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Original Article

High-Performance Liquid Chromatographic-Tandem Mass Spectrometric (HPLC-ESI MS/MS) Method For The Simultaneous Estimation Of Ethinyl Estradiol And Levonorgestrel In K_2EDTA Human Plasma

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Abstract:

A simple, sensitive and high throughput high performance liquid chromatography tandem mass spectrometry method has been developed for the simultaneous determination Ethinyl Estradiol and Levonorgestrel in K_2EDTA human plasma. The method involved double liquid liquid extraction of Ethinyl estradiol and levonorgestrel along with its deuterated analog as an internal standard (IS) from 0.600µL of human plasma. The chromatographic analysis was done on Kinetex, PFP 100 A (50 X 4.6) mm, 2.6 µm column with the mobile phase consisted Mobile phase organic mixture (Acetonitrile: Methanol, 95:05 v/v) and 2 mM Ammonium Acetate in water w/v as per gradient time program. A triple quadrupole mass spectrometer operating in the positive ionization mode was used for quantitation. In-source fragmentation of Ethinyl estradiol and levonorgestrel was selectively controlled by suitable optimization of cone voltage, cone gas flow and desolvation temperature. The method was validated over a wide concentration range of 1.000 pg/mL to 200.000 pg/mL for Ethinyl Estradiol and for Levonorgestrel 25.000 pg/mL to 5000.000 pg/mL. The Overall % recovery of was 67.39 % and 69.07% for Ethinyl estradiol and Ethinyl estradiol D4 respectively. The Overall % recovery 81.39% and 85.09% for Levonorgestrel and Levonorgestrel D6 respectively. The method was successfully applied to support a bioequivalence study Levonorgestrel and Ethinyl estradiol tablets, 0.15mg/0.03mg (T) in 48 healthy female human subjects.

Keywords: Ethinyl Estradiol, Levonorgestrel, Method validation, plasma, Liquid-Liquid Extraction, bioequivalence.

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Introduction:

Levonorgestrel is synthetic progestogen with chemical name (d (-)-13 beta-ethyl-17-alpha-ethinyl-17-beta-hydroxygon-4-en-3-one) and. Ethinyl estradiol is an estrogen with the chemical name (19-nor-17α-pregna-1, 3, 5 (10)-trien-20-yne-3, 17-diol). Levonorgestrel and Ethinyl estradiol is a used as birth control pill in combination. Oral contraceptives work primarily by preventing/inhibiting ovulation. It also suppresses the gonadotropins. Other alteration includes the changes in cervical mucus which increases difficulty for sperm to enter in the uterus and preventing changes in endometrium required for fertilization of egg. (2-6, 31)

Levonorgestrel is quickly and fully absorbed after oral administration, with a bioavailability of about 100%. It does not undergo first-pass metabolism or enterohepatic circulation, meaning its absorption remains consistent after oral intake. On the other hand, ethinyl estradiol is rapidly and almost completely absorbed by the gastrointestinal tract. However, due to first-pass metabolism in the gut mucosa and liver, its bioavailability ranges between 38% and 48%. (7)

Cytochrome P450 enzymes (CYP3A4) in the liver are responsible for the 2-hydroxylation of ethinyl estradiol, the major oxidative reaction. The 2-hydroxy metabolite is further trans-formed by methylation and glucuronidation prior to urinary and fecal excretion. Levels of cytochrome P450 (CYP3A) vary widely among individuals and can explain the variation in rates of ethinyl estradiol 2-hydroxylation. Ethinyl estradiol is excreted into the urine and feces as glucuronide and sulfate conjugates, and undergoes enterohepatic circulation. (7)

Accurate, precise and sensitive method is required to quantify these drugs form human matrix as many formulations are available in market including generic drugs. Evaluation of bioequivalence requires ensuring that generic products are just as safe and effective as their brand-name counterparts, maintaining consistent therapeutic outcomes and patient safety. (29, 30)

LC-MS-MS based methods are highly selective because they depend on the physicochemical properties of the drug for detection and quantitation. (8) There are several LC-MS-MS methods reported in literature to determine Ethinyl estradiol and levonorgestrel. The methods available are either for one drug or simultaneous estimation of both. Few assays methods have been reported for individual analysis of ethinyl estradiol and levonorgestrel, ethinyl estradiol and levonorgestrel in combination with other drugs and simultaneous estimation of ethinyl estradiol and levonorgestrel. The methods were optimized for estimation of levonorgestrel in human serum, human plasma and Rat plasma (09-12). For ethinyl estradiol assay methods were optimized in human plasma (12-17). Some assay reported for ethinyl estradiol with other drugs in human plasma and rat plasma (18-22). Methods for the simultaneous determination of oral

contraceptives concentration in human plasma were reported (23-25). However, they are lacking required sensitivity.

The method presented has the highest extensive range of linearity and lowest LLOQ of 1.000–200.000 pg/mL for ethinyl estradiol and 25.000–5000.000 pg/mL for levonorgestrel compared with the reported simultaneous estimation methods for ethinyl estradiol and levonorgestrel in human plasma. The plasma volume for sample preparation was 0.600 mL, which was considerably less than or similar to that in other reported methods (13–15). The proposed method was validated and its application to sample analysis. Subject sample and incurred sample reanalysis results are also discussed with this method, which are not presented in any other method. (23-24)

Research Methodology:

Chemical and Materials:

Reference standard Ethinyl estradiol (99.37%), Lovonogestrel (98.58%) and internal standard Ethinyl estradiol D4 (98.69%), Levonogestrel D6 (99.01%) were obtained from bio-organics. The solution were used during the development and validation Acetonitrile (HPLC/ULC/MS/LC-MS grade), Ammonium Acetate (ACS/AR/GR/ULC/MS grade), Dansyl chloride (HPLC grade), Methanol (HPLC/ULC/MS/LC-MS grade), Sodium bicarbonate (ACS grade), Sodium hydroxide (AR/LR grade), Sample container: Polypropylene vials, Water (HPLC grade/Type I Water). Blank Human plasma stored at -20ºC until used.

Liquid chromatography and mass spectrometry setting:

Chromatographic analysis was conducted on a Waters Xevo TQ-XS equipped with Acquity H-Class HPLC and Kinetex, PFP 100 A (50 X 4.6) mm, 2.6 µm column, that was maintained at 25ºC in a column oven. The mobile phase consisted Mobile phase organic mixture (Acetonitrile: Methanol, 95:05 v/v) and 2 mM Ammonium Acetate in water w/v as per gradient time program. Ionization and detection of Ethinyl estradiol, Levonorgestrel and their respective (Internal standards) IS were conducted on a Waters Xevo TQ-XS mass spectrometer, ion source ES+ .Multiple reaction monitoring (MRM), using precursor→product ion transitions of *m/z* for Ethinyl estradiol 530.11/ 170.99 (m/z), Levonorgestrel 313.09/ 245.06 (m/z) and Internal standard Ethinyl estradiol D4 534.13/170.99 (m/z) and Levonorgestrel D6 319.12/ 251.11 (m/z) respectively was used to quantify the analyte and IS. The source-dependent parameters maintained for both analytes and IS were as follows: for Capillary (kV) 1.00 , Source Temperature (°C) 150, Desolvation Temperature (°C) 600, Cone Gas Flow (L/Hr) 150, Desolvation Gas Flow (L/Hr) 1100, Nebuliser Gas Flow (Bar) 7.00, Collision Gas Flow (mL/min) 0.16.

The optimum values for compound-dependent parameters like cone voltage (V) 40 for Ethinyl estradiol, 35 for Ethinyl estradiol D4 , 34 for Levonorgestrel and for Levonorgestrel D6 was 42. The collision energy (eV) for Ethinyl estradiol, Ethinyl estradiol D4 was 35 and for Levonorgestrel 16 and Levonorgestrel D6 was 20. Dwell

time (s) was set at 0.063 ms and Span (Da) was for Ethinyl estradiol, Ethinyl estradiol D4, Levonorgestrel and Levonorgestrel D6. Data collection, peak integration and calculations were performed using Mass Lynx V. 4.2.

Standard stock solutions, calibration standards and quality control samples:

The stock solution of Ethinyl estradiol (0.200 mg/mL) was prepared by dissolving the accurately weighted reference standard in methanol. Calibration standards and quality control (QC) samples were prepared by spiking mixed CC intermediate and mixed QC intermediate solution respectively. Calibration curve standards of Ethinyl estradiol were made at concentrations of 1.000, 2.000, 12.000, 32.000, 88.000, 128.000, 168.000, 200.000 pg/mL, whereas high, medium1,medium2 and low QC samples were prepared at concentrations of 152.000, 68.000, 21.000 and 3.000 pg/mL respectively.

The stock solution of Levonorgestrel (0.200 mg/mL) was prepared by dissolving the accurately weighted reference standard in methanol. Calibration standards and quality control (QC) samples were prepared by spiking mixed CC and mixed QC intermediate solution respectively. Calibration curve standards of levonorgestrel were made at concentrations of 25.000, 50.000, 300.000, 800.000, 2200.000, 3200.000, 4200.000, 5000.000 pg/mL, whereas high, medium1, medium 2 and low QC samples were prepared at concentrations of 3800.000, 1700.000, 525.000 and 75.000 pg/mL respectively. A stock solution (0.200 mg/mL) of the Ethinyl estradiol D4 and Levonorgestrel D6 were prepared by dissolving the accurately weighted respective reference standard in methanol. Its Mixed ISTD working solution (Ethinyl estradiol D4, 1000.000 pg/mL and Levonorgestrel D6, 20000.000 pg/mL) was prepared by appropriate dilution of the stock/ ISTD intermediate solution in methanol. All solutions (standard stock, calibration standards and QC samples) were stored at 5 ºC until use.

Sample preparation:

Sample handling and processing was carried out under normal light. All frozen subject samples, calibration standards and quality control samples (in K2EDTA) were thawed and allowed to equilibrate at room temperature before extraction. To an aliquot of 0.600 mL of spiked plasma sample, 50µL of IS was added and vortex-mixed. Further, Added 2.500ml of (Extraction organic mixture) tert-Butyl methyl ether: n-Hexane, 50:50 v/v to all the samples. Sample rotated at 30 rpm for 15 minutes on Liquid-Liquid extractor. All the samples were centrifuged at 4000 rpm at 10±2˚C for 10 minutes. 2.000 mL of supernatant was withdrawn into pre-labelled tube and evaporated to dryness under nitrogen pressure at 30°C temperature. Reconstituted the samples with 0.200 mL of (Extraction buffer) 0.1M Sodium Bicarbonate pH 11.000 ± 0.150 and added 0.200 mL of Dansyl Chloride and vortexed to mix. Incubated the samples for 15 minutes at 55ºC in hot water bath of Evaporator. After Incubation samples were kept at ambient temperature for cooling. Added 2.500 mL of Extraction Organic Mixture to all the samples. Samples were rotated at 30 rpm for 15 minutes on Liquid-

Liquid extractor. Centrifuged all the samples at 4000 rpm at 10±2˚C for 10 minutes. Withdrawn 2.000 mL of supernatant into pre-labelled tubes. Evaporate to dryness under nitrogen pressure at 30°C temperature. The dried samples were reconstituted with 0.150 mL of reconstitution Solution and vortexed to mix. Appropriate volume of final solutions was transferred into pre-labeled auto-sampler vials.

Method Validation Procedures:

The method was validated for human plasma following the ICH guideline M10 on bioanalytical method validation and study sample analysis (1).

A system suitability experiment was performed by injecting six consecutive injections using aqueous standard mixtures of (Ethylene estradiol 50.000 pg/mL and levonorgestrel 1250.000 pg/mL) and IS mixture of (Ethinyl estradiol D4, 1000.000 pg/mL and levonorgestrel D6, 20000.000 pg/mL) at the start of each batch during method validation.

System Performance experiment was performed once in a day at the beginning of analytical batch or before any re-injection. Extracted Standard blank, upper limit of quantitation (ULOQ), 1st repeated acquisition of extracted standard blank, 2nd repeated acquisition of extracted standard blank, lower limit of quantitation (LLOQ), the system performance was evaluated by carryover observed in extracted Standard blank injected after ULOQ should and % Interference observed in first acquired STD BL(before ULOQ).

The selectivity of the method was established by screening separately the blank plasma (without spiking with Analyte or internal standard). One blank sample was prepared and one sample equivalent to LLOQ from each blank lot using at least 10 different lots of human plasma along with CC standards (STD 1 to STD 8) and two sets of batch qualifying QCs (at higher, middle and lower level). 10 different lots of plasma of which, six normal, two lipemic and two haemolysed plasma having anticoagulant K_2EDTA were screened.

The selectivity was evaluated by comparing the responses of interfering peak at the retention time of Analyte in the standard blank against the response of the respective extracted LLOQ sample. For internal standard (ISTD), Selectivity was evaluated by comparing the response of interfering peak at the retention time of ISTD in the blank against the mean area response of ISTD in analytical batch.

The linearity of the method was determined by the analysis of four linearity curves containing eight non-zero concentrations. The area ratio response for analyte/IS obtained from MRM was used for regression analysis. Each calibration line was analyzed by least square weighted (1/x) linear regression. The lowest standard on the calibration line was accepted as the LLOQ, having at least 10 times more response than drug-free (blank) extracted human plasma.

To determine the intra-batch accuracy and precision by using 6 replicate samples at LLOQ QC, low, middle and high quality control samples along with calibration curve standards were analyzed

on the same day. The inter-batch accuracy and precision were assessed by analyzing five precision and accuracy batches on three different days. The within run precision at each concentration level of QC samples should be ≤ 15.00% and for the LLOQ QC it should be ≤ 20.00%.

The % recovery for analyte and internal standard (ISTD was determined by comparing the mean peak area of the analyte and internal standard in 6 replicates of extracted samples against mean peak area of 6 replicates of post extracted quality control samples at high, middle and low concentrations.

Matrix effect was performed by processing three aliquots each of low and high QC samples from each ten different lots/source of previously screened biological matrix with same anticoagulant {including two hemolysed (2% hemolysed) and two lipemic lots} as per respective method SOP. The samples will be analyzed against freshly prepared CC standards and two sets of batch qualifying QC samples (at higher, middle and lower level) prepared in normal screened plasma as per the method SOP.

The matrix stability experiment were performed by using freshly prepared calibration curve standard and freshly prepared QCs samples (batch qualifying samples) at HQC and LQC levels along with the stability samples at same levels. Stock solutions and working solution of Ethinyl estradiol, Levonorgestrel and their respective IS were checked for short-term stability at room temperature and long-term stability at 5ºC. Autosampler (wet extract), bench top (at room temperature), freeze-thaw and long-term stability in plasma were performed at high and low QC levels using six replicates. The samples were considered stable if the deviation from the mean calculated concentration of QC samples was within 15.00%.

The ruggedness of the method was evaluated on three precision and accuracy batches. The first batch was analyzed by different analysts, the second batch was studied on two different columns (same make but different batch number) and third on two different equipment (same make).

The dilution integrity was determined at a concentration of 360.000 pg/mL for Ethinyl estradiol and 9000.000pg/mL for Levonorgestrel. The precision and accuracy for dilution integrity standards at 1/2 and1/10 dilution were determined by analyzing the samples against freshly prepared calibration curve standards.

Application of the method and incurred sample reanalysis (ISR):

The design of the clinical study in healthy volunteers was an open label, balanced, randomized, two treatment, two sequence, two period, crossover, truncated (for Levonorgestrel) single oral dose bioequivalence study of Levonorgestrel and Ethinyl estradiol tablets, 0.15mg/0.03mg (T) manufactured by Generic Company, India with MICROGYNON 30 (Levonorgestrel and Ethinyl estradiol tablets, 0.15mg/0.03mg) (R) of MA holder, Bayer B.V. Sirius reef 36 2132 WT Hoofddrop, Netherlands, in 48 normal, healthy, adult female subjects (18–45 years) under fasting conditions. Written consent

was taken from all subjects after informing them about the objectives and possible risks involved in the study. The study was conducted in accordance with EMA guidelines (26) and ICH E6 guidelines (27). The primary target variables of the study for levonorgestrel were C_{max}, AUC_{0-72} and AUC_{0-inf} for Ethinyl estradiol C_{max}, AUC_{0-t} and AUC_{0-inf} which were analyzed using the confidence interval approach. The secondary parameters of the study included T_{max} for levonorgestrel AUC_{0-t}/AUC_{0-inf}, Residual area, T_{max}, $t_{1/2}$ and $K_{el,}$ n points, Kel Lower and Kel upper for Ethinyl estradiol.

After an overnight fasting of at least 10.00 hrs. prior to scheduled time of dosing, all subjects were administered single oral dose of one tablet of either test product (T) or one tablet of reference product (R) orally as per randomization sequence in sitting posture with 240 ± 02 mL of water at room temperature. Blood samples of were collected at pre-dose (00.00 hr) in morning of dosing day and at 00.25, 00.50, 00.75, 01.00, 01.25, 01.50, 01.75, 02.00, 02.25, 02.50, 03.00, 04.00, 06.00, 08.00, 12.00, 16.00, 24.00, 36.00, 48.00 and 72.00 hours after dosing in each period of the study in K2EDTA Vacutainer. After collection of blood samples from the subjects at each time point, centrifuged the samples at 4000 RPM for 10 minutes at 4°C. The separated plasma was transferred in appropriate sized polypropylene screw top (previously labeled with project no., period no., subject no., sample time point, Aliquot-1 and Aliquot-2) biological sample storage vials in two aliquots. All plasma samples were stored at -70±15°C until analysis.

Statistical analysis was done on Levonorgestrel and Ethinyl estradiol pharmacokinetic data using the SAS® software (version 9.4).

Pharmacokinetic and Statistical analysis for plasma concentration vs. time profile of Levonorgestrel and Ethinyl estradiol was performed on the data obtained from the subjects completing both the study periods.

The ln-transformed pharmacokinetic parameters C_{max} , AUC_{0-72} and AUC_{0-inf} For Levonorgestrel and C_{max}, AUC_{0-t} and AUC_{0-inf} for Ethinyl estradiol was analyzed by analysis of variance (ANOVA) using SAS® Software.

For Levonorgestrel and Ethinyl estradiol, based on the statistical results of 90% confidence intervals for the geometric least square means ratio of test to reference product for the pharmacokinetic parameters C_{max} , AUC_{0-72} and AUC_{0-inf} for Levonorgestrel and C_{max}, AUC_{0-t} and AUC_{0-inf} for Ethinyl estradiol, conclusions were drawn whether test product is bioequivalent to reference product under fasting conditions. Acceptance range for comparative bioavailability is 80.00% - 125.00% for 90% confidence intervals of the geometric least square means ratio of test to reference product for C_{max} , AUC_{0-72} and AUC_{0-inf} for Levonorgestrel and C_{max} , AUC_{0-72} and AUC_{0-inf} for Ethinyl estradiol.

An incurred sample re-analysis was also conducted by the selection of 197 human samples (10% of total analyzed samples) near the maximum blood concentration (Cmax) and the elimination phase in the pharmacokinetic profile of the drug. Reanalyzed samples will be that 2/3rd

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of the reanalyzed samples must have concentrations that fall within ± 20% of the result of the original analysis.(28)

Experimental:

Method development:

The three commonly used extraction methods are Liquid-Liquid extraction (LLE), Solid phase extraction (SPE) and protein precipitation (PPT). To develop a selective, rugged and reliable method for the estimation of Ethinyl estradiol and levonorgestrel in human plasma all the extraction procedures were systematically investigated individually as well in combination. The chromatographic and mass spectrometric conditions were suitably optimized to get the desired sensitivity, selectivity and linearity in regression curves.

Mass spectrometry:

Ehinyl estradiol and levonorgestrel was tuned in the electrospray ionization (ESI) mode with both negative and positive polarity. However, the electrospray ionization (ESI) mode with positive polarity was provided a good signal. The dansyl derivatization of ethinyl estradiol (EE) introduces a basic secondary nitrogen into the molecule, which was facilitates ionization in acidic HPLC mobile phases. Under positive turbo ion spray ionization, the derivatized EE exhibits a prominent protonated molecular ion at m/z 530.11. During collision-induced dissociation, this ion produces a characteristic fragment at m/z 170.99, corresponding to the protonated 5-(dimethyl amino) naphthalene group. The multiple reaction monitoring (MRM) of the m/z 530.11 → 170.99 transition provides high specificity for ethinyl estradiol (fig. 1), with no detectable background interference from human blank plasma samples. The levonorgestrel showed an intense protonated molecular ion at m/z 313.09 under positive turbo ion spray ionization. The collision-induced dissociation of this ion formed a distinctive product ion at m/z 245.06. The multiple reaction monitoring (MRM), based on the *m/z* 313.09 -245.06 transition, was used for levonorgestrel (fig. 2).

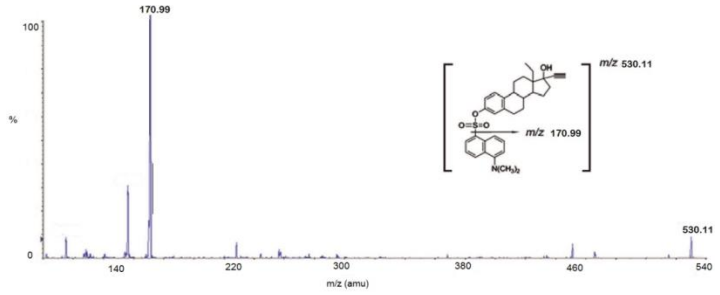

Fig. 1: Product ion mass spectra of ethinyl estradiol (m/z 530.11-170.99, scan range 100–540 amu) in the positive ionization mode.

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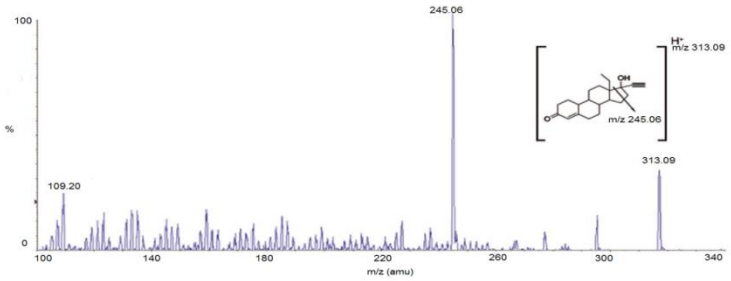

Fig. 2. Product ion mass spectra of levonorgestrel (m/z 313.09-245.06, scan range 100–350 amu) in the positive ionization mode.

This achieved an LLOQ of 1.000 pg/mL for Ethinyl estradiol and 25.000pg/ml for levonorgestrel using a 0.600 mL plasma sample and injecting 20.00 µL into Xevo TQ-XS (Waters) mass spectrometer. The source dependent and compound dependent parameters were suitably optimized to obtain a consistent and adequate response to both the analyte. A dwell time of 0.063 seconds and a span of 200 Da were determined to be sufficient for both drugs and their respective internal standards (IS), ensuring accurate and precise multiple reaction monitoring (MRM). Collision energies were optimized at 35 eV for ethinyl estradiol and its IS, ethinyl estradiol D4, 16 eV for levonorgestrel, and 20 eV for its IS, levonorgestrel D6. Additionally, to minimize in-source fragmentation, the cone voltage, desolvation, and cone gas flow were carefully fine-tuned, maintaining optimal responses for both ethinyl estradiol and levonorgestrel.

Optimization of extraction technique:

Reported procedures for the estimation of ethinyl estradiol in human plasma have used SPE, LLE, PPT followed by LLE, LLE followed by SPE for sample preparation with little or no information on ion suppression or matrix interference. Considering the steroidal moiety in chemical structures of both analytes and the high log P value LLE was tried by using the various combinations of extraction solutions like tert-Butyl methyl ether, n-hexane, n-heptane. Added tert-Butyl methyl ether combined with n-hexane in a proportion of 50:50 (v/v) added to the sample and rotated the samples Liquid-Liquid extractor. After selective extraction of both analytes, the organic supernatant layer was separated and evaporated to dryness. The dried residue was reconstituted with Sodium Bicarbonate subjected to dansyl derivatization with incubation in hot water bath. After derivatization, at ambient temperature tert-Butyl methyl ether combined with n-hexane in a proportion of 50:50 (v/v) added to the sample. Rotated the samples Liquid-Liquid extractor, centrifuged and withdrew the supernatant. After drying in evaporator reconstitution solution added to the sample. which provided help to improve the sensitivity, compatibility and reproducible response.

Optimization of chromatographic conditions

During method development to

have a rugged and efficient chromatography, different analytical columns were tested to achieve adequate run time, good peak shapes and minimum solvent consumption and matrix interference. In reported method several columns were used for Ethinyl estradiol and levonorgestrel (08-25), the different columns were evaluated in the present work, namely, zorbax SB-C8 4.6*250mm, 5µm, Exsil mono 100 C18, 3µm, 100*4.6mm, Exsil plus 100 C18-PFP, 3µm, 50*4.6mm, Zorbax Eclipse XDB-C18 4.6*150mm, 5µm, Kinetex ,2.6µm F5, 50*2.1mm and Kinetex, PFP 100 A (50 X 4.6) mm, 2.6 µm analytical columns. Furthermore, the mobile phase was optimized using Acetonitrile-formic acid and Acetonitrile-methanol and with acidic buffers like formic acid-ammonium formate , acetic acid- ammonium acetate, formic acid in water in different compositions. Separation was tried on different column. Levonorgestrel required a relatively higher portion of aqueous composition to separate closely eluting interferences on selected MRM, while derivatized ethinyl estradiol required a higher portion of organic composition to elute out in relatively shorter retention time while still maintaining the selectivity.(25)

Hence, careful optimization of chromatography was needed with gradient programming starting from lower organic portion 45% till separation of levonorgestrel peak to 50% organic portion till separation of ethinyl estradiol and providing nonpolar interference wash out with 95% of organic proportion. In the present work, the best chromatographic conditions as a function of analyte peak intensity, peak shape, adequate retention and analysis run time were achieved with Kinetex, PFP 100 A (50 X 4.6) mm, 2.6 µm using 0.01% using Acetonitrile: Methanol, 95:05 v/v, 2 mM Ammonium Acetate in water w/v (45:55 to 95:05 (v/v) gradient programming) as the mobile phase. The total chromatographic run time was 13.0 min with a retention time of 1.85 and 8.50 min for levonorgestrel and ethinyl estradiol, respectively. The LLOQ achieved for ethinyl estradiol and levonorgestrel was 1.000 pg/mL and 25.000 pg/mL, respectively, which was lowest compared with other methods reported in human plasma. Representative MRM ion chromatograms of extracted blank human plasma (double blank) at LLOQ for ethinyl estradiol (Fig. 3) and levonorgestrel (Fig. 4) demonstrated the selectivity of the method. The chromatograms showed acceptable peak shape for both the drugs. Ethinyl estradiol D4 and levonorgestrel-D6 were the deuterated compounds selected as internal standards in the present work. They had similar chromatographic behavior and were easily separated and eluted along with the analytes. There was no effect of IS on analyte recovery, sensitivity or ion suppression.

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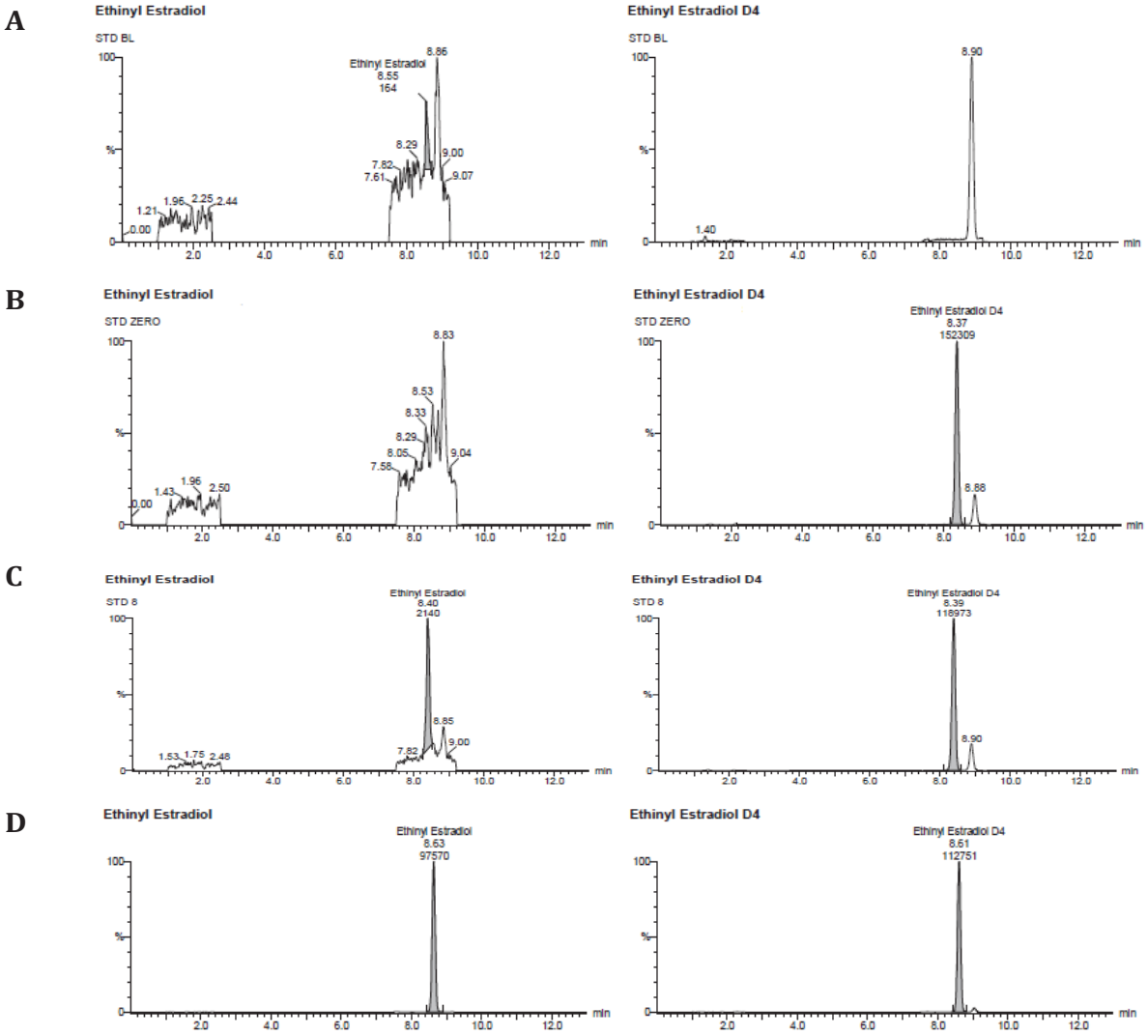
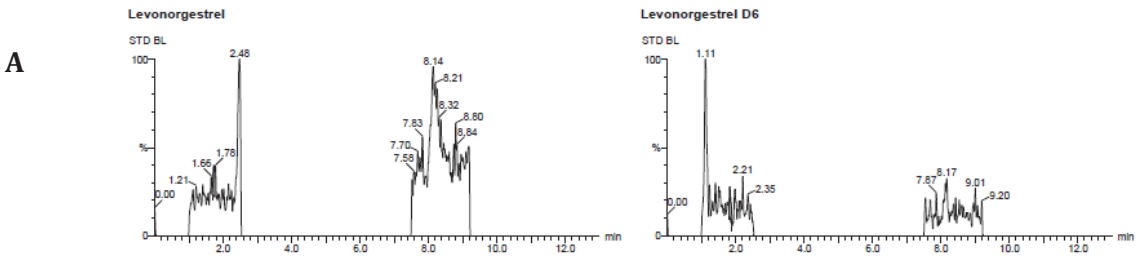


Fig.3 Representative MRM ion-chromatograms of (A) double blank (without Ethinyl estradiol and IS), (B) blank plasma with IS, (C) Ethinyl estradiol at LLOQ and IS, and (D) subject sample at 2.0 h after administration of 0.03mg dose of Ethinyl estradiol.



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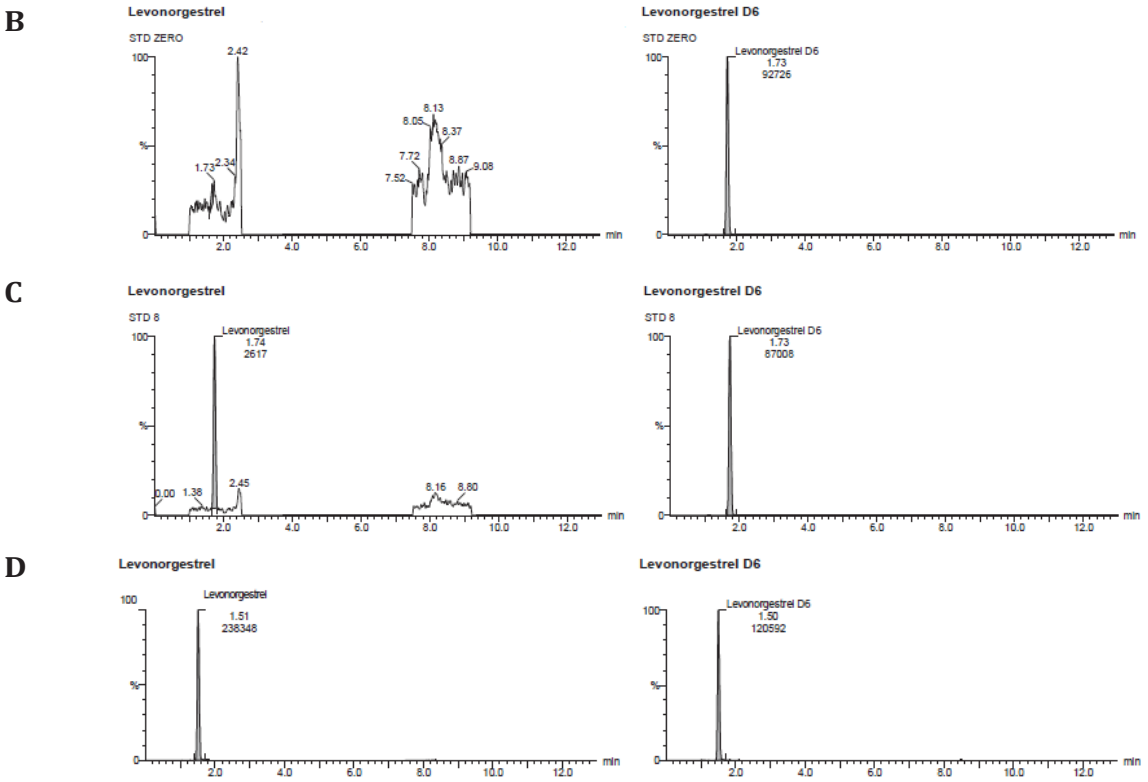

Fig.4 Representative MRM ion-chromatograms of (A) double blank (without Levonorgestrel and IS), (B) blank plasma with IS, (C) Levonorgestrel at LLOQ and IS, and (D) subject sample at 2.0 h after administration of 0.15mg dose of Levonorgestrel.

Results and Discussions:

Results for method validation:

For system suitability the % CV of analyte and ISTD Area ratio was observed as ≤ 1.05% for Ethinyl estradiol and ≤ 1.87% for Levonorgestrel. In system performance Peak area of Ethinyl estradiol (Signal of Ethinyl estradiol) was ≥ 18.1 and Peak area of Levonorgestrel (Signal of Levonorgestrel) was ≥ 28.5. Carryover evaluation was performed in each analytical run to ensure that it did not affect the accuracy and the precision of the proposed method. During the method validation levothyroxine Carryover observed for Ethinyl estradiol as ≤ 12.62 % and for Ethinyl estradiol D4 ≤ 0.00%, for levonorgestrel ≤ 5.14 % and for levonorgestrel D6 ≤ 0.04 % .The calibration lines were linear over the concentration range of 1.000–200.000 pg/mL for Ethinyl estradiol and 25.000-5000.000 pg/mL for Levonorgestrel.

The mean linear equation was y= (0.02090±0.00046) x + (0.00028± 0.00122) for Ethinyl estradiol. The mean linear equation for Levonorgestrel was y = (0.00125±0.00007) x + (0.0003963±0.0005783), where y is the peak area ratio of the analyte/IS and x is the concentration of the analyte.

The mean ± SD value for correlation coefficient (r) was 0.9997± 0.000108 for Ethinyl estradiol and 0.9998 ± 0.000191 for Levonorgestrel.

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The accuracy and precision (%CV) observed for the calibration curve standards ranged from 97.68 to 102.65% and 0.74 to 6.61, respectively for Ethinyl estradiol. The accuracy and precision (%CV) observed for the calibration curve standards ranged from 98.04 to 101.87% and 0.98 to 2.24, respectively for Levonorgestrel.

The lowest concentration in the standard curve that could be achieved with acceptable accuracy and precision was found to be 1.000 pg/mL for Ethinyl estradiol and 25.000 pg/ml for Levonorgestrel at a signal-to-noise (S/N) of about 17.05 and 75.68 respectively .Based on the high S/N values, it was possible to lower the quantitation limit.

The intra-batch precision (CV) ranged from 0.97 to 1.98% and the accuracy was within 94.16 and 99.81%. For the inter batch experiments, the precision varied from 3.46 to 7.29% and the accuracy was within 93.60 and 98.29% for Ethinyl estradiol. (Table 1).

Table 1: Intra-batch and inter-batch precision and accuracy of Ethinyl estradiol

QC	Nominal Concentration (pg/mL)	Intra batch				QC	Nominal Concentration (pg/mL)	Inter batch			
		n	Mean concentration found (pg/mL) *	Accuracy (%)	CV (%)			n	Mean concentration found (pg/mL) ↑	Accuracy (%)	CV (%)
HQC	152.000	6	148.4300	97.65	0.77	HQC	152.000	30	146.1350	96.14	2.52
MQC 1	68.000	6	66.0375	97.11	0.83	MQC1	68.000	30	65.2688	95.98	2.43
MQC2	21.000	6	20.5592	97.90	1.38	MQC2	21.000	30	20.3407	96.86	2.19
LQC	3.000	6	2.8997	96.66	3.24	LQC	3.000	30	2.8371	94.57	4.62
LLOQ QC	1.000	6	0.9253	92.53	7.82	LLOQ QC	1.000	30	0.9568	95.68	9.09

* Mean of six replicates at each concentration

↑Mean of six replicates of five Precision and accuracy batches

The intra-batch precision (CV) ranged from 1.04 to 9.70% and the accuracy was within 91.64 and 103.31%. For the inter batch experiments, the precision varied from 2.99 to 7.73% and the accuracy was within 95.14 and 99.22% for Levonorgestrel. (Table 2).

Table 2: Intra-batch and inter-batch precision and accuracy of Levonorgestrel

QC	Nominal Concentration (pg/mL)	Intra batch				QC	Nominal Concentration (pg/mL)	Inter batch			
		n	Mean concentration found (pg/mL) *	Accuracy (%)	CV (%)			n	Mean concentration found (pg/mL) #	Accuracy (%)	CV (%)
HQC	3800.000	6	3737.2582	98.35	0.96	HQC	3800.000	30	3676.6663	96.75	1.77
MQC 1	1700.000	6	1660.7887	97.69	1.19	MQC1	1700.000	30	1643.1605	96.66	1.82
MQC2	525.000	6	515.9578	98.28	0.79	MQC2	525.000	30	511.4472	97.42	1.63
LQC	75.000	6	72.9285	97.24	1.95	LQC	75.000	30	72.4354	96.58	2.51
LLOQ QC	25.000	6	25.1608	100.64	3.02	LLOQ QC	25.000	30	25.3440	101.38	3.33

* Mean of six replicates at each concentration

Mean of six replicates of five Precision and accuracy batches

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The Overall % recovery of was 67.39 **% and** 69.07% for Ethinyl estradiol and **Ethinyl estradiol D4 respectively.** The Overall % recovery 81.39**% and** 85.09% for Levonorgestrel and Levonorgestrel D6 respectively.

Matrix effect was performed in human plasma, for each individual matrix lot/source evaluated, % mean accuracy was within ±15.00% of their nominal concentration and the % CV for samples was ≤15.00% at HQC and LQC level.

The stability of the Ethinyl Estradiol, Levonorgestrel and their respective IS in human plasma and stock solutions were examined under different storage conditions. Stock solutions of Ethinyl estradiol, Levonorgestrel and their respective IS remained unchanged up to 18.0 hrs. at room temperature (short-term stability) and for a minimum of 29 days at refrigerated temperature, 5ºC (long-term stability).

Spiked plasma samples stored at -20ºC and -70ºC for the long-term stability experiment were found to be stable for a minimum period of 83 days.

The autosampler stability of the spiked quality control samples maintained at 10ºC was determined up to 145.0 hrs. without significant drug loss. All stability results in plasma at two QC levels are shown in Table 3 and Table 4.

Table 3: Stability results of Ethinyl estradiol

Stability of the Ethinyl Estradiol in human plasma (n=6)			
Storage condition	Nominal concentration (pg/mL)	Mean Stability samples ± SD	% Mean Stability
Bench top stability at ambient temperature (22 Hrs.)			
HQC	152.000	146.5720±1.40186	96.43
LQC	3.000	2.8665±0.09210	95.55
Stability of Dry extract at -20±5 ºC (22 Hrs.)			
HQC	152.000	145.8430±1.08941	95.95
LQC	3.000	2.7648± 0.14288	92.16
Freeze and Thaw stability 5 cycles at **-20±5ºC**			
HQC	152.000	146.6895±1.85537	96.51
LQC	3.000	22.8497±0.06388	94.99
Freeze and Thaw stability 5 cycles at **-70±5ºC**			
HQC	152.000	146.0317±1.24115	96.07
LQC	3.000	2.8472± 0.10025	94.91
Long term matrix stability(83 days)			
HQC	152.000	146.4668±1.16041	96.36
LQC	3.000	2.7827±0.06353	92.76

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Table 4: Stability results of Levonorgestrel

Stability of the Levonorgestrel in human plasma (n=6)			
Storage condition	Nominal concentration (pg/mL)	Mean Stability samples ± SD	% Mean Stability
Bench top stability at ambient temperature (22 Hrs.)			
HQC	3800.000	3626.4603±68.73080	95.43
LQC	75.000	72.1602± 1.18920	96.21
Stability of Dry extract at -20±5 ºC (22 Hrs.)			
HQC	3800.000	3610.1755±62.81200	95.00
LQC	75.000	71.9958±1.34028	95.99
Freeze and Thaw stability 5 cycles at **-20±5ºC**			
HQC	3800.000	3598.7198±58.38156	94.70
LQC	75.000	70.4478± 0.79900	93.93
Freeze and Thaw stability 5 cycles at **-70±15ºC**			
HQC	3800.000	3663.4210±31.33694	96.41
LQC	75.000	71.2548± 0.62612	95.01
Long term matrix stability(83 days)			
HQC	3800.000	3555.6740±55.06855	93.57
LQC	75.000	70.6405±0.94202	94.19

For method ruggedness, the precision (CV) and accuracy values for Ethinyl Estradiol with different columns ranged from 0.75 **to 3.34**% and 91.08 to 96.15%, respectively. For Levonorgestrel with different columns ranged from 0.26 **to 1.42**% and 93.70 to 95.37%, respectively.

The experiment with different analysts, the results varied from 1.07 **to 1.50**% and 99.21 to 100.96% for precision and accuracy of Ethinyl Estradiol, respectively. For Levonorgestrel the results varied from 0.68 **to 2.62** % and 97.84 to 98.86 % for precision and accuracy, respectively.

The precision for dilution integrity of 1/2 and 1/10th dilution were 0.44 and 1.87%, and the accuracy results were 96.17 and 97.12%, respectively for Ethinyl estradiol and 1.12 **to** 0.60%, and the accuracy results were 96.51 to 96.15%, respectively for Levonorgestrel, which is well within the acceptance limits of 15% for precision (CV) and 85 to 115% for accuracy.

Application to bioequivalence study and ISR results:

The developed method was used to estimate Levonorgestrel and Ethinyl estradiol concentration in human plasma samples after administration of Levonorgestrel and Ethinyl estradiol tablets, 0.15mg/0.03mg oral dose. Fig. 5 shows the mean plasma concentration vs. time profile of Levonorgestrel and Fig. 6 Ethinyl estradiol in healthy subjects. The method was sensitive enough to monitor Levonorgestrel and Ethinyl estradiol concentration up to 72.0 hrs.

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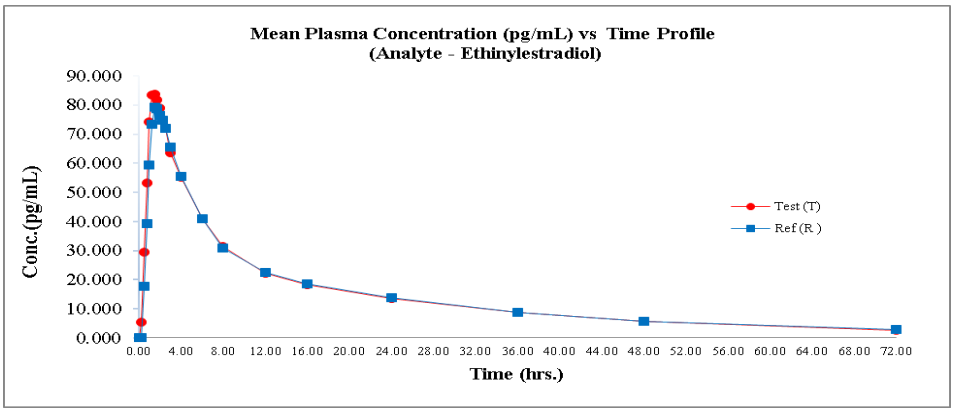

Fig. 5 Mean plasma concentration vs. time profile of Ethinyl estradiol in healthy subjects

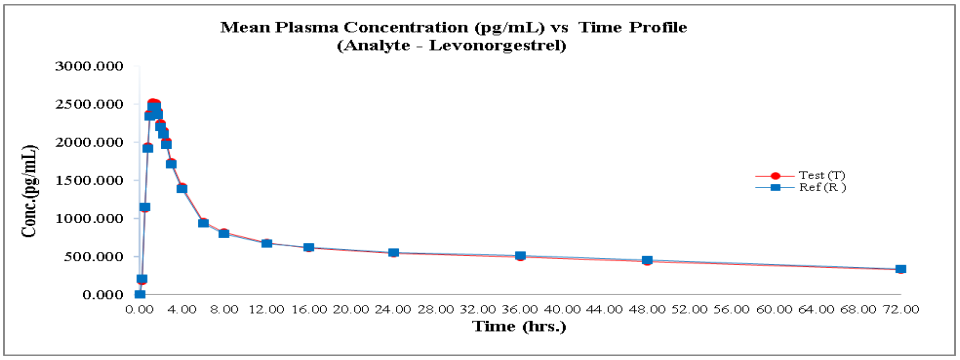

Fig. 6 Mean plasma concentration vs. time profile of Levonorgestrel in healthy subjects

The ln-transformed pharmacokinetic parameters C_{max}, AUC_{0-t} and AUC_{0-inf} For Ethinyl estradiol and C_{max}, AUC_{0-72} and AUC_{0-inf} Levonorgestrel for under fasting conditions are summarized in table 05 and 06 respectively. Furthermore, there was no adverse event during the course of the study.

ISR results showed % difference for assay reproducibility within 5% for 145 samples, 5–10% for 26 samples, 10–15% for 22 samples, while the remaining 4 samples showed % change between 15% and 20% for Ethinyl estradiol. For levonorgestrel assay reproducibility within 5% for 177 samples, 5–10% for 19 samples, 10–15% for 1 samples (Fig.7 and Fig. 8). This authenticates the reproducibility of the proposed method.

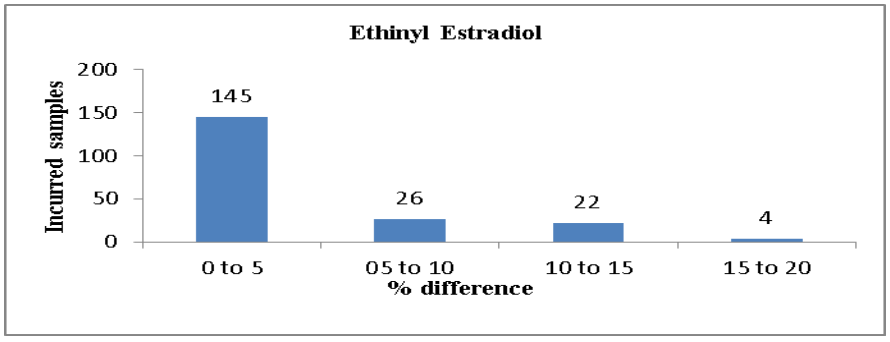

Fig. 7 % Change for assay reproducibility results with incurred study samples for Ethinyl estradiol.

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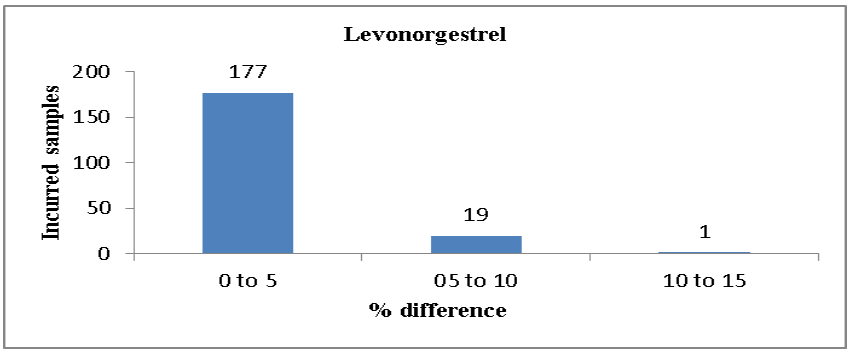

Fig. 8 % Change for assay reproducibility results with incurred study samples for Levonorgestrel.

Table 5: Mean pharmacokinetic parameters and 90% CIs of natural log (Ln)-transformed parameters following oral administration of Levonorgestrel and Ethinyl estradiol tablets, 0.15mg/0.03mg oral dose 48 healthy female subjects for Ethinyl estradiol.

Parameter	Geo LS Mean Test	Geo LS Mean Ref	Ratio (test/reference) (%)	90% confidence interval (Lower-Upper)	Intra-subject variation (%CV)
C_{max} (pg/mL)	85.13	80.74	105.44	100.80-110.30	13.20
AUC $_{0\text{-}t}$ (h pg/mL)	1010.80	972.36	103.95	98.83-109.34	14.83
AUC $_{0\text{–}inf}$ (h pg/mL)	1088.85	1058.72	102.85	98.35-107.55	13.11

Table 6 : Mean pharmacokinetic parameters and 90% CIs of natural log (Ln)-transformed parameters following oral administration of Levonorgestrel and Ethinyl estradiol tablets, 0.15mg/0.03mg oral dose 48 healthy female subjects for Levonorgestrel.

Parameter	Geo LS Mean Test	Geo LS Mean Ref	Ratio (test/reference) (%)	90% confidence interval (Lower-Upper)	Intra-subject variation (%CV)
C_{max} (pg/mL)	2803.06	2738.13	102.37	101.57-103.18	2.30
AUC $_{0\text{-}72}$ (h pg/mL)	36525.05	35919.18	101.69	100.26-103.14	4.13
AUC $_{0\text{–}inf}$ (h pg/mL)	66332.52	65698.60	100.96	98.71-103.28	6.61

Conclusion:

The HPLC-ESI MS/MS method for the quantitation of Ethinyl estradiol and levonorgestrel in human plasma was developed and fully validated as per the M10 guidelines. A total of 2016 samples were analyzed during a period of 28 days, which included calibration, QC and subject samples and the precision and accuracy were well within the acceptable limits.

The advantages of this method include high sensitivity, small sample volume for processing, and simultaneous estimation with lowest LLOQ. Based on dilution reliability results it is possible to extend the ULOQ to 360pg/mL for Ethinyl estradiol and 9000 pg/ml for levonorgestrel. In addition, assay reproducibility is effectively proved by reanalysis of 197 subject samples.

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