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Development and Validation of RP-HPLC-DAD Stability Indicating Related Substances Method for the Estimation of Impurities of Norethindrone Tablets and their Degradation Products

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Abstract

A novel and efficient stability indicating reverse phase High performance liquid chromatography with diode array detector (RP-HPLC-DAD) related substances analytical method has been developed, optimized and validated for the determination of degradation products and process impurities of Norethindrone in Norethindrone tablets: Noreandrostenedione (Imp-A), Norethindrone enolether (Imp-B), Delta-5(6)Norethindrone (Imp-C), Delta-5(10) Norethindrone (Imp-D). The impurities elution and separation was established by optimized gradient chromatographic parameter using Inertsil ODS-3V (150 mm × 4.6 mm) 5 µ analytical column, water and acetonitrile as mobile phase, with run time 55 minutes, diode array detector (DAD) was set up at 210 nm and 240 nm wavelength for the simultaneous analysis of all impurities. The developed HPLC method was validated as per the International Conference on Harmonization (ICH) guidelines. The validation parameters used are Specificity, linearity, accuracy, precision, intermediate precision, and robustness. Limit of detection (LOD) and limit of quantification (LOQ) were also obtained for all impurities. Norethindrone samples and placebo were subjected to stress conditions of hydrolysis (acid and base), oxidative, and thermal stress degradation. Sample and standard solutions stability was also performed. The proposed validated method was successfully used for the quantitative analysis of impurities and degradation products of Norethindrone tablets. The method was found to be suitable for the stability, release, in process and Quality control analysis. The developed method is stability indicating, specific, selective, simple, precise, cost effective, linear and robust.

Keywords: Norethindrone; HPLC; Degradation products; Related substances; Method validation

Abbreviations: RP-HPLC-DAD: Reverse Phase-High Performance Liquid Chromatography-Diod Array Detector; UV: Ultra Violet; PDA: Photo Diode Array; HCL: Hydrochloric acid; NaOH: Sodium hyderoxide; H_2O_2: Hyderogen Peroxide; Acc: Accuracy; nm: Nanometer; RSD: Relative Standard Deviation; SD: Standard Deviation; µ: Micrometer; LOQ: Limit of Quantitation; LOD: Limit of Detection; Imp-A: Noreandrostenedione; Imp-B: Norethindrone enolether; Imp-C: Delta-5(6)Norethindrone; Imp-D: Delta-5(10)Norethindrone and RRT: Relative Retention Time.

Introduction

Norethindrone is form of progesterone, a female hormone. Norethindrone tablet is an oral contraceptive product provides a continuous oral contraceptive regimen of 0.35 mg Norethindrone daily. Chemically Norethindrone is 17-Hydroxy-19-Nor-17α-pregn-4-en-20-yn-3-one with molecular weight 298.42 and molecular formula $C_{20}H_{26}O_2$. Norethindrone is off white, odorless, crystalline powder. It is soluble in chloroform and dioxane, sparingly soluble in ethanol, 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 [1,2].

Impurities will be quantified as known Impurities: Noreandrostenedione (Imp-A), Norethindrone enoloether (Imp-B), Delta-5(6)Norethindrone (Imp-C), Delta-5(10)Norethindrone (Imp-D). All other peaks will be considered as unknown impurities. In this work, impurities will be injected in specificity experiment to determine their retention times [3].

As per reported literature there is only one analytical method available for determination of impurities for Norethindrone in Norethindrone Tablets by HPLC [2] and by UPLC [3]. The run time is more in HPLC method while UPLC is not economic and cost effective in terms of analytical column and HPLC instrument cost and availability. However, UPLC is useful for more sample quantitation in less time. There are some analytical assay methods available for determination of steroidal hormones [4]. Norethindrone tablets are official in USP Pharmacopoeia but there is no related substances method available for determination of impurities in Norethindrone tablets [1,5-7]. 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 [8-11] for Specificity, forced degradation studies, Precision, Sensitivity (Limit of detection and Limit of Quantification), Linearity, Solution stability, Filter paper variability, Accuracy and Robustness. The Chemical structures of Norethindrone and its impurities have been illustrated in Figures 1-5.

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Chemical structures of Norethindrone and its impurities

Currently there is one UPLC and one HPLC method available to separate and quantify the Norethindrone and its impurities in Norethindrone tablets [2,3]. More than 24 hours solution stability of impurities, reference solution and test solution is not yet reported [2-4]. In this method solution stability study was established for more than 24 hours and run time is reduced to 55 minutes for test solution and 15 minutes for standard solutions.

Experimental

Materials and chemicals

HPLC grade acetonitrile was purchased from Merck Speciality Pvt. Ltd. Water purified on Millipore was used. All other chemicals taken are analytical grade. Reference standard and impurity standard of Norethindrone and Norethindrone tablets 0.35 mg were obtained as gift sample from Bayer, India.

Instrumentations and chromatographic conditions

A waters HPLC system equipped with 2695 separation module PDA 2996, Waters 2487 dual wavelength detector with empower software, Inertsil ODS-3V (150 mm × 4.6 mm) 5 µ column, digital ultra sonicator, pH meter of Thermo scientific, Mettler Toledo electronic analytical balance, Fisher scientific vacuum oven and humidity desiccator were used.

A flow rate of 1.5 mL/minute, detection wavelength of 240 nm and 210 nm, Column oven temperature 30°C, injection Volume 20 µL, run time 15 minute for diluted standard, 55 minutes for blank, system suitability, placebo and sample solutions were optimized, retention time observed for Norethindrone peak was about 9.4 minutes. Gradient programme is 0/35, 18/35, 40/80, 45/35 and 55/35 (time (min)/%B mobile phase). Water is taken as mobile phase-A and acetonitrile as mobile phase-B.

Analytical method development and optimization

The aim and objective of the present study was to develop a simple, precise, specific, accurate, stability indicating HPLC method for determination of related substances of Norethindrone tablets and their degradation products. This is done by RP-HPLC equipped with Diode array detector (DAD).

Selection of chromatographic condition

Currently there is one UPLC and one HPLC method available to separate and quantify the Norethindrone and its impurities in Norethindrone tablets [2,3]. The UPLC instrument and column used of UPLC method were not cost effective, in spite of increase in resolution, speed, and sensitivity. The HPLC method reported have 80 minutes run time for test as well as standard solutions [2]. In this present study cost effective HPLC chromatographic method with reduced usage of acetonitrile and run time of 55 minutes for test solution and 15 minutes for standard solution has been optimized. This will benefit maximum number of samples for quantification of related substances of norethindrone in norethindrone tablet in less time. The analytical column Inertsil ODS-3V (150 mm × 4.6 mm) 5 µ, cost is less compared to UPLC column used in reported work [3]. The same particle size column has been selected for the initial method development procedure. Norethindrone tablets consist of very low content of Norethindrone (0.35 mg per tablet). There are many key factors that are responsible to attain satisfactory response for the impurities present in the low dosage

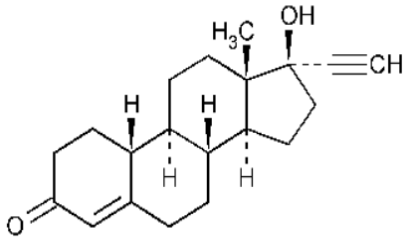

Figure 1: Norethindrone.

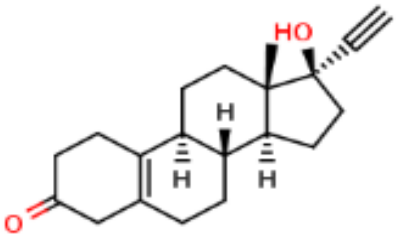

Figure 2: Delta-5(10)Norethindrone.

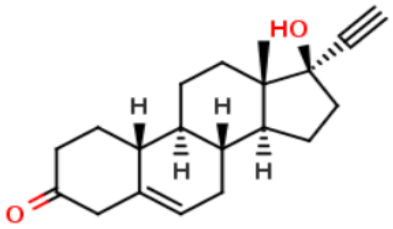

Figure 3: Delta-5(6)Norethindrone.

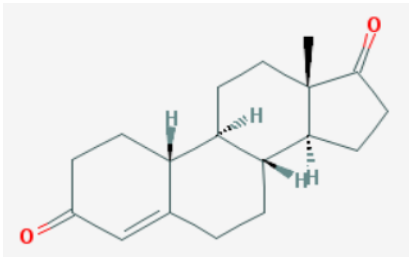

Figure 4: Noreandrostenedione.

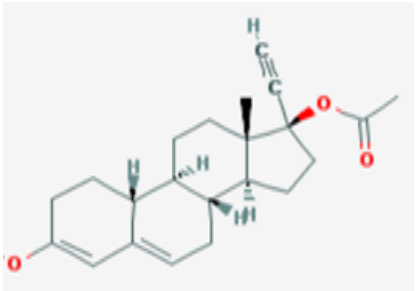

Figure 5: Norethindrone enoloether.

drug sample like selection of column, selection of mobile phase, diluent and sample concentration.

Selection of analytical column (stationary phase)

To achieve optimum area response even at low sample concentration, the universal column with optimum carbon bonded phase was used that is Inertsil ODS-3V (150 mm × 4.6 mm) 5 µ. which is compatible with 100% aqueous mobile phase and is the first choice when developing separations for polar compounds and nonpolar compounds. The same column and Inertsil C8 (150 mm × 4.6 mm) 5 µ column was used for initial trial purpose. Based on the development trial output, Inertsil ODS-3V (150 mm × 4.6 mm) 5 µ particle size column was selected. This column has a tendency to separate both polar and nonpolar components with reduced column back pressure and found to be suitable for separation of all impurities.

Selection and preparation of mobile phase

From the initial method development trials, it was observed that the component do not require any buffer for analytical method development purpose. Based on this, method development strategy was initiated using water as mobile phase-A and acetonitrile as mobile phase-B by considering the nature of drug component.

Acetonitrile and water were tried in order to find the best conditions for the elution. It was found that water and acetonitrile in the gradient chromatographic condition gave satisfactory pattern, good resolution, peak shape and desired elution was achieved. The mobile phase was tried with different proportions and using different flow rates. Finally, the optimal composition of the mobile phase was determined. Mobile phase A is water, mobile phase B is acetonitrile, with gradient chromatographic condition in HPLC.

Preparation of diluent (60:40 v/v, acetonitrile:water)

Mix 600 ml of Acetonitrile with 400 ml of water.

Selection of analytical wavelength

Impurity solutions were prepared and injected at 20 µL injection volume into RP-HPLC-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 were found to be 240 nm for Imp-A and Imp-B and 210 nm for Imp-C and Imp-D, which was finalized as working wavelength. The relative retention time is calculated with respect to norethindrone peak at corresponding wavelength.

Preparation of standard solution

Weighed accurately about 20.0 mg of Norethindrone working standard into a 20 ml volumetric flask. Added 15 ml of diluent, sonicated to dissolve and made up to volume with diluent. Diluted 5 ml of this solution to 100 ml with diluent. Further diluted 5 ml to 100 ml with diluent (0.5%).

Preparation of system suitability solution

Weighed accurately about 2.0 mg of Norandrostenedione impurity standard into a 200 ml volumetric flask. Added 50 ml of diluent, sonicated to dissolve and made up to the volume with diluent and labelled this solution as solution (A).

Weighed accurately about 10.0 mg of Norethindrone working standard into a 20 ml volumetric flask. Added 15 ml of diluent, sonicated to dissolve, then add 2 ml of above solution (A) to this solution and made up to volume with diluent.

Preparation of sample solution

Transferred 28 tablets of Norethindrone 0.35 mg into a 20 ml volumetric flask. Added 10 ml of diluent, sonicated for 20 minutes, allowed to cool and made up the volume to the mark with diluent. Filtered through Teflon filter and injected.

Preparation of placebo solution

Transferred placebo equivalent to 28 tablets into a 20 ml volumetric flask. Added 10 ml of diluent, sonicated for 20 minutes, allowed to cool and made up the volume to the mark with diluent. Filtered through Teflon filter and injected.

Evaluation of system suitability

System suitability criteria was set prior to the method validation work. The criteria are the system suitability solution at 240 nm the resolution between Norandrostenedione and Norethindrone should not be less than 1.5, and at both wavelength, tailing factor for Norethindrone peak should not be more than 2.0 and the theoretical plates for Norethindrone peak should not be less than 3000. The relative standard deviation for six replicate injections of standard solution should not be more than 5.0%.

Analytical method validation

Norethindrone tablet is single strength 0.35 mg per tablet. This strength was used for method development and validation procedure using parameters, specificity, forced degradation studies, Precision, sensitivity (Limit of detection and Limit of quantification), Linearity, Solution stability, Filter paper variability, Accuracy and Robustness.

Specificity and force degradation studies

The specificity is the ability to assess unequivocally the analyte of interest in the presence of those components which may be expected to be present in the sample matrix. Specificity/selectivity is the most crucial parameter of any analytical method used for stability study. The specificity of this method was determined by ensuring the absence of interference by any peaks in the blank diluent or placebo, and resolving any possible degradation peaks from the peaks of interest [4].

The specificity of the developed method for Norethindrone tablets 0.35 mg was determined in the presence of Norethindrone Imp-A, B, C, D at a concentration of 2.5 µg/mL. The forced conditions used for degradation studies were acid hydrolysis (5 N HCl/2 mL/70°C/3 hours), Base hydrolysis (2N NaOH/2 mL/70°C/3 hours), Oxidation (50% H_2O_2/2 mL/70°C/3 hours), Thermal (105°C/72 hours), Humidity (25°C/92%RH/72 hours) and Photolytic degradation (1.2 millionlux hours of UV light 200 watt hr/m^2 for 7 days).

Identification by retention time

Prepared representative standard solution and sample solution of each of Norethindrone tablets as per the method. Injected individual impurities, standard solution, sample solutions and spiked sample solution in an HPLC using the chromatographic system described in the method connected a photodiode array detector.

Acceptance criteria is set before specificity study is results should be comparable with respect to retention time. Peak purity should pass for Norethindrone and known impurities in control sample and spiked sample. Diluent and placebo should not show any peak at the retention

time of Norethindrone and impurity peak. Norethindrone peak should be homogeneous and there should be no co-eluting peaks. Peak purity for analyte peak and known impurity peaks should pass.

Precision

The precision of an analytical procedure is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. Precision has been divided into 'analytical repeatability' and 'intermediate precision'. Analytical repeatability includes the ability of the system to show repeatable measurements (system precision) and the method reproducibility to show reproducible sample measurements (method precision) [4].

The system precision of the method was evaluated by injecting six replicate standard preparation of Norethindrone into the HPLC. The method precision was evaluated by injecting six individual preparation of Norethindrone tablets 0.35 mg spiked with 0.15% level for Norethindrone Imp-A, B, C and D. The %RSD of each impurity was calculated. The intermediate precision (Ruggedness) of the method was evaluated by preparing and injecting Six Sample preparations of the same batch of Norethindrone Tablets 0.35 mg spiked with known impurities at 0.15% by different analyst using different column and different HPLC instrument on different day. **Results of both analysts were combined (n=12) and %RSD obtained.** Acceptance criteria is set before precision study is %RSD should not be more than 5.0% and 10% for system precision and method precision respectively.

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 further diluting the stock solution of impurity to the required concentration (Figures 6-20).

Linearity curves were made by plotting concentration versus area of the individual impurity. From these plots, LOD and LOQ were predicted from the formulae 3.3SD /S and 10SD /S respectively where SD 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. Acceptance criteria is set before sensitivity study is %RSD for LOD should not be more than 33% and for LOQ should not be more than 10%.

Linearity and range

The linearity of an analytical procedure is its ability to elicit test results that are directly proportional to the concentration of the analyte in samples over a specified range [4]. It was performed by a series of standard preparation of Norethindrone and impurity standards were prepared over a range of the LOQ to 150% of specification limits (taken as 0.2% for Norandrostenedione, 0.2% of Delta-5(6)Norethindrone, 0.3% of Delta-5(10)Norethindrone, 0.2% of Norethindrone enolether and 0.5% of Norethindrone). They are injected into HPLC.

Calibration curves were plotted by taking the peak area on the Y-axis and the concentration (ppm) on the X-axis. The specification was set up for the linearity is the coefficient of determination (r^2) from the plotted area response versus concentration curve must be ≥ 0.99 (Tables 1-6 and Figures 21-26).

Accuracy

The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value [4]. The accuracy

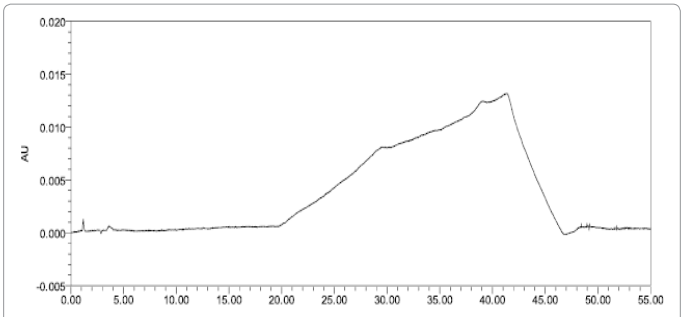

Figure 6: Chromatogram of diluent at 210 nm.

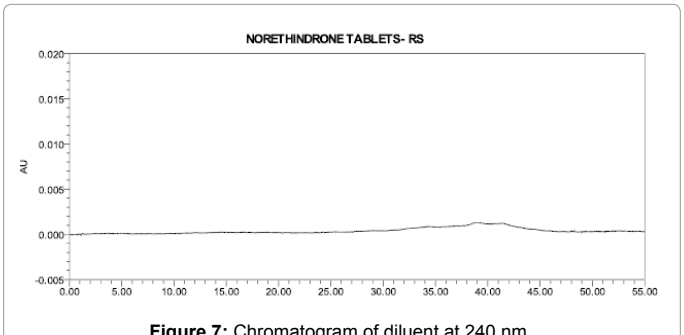

Figure 7: Chromatogram of diluent at 240 nm.

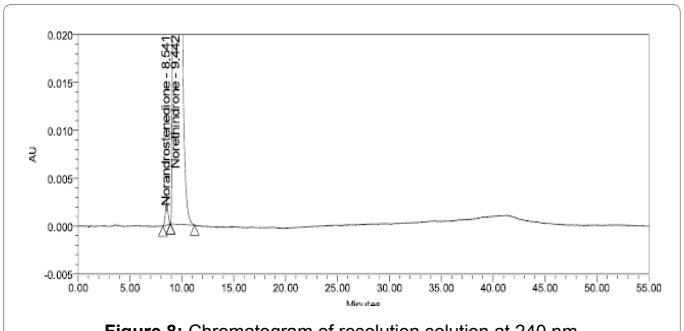

Figure 8: Chromatogram of resolution solution at 240 nm.

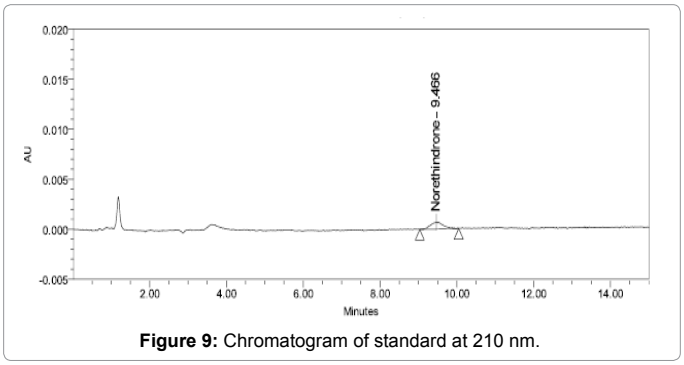

Figure 9: Chromatogram of standard at 210 nm.

of this method was confirmed by determining the recovery of a known amount of each impurity and Norethindrone added to the placebo. This is performed by the test solution of Norethindrone tablets 0.35 mg was spiked with known impurities at four different levels LOQ, 50%, 100% and 150% of the specifications limit (taken as 0.2% for Norandrostenedione, 0.2% of Delta-5(6)Norethindrone, 0.3% of Delta-5(10)Norethindrone and 0.2% of Norethindrone enoloether)

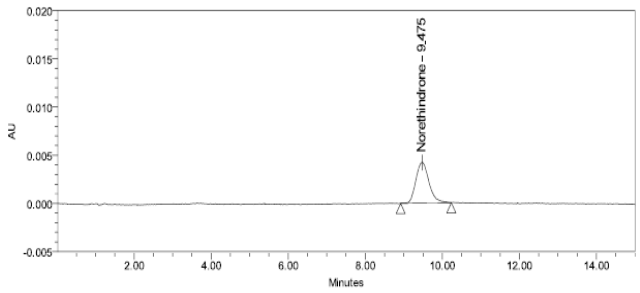

Figure 10: Chromatogram of standard at 240 nm.

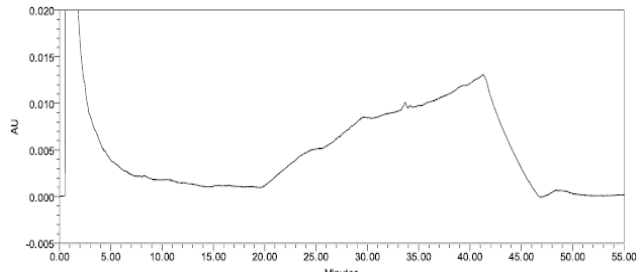

Figure 11: Chromatogram of placebo 210 nm.

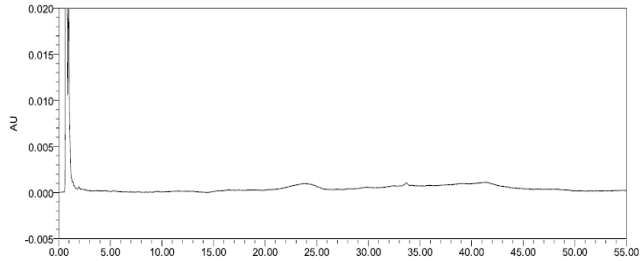

Figure 12: Chromatogram of placebo 240 nm.

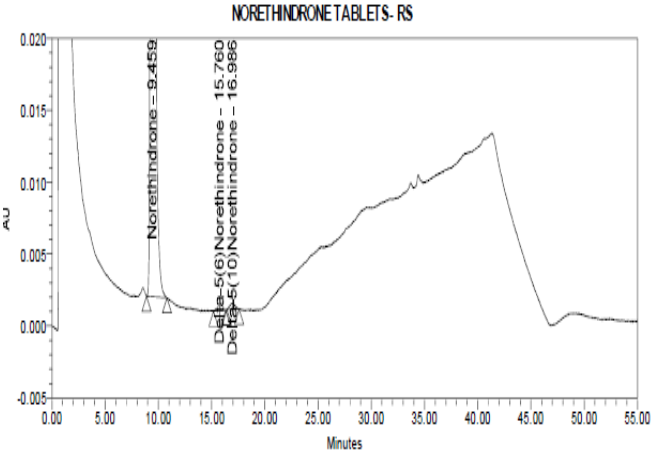

Figure 13: Chromatogram of Spiked sample 210 nm.

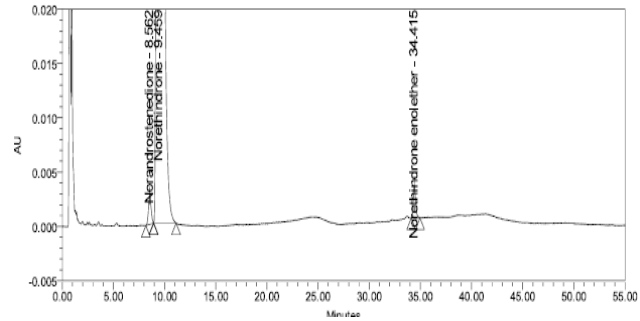

Figure 14: Chromatogram of Spiked sample at 240 nm.

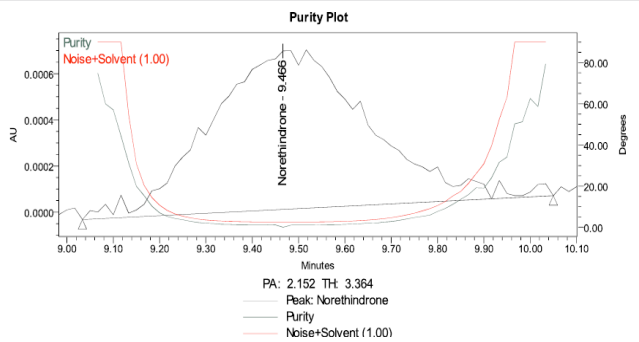

Figure 15: Spectral peak purity of Norethindrone at 210 nm.

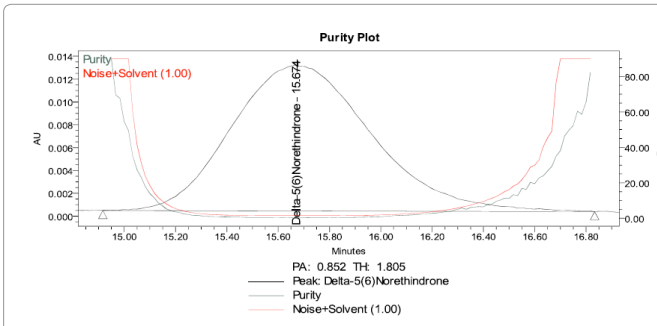

Figure 16: Spectral peak purity of Delta-5(6)Norethindrone (Imp-C) at 210 nm.

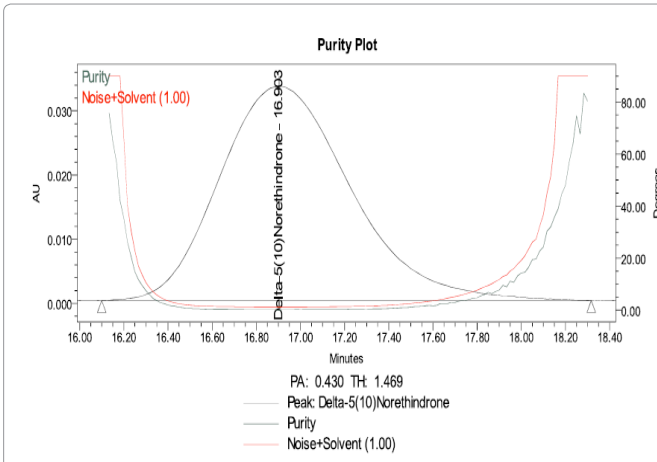

Figure 17: Spectral peak purity of Delta-5(10)Norethindrone (Imp-D) at 210 nm.

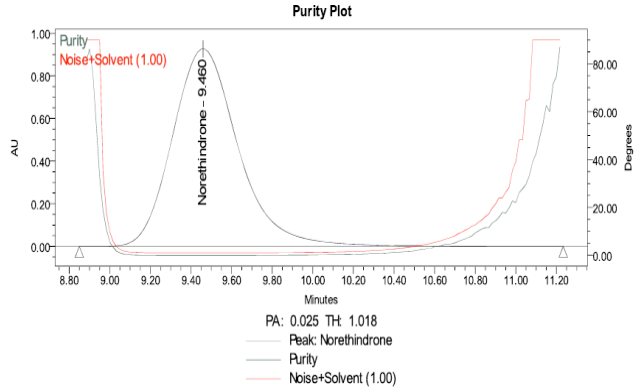

Figure 18: Spectral peak purity of Norethindrone at 240 nm.

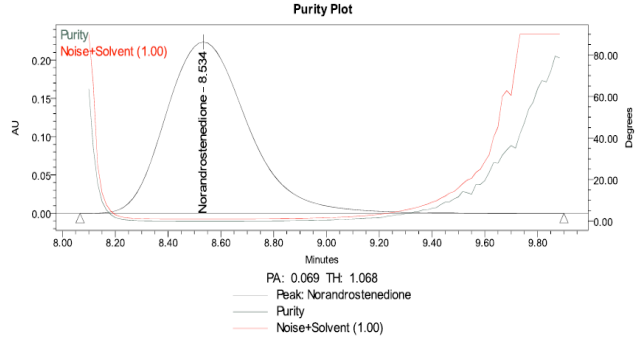

Figure 19: Spectral peak purity of Noreandrostenedione (Imp-A) at 240 nm.

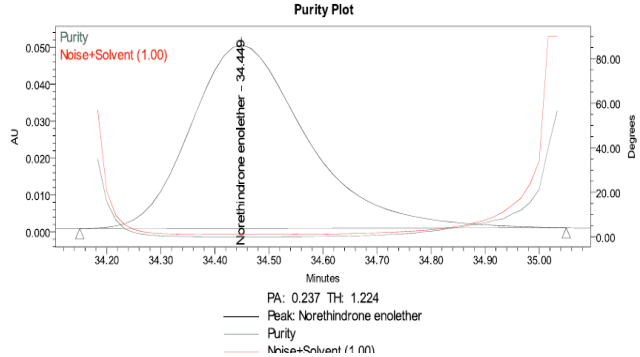

Figure 20: Spectral peak purity of Norethindrone enoloether (Imp-B) at 240 nm.

in triplicate in total twelve determinations along with control sample (without spiking). Injected into HPLC system and run the chromatogram as per the method developed. The data were evaluated the amount spiked versus the amount recovered and expressed as a percentage recovery.

Acceptance criteria is set before accuracy study is mean recovery should be in the range of 90.0% to 110.0% for 50%, 100% and 150% levels. Mean recovery should be in the range of 85% to 115% for LOQ level. The %RSD should not be more than 5%.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations to the procedure listed in the method and provides an indication of its reliability during normal usage. The system suitability parameters (% RSD of peak area response at the beginning and throughout the run were evaluated after every adjustment to ensure system suitability met the criteria. Average results obtained with modified conditions were compared to results obtained using the eventual method, and the % recovery between the two was determined [4].

This is performed by altering experimental conditions and the effect of the variation on each impurity and Norethindrone was evaluated. The flow rate of the mobile phase is 1.5 mL/minute. To study the effect of flow rate ± 10% changed as 1.35 mL/min. and 1.65 mL/min along with 1.5 mL/min. The effect of column temperature is studied at 25°C and 35°C along with 30°C. For wavelength change, ± 5 nm was changed. In all the robustness conditions, only one parameter changed by keeping all remaining conditions unchanged. Control sample, standard, placebo and sample spiked with known impurities were injected. Placebo prepared as per sample preparation.

Acceptance criteria is set before robustness study is system suitability should meet as per the developed method at each variable condition.

Solution stability study

In order to demonstrate the solution stability, standard solution, test solution and spiked test solutions were analyzed initially and at different time intervals at room temperature (ambient) as per developed method. Acceptance criteria is set before solution stability study, is cumulative %RSD should not be more than 10.0% for all the known impurities.

Filter equivalency

In order to evaluate the filter compatibility, test solution spiked with known impurities at 0.15%. This sample was centrifuged in triplicate and filtered in triplicate through one or more different membrane filters such as Teflon 0.45 and Nylon 0.45 µ filters discarded first few mL of the filtrate. Results were compared with centrifuged solutions.

Acceptance criteria is set before filter study is Overall RSD should not be more than 10.0% for the results obtained at centrifuged and with different filters (Tables 7-12).

Result and Discussions

System suitability

Norethindrone sample spiked with all impurities and injected in to the HPLC system and confirmed that separation between impurities was found to be satisfactory at the developed specified conditions. Trials were initiated with different gradient programme to achieve best separation of impurities and based on output the gradient programme was finalized as 0/35, 18/35, 40/80, 45/35 and 55/35 (time (min)/%B mobile phase). In the optimized conditions, the typical relative retention times of Imp-A, Imp-B, Imp-C and Imp-D with respect to Norethindrone at 240 nm and 210 nm were 0.93, 3.15, 1.74 and 1.88 respectively.

For the system suitability solution at 240 nm the resolution between norandrostenedione and Norethindrone was found more than 1.5, tailing factor for Norethindrone peak was less than 2.0 and the theoretical plates for Norethindrone peak was more than 3000. The relative standard deviation for six replicate injections of diluted

Sr. No.	Concentration (ppm)	Area response
1	0.25	1490
2	1.28	7378
3	2.04	14166
4	2.55	17037
5	3.07	21604
6	3.83	26728
Correlation Coefficient		**0.99598**

Table 1: Linearity solution levels of Norethindrone at 210 nm.

Sr. No.	Concentration (ppm)	Area response
1	0.10	4499
2	1.27	57835
3	2.03	90810
4	2.54	112384
5	3.05	139047
6	3.81	171837
Correlation Coefficient		**0.99957**

Table 2: Linearity solution levels of Norethindrone at 240 nm.

Sr. No.	Concentration (ppm)	Area response
1	0.10	4839
2	0.51	25828
3	0.82	40474
4	1.03	50164
5	1.24	61903
6	1.54	76378
Correlation Coefficient		**0.99960**

Table 3: Linearity solution levels of Norandrostenedione at 240 nm.

Sr. No.	Concentration (ppm)	Area response
1	0.23	733
2	0.48	2373
3	0.76	3626
4	0.95	4667
5	1.14	5315
6	1.43	6652
Correlation Coefficient		**0.99269**

Table 4: Linearity solution levels of Delta-5(6) Norethindrone at 210 nm.

Sr. No.	Concentration (ppm)	Area response
1	0.24	1630
2	0.75	6065
3	1.20	10369
4	1.50	13540
5	1.80	17205
6	2.25	20817
Correlation Coefficient		**0.99722**

Table 5: Linearity solution levels of Delta-5(10) Norethindrone at 210 nm.

Sr. No.	Concentration (ppm)	Area response
1	0.10	4358
2	0.50	24909
3	0.80	38939
4	1.00	48118
5	1.20	59474
6	1.50	72985
Correlation Coefficient		**0.99940**

Table 6: Linearity solution levels of Norethindrone enoloether at 240 nm.

standard solution was less than 5.0% at both the wavelengths 240 nm and 210 nm (Table 12).

Specificity and forced degradation studies

Forced degradation studies on Norethindrone tablets 0.35 mg under different stress conditions shown degradation results in Table 7. The peak purity data of Norethindrone peak in every degradation sample shows that the Norethindrone peak and all known impurity peaks are homogeneous and there are no co-eluting peaks indicating that the method is stability indicating and specific. Purity angle was found less than the purity threshold hence peak purity passes for Norethindrone and known impurities in control sample and spiked sample. Hence the method is Selective. No interference was observed from diluent and placebo at the retention time of Norethindrone and known impurity peaks (Tables 7 and 8).

Identification by retention time

The retention time (in minutes) obtained from chromatogram for Norethindrone at 210 nm, Norethindrone at 240 nm, Noreandrostenedione, Delta-5(6)Norethindrone, Delta-5(10) Norethindrone, Norethindrone enolether, is 9.47, 9.47, 8.53, 15.67, 16.90, 34.45 respectively.

Precision

The % RSD of Norethindrone standard solution at 210 nm and 240 nm was 1.09% and 0.86% respectively which is less than 5% indicating the method is precise. The %RSD of Norethindrone Imp-A, B, C, D was 0.48, 0.47, 1.50 and 1.40 respectively which is less than 10% confirming the developed method is reproducible. The %RSD obtained in intermediate precision for Norethindrone Imp-A, B, C, D was 2.23, 1.56, 4.18 and 1.56. The Overall %RSD for twelve results is less than 10.0% indicating the method is rugged.

Sensitivity (Limit of detection and limit of quantification)

%RSD for LOD was found less than 33% and for LOQ was less than 10%. The Limit of detection for Norethindrone Imp-A, B, C and D was 0.01, 0.01, 0.024 and 0.025 respectively. The Limit of Quantification for Norethindrone Imp-A, B, C and D was 0.02, 0.02, 0.05 and 0.05 respectively. This reveal that the method is sensitive.

Linearity and range

Results show an excellent correlation obtained between peak area and concentration of Norethindrone and all the impurities. Linear calibration plot for the related substances method was obtained over the calibration range LOQ to 150%. The Correlation coefficient for Norethindrone and known impurities is more than 0.99. Therefore, the HPLC method for the determination of related substances in Norethindrone Tablets is linear. The response of Impurity A, B, C and D was found to be 0.93, 0.97, 1.45 and 0.71 respectively (Tables 1-6).

Accuracy

Accuracy was assessed from three replicate determinations of four different levels including LOQ, 50%, 100% and 150% of the specified level of the impurities. The mean recovery results for LOQ was found in the range between 85 to 115% within the % RSD of 5.0% and for 50%, 100% and 150% was found in the range between 90 to 110% within the %RSD of 5.0% demonstrating that the recovery is within the desired range. The mean recovery for all impurities is meeting the acceptance

Sr. No.	Experiment	Degradation Condition	Purity Angle	Purity Threshold
			Norethindrone	
1	Control sample 210 nm	-	0.024	1.014
2	Control sample 240 nm	-	0.025	1.018
3	Acid Degradation 210 nm	5N HCl/70°C/3 hours	0.236	1.218
4	Acid Degradation 240 nm	5N HCl/70°C/3 hours	0.270	1.268
5	Base Degradation 210 nm	2N NaOH/70°C/3 hours	0.032	1.014
6	Base Degradation 240 nm	2N NaOH 70°C/3 hours	0.036	1.022
7	Peroxide Degradation 210 nm	50% H2O2 70°C/3 hours	0.090	1.073
8	Peroxide Degradation 240 nm	50% H2O2 70°C/3 hours	0.106	1.088
9	Thermal Degradation 210 nm	105°C 72 hours	0.025	1.015
10	Thermal Degradation 240 nm	105°C 72 hours	0.026	1.020
11	Humidity Degradation 210 nm	25°C/92%RH 72 hours	0.031	1.021
12	Humidity Degradation 240 nm	25°C/92%RH 72 hours	0.026	1.018
13	Photolytic Degradation 210 nm	1.2 million lux hours	0.030	1.014
14	Photolytic Degradation 240 nm	1.2 million lux hours	0.031	1.015

Table 7: Table for forced degradation studies.

Sr. No.	Experiment	Degradation Condition	% Norandro-stenedione	% Norethind rone enolether	% Single max. unknown	% Total
1	Control	-	0.00	0.00	0.00	0.00
2	Acid Degradation	5N HCl/70°C/3 hours	0.00	0.00	0.381	0.68
3	Base Degradation	2N NaOH /70°C/3 hours	0.00	0.026	0.00	0.03
4	Peroxide Degradation	50% H_2O_2/70°C/3 hours	0.456	0.00	3.270	12.93
5	Thermal Degradation	105°C/72 hours	0.00	0.026	0.00	0.03
6	Humidity Degradation	25°C/92%RH/72 hours	0.00	0.00	0.00	0.00
7	Photolytic Degradation	1.2 million lux hours	0.00	0.00	0.00	0.00

Table 8: Table for impurities in forced degradation studies.

Impurity Name	Accuracy levels	Amount added(mg)	Amount recovered (mg)	Mean% Recovery	Mean % RSD
Norandrostenedione	LOQ	0.00202	0.00198	98.0	1.35
	50%	0.01010	0.01054	103.8	1.43
	100%	0.02020	0.02063		
	150%	0.03030	0.03176		
Delta-5(6) Norethindrone	LOQ	0.00392	0.00408	104.1	1.25
	50%	0.00871	0.00896	99.9	3.74
	100%	0.01743	0.01765		
	150%	0.02614	0.02500		
Delta-5(10) Norethindrone	LOQ	0.00692	0.00713	103.1	1.49
	50%	0.01457	0.01469	101.4	1.04
	100%	0.02914	0.02986		
	150%	0.04371	0.04411		
Norethindrone enolether	LOQ	0.00206	0.00210	102.1	0.28
	50%	0.01028	0.01100	106.9	1.04
	100%	0.02056	0.02190		
	150%	0.03083	0.03300		

Table 9: Accuracy results of Norethindrone and its related substances (known impurities).

Sr. no.	Parameters	Variations	RRT of Related Compounds			
			Norandros tenedione	Delta-5(6)Norethindrone	Delta-5(10)Norethindrone	Norethindrone enol ether
1	Control	-	0.90	1.66	1.79	3.59
2	Column Temperature	-5°C	0.90	1.69	1.82	3.53
		+5°C	0.90	1.50	1.75	3.69
3	Flow rate	-10%	0.90	1.58	1.67	3.40
		+10%	0.90	1.65	1.79	3.75
4	Wavelength	-5 nm	0.90	1.67	1.80	3.64
		+5 nm	0.90	1.66	1.79	3.64

Table 10: Robustness results obtained by varying conditions of method parameters, RRT.

Sr. No.	Experiment	Resolution between Norandrostenedione and Norethindrone	Tailing of Norethindrone in resolution solution	Tangent of Norethindrone in resolution solution
1	Control	1.88	1.28	5522
2	Temperature -5°C	1.81	1.25	4995
3	Temperature +5°C	1.91	1.25	5783
4	Flow -10%	1.85	1.26	5458
5	Flow +10%	1.82	1.25	5074
6	Wavelength -5nm	1.58	1.23	3784
7	Wavelength +5nm	1.59	1.23	3785

Table 11: Table for System suitability in robustness study.

Sr. No.	Parameters	% RSD of standard 210 nm	% RSD of standard 240 nm	Resolution between Norandrostenedione and Norethindrone	Tailing of Norethindrone in resolution solution	Tangent of Norethindrone in resolution solution
1	Specificity	2.82	0.78	1.73	1.29	4604
2	Forced Degradation	2.22	2.41	1.60	1.23	3781
3	LOD/LOQ	0.41	0.09	1.91	1.06	7255
4	Linearity	0.89	0.21	2.04	1.05	8487
5	Accuracy	2.54	0.37	1.60	1.28	3933
6	Method Precision	1.09	0.86	2.00	1.29	6235
7	Ruggedness	1.60	0.37	1.88	1.28	5522

Table 12: System suitability in overall validation.

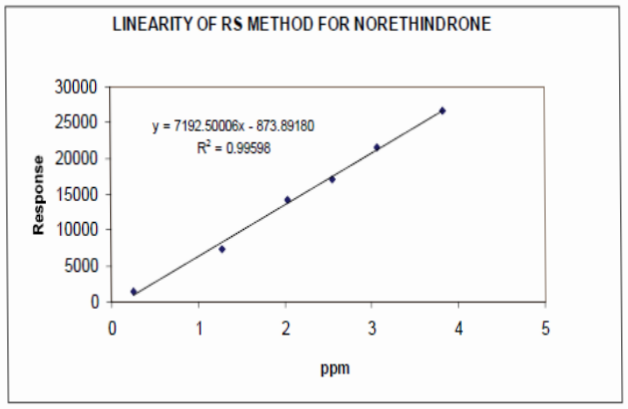

Figure 21: Linearity graph of Norethindrone at 210 nm.

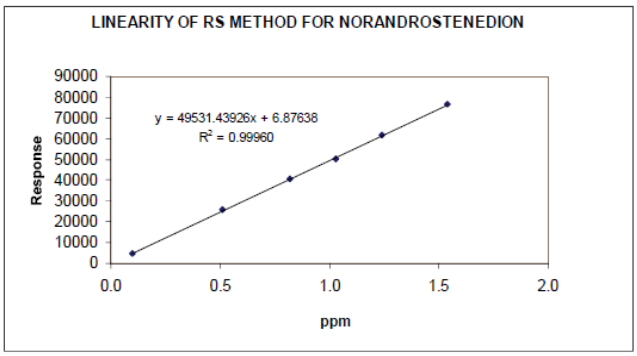

Figure 23: Linearity graph of Norandrostenedione at 240 nm.

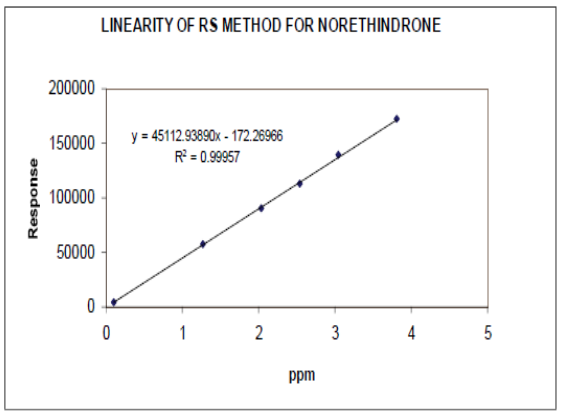

Figure 22: Linearity graph of Norethindrone at 240 nm.

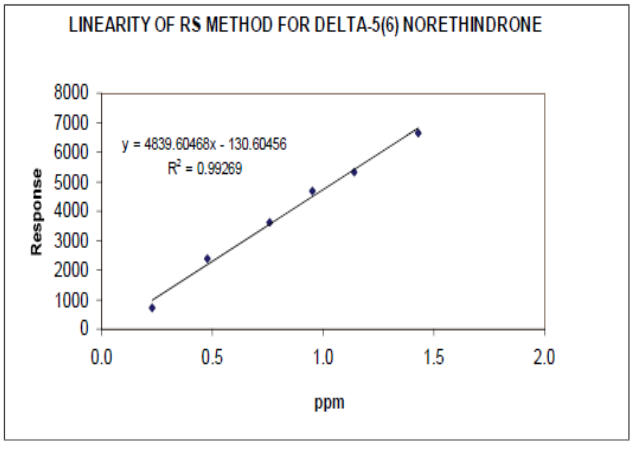

Figure 24: Linearity graph of Delta-5(6) Norethindrone at 210 nm.

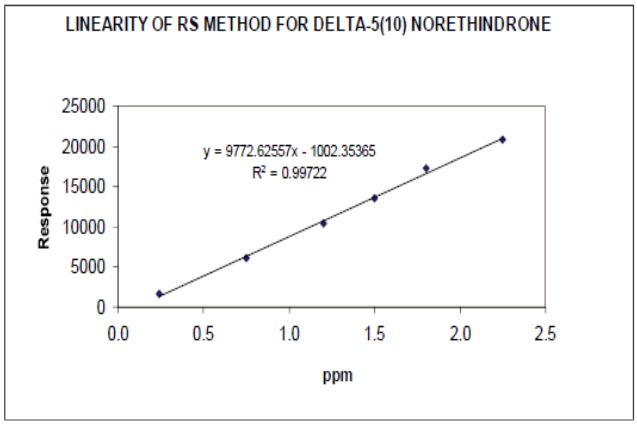

Figure 25: Linearity graph of Delta-5(10) Norethindrone at 210 nm.

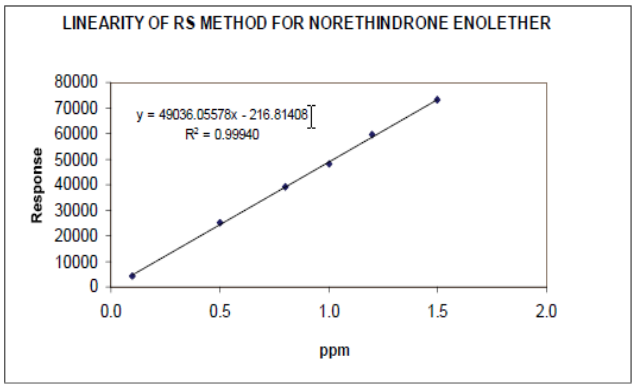

Figure 26: Linearity graph of Norethindrone enolether at 240 nm.

criteria. Therefore, the HPLC method for the determination of related substances in Norethindrone tablets is accurate (Table 9).

Robustness

This HPLC method for determination of related substances in Norethindrone tablets is robust for small changes in flow rate, column temperature and wavelength. But the method is sensitive for small changes in mobile phase composition in gradient run. Imp-A merges with Norethindrone peak. Therefore, proper care must be taken. There is no significant change observed in the relative retention times of the Norethindrone and their related substances at this gradient condition (Tables 10 and 11).

Solution stability study

Evaluation of standard solution, control sample and impurity spiked sample solutions at their respective wavelength shown that the cumulative %RSD is within limits (10%). No significant changes are observed in the area of Norethindrone, Imp A, B, C and D during solution stability experiment. Therefore, standard solution and sample solutions are stable for 35 hours at room temperature.

Filter equivalency

The results of the filter equivalency were evaluated for sample spiked with known impurities at 0.15% then was centrifuged in triplicate and filtered in triplicate through two different membrane filters such as Teflon 0.45 and Nylon 0.45 µ filters discarding first few mL of the filtrate. The centrifuged and filtered samples results were compared. Overall %RSD is within limits (10%). Therefore, Nylon and Teflon 0.45 µ filters are suitable for filtration of samples.

In this study cost effective gradient HPLC chromatographic method with reduced usage of acetonitrile and run time of 55 minutes was validated. This has advantage of analysing maximum number of samples for quantification of related substances of norethindrone in norethindrone tablet in less time and less cost. The analytical column cost is less compared to UPLC column [3]. Solution stability study was established for standard and sample solutions up to 35 hours.

Conclusion

The developed analytical RP-HPLC-DAD method is validated as per International conference of Hormonization guideline. The forced degradation studies indicated that method is selective and stability indicating. UV detection at 240 nm and 210 nm was found to be suitable without any interference from placebo excipients. All the calibration curves obtained were found to be linear with values of correlation coefficients more than 0.99. The response factor of individual known impurity is useful for accurate quantification of finished and final marketed formulations. Recovery tests confirmed the accuracy of the method. The proposed HPLC method is sensitive, precise, accurate, specific, robust, rugged, stability indicating, cost effective and efficient.

The proposed HPLC method enables the separation and quantitative determination of specified and unspecified impurities of Norethindrone in Norethindrone tablets by HPLC techniques. This confirms the quality of Norethindrone tablet and eventually reduce the side effects of Norethindrone tablets.

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