD) values were consistently below **5%**, indicating that the method is precise, reproducible, and reliable for routine analysis.

Accuracy Results (Average of Triplicate analysis)					
Recovery of Levonorgestrel					
Sample / **Conc.**	**Sample I**	**Sample II**	**Sample III**	**Average**	**Limit**
	Assay in%				
80%	99.94	99.16	99.99	99.70	**(98%to 102%)**
100%	100.39	100.36	99.05	99.93	
120%	100.16	99.38	99.87	99.80	
Average (in %)				**99.81**	

Precision Analysis by1st Analyst	**Precision Analysis by2nd Analyst**
Analysis done on:24.07.2025	**Analysis done on:25.07.2025**

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Assay of Levonorgestrel					
Sample No.	**Assay in mg**	**Assay in %**	**Sample No.**	**Assay in mg**	**Assay in %**
Sample I	1.5049	100.32	**Sample I**	1.4938	99.58
Sample II	1.4986	99.91	**Sample II**	1.4936	99.57
Sample III	1.5006	100.04	**Sample III**	1.4950	99.66
Sample IV	1.5018	100.12	**Sample IV**	1.4899	99.32
Sample V	1.5019	100.12	**Sample V**	1.4977	99.84
Sample VI	1.4952	99.68	**Sample VI**	1.4956	99.71
Average	1.5005	100.03	**Average**	1.4942	99.62
RSD%	0.220	0.220	**RSD%**	0.175	0.175
RSD Difference between two analyst=0.045%					

Robustness: Test the standard and samples at changed flow rate.±0.2ml

Flow rate change 1.3 ml/Min: Prepare the standard, sample and mobile phase and inject the sample set the flow rate1.3ml/Min calculate the average assay of 6 determinations the difference between normal assay and change condition results is NMT 2.0%. And the results found within the acceptance criteria.

Parameter	**Parameter as per method**	**Change parameter**	**Result**	**Initial result**	**Difference with Initial result (NMT 2.0)**
Flow rate Change	1.5ml/min.	1.3ml/min.	99.54%	99.67%	0.13%

Flow rate change 1.7 ml/Min: Prepare the standard, sample and mobile phase and inject the sample. Set the flow rate 1.7 ml/Min. calculate the average assay of 6 determinations, the Difference between normal assay and change condition results is NMT 2.0% And the results found within the acceptance criteria.

Para mete r	**Parameter as per method**	**Change parameter**	**Result**	**Initial result**	**Difference with Initial result (NMT 2.0)**
Flow rate Change	1.5ml/min.	1.7ml/min	98.76%	99.67%	0.91%

Limit of Detection (LOD) and Limit of Quantitation (LOQ): The LOD for all target drugs was determined to be below 1 ng/mL, and the LOQ ranged from5 µg/mL to

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20 µg/mL. These values are well below typical cleaning validation limits, allowing for highly sensitive residue detection. The LOD was confirmed by the six replicate injection of diluted solution at detection level and six replicate injection of diluted solution at quantification level with relative standard deviations (RSD) of less than 5%.

LOQ Level	Standard Solution Taken in ml	Diluted to volume methanol (ml)	Concentration in ppm of Levonorgestrel	RT	Area
1	5	200	1.25	7.494	19964
2	1	20	2.5	7.492	39462
3	2	20	5	7.493	77855
4	5	25	10	7.496	162524
5	4	10	20	7.501	357957
6	Standard sol.(Stock solution as such)		50	7.500	789228
LOD			0.22		
LOQ			0.67		

7.0 CONCLUSION

This research successfully validated an RP-HPLC method for the sensitive detection of potent hormonal drug residues on pharmaceutical manufacturing equipment surfaces. The method demonstrates high specificity, accuracy, and sensitivity, even for trace- level residues, making it a valuable tool for cleaning validation in the pharmaceutical industry with respect to the residue analysis. It is capable of meeting regulatory requirements for analytical validation and ensuring that equipment is free from harmful drug residues ultimately safe guarding patient safety and maintaining the integrity of subsequent pharmaceutical products.

The implementation of this RP-HPLC method provides pharmaceutical companies with a reliable and effective means of ensuring the effectiveness of their cleaning validation procedures, contributing to the ongoing efforts to uphold the highest standards of pharmaceutical manufacturing.

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