REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF NORETHINDRONE ACETATE AND ETHINYL ESTRADIOL IN PHARMACEUTICAL FORMULATION

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ABSTRACT
RP-HPLC method has been proposed on phenomex BDS C18 column (250 × 4.6 mm, 5µ) using the mixture of methanol: phospahte buffer, 10 mM (pH adjusted to 2.0 with 0.1% O-phosphoric acid) in the ratio of 84:16 v/v as mobile phase. The flow rate was maintained at 1mL/min and the analytes were monitored at 220 nm. Ethinyl Estradiol and Norethindrone Acetate eluted at 3.5 and 4.7 min respectively with resolution of 4.17 min. The peaks obtained were symmetrical with tailing factor less than 1.5 and theoretical plates more than 2000. The developed method was validated in accordance with ICH guidelines and the results of all parameters were found within acceptable limits. The method was found linear in the concentration range of 5-1000 µg/mL and relative standard deviation of the precision study was found about 2.0%. The assay results was found 102.94 (±2.01) and 105.37 (±1.94) % with mean % Recovery 106.30 and 95.45 for Ethinyl Estradiol and Norethindrone Acetate respectively. The developed method has been found suitable for routine analysis of Ethinyl Estradiol and Norethindrone Acetate in mixture and formulation.

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INTRODUCTION

The average population change is currently growing at a rate of about 1.14% per year as reported by World Meters (Population). [1] Because of this alarming trend, contraceptives are the need of the day. In the earlier part of 20th century, methods of contraception used were condoms, diaphragms, spermicidal creams, foam tablets etc. which intimately related to sexual intercourse with high failure rate, therefore despised by most couples.

WHO stated that about 85 % of undesired pregnancies occurring in 35 countries could have been prevented with the optimal use of modern methods of contraception. [2] Hormonal contraception refers to birth control methods that act on the endocrine system that contains steroidal hormones either Progestogen, Estrogen or both. The successful use of an oral Progestogen for contraception was proved in 1955 and the combined oral contraceptive pill (COCPs) containing Progestogen and Estrogen was first marketed in 1960. [3, 4]

Steroidal synthetic derivatives of Estrogen (Ethinyl Estradiol, Mestranol, Quinestrol) and Progestogen (Norethindrone Acetate, Levenorgestrel, Norgestel, Ethynodiol Diacetate, Lynestrenol, Dosogestrel) are commonly used as COCPs. Norethindrone Acetate (NEA) is 17-Hydroxyl-19-nor-17a-pregn-4-en-20-yn-3-one acetate [5] and works by decreasing the pulse frequency of gonadotropin-releasing hormone (GnRH).

![Fig. 1: Structure of Norethindrone Acetate (NEA).](image1)

Ethinyl Estradiol (EE) is 19-Nor-17a-pregna-1, 3, 5(10)-triien-20-yn-3-diol [6] which decreases the secretion of follicle stimulating hormone (FSH).

![Fig. 2: Structure of Ethinyl Estradiol (EE).](image2)

The combined effect prevents a mid-cycle luteinizing hormone (LH) surge and inhibits follicular development and thereby prevents ovulation. Tablet formulations containing EE and NEA like Femhrt (2.5 µg:0.5 mg), Jintel (5 µg:1 mg), Activella (1 mg: 0.5 mg), Estrostep (20 µg:1 mg) and Junel (30 µg:1.5 mg) are available in US market and prescribed as COCPs. [7]

Literature review reveals that there are RP–HPLC [8–10] Stability indicating liquid chromatographic [11,12], Partial Least Squares and Principal Component Regression Multivariate Calibration [13] spectrophotometric [14] methods available for estimation of EE and NEA individually and in combination with other drugs. A LC-MS/MS method has been reported for simultaneous estimation of EE and NEA in human plasma. [15] No method has reported so far simultaneous estimation EE and NEA in formulation. Hence the objective of the present study was to develop and validate simple RP–HPLC method for simultaneous estimation of EE and NEA in COCPs.

EXPERIMENTAL

Instrumentation

Analysis was performed with a Shimadzu chromatograph equipped with an LC-10 AT solvent-delivery module, an SPD-10A UV–visible detector and a Rheodyne injector with 20-µL sample loop. The equipment was controlled by a LC Solution Software. Acculab Balance, Ultrasonicator bath, Rotary vacuum pump and Digisun Electronics Digital pH meter (7007) were used throughout the studies.

Chemicals and Reagents:

Norethindrone Acetate (Reference Standard) and Ethinyl Estradiol (Reference Standard) were obtained in the form of gift samples from Cipla Ltd, Mumbai. Norethindrone Acetate Tablets (Regestrone, 5 mg, Sandoz a Novarits company), Ethinyl Estradiol Tablets (Lynoral, 0.1 mg, Organon (India) private Ltd) were procured from local pharmacy. HPLC grade Methanol, Acetonitrile, Water, Potassium dihydrogen orthophosphate and Ortho-phosphoric acid were used throughout the analysis.
Chromatographic conditions

Reversed-phase chromatography was performed using Phenomenex BDS C18 column (5μ, 250×4.6 mm i.d.) as a stationary phase and the mixture of methanol and phosphate buffer (pH 2.0 adjusted with 0.1% o-phosphoric acid) in the ratio of 84:16 (v/v) as mobile phase. The mobile phase was filtered through 0.2 μ nylon membrane filter paper and degassed by sonication before use. The flow rate of mobile phase was maintained at 1.0 mL/min. After equilibration of column with the mobile phase indicated by a stable baseline, aliquots of sample (20 μl) were injected and the total run time was kept for 10 min. All analytes were monitored at 220 nm wavelength.

Preparation of mobile phase:

Potassium dihydrogen orthophosphate buffer (KH$_2$PO$_4$), 10mM was prepared by dissolving 0.68 mg of potassium dihydrogen orthophosphate in few ml of water in 500 mL volumetric flask and the volume was made up to mark with water. The pH of the buffer was adjusted to 2.0 by using 0.1% ortho-phosphoric acid and mixed with methanol. The mobile phase was filtered through membrane filter (0.2μ) and sonicated for 10 min before use.

Preparation of Standard Ethinyl Estradiol and Norethindrone Acetate Solution:

Accurately weighed 50 mg of EE and NEA was transferred separately into a clean and dry 10 mL volumetric flask and dissolved in few mL of methanol. The volume was made up to mark with methanol to get concentration 5000 µg/mL.

Preparation of Mixed Standard Solution:

Accurately 50 mg of NEA was weighed, transferred into 10 mL volumetric flask along with 1 mL of 1000 µg/mL methanolic solution of EE, dissolved in methanol by sonication for 10 minutes and made up the volume to the mark to get concentration of 100:5000 µg/mL of EE and NEA. This solution was further diluted with mobile phase to get final concentration 10 µg/mL of EE and 500 µg/mL of NEA.

Preparation of serial standard solutions for calibration curves:

The standard EE solution was diluted to get concentration 2000, 1000, 500, 250, 100, 50, 10, 5, 2, 1, 0.5 and 0.2 µg/mL and similarly the standard NEA solution was diluted to get a concentration 1000, 500, 250, 125, 100, 50, 10, 5 and 1 µg/mL with mobile phase. These solutions were used for determination of linearity and range.

Stability of the solution:

Stability of the solution was evaluated by analyzing mixed standard solution (n=3) of the concentration 10: 500 µg/mL of EE and NEA at different time intervals (0, 2, 4, 24, 48 hrs) under the same experimental conditions on the same day.

Assay:

Ten tablets of NEA (Regestrone, 5 mg) and EE (Lynarol 0.1mg) were transferred into 100 mL volumetric flask; about 50 mL of methanol was added and sonicated for 20 minutes. The volume was made up to the mark with methanol and filtered through whatmann filter paper (no 41). First few mL of the filtrate was discarded and rest of the filtrate was used for the analysis. The concentration of the obtained filtrate was 10: 500 µg/mL of EE and NEA respectively which was filtered through 0.45 µ cellulose acetate filter using syringe. Sample and mixed standard solution were injected into the Rheodyne injector (20µl) of HPLC system and their chromatograms were recorded under the finalized chromatographic conditions after getting a stable baseline. Peak areas were recorded for all the peaks and the amount of EE and NEA present in the mixture was calculated following formulae.

\[
\text{Assay (%) = } \frac{\text{AUC of sample} \times \text{Concentration of standard} \times \text{Dilution factor} \times 100}{\text{AUC of Standard} \times \text{Label claim}}
\]

Validation procedure

The proposed method of RP-HPLC analysis for EE and NEA in combination were validated as per the recommendations of ICH guidelines (Q2R1) for the parameters like specificity, linearity and range, accuracy, precision, detection limit and quantitation limit, robustness and system suitability parameters. [16]

Specificity

The peak purity of NEA and EE were assessed by comparing the chromatogram of standard, sample, mobile phase (blank) and excipient solutions.

Linearity and Range:

Serial dilutions of EE (0.2-2000 µg/mL) and NEA (1-1000 µg/mL) were injected and linearity was determined by plotting area under curve (AUC) vs concentration whereas range was determined by plotting response (AUC/Concentration) vs log concentration.
Accuracy/Recovery:
To study the accuracy of the proposed method, recovery studies were carried out by standard addition method at three different levels (50%, 100% and 150%). A known amount of EE and NEA were added to pre-analysed tablet solution, analysed and per cent recoveries were calculated. The results are presented in Table 1.

Precision
Precision commonly expressed as the standard deviation or relative standard deviation (coefficient of variation) and was performed as explained below.

Intra-day precision
Intra-day precision was evaluated by analyzing mixed standard solution (n=3) of the concentration 10: 500 μg/ml of EE and NEA at three different time intervals (0, 2, 4 hrs) under the same experimental conditions on the same day.

Inter-day precision
Intermediate precision (inter-day precision) was determined by analyzing mixed standard solution (n=3) of the concentration 10: 500 μg/ml of EE and NEA on three consecutive days.

Reproducibility:
Reproducibility was evaluated by analyzing the mixed standard solution of the concentration range of 10: 500 μg/ml of EE and NEA prepared by two different analysts. The t and F-test were performed to determine significant variation between the results and precision obtained by two analysts.

Sensitivity
Sensitivity of the method was determined by means of the detection limit (LOD) and quantification limit (LOQ). Calculations for LOD and LOQ were based on the standard deviation of the intercept from the calibration curve (σ) and the mean slope of curve (S), using the equation LOD = 3.3 × σ/S and the equation LOQ = 10 × σ/S.

Robustness:
Robustness was determined by comparing the system suitability parameters of mixed standard solution of concentration 10: 500 μg/ml of EE and NEA with small change in pH of buffer from 2.0 to 1.94 and 2.06, in flow rate from 1 mL/min to 0.97 and 1.03 mL/min, in organic phase composition from 84:16 to 83:17 and 85:15 v/v. Their effects on the retention volume, theoretical plates, tailing factor and resolution of the peaks were studied. These facts suggest that the method did not change with time and experimental conditions.

RESULTS AND DISCUSSION:
Development and optimization of the chromatographic method
The chromatographic conditions were adjusted to provide the best performance of the assay. For system optimization the important parameters such as type and concentration of organic solvents and buffer, ultra-violet detection, pH, and flow rate were investigated.

Detection wavelength:
The solutions of analytes in the mobile phase were scanned by UV–Visible spectrophotometer in the region of 200–400 nm (Figure 3) and an isobestic wavelength 220 nm was selected for monitoring analytes as the most appropriate wavelength for analysis considering the response and the sensitivity.

Figure 3: Overlaid UV spectra of EE and NEA.
Effect of organic phase composition:
The mobile phases composing of methanol:water (70:30) and acetonitrile:water (70:30) were tested on C18 column at flow rate 1mL/min. The mobile phase containing methanol was showing better peak symmetry and theoretical plates than acetonitrile for the analytes, hence methanol was selected as organic phase.

Effect of mobile phase composition:
Methanol: Phosphate buffer, 10 mM in different ratios of 70:30, 50:50, 60:40, 80:20 and 84:16 v/v were analysed using C18 column at flow rate 1mL/min. The mobile phase of methanol: phosphate buffer in the ratio 84:16 v/v was finalized as best resolution between EE and NEA with good peak symmetry was found as shown in Figure 4.

![Figure 4: The chromatogram of mixed standard solution of EE and NEA.](image)

Effect of pH:
The mobile phase with pH values 2.0 and 3.0 were checked to establish the optimum separation and highest analytical sensitivity for EE and NEA. The best results were obtained at 2.

Stability of the solution
The solution was found stable for 48 hrs as RSD of the results obtained at 0, 24 and 48 hrs was found about 2%. The best results can be obtained within 24 hrs of preparation of solution as % RSD of the results obtained at 0, 2, 4 and 24 hrs was found less than 1.

Assay
Tablet formulation containing EE and NEA in the ratio 1: 50 was selected for analysis considering the linearity and range of the method. To achieve the best possible accuracy and precision, the concentration of EE selected for assay was about three times of LOQ (10 µg/mL of EE). The formulation is not available in India, hence a mixture of individual tablet formulation was used for analysis and the assay results was found 102.94 (±2.01) and 105.37 (±1.94) % for EE and NEA respectively as presented in table 1.

<table>
<thead>
<tr>
<th>Level of Addition</th>
<th>Amount spiked (µg/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EE</td>
<td>NEA</td>
</tr>
<tr>
<td>Assay (n=6)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50%</td>
<td>5</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>10</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150%</td>
<td>15</td>
<td>750</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean % Recovery (%RSD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>106.3 (2.84)</td>
<td>95.45 (2.92)</td>
</tr>
</tbody>
</table>

Method validation
Specificity
The overlay chromatograms of standard, sample, blank and excipient solution were studied and the method was found specific as the peaks found in the chromatogram of blank and excipients were not overlapping with the analytes peaks.
Linearity and range:
To determine linearity and range, RP-HPLC analysis was performed for concentration range 1-1000 µg/ml for NEA and 0.1-2000 µg/ml for EE. The range was determined from the linear part of the graph of response (AUC/Concentration) vs log concentration as shown in figure 5. The linearity of RP-HPLC method was determined from the plot AUC vs concentration as shown in figure 6 and 7 for EE and NEA respectively and it was found in the concentration ranges of 5–1000 µg/mL for EE and NEA with correlation coefficients of 0.98 and 0.982 respectively.

Accuracy:
Accuracy by standard addition method was determined by calculating percentage recovery and the mean recovery was found to be 106.3 % (±2.84 %) for EE and 95.45 % (±2.92%) for NEA as presented in table 1.

Precision:
The mixed standard solution of concentration 10 µg/mL of EE and 500 µg/mL of NEA were selected for intra-day and inter-day precision. The RSD of the study was found about 2%. Reproducibility was determined by applying t-test and F-test for the results obtained from two different analysts and there was no significant difference observed between precision and results of the assay of two different analyst as calculated t and F values were found less than tabulated values at degree of freedom 2, 2 (P=0.10).

Limits of detection and quantification:
LOD was found to be 1.025 µg/mL and 0.273 µg/mL while LOQ was found to be 3.1062 µg/mL and 0.8296 µg/mL for EE and NEA respectively at which the analytes could be readily detected and quantified with accuracy.
Robustness:

There was no significant variation found for the retention volumes, theoretical plates, asymmetric factor and resolution on deliberate change in flow rate, organic composition and pH in the robustness study. The results of the robustness study are presented in table 3.

Table 3: Robustness Data of EE and NEA obtained from (10:500 µg/mL).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Parameters</th>
<th>Flow Rate</th>
<th>Organic Phase</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE</td>
<td>Retention volume (ml)</td>
<td>0.978</td>
<td>1.038</td>
<td>0.202</td>
</tr>
<tr>
<td></td>
<td>Theoretical plates</td>
<td>1.998</td>
<td>1.946</td>
<td>1.975</td>
</tr>
<tr>
<td></td>
<td>Tailing Factor(min)</td>
<td>1.411</td>
<td>1.457</td>
<td>1.86</td>
</tr>
<tr>
<td>NEA</td>
<td>Retention volume (ml)</td>
<td>0.956</td>
<td>1.952</td>
<td>0.409</td>
</tr>
<tr>
<td></td>
<td>Theoretical plates</td>
<td>1.849</td>
<td>1.629</td>
<td>1.947</td>
</tr>
<tr>
<td></td>
<td>Tailing Factor (min)</td>
<td>1.345</td>
<td>1.457</td>
<td>0.708</td>
</tr>
<tr>
<td></td>
<td>Resolution between EE and NEA (%RSD, n=9)</td>
<td>1.535</td>
<td>1.601</td>
<td>1.547</td>
</tr>
</tbody>
</table>

Limits: RSD <2

System suitability

System suitability was performed to confirm that the system was appropriate for the analysis to be performed. The test was carried out by making six replicate injections of mixed standard solution containing 500 µg/mL NEA and 10 µg/mL EE and analysing each analyte for its theoretical plates (5264 and 6322), resolution (4.17 min) and tailing factor (1.199 and 1.429 min).

The developed and validated method was found to be accurate (% mean recovery 95-106) and precise (%RSD ~ 2.0) for analysis of EE and NEA in tablet mixture (1: 50).

CONCLUSION

A RP-HPLC method was developed on the column Phenomenex BDS C18 (250 × 4.6 mm, 5µ) using the mixture of Methanol : Phosphate buffer, 10 mM (pH 2.0,) in the ratio of 84: 16 v/v as a mobile phase at a flow rate of 1 mL/min and the analytes were monitored at 220 nm. The peaks of Norethindrone Acetate and Ethinyl Estradiol were found well resolved with retention time of 3.5 and 4 min respectively. The system suitability parameters like theoretical plates, tailing factor and resolution were found complying with the recommended specification.

The method was validated as per ICH (Q2R1) recommendation and the results for validation parameters and assay in tablet mixture were found within the acceptance criteria. Thus, the proposed method can be successfully applied for the routine analysis of Norethindrone Acetate and Ethinyl Estradiol in pharmaceutical formulation.

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