Cost Effective, Efficient and Stability indicating Method Development and Validation for determination of related substances for Levonorgestrel and Ethinyl Estradiol Tablets

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INTRODUCTION:

One method of contraception is the method of low-dose combined oral contraceptives (COCs), a combination of low-dose COCs containing synthetic estrogens such as ethinyl estradiol and synthetic progestogens, such as levonorgestrel or Norethisterone. Examples of contraceptive drugs, including COCs, are a combination of oral contraceptives of ethinyl estradiol and levonorgestrel. This combination of drugs works synergistically by suppressing gonadotropin and inhibiting ovulation1-3.

Levonorgestrel and Ethinyl Estradiol Tablets product is official in USP. But the Assay, Dissolution method is given in USP-32 but method for related substances test was not available in USP. Hence in-house method was developed for related substances test using HPLC with UV detector. The API sources used in the product are Levonorgestrel and Ethinylestradiol (Schering)4-6. For both the API’s all the impurities given in API supplier COA and the potential degradant impurities mentioned in DMF are considered for method development. Different trials were taken to separate all impurities and it is discuss in details under RS development. All the impurities checked for stability indicating nature of the method and finalized the related substances method by HPLC with UV detector. The method was checked for adequacy as per the ICH requirement before implementation7-9.

List of Abbreviations:

| Abbreviation | Description |
|--------------|-------------|
| % w/w         | Percentage weight by weight w.r.t |
| EE           | Ethinylestradiol |
| Dil Std      | Diluted Standard |
| RT           | Retention time |
| RRT          | Relative Retention time |
| SD           | Standard Deviation |
| RSD          | Relative Standard Deviation |
| NLT          | Not Less Than |
| NMT          | Not More Than |
| PPM          | parts per million |
| PA           | Purity Angle |
| PT           | Purity Threshold |
| MP           | Method precision |
| IP           | Intermediate precision |
Drug Substance and Drug Product Information

**Name of Drug Product:** Levonorgestrel and Ethinyl Estradiol Tablets (0.1 mg/ 0.02 mg)

**Description of Drug Product:** Light yellow colored, round, flat tablets debossed with “105” on one side and other side plain.

**Name of Drug Substance:**
- Levonorgestrel USP
- Ethinyl Estradiol USP

**Description of Drug Substance:**
- Levonorgestrel is White to off-white powder.
- Ethinyl Estradiol is a white to faintly yellowish white crystalline powder.

**Chemical Name:**
- Levonorgestrel: 17-Hydroxy-6β,7β:15β,16β-dimethylene-3-oxo-17α-pregn-4-ene-21-carboxylic acid, Y-lactone
- Ethinyl Estradiol: 19-Nor-17α-pregna-1,3,5(10)-triien-20-yn-3,17-diol

**Structure:**

![Chemical Structure](image)

**Molecular Formula:**
- Levonorgestrel: C_{24}H_{30}O_{3}
- Ethinyl Estradiol: C_{20}H_{24}O_{2}

**Molecular Weight:**
- Levonorgestrel: 366.49
- Ethinyl Estradiol: 296.40

**CAS No.:**
- Levonorgestrel: 67392-87-4
- Ethinyl Estradiol: 57-63-6

**METHODOLOGY:**

**Reagents and Solvents:**

| Sr.No | Reagent/Solvent         | Grade     | Make                  |
|-------|-------------------------|-----------|-----------------------|
| 1     | Water                   | HPLC grade| NA                    |
| 2     | Acetonitrile            | HPLC grade| J.T. Baker or equivalent |
| 3     | Methanol                | HPLC grade| J.T. Baker or equivalent |
| 4     | Conc. Hydrochloric Acid | AR grade  | Rankem or equivalent |
| 5     | Sodium Hydroxide Pellets| AR grade  | Merck or equivalent |
| 6     | Hydrogen Peroxide)      | AR grade  | Merck or equivalent |

**Sample Details:**

| Sr. No | Name                              | Batch No.  | Strength      |
|--------|-----------------------------------|------------|---------------|
| 1      | Levonorgestrel+Ethinylestradiol   | LUTE/US/026| 0.1 mg / 0.02 mg |
| 2      | Plain Placebo                     | NA         | NA            |
| 3      | Placebo+ Levonorgestrel           | NA         | NA            |
| 4      | Placebo + Ethinylestradiol        | NA         | NA            |

**Working Standard Details:**

| Sr.No | Standard Name       | Batch No.  | WS No.       | % Purity | Retest Date |
|-------|---------------------|------------|--------------|----------|-------------|
| 1     | Levonorgestrel WS   | M1482M     | AR/WRS/024/00| 99.7     | 28-11-2010  |
| 2     | Ethinylestradiol WS | L00030258  | AR/WRS/022/00| 99.4     | 15-11-2010  |
Levonorgestrel Impurities

| Sr. No | Name of the Impurity               | Reason for selecting the impurity                                 |
|--------|------------------------------------|------------------------------------------------------------------|
| 1.     | 6-β Hydroxy Levonorgestrel         | Potential degradant Impurity as per DMF                         |
| 2.     | 6-Keto Levonorgestrel              | Potential degradant Impurity as per DMF                         |
| 3.     | Δ 8(14) Levonorgestrel             | Process Impurity as per DMF                                     |
| 4.     | Δ 6 Levonorgestrel                 | Potential degradant Impurity as per DMF                         |
| 5.     | 18-Methylmamandroloene             | Process Impurity as per DMF                                     |
| 6.     | *4-5 Dihydro(5)α-Methoxy Levonorgestrel (210nm) | Process Impurity as per DMF                                      |
| 7.     | * 8-α-D5(10) Levonorgestrel (210nm) | Process Impurity as per DMF                                     |
| 8.     | Levonorgestrel 3-Methylidenolether | Process Impurity as per DMF                                     |

Ethinyl Estradiol Impurities

| Sr. No | Name of the Impurity               | Reason for selecting the impurity                                 |
|--------|------------------------------------|------------------------------------------------------------------|
| 1.     | Δ-9(11)-Ethinyl Estradiol          | Potential degradant mentioned in COA                             |
| 2.     | 17-β-Ethinyl Estradiol             | Process Impurity mentioned in COA                                |
| 3.     | Estrone                            | Process Impurity mentioned in COA                                |
| 4.     | 6-α-hydroxy Ethinyl Estradiol      | Potential degradant available in other source API's             |
| 5.     | 6-β-hydroxy Ethinyl Estradiol      | Potential degradant available in other source API's             |
| 6.     | Δ-6-Ethinyl Estradiol              | Potential degradant available in other source API's             |
| 7.     | 6-keto-Ethinyl Estradiol           | Potential degradant available in other source API's             |
| 8.     | 1-methyl-Ethinyl Estradiol         | Process Imp available in other source API's                     |
| 9.     | 4-methyl-Ethinyl Estradiol         | Process Imp available in other source API's                     |

Limits for considered: As per the label claim of the product all known impurities fall in the < 10mg Qualification threshold of ICH guidelines, hence the limits taken for all impurities are Not More Than 1.0% as per ICH.

Preparation of Standard Stock Solution: Weigh accurately about 15.0 mg of Levonorgestrel working standard & about 3.0 mg of Ethinyl estradiol working standard into 100 ml volumetric flask. Add to this 80ml diluent and sonicate to dissolve. Dilute up to the mark with diluent Solution C. (Concentration: 60 µg per mL).

Preparation of Diluted Standard Solution: Pipette out 1.0 ml of standard stock solution in 100ml volumetric flask and dilute up to mark with diluent. (Concentration of Levonorgestrel about 1.5 ppm & Ethinyl estradiol about 0.3 ppm)

Preparation of Sensitivity Solution: Dilute 1 ml of the above diluted standard solution to 10 ml with diluent.

Preparation of Impurities Standard Stock Solution: Weigh accurately about 3.0 mg of 17 β Ethinyl estradiol & 3.0 mg of Estrone into 100ml volumetric flask Add to this 80ml diluent and sonicate to dissolve. Dilute up to the mark with diluent.

Preparation of System Suitability Solution: Weigh accurately about 15.0 mg of Levonorgestrel working standard & about 3.0 mg of Ethinyl estradiol working standard into 100ml volumetric flask. Add to this 80ml diluent and 1.0ml of impurities standard stock solution sonicate to dissolve. Dilute up to the mark with diluent.

Preparation Placebo solution I: (Without Levonorgestrel & Ethinylestradiol): Weigh & transfer 15 tablets of placebo or placebo powder blend equivalent to 15 tablets into a 10ml volumetric flask, add to this 5.0 ml of diluent, sonicate for 30 minutes with intermediate shakings, cool and make up to the volume with diluent. Centrifuge for 10 minutes at 3500 rpm, filter though 0.45 nylon filter and inject.

Preparation Placebo solution II: (With Levonorgestrel): Weigh & transfer 15 tablets of placebo or placebo powder blend equivalent to 15 tablets into a 10ml volumetric flask, add to this 5.0 ml of diluent, sonicate for 30 minutes with intermediate shakings, cool and make up to the volume with diluent. Centrifuge for 10 minutes at 3500 rpm, filter though 0.45 nylon filter and inject.

Preparation Placebo III: (With Ethinylestradiol): Weigh & transfer 15 tablets of placebo or placebo powder blend equivalent to 15 tablets into a 10ml volumetric flask, add to this 5.0 ml of diluent, sonicate for 30 minutes with intermediate shakings, cool and make up to the volume with diluent. Centrifuge for 10 minutes at 3500 rpm, filter though 0.45 nylon filter and inject.

Preparation Sample Solution: Weigh & transfer 15 tablets into a 10ml volumetric flask, add to this 5.0 ml of diluent, sonicate for 30 minutes with intermediate shakings, cool and make up to the volume with diluent. Centrifuge for 10 minutes at 3500 rpm, filter though 0.45 nylon filter and inject.

Procedure: Inject the specified volume of Diluent, Placebo solution-I, Placebo solution-II, Placebo solution-III and Sample solution into the chromatograph and record the chromatogram. Disregard peaks due to blank and placebo.
solution. Calculate all known impurities at corresponding wavelength as mentioned in below table. Calculate unknown impurities of Ethinylestradiol at 210 nm, and calculate unknown impurities of Levonorgestrel at both the wavelengths. Calculate any other unknown impurities at 210 nm against diluted standard area of Ethinylestradiol at 210 nm.

Table 2: showing RRT’s for known impurities w.r.t Levonorgestrel:

| Sr. No. | Known impurities       | Ethinylestradiol | RRT  |
|---------|------------------------|------------------|------|
| 1       | Levonorgestrel         | Ethinylestradiol | EE   |
| 2       | Levonorgestrel         | Ethinylestradiol | Levo |
| 3       | 6-β Hydroxy Levonorgestrel | 6-α Hydroxy Ethinylestradiol | 0.31 | 0.34 |
| 4       | 6-Keto Levonorgestrel  | 6- β Hydroxy Ethinylestradiol | 0.41 | 0.50 |
| 5       | Δ 8(14) Levonorgestrel | 6-Keto Ethinylestradiol | 0.51 | 0.87 |
| 6       | Δ 6 Levonorgestrel     | Δ 9,11 Ethinylestradiol | 0.89 | 0.95 |
| 7       | 18-Methynanodrolone    | Δ 6 Ethinylestradiol | 0.93 | 0.97 |
| 8       | * 4-5 Dihydro(5)α-Methoxy Levonorgestrel | Estrone | 0.95 | 1.05 |
| 9       | * 8-α-Δ5(10) Levonorgestrel | 1-Methyl Ethinylestradiol | 1.12 | 1.18 |
| 10      | Levonorgestrel 3 Methylidionelether | 17 β Ethinylestradiol | 1.20 | 1.50 |

Evaluation of System Suitability:

For Sensitivity Solution: Inject the specified volume of sensitivity solution and record the chromatogram at 210 nm & 254 nm. The area counts of Ethinylestradiol peak at 210 nm and Levonorgestrel peak at 254 nm & 210 nm in sensitivity solution should be in the range of 0.09 to 0.11 times of the average area counts of Ethinylestradiol peak and Levonorgestrel peak in the diluted solution at corresponding wavelengths.

For System Suitability Solution: Inject the specified volume of system suitability solution and record the chromatogram at 210 nm & 254 nm. Resolution between Estrone peak and Ethinylestradiol peak should not be less than 1.5 at 210 nm. Resolution between 17β-Ethinylestradiol peak and Levonorgestrel peak should not be less than 1.2 at 210 nm. The tailing factor for Ethinylestradiol and Levonorgestrel peak should not be more than 2.0 at both the wavelengths and Theoretical plate for Ethinylestradiol and Levonorgestrel peak should not be less than 25000 at both the wavelengths.

For Diluted Standard Solution: Inject the specified volume of diluted standard solution for 6 times, the relative standard deviation for Levonorgestrel at both the wavelengths and Ethinylestradiol peak at 210 nm the area counts from 6 replicate injections of standard solution should not be more than 5.0 %.

METHOD DEVELOPMENT

Experiment No. 1: Sample and Standard preparation: prepared sample and standard solution as above mentioned in methodology section.

Chromatographic Condition:

Column : C18, 150 x 4.6, 5µ
Column Make : Peerless,
Mobile Phase : A: Water  B: Methanol:Acetonitrile (80:20) ([A+B] (42:58))
Flow : 1.0 ml/min.
Column Temperature : 25°C
Injection Volume : 50µl
Wavelength : 215 nm for Ethinylestradiol & 240nm for Levonorgestrel
Run Time : 45 minute

Figure 1: Reference Chromatogram of Experiment trail 1
**Experiment No. 2**: Sample and Standard preparation: prepared sample and standard solution as above mentioned in methodology section.

**Chromatographic Condition:**
- **Column**: 250 x 4.6, 5µm
- **Column Make**: YMC pack ODS-AM
- **Mobile Phase**: A: Water B: Methanol
- **Flow**: 1.0 ml/min.
- **Column Temperature**: 25°C
- **Sample Temperature**: 15°C
- **Injection Volume**: 50µl
- **Wavelength**: 210 nm for Ethinylestradiol & Levonorgestrel
- **Run Time**: 95 minute

**Gradient Program:**

| Time in min. | Flow ml/min | Mobile Phase A Water | Mobile Phase B Methanol |
|--------------|-------------|----------------------|-------------------------|
| 0            | 1.0         | 90                   | 10                      |
| 7            | 1.0         | 90                   | 10                      |
| 12           | 1.0         | 60                   | 40                      |
| 20           | 1.0         | 55                   | 45                      |
| 55           | 1.0         | 55                   | 45                      |
| 60           | 1.0         | 40                   | 60                      |
| 65           | 1.0         | 40                   | 60                      |
| 70           | 1.0         | 15                   | 85                      |
| 80           | 1.0         | 15                   | 85                      |
| 85           | 1.0         | 90                   | 10                      |
| 95           | 1.0         | 90                   | 10                      |

*Figure 2: Reference Chromatogram of Experiment trail 2*
Experiment No. 3: Sample and Standard preparation: prepared sample and standard solution as above mentioned in methodology section.

Chromatographic Condition:
- Column: 250 x 4.6, 5µm
- Column Make: YMC pack ODS-AM
- Mobile Phase: A: Water, B: Methanol, C: Acetonitrile
- Flow: 1.0 ml/min.
- Column Temperature: 30°C
- Sample Temperature: 15°C
- Injection Volume: 50μl
- Wavelength: 220 nm for Ethinylestradiol & Levonorgestrel
- Run Time: 100 minute

Gradient Program:

| Time in min. | Flow ml/min | (A) Water | (B) Methanol | (C) Acetonitrile |
|--------------|-------------|-----------|--------------|-----------------|
| 0            | 1.0         | 75        | 0            | 25              |
| 5            | 1.0         | 75        | 0            | 25              |
| 10           | 1.0         | 62        | 0            | 38              |
| 20           | 1.0         | 45        | 40           | 15              |
| 50           | 1.0         | 45        | 40           | 15              |
| 60           | 1.0         | 40        | 10           | 50              |
| 70           | 1.0         | 20        | 0            | 80              |
| 85           | 1.0         | 20        | 0            | 80              |
| 90           | 1.0         | 75        | 0            | 25              |
| 100          | 1.0         | 75        | 0            | 25              |

Conclusion of Experiment No 3: In this trial Ethinylestradiol & Estrone peak are not resolved & also 8-α (14) Levonorgestrel & 1-Methyl Ethinylestradiol impurities are not separated.

Figure 3: Reference Chromatogram of Experiment trail 3
**Experiment No. 4:** Sample and Standard preparation: prepared sample and standard solution as above mentioned in methodology section.

Chromatographic Condition:

- **Column:** 250 x 4.6, 5µm
- **Column Make:** YMC pack ODS-AM
- **Mobile Phase:** A: Water, B: Methanol: Acetonitrile (90:10)
- **Flow:** 1.0 ml/min.
- **Column Temperature:** 25°C
- **Sample Temperature:** 15°C
- **Injection Volume:** 50μl
- **Wavelength:** 210 nm for Ethinylestradiol & 254 nm for Levonorgestrel
- **Run Time:** 125 minute

**Gradient Program**

| Time in min. | Flow ml/min | (A) Water | (B) Methanol: ACN (90:10 v/v) |
|--------------|-------------|-----------|-------------------------------|
| 0            | 1.0         | 90        | 10                            |
| 2            | 1.0         | 90        | 10                            |
| 5            | 1.0         | 62        | 38                            |
| 7            | 1.0         | 55        | 45                            |
| 20           | 1.0         | 52        | 48                            |
| 42           | 1.0         | 48        | 52                            |
| 70           | 1.0         | 42        | 58                            |
| 80           | 1.0         | 35        | 65                            |
| 85           | 1.0         | 30        | 70                            |
| 92           | 1.0         | 20        | 80                            |
| 112          | 1.0         | 15        | 85                            |
| 115          | 1.0         | 90        | 10                            |
| 125          | 1.0         | 90        | 10                            |

At 210 nm

![Reference Chromatogram at 210 nm](image)

At 254 nm

![Reference Chromatogram at 254 nm](image)
Method Finalization Summary: The Related substances method was finalized based on the below observations.

Injection Volume Selection: During the time of development study, 25µL and 50µL of 1% imp spike solution was injected separately. It was observed that in 50µL injection volume, the LOQ of all peak increased. Whereas in 25µL injection volume, the LOQ of all peak decreased. Hence an injection volume was finalized to 50 µL.

Diluent Selection: Following different diluents were tried for sample preparation,
1) Acetonitrile:Water (50:50)
2) Acetonitrile:Water (70:30)
3) Acetonitrile:Water (90:10)
The % recovery of Levonorgestrel and Ethinylestradiol in 50:50 compositions was lesser than other compositions, whereas hump was observed after Levonorgestrel peak in (90:10) diluent. Hence final (70:30) diluent was selected as a diluent for sample preparation.

Solvent Make Selection: By using two different make solvents, same method was run separately. A gradient induced hump was observed due to Rankem solvent just before Delta-9, 11 EE, which was not seen in J.T Baker solvents. So J.T. Baker solvents were finalized.

Wavelength Selection: Wavelength of Ethinylestradiol and Levonorgestrel was taken from API method of analysis (DMF), provided by API vendor. Also unknown impurities due to Levonorgestrel have average maxima at 254 nm whereas Ethinylestradiol impurities at 210 nm. Hence we finalized Wavelength of Ethinylestradiol at 210 nm and Levonorgestrel at 254 nm.

Figure 5: Reference Chromatogram Wavelength of Ethinylestradiol & Levonorgestrel (254 nm)

Figure 6: Reference Chromatogram Wavelength of Ethinylestradiol & Levonorgestrel (210 nm)

Conclusion of Development Trails:
Conclusion of experiment no 1: In this method resolution between δ 9, 11 Ethinylestradiol & δ 6 Ethinylestradiol are not resolved & also Estrone merge with δ 6 Ethinylestradiol. And also Levonorgestrel process impurities are not include.

Conclusion of experiment no 2: In this trial Levonorgestrel impurities are not separated.

Conclusion of Experiment No 3: In this trial all impurity of Levonorgestrel & Ethinylestradiol are resolved well. Based on the all experiment trails it its concluded that in experiment trail no 3 was separated each impurities very well and consider method trail no 3 for further method optimization/method validation.

METHOD VALIDATION:
Method optimization of the given method was performed to check the stability indicating nature of the method. Validation parameters Specificity, Accuracy (Recovery), Limit of Detection and Limit of Quantitation, Forced Degradation study, Solution Stability, Precision, Filter Study were considered while an optimization of the Method.
Specificity:

Table 1: Specificity Study of Levonorgestrel and Ethinyl Estradiol

| Parameter                     | Specification                                                                 | Ethinyl Estradiol | Levonorgestrel |
|-------------------------------|-------------------------------------------------------------------------------|-------------------|---------------|
| Identification                | Results should be comparable with respect to Retention time.                  |                   |               |
| Placebo Interference          | Diluent and Placebo should not show any peak at the retention time of Active and its impurity peaks. |                   |               |
| Individual Active Ingredients  | Peak purity should pass. Main peaks should be pure and homogeneous and there should be no co-eluting peaks. |                   |               |

Accuracy (Recovery): Overall Mean recovery for all Ethinyl & Levo and their all impurities should be in the range of 90.0 % to 110.0 %. Table-2, shows Tentative Recovery of known impurities for Ethinylestradiol & Levonorgestrel.

Table 2: Recovery of known impurities for Ethinylestradiol & Levonorgestrel

| Ethinylestradiol Impurities | % Recovery | % Recovery | RF |
|-----------------------------|------------|------------|----|
| 6-αHydroxy Ethinylestradiol | 102.2      | 6-β Hydroxy Levonorgestrel | 91.4 |
| 6- β Hydroxy Ethinylestradiol | 98.1      | 6-Keto Levonorgestrel | 99.2 |
| 6-Keto Ethinylestradiol     | 100.6      | Δ 8(14) Levonorgestrel | 102.1 |
| Δ 9,11 Ethinylestradiol     | 100.5      | Δ 6 Levonorgestrel | 96.0 |
| Δ 6 Ethinylestradiol        | 97.6       | 18-Methylnanodroloene | 103.6 |
| Estrone                     | 97.3       | * 4-5 Dihydro(5)α-Methoxy Levonorgestrel | 91.6 |
| 1-Methyl Ethinylestradiol   | 99.8       | * 8-α-Δ5(10) Levonorgestrel | 93.0 |
| 17 β Ethinylestradiol       | 94.3       | Levonorgestrel 3-Methylidenolether | 107.7 |
| 4-Methyl Ethinylestradiol   | 99.0       |            |    |

Limit of Detection & Limit of Quantification: LOD Ethinyl & all impurities should be in the range of 0.1 % i.e. Test Concentration. Table 3, shows Tentative RF, LOQ and LOD for known impurities of Ethinylestradiol & Levonorgestrel.

Table 3: RF, LOQ and LOD for known impurities of Ethinylestradiol

| Ethinylestradiol Impurities | % LOD | % LOQ | RF |
|-----------------------------|-------|-------|----|
| 6-αHydroxy Ethinylestradiol | 0.033 | 0.099 | 2.45 |
| 6- β Hydroxy Ethinylestradiol | 0.030 | 0.092 | 2.46 |
| 6-Keto Ethinylestradiol     | 0.034 | 0.104 | 1.07 |
| Δ 9,11 Ethinylestradiol     | 0.049 | 0.102 | 0.92 |
| Δ 6 Ethinylestradiol        | 0.026 | 0.078 | 0.77 |
| Estrone                     | 0.042 | 0.100 | 1.03 |
| 1-Methyl Ethinylestradiol   | 0.045 | 0.107 | 0.50 |
| 17 β Ethinylestradiol       | 0.048 | 0.145 | 1.52 |
| 4-Methyl Ethinylestradiol   | 0.045 | 0.102 | 1.16 |
Table 4: RF, LOQ and LOD for known impurities of Levonorgestrel.

| Levonorgestrel Impurities                        | % LOD | % LOQ | RF   |
|-------------------------------------------------|-------|-------|------|
| 6-β Hydroxy Levonorgestrel                      | 0.035 | 0.106 | 1.82 |
| 6-Keto Levonorgestrel                           | 0.030 | 0.090 | 1.84 |
| Δ 8(14) Levonorgestrel                          | 0.025 | 0.075 | 1.61 |
| Δ 6 Levonorgestrel                              | 0.039 | 0.117 | 2.69 |
| 18-Methylnandrolone                             | 0.013 | 0.040 | 1.75 |
| * 4-5 Dihydro(5)α-Methoxy Levonorgestrel        | ND    | 0.50  | --   |
| * 8-α-Δ5(10) Levonorgestrel                     | 0.035 | 0.106 | 1.41 |
| Levonorgestrel 3-Methyldienolether              | 0.031 | 0.095 | 3.31 |

The forced degradation experiment condition: Forced degradation studies were carried out for related substances at following conditions and the degradants were well separated by this method and the peak was found to be spectrally pure for known impurities and main analyte peaks.

Table 5: Forced degradation Study

| Condition                                                                 | % Degradation Ethinyl Estradiol | % Degradation Levonorgestrel | Peak purity |
|---------------------------------------------------------------------------|---------------------------------|-------------------------------|-------------|
| Initial sample                                                            | ---                             | ---                           | Passed      |
| Fifteen tablets + 1 ml of 1M HCl solution in a 10 ml volumetric flask, add 1 ml of diluent and sonicate for 30 min, with intermediate shaking Neutralized by adding 1 ml of 1M NaOH solution and add 3 ml water shake & dilute up to mark with diluent. | 18.4 %                          | 15.1 %                       | Passed      |
| Fifteen tablets + 1 ml of 0.5 M NaOH solution in a 10 ml volumetric flask, add 1 ml of diluent and sonicate for 30 min. with intermediate shaking. Neutralized by adding 1 ml of 0.5 M HCL solution and add 3 ml water shake & dilute up to mark with diluent. | 25.5 %                          | 25.0 %                       | Passed      |
| Fifteen tablets + 1 ml of Hydrogen peroxide (30 %) solution in a 10 ml volumetric flask, sonicate for 30 min, with intermediate shaking and add 3 ml water shake & dilute up to mark with diluent. | 10.0 %                          | 7.7 %                        | Passed      |
| Sample exposed to heat at 105°C for 24 hours on open Petri dish. Fifteen tablets + 3 ml water in a 10 ml volumetric flask, sonicate for 30 min, with intermediate shaking shake & dilute up to mark with diluent. | 9.6 %                           | 7.5 %                        | Passed      |
| Sample exposed in photo stability chamber for 1.2 million lux hour. Fifteen tablets + 3 ml water in a 10 ml volumetric flask, sonicate for 30 min, with intermediate shaking shake & dilute up to mark with diluent. | 8.7 %                           | 2.0 %                        | Passed      |

Solution Stability: Mean recovery for all Ethinyl & Levo and their all impurities should be in the range of 90.0 % to 110.0 %. Table 6 shows solution stability for 48 hrs for known impurities of Ethinylestradiol & Levonorgestrel.

Table 6: Solution stability for known impurities of Ethinylestradiol & Levonorgestrel

| Ethinylestradiol         | % Recovery | Levonorgestrel          | % Recovery |
|--------------------------|------------|-------------------------|-----------|
| 6-αHydroxy Ethinylestradiol | 92.3       | 6-β Hydroxy Levonorgestrel | 92.8      |
| 6-β Hydroxy Ethinylestradiol | 92.6       | 6-Keto Levonorgestrel     | 99.5      |
| 6-Keto Ethinylestradiol  | 100.9      | Δ 8(14) Levonorgestrel    | 98.6      |
| Δ 9,11 Ethinylestradiol  | 98.5       | Δ 6 Levonorgestrel        | 91.3      |
| Δ 6 Ethinylestradiol     | 97.6       | 18-Methylnandrolone      | 97.7      |
| Estrone                  | 105.5      | 4-5 Dihydro(5)α-Methoxy Levo | 88.9     |
| 1-Methyl Ethinylestradiol | 101.0      | * 8-α-Δ5(10) Levonorgestrel | 96.8      |
| 17 β Ethinylestradiol    | 91.2       | Levonorgestrel 3-Methyldienolether | 110.1    |
| 4-Methyl Ethinylestradiol | 93.7       | Levonorgestrel           | 100.20    |
| Ethinylestradiol         | 100.8      | NA                      |           |
**Precision:** RSD should not more than 15.0%. Table 7, showing precision study for known impurities of Ethinylestradiol & Levonorgestrel.

| Ethinylestradiol | % RSD | Levonorgestrel | % RSD |
|-----------------|-------|----------------|-------|
| 6α-Hydroxy Ethinylestradiol | 7.9 | 6β-Hydroxy Levonorgestrel | 2.0 |
| 6β-Hydroxy Ethinylestradiol | 12.8 | 6-Keto Levonorgestrel | 1.0 |
| 6-Keto Ethinylestradiol | 0.7 | Δ 8(14) Levonorgestrel | 1.6 |
| Δ 9,11 Ethinylestradiol | 3.2 | Δ 6 Levonorgestrel | 1.7 |
| Δ 6 Ethinylestradiol | 1.8 | 18-Methylnanodroloene | 1.2 |
| Estrone | 2.0 | * 4-5 Dihydro(5)α-Methoxy Levonorgestrel | 4.1 |
| 1-Methyl Ethinylestradiol | 1.8 | * 8-α-Δ5(10) Levonorgestrel | 7.5 |
| 17β Ethinylestradiol | 10.1 | Levonorgestrel 3-Methylidenoether | 3.7 |
| 4-Methyl Ethinylestradiol | 4.1 | Levonorgestrel | 1.1 |
| Ethinylestradiol | 11.1 | - | - |

**Filter compatibility:** Should be between 90% - 110%. Table 8, shows Filter study for known impurities of Ethinylestradiol & Levonorgestrel.

| Ethinylestradiol | % Assay | Levonorgestrel | % Assay |
|-----------------|---------|----------------|---------|
| 6α-Hydroxy Ethinylestradiol | 108.7 | Levonorgestrel | % |
| 6β-Hydroxy Ethinylestradiol | 107.6 | 6β-Hydroxy Levonorgestrel | 103.0 |
| 6-Keto Ethinylestradiol | 102.1 | 6-Keto Levonorgestrel | 101.4 |
| Δ 9,11 Ethinylestradiol | 99.9 | Δ 8(14) Levonorgestrel | 102.2 |
| Δ 6 Ethinylestradiol | 100.4 | Δ 6 Levonorgestrel | 101.1 |
| Estrone | 98.3 | 18-Methylnanodroloene | 101.8 |
| 1-Methyl Ethinylestradiol | 100.3 | 4-5 Dihydro(5)α-Methoxy Levo | 104.8 |
| 17β Ethinylestradiol | 103.0 | 8-α-Δ5(10) Levonorgestrel | 104.1 |
| 4-Methyl Ethinylestradiol | 101.7 | Levonorgestrel 3-Methylidenoether | 104.8 |

**CONCLUSION:**
The method is Specific, Linear, Precise and Accurate for Related substances, for Levonorgestrel 0.10 mg and Ethinyl Estradiol 0.02 mg Tablets. For one of the known impurity of Levonorgestrel, (4, 5-Dihydro-5-alpha-methoxy-levonorgestrel) LOQ is about 0.5%. This is a process impurity with limit specification of 0.3% in the API. Degradation for this impurity in the formulation will be confirmed by alternative method.

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