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Ultra performance liquid chromatography (UPLC) method development and validation fordetermination of impuritiesof Norethindrone tablets using advanced T3 Bonding process

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ABSTRACT

An advanced Ultra performance liquid chromatography (UPLC) method has been developed and validated for determination of process and degradation impurities in Norethindrone tablets. The chromatographic separation was achieved by using Acquity UPLC HSS T3 (100 mm × 2.1 mm), 1.8µ column, with shorter run time 11 minutes. Method validation parameters such as specificity, linearity, precision, accuracy, determination of LOD and LOQ and robustness were evaluated as per ICH guidelines. The validated UPLC method was successfully applied to the quantitative determination of impurities of Norethindrone in tablet dosage form. The method was found to be suitable for the quality control and to assure therapeutic efficacy. The described method is simple, rapid, linear, precise and robust.

Keywords: Norethindrone, UPLC, Acquity UPLC HSS, degradation

INTRODUCTION

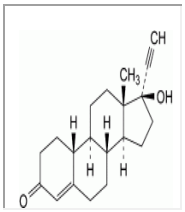
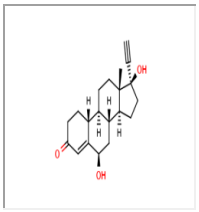
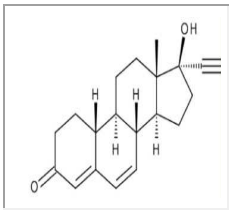
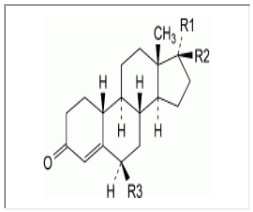
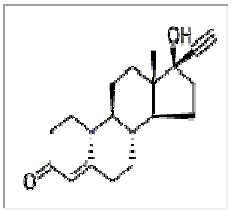
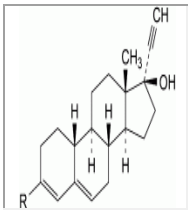
Norethindrone is form of progesterone, a female hormone. Norethindrone tablet is an oral contraceptive product provides a continuous oral contraceptive regimen of 0.35mg Norethindrone daily. Norethindrone chemically described as 17-Hydroxy-19-Nor-17α-pregn-4-en-20-yn-3-one with molecular weight 298.42 and molecular formula C20H26O2. Norethindrone is a white to creamy white, odourless, crystalline powder. It is soluble in chloroform and dioxane, sparingly soluble in alcohol, slightly soluble in ether, practically insoluble in water. Norethindrone tablets works by suppressing ovulation, thickening cervical mucus to prevent sperm penetration, and altering the lining of the uterus.

As per reported literature there is only one analytical method available by HPLC for determination of impurities for Norethindrone in Norethindrone Tablets [1]. There are some analytical methods available for determination of steroidal hormones [2-6]. Norethindrone tablets are official in USP Pharmacopoeia but there is no related substances method available for determination of impurities in Norethindrone tablets [7]. According to the ICH stability testing guideline stress testing of the drug products [8-9] is considered necessary to establish the degradation pathway and intrinsic stability of the molecule which is important to detect the degradation products and stability indicating nature of the analytical procedure. The developed method is validated as per guidelines and ICH recommendations [10-16] for Specificity, Forced degradation studies, Precision, Sensitivity (Limit of detection and Limit of Quantification), Linearity, Solution stability, Filter paper variability, Accuracy and Robustness.

The possible process and degradation impurities of Norethindrone are 6-Beta Hydroxy Norethindrone (Nor Imp-1), Norethindrone Ph.Eur. Imp-A (Nor Imp-2),Norandrostenedione (Nor Imp-3), 17-Epi-Norethindrone(Nor Imp-4), Norethindrone Enolether (Nor Imp-5).

The Chemical structures of Norethindrone and its impurities have been illustrated in **Fig. 1**.

Fig. 1. Chemical structures of Norethindrone and its impurities

					
NORETHINDRONE	NOR IMP-1 (6-BETA HYDROXY NORETHINDRONE)	NOR IMP-2 (NORETHINDRONE PH.EUR.IMP-A)	NOR IMP-3 (NORANDROSTENEDIONE)	NOR IMP-4 (17-EPI-NORETHINDRONE)	NOR IMP-5 (NORETHINDRONE ENOLETHER)

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and reagents:

Norethindrone drug substance, impurities of Norethindrone, Norethindrone tablets generously sponsored by Aurobindo pharma limited. HPLC grade Acetonitrile is obtained from Merck chemicals. Ultrapure water is prepared by using Millipore Milli-Q plus water purification system.

2.1.2. Equipments

The Waters-Acquity UPLC system used for method development and validation consists of gradient pump (Binary) system with auto Sampler, thermostatic column oven compartment and Photo Diode Array detector (PDA). Signals were monitored through Empower-3 Method validation manager software connected with windows based computer.

2.2. Method development and optimization

Currently there is no UPLC method available to separate and quantify the Norethindrone and its impurities in Norethindrone tablets. Introduction of Ultra performance liquid chromatography (UPLC) is revolutionary change in the separation science. The design of instrumentation and column technology of UPLC were made to achieve remarkable increase in resolution, speed, and sensitivity in liquid chromatography. Different strategies can be implemented in order to perform faster procedures, while maintaining acceptable chromatographic performance. 1.8µm High Strength Silica (HSS) particles are the first and only columns that allow separation scientist to achieve maximum speed sensitivity and resolution without compromise. The same particle size column has been selected for the initial method development procedure.

From the initial method development trials, it was observed that the component do not require any buffer for analytical method development purpose. Keeping in view of this, method development was initiated using Water as Mobile phase-A and Acetonitrile as Mobile phase-B by considering the nature of drug component.

Norethindrone tablets consist of very low content of Norethindrone (0.35mg per tablet). There are many key factors that are responsible to attain satisfactory response for the impurities present in the low dosage drug sample matrix like selection of column and sample concentration. To attain sharper peaks and optimal area counts even at low sample concentration it is preferable to choose column with small particle size. The universal silica based bonded phase used for the Acquity HSS T3 sorbents is compatible with 100% aqueous mobile phase and is the first choice when developing separations for polar compounds and non polar compounds. The same column was used for initial trial purpose. Based on the development trial output, Acquity UPLC HSS T3 (100 mm × 2.1 mm), with 1.8µm particle size column was selected. This column have a tendency to separate both polar and non polar components with reduced column back pressure and found to be suitable for separation of all impurities.

Impurity solutions were prepared and injected at 5µL injection volume into DAD (Diode Array Detector) to know the elution pattern of Norethindrone impurities. From the spectral characteristics of both specified impurities and Norethindrone, the absorption maxima was found to be 254nm, which was finalized as working wavelength.

Norethindrone sample spiked with all impurities and injected in to the UPLC system and confirmed that separation between impurities was found to be satisfactory at the above specified conditions. Trials were initiated with different gradient programmes to achieve best separation of impurities and based on output the following gradient programme was finalized i.e.,0/30, 1.2/30, 3/40, 5/40, 7.5/90, 9.5/90, 9.7/30 and 11.0/30 (time (min) /%B). In the optimized conditions, the typical retention times of Nor Imp-1, Nor Imp-2, Nor Imp-3, Norethindrone, Nor Imp-4 and Nor Imp-5 were 1.79, 4.73, 4.97, 5.27, 6.39 and 8.14 respectively.

2.3. Chromatographic Conditions

Analytical work was executed on an Acquity UPLC HSS T3 (100 mm × 2.1 mm), 1.8µ particle size column. The Mobile phase- A contains Water and Acetonitrile is used as mobile phase-B. The flow rate of the mobile phase is 0.5 mL/min with a gradient program of 0/30, 1.2/30, 3/40, 5/40, 7.5/90, 9.5/90, 9.7/30 and 11.0/30 (time (min)/ %B).The column temperature is maintained at 25°C and the detection is monitored at wavelength of 254 nm. The injection volume is 5 µL. Diluent consists of Water and Acetonitrile in the ratio 40:60 v/v.

2.4. Preparation of Solutions

2.4.1. Preparation of standard solution:

Standard stock solution of Norethindrone (0.6 mg/mL) was prepared with diluent. This stock solution was further diluted to obtain a concentration of 0.96µg/mL of Norethindrone. All impurities were prepared in diluent i.e., Acetonitrile and water 60:40 v/v.

2.4.2 Preparation of sample solution:

Transferred 14 intact tablets into a 50 mL clean, dry volumetric flask. Added about 35mL of diluent and sonicated for about 30 minutes with intermediate shaking. Allowed the solution to cool to room temperature and diluted to volume with diluent and mixed. The solution was centrifuged at 10,000RPM and filtered to get the clear solution through 0.22 µ membrane filter.

2.5. Analytical method validation

Norethindrone tablets were available in single strength 0.35mg per tablet in generic version. The same strength is used for experimentation and validation procedure.

The developed method is validated for Specificity, Forced degradation studies, Precision, Sensitivity (Limit of detection and Limit of Quantification), Linearity, Solution stability, Filter paper variability, Accuracy and Robustness.

2.5.1. Specificity and Stress studies

The specificity of the developed method for Norethindrone tablets was determined in the presence of Nor Imp-1, Nor Imp-2, Nor Imp-3, Nor Imp-4 and Nor Imp-5 at a concentration of 5 µg/mL.The stress conditions used for degradation studies were Acid hydrolysis (0.5 M HCl /0.5mL/RT / 20hours), Base hydrolysis (0.1M NaOH / 0.5mL/RT / 20 hours), Oxidation (30% H_2O_2 / 0.5mL/ RT / 20hours), Thermal (85°C / 48 hours), Humidity (95% RH / 24 Hours) and Photolytic (White fluorescent 1.2 million lux hours UV 200 watt hr/m^2 for 7 days).

2.5.2. Precision

The precision of the method is checked by injecting six individual preparation of Norethindrone tablets spiked with 1% level for Nor Imp-1, Nor Imp-2, Nor Imp-3, Nor Imp-4 and Nor Imp-5. The percentage RSD for % w/w of each impurity is calculated.

The intermediate precision (Ruggedness) of the method was evaluated by different analyst using different column and different UPLC instrument on different day.

2.5.3 .Sensitivity (Limit of detection and Limit of Quantification)

For the establishment of LOD and LOQ levels, a series of standard solutions were prepared from 1% to 150% with respective impurity specification level by diluting the impurity stock solution to the required concentration. Linearity curves were drawn by plotting concentration versus area of the individual impurity. From these plots, LOD and LOQ were predicted from the formulae 3.3σ/S and 10σ/S respectively where σ is the standard deviation of the response and S is the slope of the linearity curve. Precision was performed at predicted LOD and LOQ values and finalized the LOD and LOQ levels.

2.5.4 Linearity

Linearity curves were plotted from the finalized LOQ level to 150% of the impurity specification level. The correlation coefficient, slope and Y-intercept of the Linearity curve were calculated for each impurity.

2.5.5 .Accuracy
A known amount of the impurity stock solutions are spiked to the samples at LOQ, 50%, 100% and 150% of the analyte concentration. The % w/w of recoveries for all the impurities were calculated. Each concentration level is prepared for triplicate preparation.

2.5.6. Range
The range of the analytical method was demonstrated from LOQ to 150% of the impurity specification levels.

2.5.7 Solution Stability
In order to demonstrate the solution stability, both standard and sample solutions were injected immediately and at periodical intervals by maintaining room temperature.

2.5.8. Filter paper variability
In order to demonstrate the effect of filters used for standard and sample solutions, these solutions were filtered using Millipore PVDF and mdi Nylon membrane filters by initially discarding 2-3 mL of aliquots from the filters. Results were compared with centrifuged solutions.

2.5.9. Robustness
To determine the robustness of the developed method, experimental conditions are deliberately changed and the impact of the variation on each impurity was evaluated. The flow rate of the mobile phase is 0.5 mL/min. To study the effect of flow rate 0.1 unit changed i.e., 0.4 and 0.6 mL/min. The effect of column temperature is studied at 20°C and 30°C instead of 25°C. For gradient programme variation, the composition of Mobile phase-B was changed ± 2 absolute. For wavelength variation, ± 5 nm was changed from the working wavelength i.e., 254nm. In all the robustness conditions, only one parameter changed by keeping all remaining conditions unchanged.

RESULTS AND DISCUSSION

3.1. Specificity and Stress studies
Stress studies on Norethindrone tablets under different stress conditions suggested the following degradation behaviour.

3.1.1. Degradation in Acid stress condition
Norethindrone significantly undergone degradation in acid stress condition and prominent degradation is observed for Nor Imp-1. There is no significant degradation observed for other specified and unspecified impurities.

3.1.2. Degradation in Base stress condition
There is no significant degradation observed for specified and unspecified impurities

3.1.3 .Degradation in Peroxide stress condition
Norethindrone significantly undergone degradation in Peroxide stress condition and prominent degradation is observed for Nor Imp-1.

3.1.4. Degradation in Thermal stress condition
There is no significant degradation observed for specified and unspecified impurities**.**

3.1.5. Degradation in Photolytic stress condition
There is no significant degradation observed for specified and unspecified impurities**.**

3.1.6. Degradation in Humidity stress condition
There is no significant degradation observed for specified and unspecified impurities**.**(**Fig.3, Table 1**)

3.2 Method Validation
3.2.1 .Precision
The percentage RSD of Nor Imp-1, Nor Imp-2, Nor Imp-3, Nor Imp-4 and Nor Imp-5 is 1.0, 2.7, 0.9, 2.2 and 3.0 respectively confirming the good precision of the developed method. The % RSD obtained in intermediate precision study for Nor Imp-1, Nor Imp-2, Nor Imp-3, Nor Imp-4 and Nor Imp-5 was 4.1, 3.6, 4.2, 4.8 and 6.0 respectively confirming the intermediate precision.

3.2.2. Sensitivity (Limit of detection and Limit of Quantification)

The Limit of Detection for Nor Imp-1, Nor Imp-2, Nor Imp-3, Nor Imp-4 and Nor Imp-5 were 0.013%, 0.015%, 0.017%, 0.015% and 0.013% respectively (of analyte concentration, i.e. 5 µg/mL). The Limit of Quantification for Nor Imp-1, Nor Imp-2, Nor Imp-3, Nor Imp-4 and Nor Imp-5 is 0.045%, 0.050%, 0.057%, 0.052% and 0.044% respectively (of analyte concentration, i.e. 5 µg/mL)

3.2.3. Linearity and Range

Linear calibration plot for the related substances method was obtained over the calibration range LOQ to 150%. The results show an excellent correlation obtained between peak area and concentration of Norethindrone and all the impurities. (**Table 2**)

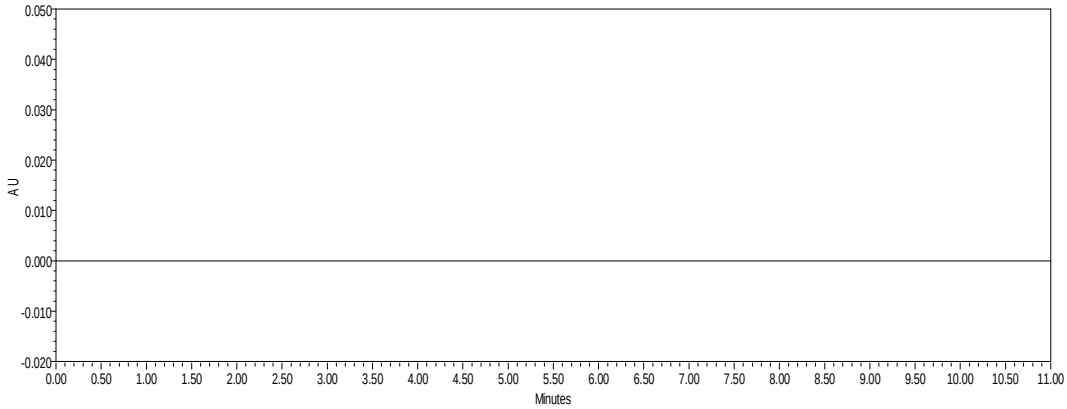
3.2.4. Accuracy

Accuracy was assessed from three replicate determinations of four different levels including LOQ, 50%, 100% and 150% of the specification level of the impurities. The observed recovery results were found in the range between 90 to 110% with the % RSDs lower than 5.0% demonstrating that the method is accurate within the desired range. (**Table 3.a. & 3.b.**)

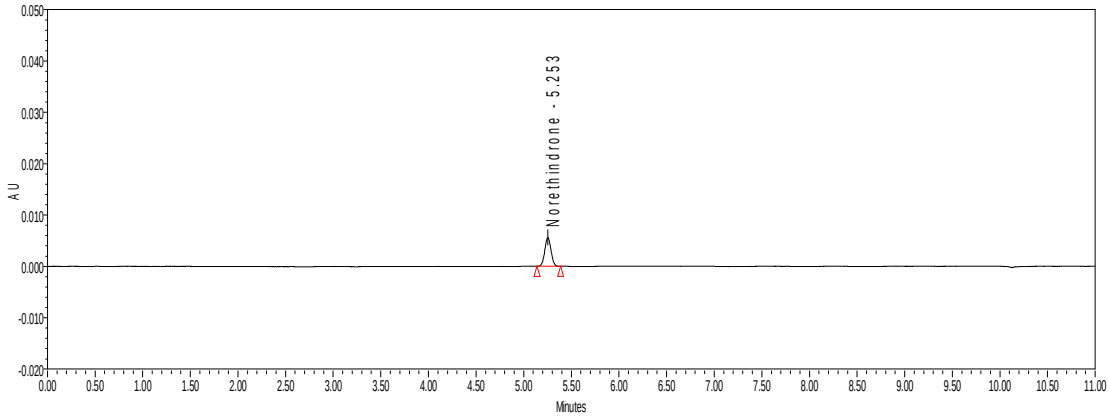
3.2.5. Solution Stability:

No significant changes are observed in the area of Nor Imp-1, Nor Imp-2, Nor Imp-3, Nor Imp-4 and Nor Imp-5 during solution stability experiment. The solution stability experiment data confirms that standard and sample solutions are stable up to the study period of 24 hours at 25°C.

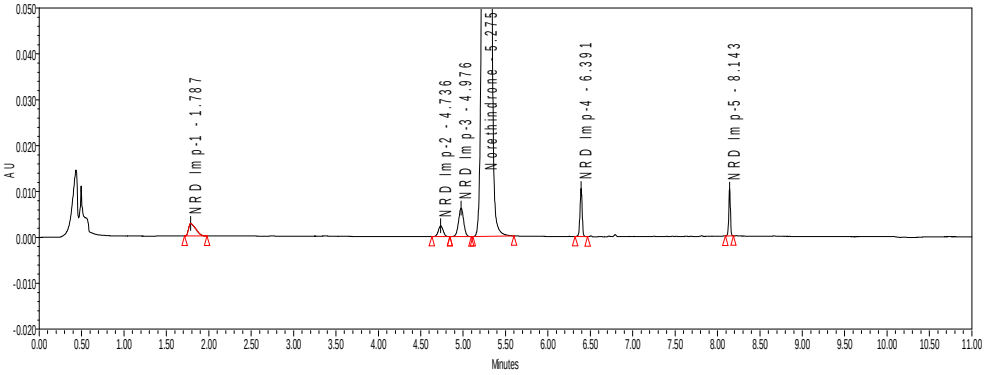
Fig.2: Typical chromatogram of Diluent, Standard solution, Norethindrone spiked with impurities & Stress sample chromatograms of Acid, Base, Peroxide, Thermal, Humidity and Photolytic conditions



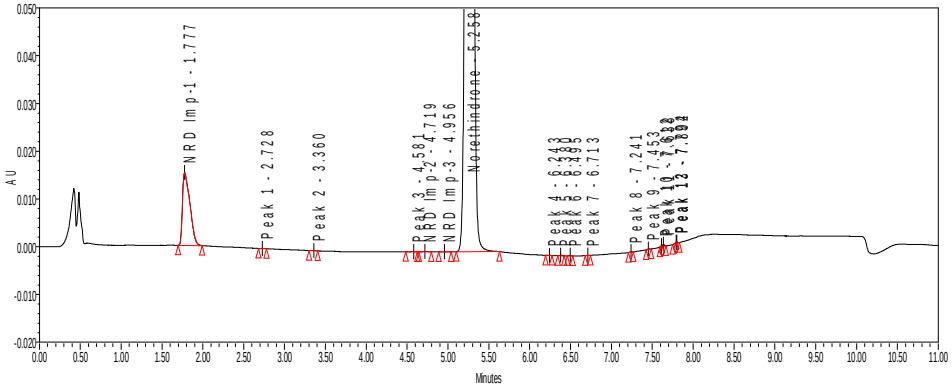
(a) Diluent



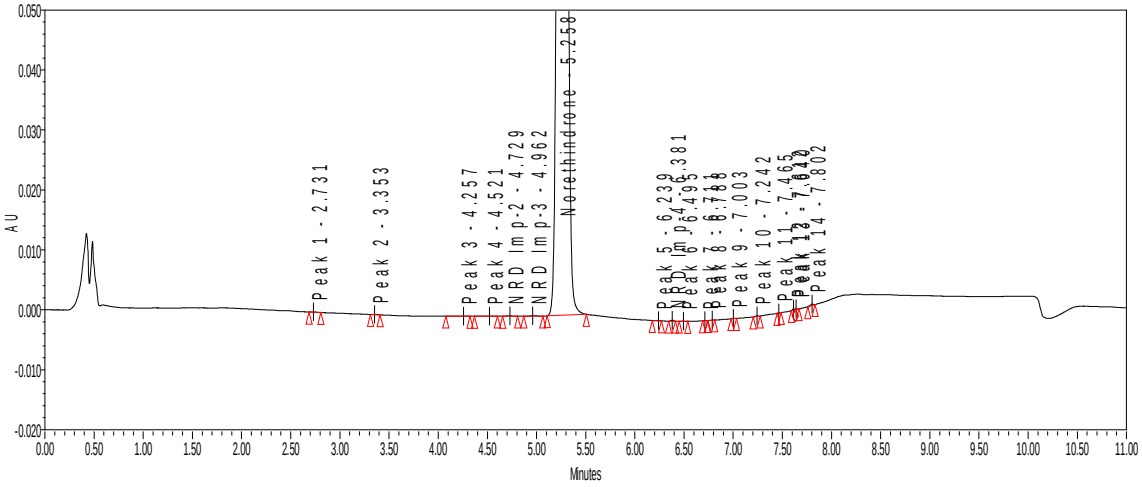
(b) Diluted Standard



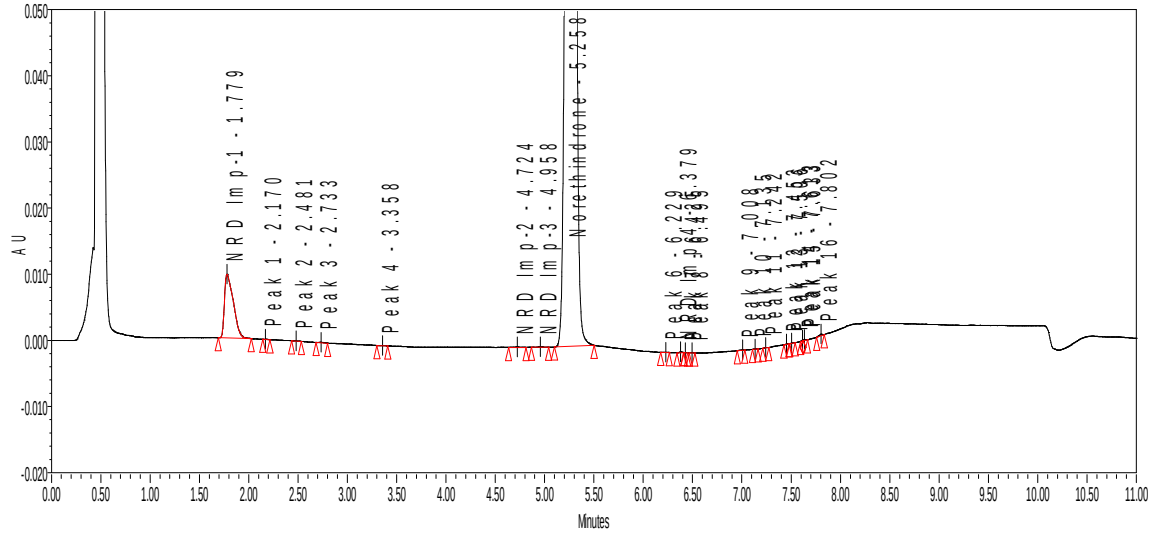
(c)Spiked sample



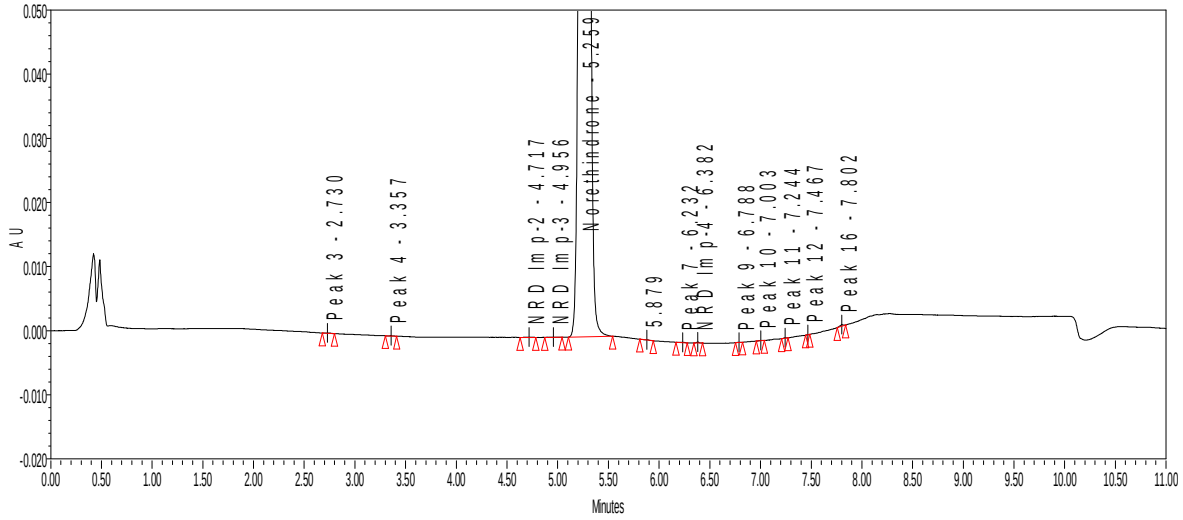
(d) Acid degradation sample



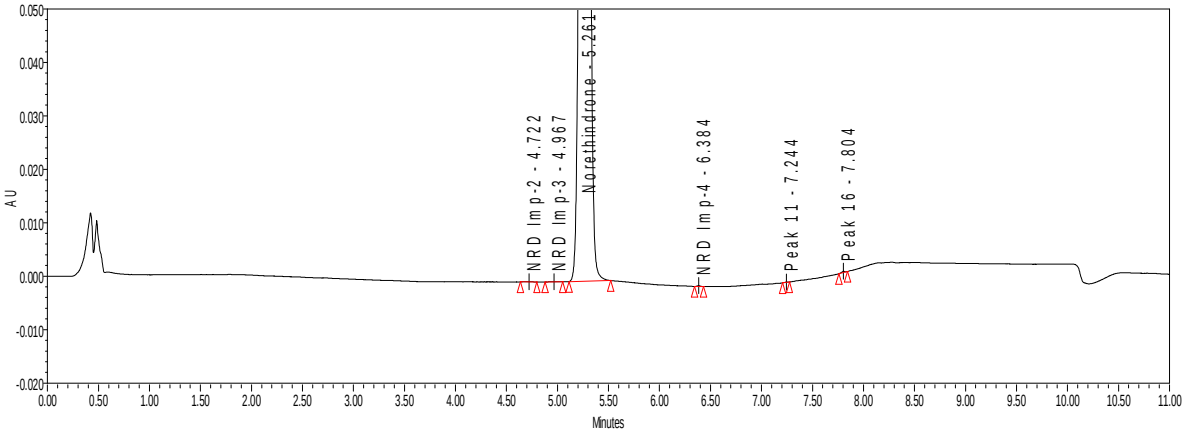
(e) Base degradation sample



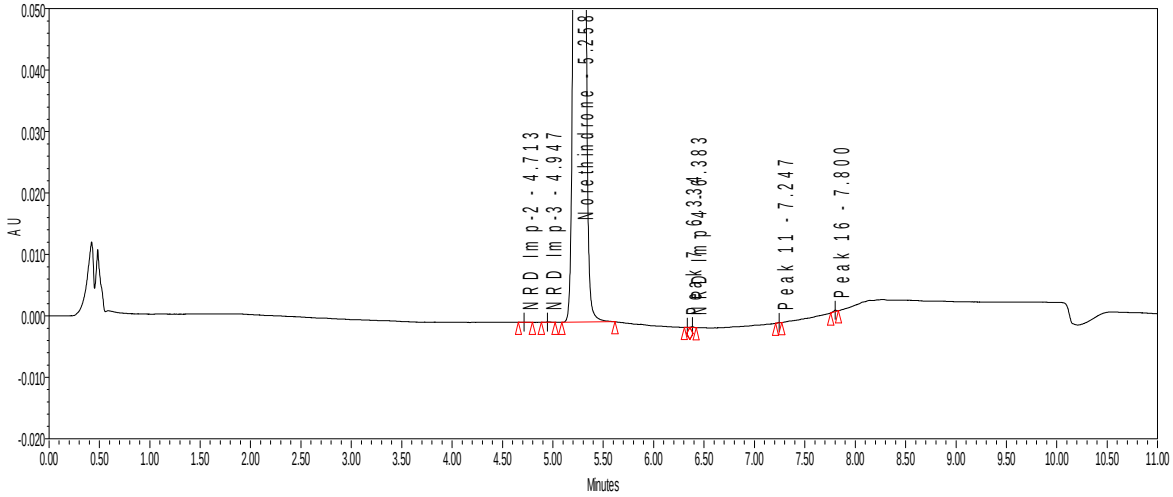
(f) Peroxide degradation sample



(g) Thermal degradation sample



(h) Humidity degradation sample



(i) Photolytic degradation sample

Table 1. Forced degradation table

Stress condition and Time	% Assay of Norethindrone	% imps+ %Deg. products	mass balance (%Assay+ Imps+% Deg products)	Major Appeared impurities
Acid hydrolysis -0.5 M HCl/0.5 mL/RT	93.4	6.29	99.69	Nor Imp-1
Alkali hydrolysis-0.1 M NaOH/0.5 mL/RT	98.9	0.12	99.02	No significant degradation
Oxidation – 30% H_2O_2 /0.5mL/RT	96.9	3.94	100.84	Nor Imp-1
Thermal deg – (Heated at 85°C)	100.6	0.08	100.68	No significant degradation
Humidity deg - (95%RH)	101.4	0.06	101.46	No significant degradation
Photolytic deg - (white fluorescent 1.2 million lux hours UV 200 watt hr/m^2 for 7 days).	101.4	0.06	101.46	No significant degradation

Table 2. Linearity table

Name of the component	Trendline equation	Range	Correlation coefficient	Intercept	Residual sum of squares
Nor Imp - 1	y = 17129x +52	0.010-1.480	0.99981	52	129
Nor Imp - 2	y = 10110x -20	0.010-1.449	0.99990	-20	74
Nor Imp - 3	y = 27843x +210	0.010-1.506	0.99991	210	210
Norethindrone	y = 27395x -255	0.010-1.475	0.99979	-255	302
Nor Imp -4	y = 20733x + 27	0.010-1.444	0.99990	27	153
Nor Imp -5	y = 15586x + 28	0.010-1.482	0.99993	28	98

Table 3.a. Table for Accuracy study for Norethindrone and its impurities

	Nor Imp-1			Nor Imp-2			Nor Imp-3		
Sample spiked at level	Amount added (%w/w)	Amount recovered (%w/w)	% Recovery	Amount added (%w/w)	Amount recovered (%w/w)	% Recovery	Amount added (%w/w)	Amount recovered (%w/w)	% Recovery
LOQ Sample-1	0.0545	0.0559	102.6	0.0520	0.0514	98.8	0.541	0.0548	101.3
LOQ Sample-2	0.0545	0.0554	101.7	0.0520	0.0525	101.0	0.541	0.0577	106.7
LOQ Sample-3	0.0545	0.0546	100.2	0.0520	0.0538	103.5	0.541	0.0529	97.8
50% sample -1	0.503	0.476	94.6	0.465	0.453	97.4	0.484	0.451	93.2
50% sample -2	0.503	0.499	99.2	0.465	0.478	102.8	0.484	0.494	102.1
50% sample -3	0.503	0.525	104.4	0.465	0.489	105.2	0.484	0.510	105.4
100% sample -1	1.007	0.982	97.5	0.931	0.942	101.2	0.968	0.973	100.5
100% sample -2	1.007	1.030	102.3	0.931	0.972	104.4	0.968	1.016	105.0
100% sample -3	1.007	1.011	100.4	0.931	0.966	103.8	0.968	1.001	103.4
150% sample -1	1.510	1.513	100.2	1.396	1.446	103.6	1.451	1.503	103.6
150% sample -2	1.510	1.537	101.8	1.396	1.471	105.4	1.451	1.519	104.7
150% sample -3	1.510	1.542	102.1	1.396	1.460	104.6	1.451	1.520	104.8

Table 3.b. Table for Accuracy study for Norethindrone and its impurities

Sample spiked at level	Nor Imp-4			Nor Imp-5		
	Amount added (%w/w)	Amount recovered (%w/w)	% Recovery	Amount added (%w/w)	Amount recovered (%w/w)	% Recovery
LOQ Sample-1	0.0519	0.0484	93.3	0.0515	0.0533	103.5
LOQ Sample-2	0.0519	0.0488	94.0	0.0515	0.0547	106.2
LOQ Sample-3	0.0519	0.0503	96.9	0.0515	0.0528	102.5
50% sample -1	0.464	0.469	101.1	0.461	0.428	92.8
50% sample -2	0.464	0.477	102.8	0.461	0.469	101.7
50% sample -3	0.464	0.490	105.6	0.461	0.491	106.5
100% sample -1	0.928	0.961	103.6	0.921	0.940	102.1
100% sample -2	0.928	0.991	106.8	0.921	0.977	106.1
100% sample -3	0.928	0.959	103.3	0.921	0.955	103.7
150% sample -1	1.392	1.457	104.7	1.382	1.455	105.3
150% sample -2	1.392	1.497	107.5	1.382	1.471	106.4
150% sample -3	1.392	1.474	105.9	1.382	1.456	105.4

3.2.6. Filter paper variability

The results of the filter evaluation reveal that the absolute differences between the concentrations of standard samples and filtered/centrifuged samples were within range. This demonstrates the absence of Norethindrone adsorption by the filter and the suitability of PVDF or Nylon filter paper in the related substances test.

3.2.7. Robustness

Close observation of analysis results for deliberately changed chromatographic conditions flow rate, column temperature and change of organic component in gradient programme revealed that there is no significant change observed in the relative retention times of the main analytes and their corresponding impurities illustrating the robustness of the method.

CONCLUSION

The proposed UPLC method enables the separation and quantitative determination of specified and unspecified impurities of Norethindrone in Norethindrone tablets. The developed method is validated as per ICH requirements. The stress studies indicated that method is selective and stability indicating. UV detection at 254nm was found to be suitable without any interference from excipients. All the calibration curves obtained were found to be linear with values of correlation coefficients greater than 0.995. Recovery tests confirmed the accuracy of the method. The proposed UPLC method is fast, precise, accurate, sensitive and efficient.

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