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Simultaneous determination of etonogestrel and ethinyl estradiol in human plasma by UPLC-MS/MS and its pharmacokinetic study

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Abstract

A selective, sensitive and rapid ultra-performance liquid chromatography tandem mass spectrometry method was developed and validated for the simultaneous determination of etonogestrel (ENG) and ethinyl estradiol (EE) in human plasma. The analytes and their deuterated internal standards, ENG-d7 and EE-d4, were extracted from plasma samples by solid-phase extraction on HyperSepTM Retain PEP cartridges. The chromatographic analysis was performed on an Acquity UPLC HSS Cyano column, 100 Å (50 × 2.1 mm, 1.8 µm), column using gradient mobile phase, acetonitrile and 2.0 mM ammonium trifluoroacetate at 0–1.7 min (65:35, v/v) and 1.8–2.7 min (95:5, v/v) with 0.250 mL/min flow rate. Analytes and IS protonated precursor → product ion transitions (ENG, m/z 325.2 → 257.2; EE, m/z 530.2 → 171.2; ENG-d7, m/z 332.2 → 263.2; EE-d4, m/z 534.2 → 171.2) were monitored on a Triple Quadrupole Mass spectrometer (TQMS), operating in multiple reaction monitoring and positive ionization mode. The calibration curves were established at 10.00–2500 pg/mL for ENG and 1.500–150.0 pg/mL for EE with a correlation coefficient (r^2) 0.9996 for both. The validated method was successfully applied to support a bioequivalence study of 0.15 mg ENG and EE 0.03 mg tablet formulation, administered in 24 healthy Indian females. Method reliability was assessed by reanalysis of 94 incurred study samples.

Keywords

ethinyl estradiol; etonogestrel; human plasma; low pg/mL; UPLC-MS/MS

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

1 | INTRODUCTION

11-Methylene levonorgestrel or 3-keto-desogestrel, normally known as etonogestrel (ENG), is a steroid progestin utilized as a part of hormone contraceptives alongside ethinyl estradiol (EE; Berkowitz, Barbieri, Dunaif, Ryan, & Barbieri, 1999). After its first use in Indonesia in 1998 as implanon (Carcio & Secor, 2014; Mayeaux, 2012), an increase in its use was seen in the UK (Glasier & Winikoff, 1999) and USA in 2006 (Carcio & Secor, 2014; Mayeaux, 2012). Desogestrel, a prodrug, is quickly metabolized into 3-keto-desogestrel, which is the biologically active metabolite while EE likewise is absorbed in the third cycle of utilization after the single measurement of desogestrel and EE, with a relative bioavailability of around 83% [5] Desogestrel and Ethinyl Estradiol, National Drug Code List, NDC16714. The utilization of ENG and EE has expanded as a combined contraceptive pill since etonogestrel is less androgenic than levonorgestrel and norethisterone (Danby, 2015; Golan, Tashjian, & Armstrong, 2011). Furthermore, it does not tend to decrease in the sex-hormone binding globulin levels (Speroff & Darney, 2011). Etonogestrel works by avoiding ovulation and creates changes in the uterine covering and cervical bodily fluid, making it harder for the sperm to enter the uterus and moreover for the fertilized egg to connect to the uterus (Dionne & Rahman, 2008; Drugsite Trust, 2000; Funk et al., 2005; Thomson Healthcare, 2007). Comparison the combined oral contraceptive pills and contraceptive vaginal rings led to increased use of the rings owing in part to long length of activity and low hormone variances. However, because of side effects, including increased prevalence of stroke, heart-attack and blood clotting, combined contraceptive pills are more ideal than rings.

Bioavailability and bioequivalence from two formulations, Cerazette and Liseta was studied using Radio-Immunoassay (RIA) (Timmer, Srivastava, Dieben, & Cohen, 1999). Simultaneous determination of ENG and EE in vaginal ring was done by extraction and derivative spectrophotometry (Inés, Nacaratte, & Nova, 2015) determined by solid-phase extraction extraction with HPLC-MS (Moser, Zoderer, & Luef, 2012) while others (Andrasi, Helenkar, Zaray, Vasanits, & Molnar-Perl, 2011) measured aqueous samples by derivatization of analytes through GC-MS. A comparative study of Nuva ring with oral contraceptive containing ENG and EE was studied in uterine and serum samples and measured by RIA (Frans Roumen & Thom Dieben, 2006). Thomas et al. (2013) studied the pharmacokinetics of ENG during vaginal ring use in serum samples using D8 progesterone as an internal standard. Anari et al. (2002) reported a chemical derivatization procedure with dansyl chloride for the determination of EE2 in monkey plasma to achieve low pg/mL-range sensitivity using LC-MS/MS analysis. Shou, Jiang, and Weng (2004) described an LC-MS/MS method for the analysis of EE2 with dansyl chloride derivatization. EE in combination with norethindrone has been determined by HPLC-MS/MS in human plasma using a derivatization agent by Li et al. (2010). A bioequivalence study of generic formulation having EE and chlormadinone acetate has been reported by Bonn, Urte, Martin, and Lucio (2009) in healthy female subjects. Determination of EE along with Gestodene and etonogestrel by LC-MS/MS using derivatization has been reported by Lui, Ding, Qing-H, Zhou, and Zhi (2010). Xiong et al. (2010) reported a UPLC-MS/MS method for the simultaneous determination of Gestodene and ethinyl estradiol in rat plasma. Wheaton, Chambers, and Fountain (2012) reported a method taking into consideration the challenges

involved in developing an ultra-sensitive method for the determination of EE2 in human plasma. A UPLC-MS/MS method has been reported for the simultaneous determination of EE with norgestimate and 17-desaetyl norgestimate in human plasma by Huang et al. (2016).

Although an LC-MS/MS method has been reported for the determination of EE2 in human plasma, the reported methods for the simultaneous determination of ENG and EE in human plasma are based on vaginal rings, and to the best of our knowledge, there are currently no methods for the simultaneous determination of ENG and EE in human plasma for contraceptive pills. Thus, a highly sensitive UPLC-MS/MS method was developed to quantify ENG and EE in human plasma. The proposed method required 400 μ L of human plasma for sample processing and demonstrates acceptable chromatographic performance in a run time of 2.7 min. It was extensively validated under current regulatory guidelines and applied to support a bioequivalence study in 24 healthy Indian subjects after the administration of oral tablet formulation.

2 | EXPERIMENTAL

2.1 | Chemicals and materials

Reference standards of etonogestrel (ENG, >98.0%), etonogestrel-d7 (ENG-d7, IS, >98.0%), ethinyl estradiol (EE, >99.38%) and 17- α -ethinyl estradiol-d4 (EE-d4, IS, >98.35%) were obtained from Clearsynth Labs (P) Ltd (Mumbai, India). HPLC-grade methanol, acetonitrile and *n*-hexane were obtained from Thermo Fisher Scientific India (P) Ltd (Mumbai, India). Mass spectrometry-grade ammonium trifluoroacetate, sodium bicarbonate and HPLC-grade dansyl chloride were purchased from Sigma-Aldrich (St Louis, MO, USA). Samples were extracted by solid-phase extraction using HyperSep™ Retain PEP cartridges (30 mg, 1 mL) and an extraction cartridge from Thermo Fisher Scientific India (P) Ltd (Mumbai, India). Water was purified using a Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma was obtained from Supratech Micropath (Ahmedabad, India) and was stored at -20°C until use.

2.2 | Calibration standards and quality control samples

The standard stock solutions of ENG (400.0 $\mu\text{g/mL}$) and EE (400.0 $\mu\text{g/mL}$) were prepared by dissolving accurate amounts in methanol. Working solutions were prepared using intermediate solutions of 1000 ng/mL for ENG and 5000 and 5.000 ng/mL for EE in methanol–water (50:50, v/v). Calibration standards and quality control (QC) samples were prepared by spiking blank plasma with stock/intermediate solutions. Calibration standards were made at 10.00, 25.00, 100.0, 200.0, 400.0, 800.0, 1200, 1600, 2000, 2500 pg/mL and 1.500, 3.000, 5.000, 10.00, 20.00, 40.00, 80.00, 100.0, 120.0, 150.0 pg/mL concentrations for ENG and EE respectively. While the QC samples were prepared at 2100/125.0 pg/mL (HQC, high quality control), 1500/90.00 pg/mL (MQC-1, medium quality control-1), 300.0/15.00 pg/mL (MQC-2, medium quality control-2), 30.00/4.500 pg/mL (LQC, low quality control) and 10.00/1.500 pg/mL (LLOQ QC, lower limit of quantification quality control) for ENG and EE, respectively. The stock solutions were stored at 5°C , while calibration standards and QC samples were stored at -70°C until use. Individual stock

solutions 100.0 µg/mL of the internal standards were prepared by dissolving 1.0 mg of reference standards in 10 mL of methanol. The combined working solution (12.0 ng/mL for ENG-d7 and 0.60 ng/mL for EE-d4) was prepared from the stock solutions in methanol–water (50:50, v/v). All of the stock and working solutions were stored at 2–8°C until use.

2.3 | Sample preparation

Prior to analysis, all frozen subject samples, calibration standards and QC samples were thawed and allowed to equilibrate at room temperature for 30 min. To an aliquot of 400 µL of spiked plasma/subject samples, 25 µL of combined ISs solution was added. After vortex mixing for 30 s, the samples were centrifuged at 13,148 g for 5 min at 10°C. Plasma samples were then loaded on HyperSep™ Retain PEP cartridges (30 mg, 1 mL), which were pre-conditioned with 1.0 mL methanol followed by 1.0 mL of water. The samples were washed sequentially with 1.0 mL *n*-hexane, 1.0 mL water and 1 mL of 10% methanol in water. The cartridges were dried for 1.0 min by applying nitrogen (1.72×10^5 Pa) at 2.4 L/min flow rate. The analytes and ISs were eluted with 0.5 mL of acetonitrile. The eluent was evaporated to dryness into pre-labeled vials under a gentle stream of nitrogen and then reconstituted with 50 µL of sodium bicarbonate in water (100 mM, pH 10.5). The samples were vortexed after adding 150 µL of dansyl chloride solution (1.500 mg/mL in acetone) and then incubated at 55°C. To all incubated samples 1.0 mL of *n*-hexane was added and vortexed, and the organic layer was separated and evaporated to dryness under a gentle stream of nitrogen. All samples were reconstituted with 125 µL of acetonitrile–5.0 mM ammonium trifluoroacetate (75:25, v/v), briefly vortexed for 15 s and 10 µL injected into the chromatographic system.

2.4 | Liquid chromatography and tandem mass spectrometry conditions

All samples were analyzed on Waters Acquity UPLC HSS Cyano column, 100 Å (50 × 2.1 mm, 1.8 µm), coupled to a Waters Quattro Premier triple quadrupole mass spectrometer (MA, USA). The analytes were separated on the reversed-phase column using gradient mobile phase, acetonitrile and 2.0 mM ATFA at 0–1.7 min (65:35, v/v) and 1.8–2.7 min (95:5, v/v) with 0.250 mL/min flow rate. The temperature of the column was maintained at 40°C in a column oven. The electrospray ionization in the mass spectrometer was operated in the positive ionization mode and the quantitation of analytes was performed using multiple reaction monitoring (MRM). The MRM transitions and mass parameters optimized for the analytes and ISs are given in Table S1 in the Supporting Information.

2.5 | Validation procedures

The method was validated for system suitability, system performance, selectivity, carryover, linearity, accuracy and precision, recovery, matrix effect, stability, dilution reliability and ruggedness as per the draft guidance of the US Department of Health and Human Services, US Food and Drug Administration (2013). The procedures were similar to those in our previous report (Patel, Sharma, Sanyal, Singhal, & Shrivastav, 2012) and are briefly described in Appendix A in the Supporting Information.

3 | RESULTS AND DISCUSSION

A technique that is sensitive and selective is required to quantify the analytes of ENG and EE in human plasma. The prevailing methods have centered on the bioequivalence and X-ray visibility of ENG alone (Schnabel et al., 2012), the bioavailability and bioequivalence of ENG from two formulations (Timmer et al., 1999), and a pharmacokinetics study of ENG and EE within the vaginal ring (Timmer & Mulders, 2000). Most studies were performed on the vaginal mode of contraceptive method with ENG and EE. In the present work, a simultaneous method for the determination of the ENG and EE at the low pg/mL level (10.00 and 1.500 pg/mL) in human plasma was developed for use in pharmacokinetic studies of ENG and EE in healthy Indian females. The key challenge featured throughout the course of methodology development was overcoming issues with endogenous matrix components that interfere with the measurement of analytes at sub pg/mL levels and thereby increasing the accuracy and precision of the method.

Analysis of ENG and EE was performed using MRM and in positive electrospray ionization mode. Initially, the precursor and products ions of the analytes and their deuterated ISs were optimized by infusing 50 ng/mL solutions within the mass range of 50–400 amu for ENG and 70–560 amu for EE. Dansyl chloride was used for the derivatization of EE. The hydroxyl group of the benzene ring allows derivatization of EE and gives a molecular ion at 529.1, whereas the stable ketonic ($-\text{C}=\text{O}-$) group present in ENG cannot react with dansyl chloride even though it has an identical position of the $-\text{OH}$ group to EE. This gives protonated precursor $[\text{M} + \text{H}]^+$ ions at m/z 325.2. Therefore, on applying adequate collision energy for fragmentation, the most stable and superabundant product ions in Q3 MS were found at m/z 257.2 and 263.2 for ENG and ENG-d7, whereas they were at 171.2 for both the analytes and ISs of EE (Figure 1). A cross-talk experiment was performed on each of the analytes since the ISs of EE have the same product ion. The results showed no memory effects within the collision cell attributable to identical mass in their protonated precursor–product ion channels.

To have a much better control of matrix effect, sample preparation was initiated using deuterated analogs of the drug. Protein precipitation with methyl alcohol, ethanol and acetonitrile were carried out; however, extensive matrix interference for both the analytes with poor recovery was found for ENG and EE and thus liquid–liquid extraction was tested with methyl *tert*-butyl ether, dichloromethane, ethyl acetate and *n*-hexane in the presence of hydrochloric acid and ammonium hydroxide/sodium hydroxide and under neutral conditions. The results were unsatisfactory. Therefore, sample clean-up was initiated using solid-phase extraction on HyperSep™ Retain PEP cartridges through careful choice of solvents for sample loading and washing and extraction of analytes. It was found that neutral conditions were best suited to optimum retention of ENG and EE with virtually complete removal of matrix components using 1.0 mL acetonitrile, 1.0 mL water, 1.0 mL *n*-hexane and 1 mL, 10% methanol in water sequentially as solvents for washing. The analytes were eluted from the column using acetonitrile. The recovery ratios obtained were extremely consistent for ENG/ENG-d7 and EE/EE-d4 at 95.7–104.6 and 91.9–97.7%, respectively (Table 1).

Reversed-phase chromatographic conditions were optimized on an Acquity UPLC HSS Cyano column, 100 Å (50 × 2.1 mm, 1.8 µm), to attain sub-pg/mL quantitation limits by complete separation of analytes from endogenous matrix components. Throughout the trials, it was possible to separate ENG and EE; however, some endogenous matrix components were found to interfere with the retention times of the analytes. Consequently, the chromatographic conditions were suitably optimized to maximize peak area response for the analytes and minimize interference of endogenous peaks within a brief analysis time. Several additives like formic acid, acetic acid and ATFA were added along with organic diluents (methanol and acetonitrile) in different proportions to enhance the signal-to-noise ratio and separate interfering peaks from the analyte peaks. The optimum conditions were established using methanol–water (78:22, v/v), acetonitrile–water (78:22, v/v) and acetonitrile–2.0 mM ATFA (75:25, v/v) as the mobile phase. The obtained retention time for ENG was ~1.3 min with methanol–water (78:22, v/v), acetonitrile–water (78:22, v/v) and acetonitrile–2.0 mM ATFA (75:25, v/v) under isocratic elution. However, with these isocratic conditions we were not able to achieve chromatography for dansyl EE for simultaneous determination. We finalized the gradient mobile phase, acetonitrile and 2.0 mM ATFA at 0–1.7 min (65:35, v/v) and 1.8–2.7 min (95:5, v/v) to retain and improve the results for EE after applying 150 µL of dansyl chloride as it provided a very stable ion formation complex for ionization in the positive mode. The retention times for ENG and EE were 1.44 and 2.14 min, respectively. The representative UPLC-MS/MS chromatograms of double blank plasma, blank plasma spiked with ISs, at LLOQ concentration of analytes and at maximum plasma concentration ($C_{\max}$) level concentration of analytes in subject samples are shown in Figure 2. Further, there was no interference by other medications concurrently used by test subjects at the retention time of the analytes. Post-column infusion chromatograms showed significant ion suppression that was abundant ahead of the analyte peaks and failed to influence their quantitation (Figure S1 in the Supporting Information).

To assess the extent of matrix interference, matrix factors (MF) were calculated from the peak area response of the analyte and their respective IS (Table 1). The IS-normalized MF calculated from the ratio of the MF of the analyte/IS ranged from 0.923 to 0.973 for both analytes, indicating minimal interference by the matrix components throughout the analysis. The variation in several plasma sources for matrix effect was additionally checked in six heparinized, one hemolyzed and one lipemic plasma. The precision (CV, %) within the measurement of slope of standardization curves prepared in these plasma sources was 1.29 for ENG and 1.49 for EE (Table S2 in the Supporting Information), that is within the acceptance criteria of 3.0%.

The UPLC-MS/MS methodology performance characteristics associated with system suitability, system performance, selectivity, autosampler carryover, linear range, dilution reliability and ruggedness were all within the acceptance criteria (Table 2). The method showed excellent limit of quantitation of both analytes (10.0 pg/mL, ENG and 1.50 pg/mL, EE) and a good intra-batch and inter-batch precision for both of the analytes (Table S3). The stock solutions of analytes and ISs in methanol were stable at room temperature and under refrigerated conditions for 16 h and 2 weeks, respectively. The bench-top stability of analytes in plasma was established up to 10 h and both analytes were stable throughout five freeze–thaw cycles and for a minimum of 24 h within the autosampler. Dry extract

(processed sample) stability of analytes was established up to 22 h at 5°C with no significant modification within the concentration of ENG and EE. Spiked plasma samples stored at –20 and –70°C for long-term stability were stable for a minimum period of 125 days. The obtained accuracies at LQC (lower quality control) and HQC (high quality control) of bench-top, auto-sampler, dry extract, wet extract, freeze–thaw and long-term stability results are presented in Figure S2.

The projected methodology is extremely sensitive, very rapid and consistent for real subject sample analysis. To the best of our knowledge, this is the first description of the simultaneous analysis of ENG and EE in human plasma. Additionally, the method employs deuterated analogs as ISs which further helps to maintain the overall efficiency of the analysis and thereby the accuracy of the data.

3.1 | Application of the method and assay reliability

Currently there are no reports on the pharmacokinetics of ENG and EE in Indian female subjects. Thus, the validated method was applied to determine the concentrations of ENG and EE in human plasma for a bioequivalence study between generic and reference oral tablet formulations of 0.15 mg desogestrel (active metabolite ENG) and 0.03 mg EE administered in 24 healthy Indian female subjects under fasting conditions. The study was conducted as per International Conference on Harmonization, E6 Good Clinical Practice guidelines (Guidance for Industry: ICH E6 Good Clinical Practice, U.S. Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), Centre for Biologics Evaluation and Research (CBER), 1996).

A representative MRM chromatogram obtained for a subject sample at $C_{\max}$ after administration of 0.15 mg desogestrel (active metabolite ENG) and 0.03 mg is illustrated in Figure 2. The mean plasma concentration–time profiles (AUC) of ENG and EE for the test and reference formulation are shown in Figure 3. Additionally, the essential pharmacokinetic parameters obtained showed close similarity obtained between the two formulations, and did not significantly deviate (Table 3). Thus, the two tablet formulations can be considered as bioequivalent in terms of rate and extent of absorption. The method was sensitive enough to monitor the ENG–EE plasma concentration up to 120 h. The point estimates and 90% confidence intervals (CI) for $C_{\max}$, AUC_{0-120h} and AUC_{0-inf} for ENG–EE were each within the 80–125% range, as represented in Table 3, demonstrating the bioequivalence of the two formulations. There was no adverse event during study. Furthermore, after administration of 0.15 mg desogestrel and ethinyl estradiol 0.03 mg in health female volunteers, obtained $C_{\max}$, $T_{\max}$ (time point of maximum plasma concentration) and $t_{1/2}$ (half-life of drug elimination during the terminal phase) values were in completely agreement with previously reported results for Caucasian (Bonn et al., 2009) and Dutch (Huang et al., 2016; Timmer et al., 1999) volunteers under fasting conditions. Competitive evolution of mean bioequivalence parameters for the present and previous studies is represented in Table S5.

Further to prove the assay reproducibility, sample reanalysis was performed using 94 subject samples as described previously (Yadav & Shrivastav, 2011). The results confirmed method reproducibility as the percentage change in the concentration from the original results ranging from –11 to 13%, which is within the acceptance criterion of $\pm 20\%$ (Figure S3).

4 | CONCLUSION

There is expanded interest in contraceptive strategy because of the present worldwide trend towards small families. While contraceptions like vaginal rings and intrauterine devices have been produced and are used in the clinic, there remains an inconvenience of the long-term contraception with the reliance on trained health care personnel to start and end treatment. There is also the prerequisite of minor surgery for insertion and expulsion of implants, a high rate of bleeding abnormalities and initial high cost, making the oral method of contraception more widely used.

In this study, we have developed a solid-phase extraction method for the simultaneous determination of ENG and EE in human plasma that was employed in a pharmacokinetic investigation of ENG and EE in healthy adult females with precision and accuracy. A short run time of 2.7 min highlights the utility of the strategy in an expansive clinical setting. The technique has excellent reliability, as apparent from the incurred sample reanalysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ATFA	ammonium trifluoroacetate
EE	ethinyl estradiol
ENG	etonogestrel
MF	matrix factors
MRM	multiple reaction monitoring

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FIGURE 1.

Product ion mass spectra, scan range 50–400 amu of (a) etonogestrel (m/z 325.2 ? 257.2) (b) etonogestrel-d7 (m/z 332.2 ? 263.2) and scan range 70–560 amu (c) ethinyl estradiol (m/z 530.2 ? 171.2) (d) 17- α ethinyl estradiol-d4 (m/z 534.2 ? 171.2) in positive ionization mode

FIGURE 2.

MRM ion-chromatograms of etonogestrel (ENG), etonogestrel-d7 (ENG-d7), ethinyl estradiol (EE) and 17-ethinyl estradiol-d4 (EE-d4) in (a) double blank plasma, (b) blank plasma spiked with ENG-d7 and EE-d4, (c) ENG (10.00 pg/mL) & ENG-d7 and EE (1.500 pg/mL) & EE-d4 respectively and (d) ENG and EE (with spiked ENG-d7 and EE-d4) subject sample at $C_{\max}$ after oral administration of fixed dose formulation

FIGURE 3.

Mean plasma concentration-time profile of etonogestrel (ENG) and ethinyl estradiol (EE) after administration of 0.15 mg desogestrel and ethinyl Estradiol 0.03 mg in healthy female volunteers

TABLE 1

Extraction recovery and matrix factor

QC level (pg/mL)	Area response ratio (area response), $n = 6$			Extraction recovery (%) (A/B)	Matrix factor		IS normalized
	A (A')	B (B')	C (C')		Analyte (B'/C')	IS	
ENG							
LQC	0.0182 (2187)	0.0188 (3105)	0.0186 (2886)	97.2	1.076	1.064	1.011
MQC-2	0.1917 (23490)	0.1833 (31786)	0.1775 (29076)	104.6	1.093	1.059	1.032
MQC-1	0.8832 (110786)	0.8969 (155646)	0.8682 (150675)	98.5	1.033	1.070	0.966
HQC	1.338 (158922)	1.399 (235646)	1.311 (212565)	95.7	1.109	1.039	1.067
EE							
LQC	0.0444 (4350)	0.0455 (5120)	0.0473 (4985)	97.7	1.027	1.067	0.962
MQC-2	0.1446 (14489)	0.1500 (16543)	0.1533 (16153)	96.4	1.024	1.047	0.978
MQC-1	0.8905 (87673)	0.9686 (101276)	0.953 (97543)	91.9	1.038	1.022	1.016
HQC	1.338 (123543)	1.386 (141290)	1.290 (135765)	96.5	1.041	0.969	1.074

A, Analyte/internal standard mean area response ratio of six replicates prepared by spiking before extraction.

B, Analyte/internal standard mean area response ratio of six replicates prepared by spiking in extracted blank plasma.

C, Analyte/internal standard mean area response ratio of six replicates prepared by spiking in mobile phase (neat samples).

A', Analyte mean area response of six replicates prepared by spiking before extraction.

B', Analyte mean area response of six replicates prepared by spiking in extracted blank plasma.

C', Analyte mean area response of six replicates prepared by spiking in mobile phase (neat samples).

n, Number of replicates; IS, internal standard; LQC, low quality control; MQC, medium quality control; HQC, high quality control.

TABLE 2

UPLC-MS/MS assay performance

Assay performance	ENG	EE	Linearity assessment	ENG (pg/mL)	EE (pg/mL)
<i>System suitability^a</i>			Linearity range	10.00–2500	1.500–150.0
Precision (CV, %)	1.42/2.98 for retention time/area response	2.16/4.07 for retention time/area response	Calibration standards	10.00, 25.00, 100.0, 200.0, 400.0, 800.0, 1200, 1600, 2000, 2500	1.500, 3.000, 5.000, 10.0, 20.00, 40.00, 80.0, 100.0, 120.0, 150.0
Accuracy (change, %)	95.3–102.2	97.1–104.3	Quality control samples	10.00, 30.00, 300.0, 1500, 2100	1.500, 4.500, 15.00, 90.00, 125.0
<i>System performance</i>			Weighting factor	1/x ²	1/x ²
S/N at LLOQ	44	40	Mean regression line ($y = mx + c$)	$y = (0.001657 \pm 0.000003)x + (-0.000705 \pm 0.000338)$	$y = (0.011205 \pm 0.000177)x + (-0.000162 \pm 0.000376)$
<i>Autosampler carry-over^b</i>			Correlation coefficient (r^2)	0.9998 $\pm$ 0.0001	0.9996 $\pm$ 0.0001
Blank plasma area response	0.89 (0.18% of LLOQ response)	2.67 (0.08% of LLOQ response)	Precision (CV, %)	0.23–0.53	0.53–1.77
<i>Method ruggedness^c</i>			Accuracy (change, %)	97.9–102.2	98.4–101.6
Precision (CV, %)	2.12–3.86	1.38–2.98	LLOQ, S/N ratio	10.0 pg/mL, 47	1.500 pg/mL, 38
Accuracy (change, %)	96.0–103.4	97.4–104.5	LOD, S/N ratio	2.5 pg/mL, 18	0.500 pg/mL, 12
<i>Dilution integrity^d</i>					
Precision (CV, %)	0.76–3.12	1.87–3.87			
Accuracy (change, %)	95.8–101.9	96.2–103.9			

^a Aqueous standard mixture of analytes (at ULOQ) and ISs injected as six consecutive injections at the start of each batch.

^b Samples analyzed in the order: extracted blank plasma $\rightarrow$ ULOQ $\rightarrow$ extracted blank plasma $\rightarrow$ LLOQ $\rightarrow$ extracted blank plasma.

^c One QC batch analyzed on two HSS Cyno (CN) columns with different batch numbers, while the second batch was analyzed by different analysts.

^d Blank human plasma spiked with 1:5 and 1:10 dilution of the stock solution prepared at 5000 pg/mL for ENG and 300.0 pg/mL for EE. EE, Ethinyl estradiol; ENG, etonogestrel; S/N, signal-to-noise ratio.

TABLE 3

Mean pharmacokinetic parameters and comparison of treatment ratios and 90% CIs of natural log(ln)-transformed parameters following oral administration of 0.15mg desogestrel and ethinyl estradiol 0.03mg tablet formulation in 24 healthy Indian female subjects under fasting conditions

Parameter	Test (mean $\pm$ SD)	Reference (mean $\pm$ SD)	Ratio (test/reference), %	90% CI (lower - upper)	Power	Intra subject variation, % CV
<i>ENG</i>						
C_{max} (pg/mL)	1730.2 $\pm$ 615.5	1780.1 $\pm$ 638.8	97.2	90.5–101.4	0.9998	7.76
AUC_{0-120h} (h pg/mL)	17,564.9 $\pm$ 2631.2	18,443.3 $\pm$ 5874.1	95.2	86.3–102.3	0.9996	8.96
AUC_{0-inf} (h pg/mL)	17,697.9 $\pm$ 2673.5	18,662.9 $\pm$ 5943.8	94.8	87.9–104.7	0.9991	10.1
T_{max} (h)	1.74 $\pm$ 0.62	1.62 $\pm$ 0.74	-	-	-	-
$t_{1/2}$ (h)	28.6 $\pm$ 5.7	28.1 $\pm$ 6.3	-	-	-	-
K_{el} (1/h)	0.024 $\pm$ 0.122	0.025 $\pm$ 0.110	-	-	-	-
<i>EE</i>						
C_{max} (pg/mL)	92.6 $\pm$ 38.8	98.1 $\pm$ 31.1	94.4	90.9–100.2	0.9997	9.54
AUC_{0-120h} (h pg/mL)	975.7 $\pm$ 364.9	1068.0 $\pm$ 391.2	91.4	87.2–97.9	0.9999	8.57
AUC_{0-inf} (h pg/mL)	976.1 $\pm$ 365.1	1069.0 $\pm$ 391.6	91.3	86.1–98.7	0.9993	7.98
T_{max} (h)	1.69 $\pm$ 0.51	1.53 $\pm$ 0.68	-	-	-	-
$t_{1/2}$ (h)	24.7 $\pm$ 22.8	25.3 $\pm$ 21.6	-	-	-	-
K_{el} (1/h)	0.028 $\pm$ 0.030	0.027 $\pm$ 0.032	-	-	-	-

CI, Confidence interval; CV, coefficient of variation; C_{max} , maximum plasma concentration; AUC_{0-120h} , area under the plasma concentration–time curve from 0 to 120h; AUC_{0-inf} , area under the plasma concentration–time curve from zero hour to infinity; T_{max} , time point of maximum plasma concentration; $t_{1/2}$, half-life of drug elimination during the terminal phase; K_{el} , elimination rate constant; SD, standard deviation.