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A semi-automated 96-well plate method for the simultaneous determination of oral contraceptives concentrations in human plasma using ultra performance liquid chromatography coupled with tandem mass spectrometry

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Abstract

Two semi-automated, relatively high throughput methods using ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC–MS/MS) were developed for the simultaneous determination of ethinyl estradiol (EE) in combination with either 19-norethindrone (NE) or levonorgestrel (LN) in human plasma. Using 300 μL plasma, the methods were validated over the concentration ranges of 0.01–2 ng/mL and 0.1–20 ng/mL for EE and NE (or LN), respectively. The existing methods for the determination of the oral contraceptives in human plasma require large volumes of plasma (≥500 μL), and sample extraction is labor-intensive. The LC run time is at least 6 min, enabling analysis of only about 100 samples a day. In the present work the throughput was greatly improved by employing a semi-automated sample preparation process involving liquid–liquid extraction and derivatization with dansyl chloride followed by UPLC separation on a small particle size column achieving a run time of 2.7 min. The validation and actual sample analysis results show that both methods are rugged, precise, accurate, and well suitable to support pharmacokinetic studies where approximately 300 samples can be extracted and analyzed in a day.

Keywords: Ethinyl estradiol; 19-Norethindrone; Levonorgestrel; Oral contraceptives; Ultra performance liquid chromatography; Mass spectrometry; Quantitative; Human plasma

1. Introduction

Ethinyl estradiol (EE), an extremely potent synthetic estrogen, in combination with the progestrogen of 19-norethindrone (NE) or levonorgestrel (LN), has been widely used as oral contraceptives (OC) to prevent pregnancy in women [1,2]. Despite the popularity of these OC drugs in the developed world, many women discontinue using contraceptive pills primarily due to tolerability issues such as cycle control (bleeding irregularities), mood changes, nausea, bodyweight gain, breast tenderness, headaches, hypertensions and fluid retention [2]. This has led to the further reduction of the effective dose for OC to generally 150–1500 μg/tablet/day for NE (or LN) and 10–50 μg/tablet/day for EE [1,3]. With the introduction of low dose combination of these compounds, there has been a growing concern about the possible interaction with co-administered drugs, and potential failure of contraception in women using OC. A number of studies have been conducted to investigate interactions between OC and co-administered drugs [4–8]. The enhanced clearance of OC drugs due to the induction of drug-metabolizing enzymes, such as cytochrome P450 CYP3A4, has been suggested as a major mechanism of OC-drug interactions [9,10]. For example, inducers of cytochrome P450 have been reported to increase the incidence of breakthrough bleeding and unwanted pregnancies in women using OC [11]. Thus, it is very important to explore the potential interaction of new drug candidates with low dose OC during drug development process to ensure the optimum OC exposure is maintained during the concomitant therapy. To meet this need, a highly sensitive analytical method with a low limit of quantification (LLQ) in pg/mL level

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for EE and NE (or LN) is required to accurately measure analyte concentrations in human plasma samples.

A number of bioanalytical techniques including radioimmuno assays (RIA), gas chromatography/mass spectrometry (GC/MS or GC/MS/MS), and liquid chromatography/tandem mass spectrometry (LC/MS/MS), have been reported in the literature for the determination of EE, NE and LN in biological specimens [12–21], aquatic environmental samples [22,23], and in sediments [24,25]. RIA methods have been used for pharmacokinetic studies reaching detection limits in the low pg/mL range [12–15]. However, these methods often involved the time consuming solid phase extraction (SPE) or liquid–liquid extraction (LLE) sample extraction procedures and also required handlings of radioactive materials. Furthermore, RIA methods were known to suffer from the lack of specificity due to cross reactivity from their polar metabolites, endogenous steroids, and non-specific binding. GC/MS or GC/MS/MS methods generally employed LLE or SPE, and one or multiple steps of derivatization were also required prior to analysis [21,23–25]. Although these methods were selective and sensitive enough to support pharmacokinetic studies when low doses of OC drugs were administered, sample preparation was usually very time-consuming and run times may exceeded 20 min per sample, thus making them less suitable for the high-throughput analysis. Because of its high sensitivity and selectivity, LC–MS/MS has become the method of choice for the analysis of OC drugs in plasma or serum [16–20]. Analytes were generally analyzed after LLE followed by derivatization of EE with dansyl chloride. Recently, Li et al., reported an impressive method for the simultaneous determination of EE and NE in human plasma with excellent sensitivity (an LLQ level of 0.0025 ng/mL for EE and 0.05 ng/mL for NE) [20]. However, a plasma volume of 500 μL or more is needed in all the currently reported methods in order to achieve the LLQ of low pg/mL. Consequentially, a large volume of organic solvents are used for LLE, limiting the sample preparations in tube format and thus difficult to automate. Besides the labor intensive sample preparation method, the LC run time was at least 6 min per injection allowing analysis of less than 100 samples, along with calibration standards and quality control samples, per day and thus making it unfit for the high-throughput quantitative bioanalysis.

In this paper, we will describe a relatively high-throughput LC/MS/MS method for the simultaneous determination of EE and NE (or LN) in human plasma with the LLQ of 0.01 and 0.1 ng/mL for EE and NE (or LN), respectively. A semi-automated LLE sample preparation method is employed to extract the analytes from 300 μL plasma in 96-well plate format. In addition, sample analysis throughput is dramatically improved through the utilization of ultra high pressure chromatography (UPLC). UPLC using 1.7 μm particle LC columns has already been proven to be a great tool to increase productivity while maintaining or improving assay selectivity and sensitivity [26–30]. One drawback when using the smaller particle size columns is the increased system backpressure. Reducing the particle size by a factor of 3 results in an increase in the backpressure by a factor of 27 [26], which traditional HPLC systems cannot tolerate. The current Waters Acquity UPLC technology can withstand a backpressure of up to 15,000 psi.

2. Experimental

2.1. Chemicals and reagents

Ethinyl estradiol (EE, free base, 99% chemical purity), 19-norethindrone (NE, free base, 99% chemical purity), and levonorgestrel (LN, free base, 99% chemical purity) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Internal standards, EE-d_4 (free base, 99% chemical purity), LN-d_6 (free base, 99% chemical purity) and NE-d_6 (free base, 99% chemical purity) were obtained from CDN Isotopes (Quebec, Canada). Dansyl chloride, sodium bicarbonate and *N,N*-dimethylformamide were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade solvents of acetonitrile and acetone were purchased from Burdick and Jackson (Muskegon, MI, USA). HPLC grade solvents of methyl tertiary-butyl ether (MTBE), hexane and formic acid were purchased from EMD Scientific (Gibbstown, NJ, USA). EDTA human plasma was obtained from Bioreclamation Inc (East Meadow, NY, USA).

2.2. Equipment

An Eppendorf 5810R centrifuge with a four 96-well plate rotor (Brinkmann Instrument, Westbury, NY, USA), a Mettler UMX2 balance (Hightown, NJ, USA), 1.2 mL polypropylene 96-well tubes, pierceable TPE capclusters (Micronic Systems, Lelystad, Holland), and a Harvard Apparatus Model 22 infusion pump (South Natick, MA, USA) were used. Hamilton Mircrolab STAR liquid handler (Reno, NA, USA) was used for plasma transfer; TomTec Quadra 3 SPE (Hamden, CT, USA) was used for liquid transfer. Varian Combilute 96-well plate Hydromatrix (260 mg per well) LLE plate (Palto Alto, CA, USA), Arctic White LLC 96-well round 2 mL plates and silicone with PTFE film seal mats (Bethlehem, PA, USA) were used to extract analytes and their internal standards from plasma. 1.2 mL deactivated (silanized) glass vials from Waters (Milford, MA, USA) along with Varian 96-well plate covers were used in the UPLC for sample introduction. The SPE sample extraction experiment performed during method development was carried out on the Oasis HLB 96-well plates from Waters (Milford, MA, USA).

2.3. Preparation of standards and quality control/validation samples (QC)

Stock solutions of EE and NE were prepared in dimethylformide at concentrations of 1.0 mg/mL and stored at 4 °C. The stock solution was further diluted with 50/50 acetonitrile/water to make sub-stock solutions of NE at 10 μg/mL and EE at 1 μg/mL. A working solution (WS1) for the standard curve was prepared at a concentration of 1000/100 ng/mL of NE/EE in 50/50 acetonitrile/water. WS1 and serial dilution were used to make duplicate calibration standards at 20/2, 10/1, 5/0.5, 2/0.2, 1/0.1, 0.5/0.05, 0.2/0.02 and 0.1/0.01 ng/mL of NE/EE. Working solutions (WQ1–WQ3) for the QC samples were pre-

pared at concentrations of 1000/100, 400/40 and 100/10 ng/mL of NE/EE. The sub-stock and WQ1–WQ3 were used to make QC samples at, 20/2, 16/1.6, 1/0.1, 0.4/0.04 and 0.1/0.01 ng/mL of NE/EE. Samples were transferred to polypropylene tubes in 2.5 mL aliquot and frozen at −80 °C or extracted immediately. In the first run, freshly prepared QC samples were analyzed against freshly prepared calibration standards. For each subsequent validation run, frozen replicate aliquots of the QC samples were thawed at room temperature and analyzed against a freshly prepared standard curve. The standards and QC preparation for EE and LN method was identical as described above except NE was replaced with LN.

2.4. Sample preparation

At first, 1 mL of MTBE was added to each well of the 2 mL ArcticWhite 96-well polypropylene plate. The plate was then sealed with the ArctiSeal mat and vortexed in an inverted position for 2 min and the MTBE discarded and the plate dried. This wash step removed plastic residue from the plates and seals. Next 300 μL of plasma samples was transferred to the cleaned 96-well plate using a Hamilton STAR liquid handler. A volume of 25 μL of internal working standard solution (100 ng/mL for NE-d_6/LN-d_6, and 10 ng/mL for EE–d_4) was added to all tubes with the exception of the blanks, which received 25 μL of 50/50 acetonitrile/water instead. All wells were capped and vortex-mixed for 1 min and 1 mL of MTBE was added to all wells. The wells were capped with the ArctiSeal mat and vortex mixed for 3 min, followed by centrifugation at 3220 × *g* for 5 min. The MTBE was transferred to 1.2 mL polypropylene 96-well tubes using a TomTec liquid handler and evaporated under a stream of nitrogen at 45 °C. After evaporation, 100 μL of 100 mM sodium bicarbonate (pH 11) was added to all tubes, followed by the addition of 100 μL of 1 mg/mL dansyl chloride in acetone. The tubes were sealed and vortexed for 3 min, followed by incubation at 60 °C for 5 min. After incubation the samples were allowed to cool to room temperature, then loaded onto the Varian Hydromatrix LLE plate, and allowed to sit for 5 min. The analytes were subsequently eluted with 750 μL of hexane twice and collected into 96-well plate containing 1.2 mL deactivated (silanized) glass-inserts. These sample extracts were evaporated at 45 °C under a stream of nitrogen. The analytes were then eluted with a third 750 μL of hexane and collected into the same deactivated (silanized) glass-inserts and evaporated. Finally, the samples were reconstituted with 100 μL of 50/50 acetonitrile/water, caped and mixed before analysis.

2.5. Chromatographic conditions

An ACQUITY™ UPLC integrated system from Waters (Milford, MA, USA), consisting of an autosampler combined with a sample organizer capable of holding ten 96-well deep well plates, binary solvent manager, and an ACQUITY UPLC™ BEH C18 column (50 × 2 mm, 1.7 μm particle size) was used. The column temperature was maintained at 45 °C and the sample compartment was maintained at 10 °C. The mobile phase A consisted of 0.1% formic acid in acetonitrile and mobile phase B consisted of 50:50 acetonitrile in water. The LC system was held at 0% A for 0.65 min at a flow rate of 0.75 mL/min followed by a nonlinear gradient (concave with steep initial gradient) profile from 0% A at 0.65 min to 60% A at 1.85 min at 0.75 mL/min and then from 60% A at 1.85 min to 90% A at 2.30 min at 1.00 mL/min. The LC was returned back to 0% A at 2.35 min and was allowed to equilibrate till 2.7 min with a flow rate of 0.75 mL/min before next injection.

Analysis using the conventional HPLC was performed on a quaternary Rheos 2000 pump (Flux Instruments, Basel, Switzerland) coupled with CTC HTS autosampler (CTC Analytics AG, Zingen, Switzerland). The separation was achieved using Genesis C18 (50 × 2 mm) column packed with 3 μm particles. The mobile phase A consisted of 100% water, mobile phase B was 100% acetonitrile, and mobile phase C consisted of in 0.1% formic acid in water. The LC system was held at 30:70:0% of A:B:C for 0.8 min followed by a linear gradient profile to 0:90:10% A:B:C at 3.0 min and then held isocratic at 0:90:10% A:B:C for one and a half min. The LC was returned back to 30:70:0% of A:B:C at 4.5 min and was allowed to equilibrate till 6 min before next injection. The flow rate was kept constant at 0.5 mL/min.

2.6. Mass spectrometric conditions

A triple quadrupole mass spectrometer API 4000 (Applied Biosystems/MDS–Sciex, Concord, Ontario, Canada) with a turboionspray interface (TIS) operated in the positive ionization mode was used. The instrument was optimized for NE, NE-d_6, LN, LN-d_6, dansyl-EE, and dansyl-EE-d_4 by infusing a 20 ng/mL solution in acetonitrile:water:formic acid (50:50:0.1 v/v/v) at 500 μL/min through an Agilent pump 1100 series (Palo Alto, CA USA) directly connected to the mass spectrometer. Dansyl-EE, and dansyl-EE-d_4 were further purified using LLE with hexane as described in Section 2.4 before infusion. The MRM transitions monitored were *m/z* 530 to 171, *m/z* 534 to 171, *m/z* 299 to 231, *m/z* 305 to 237, *m/z* 313 to 245, and *m/z* 319 to 251 for dansyl-EE, dansyl-EE-d_4, NE, NE-d_6, LN, and LN-d_6, respectively. The optimized mass spectrometric conditions were: TIS source temperature, 700 °C; TIS voltage, 5000 V; curtain gas, 20 psi (nitrogen); nebulizing gas (GS1), 75 psi (zero air); TIS gas (GS2), 75 psi (zero air); collision energy, 26 eV for NE, LN and their internal standards and 50 eV for dansyl-EE and dansyl-EE-d_4. The dwell times were 100 ms and 50 ms for the analytes and the internal standards, respectively.

2.7. Data analysis

MS data were acquired and processed (integrated) using the proprietary software application Analyst™ (Version 1.1 for acquisition and Version 1.4.1 for processing, Applied Biosystems/MDS Sciex, Canada). Calibration plots of analyte/internal standard peak area ratio versus EE and NE (or LN) concentrations were constructed and a weighted $1/x^2$ linear regression was applied to the data. Concentrations of EE and NE (or LN) in validation samples were determined from the appropriate cal-

ibration line, and used to calculate the bias and precision of the method with an in-house LIMS (Study Management System, SMS2000, version 1.4, GlaxoSmithKline).

3. Results and discussion

3.1. Method development

Low throughput on the currently available assays is generally due to labor-intensive sample preparation and long LC run time. Both limiting factors were significantly improved in this paper. The sample processing procedures reported can be divided into three steps: extraction of EE and NE (or LN) from plasma, derivatization of EE with dansyl chloride to enhance the ionization efficiency of EE, and extraction of dansyl-EE and NE (or LN) from the derivatization mixture. Other methods such as protein precipitation, SPE and LLE were tested for the extraction of the analytes from plasma. However, protein precipitation was eliminated due to its inadequacy to remove endogenous interferences and long evaporation time of the supernatant containing a mixture of acetonitrile and water. The SPE method using Waters Oasis HLB 96-well plates was also discarded due to presence of an unexpected interference peak that coeluted with the analyte.

The most commonly used extraction method for the oral contraceptives in human plasma has been LLE [18–21]. However, a plasma volume of 500 μL or more is needed in the currently reported methods in order to achieve the LLQ of low pg/mL. Consequentially, a large volume of organic solvents are used for LLE, limiting the sample preparations in tube format and therefore difficult to automate. In order to increase sample throughput, two semi-automated LLE methods in 96-well plate format were investigated. First, a Combilute 96-well plate packed with hydromatrix diatomaceous earth (260 mg/well) from Varian was tested to assist in automating the LLE. Historically diatomaceous earth has been used to extract nonpolar compounds biological specimens [31,32]. Polar compounds are generally absorbed by the silica while nonpolar are exposed to the surface and are easily extracted into the organic solvent which is water immiscible. For this experiment the plasma samples were loaded in the hydromatrix 96-well plate, allowed to interact with the sorbent bed for 5 min and then MTBE was applied to elute the analytes. Neither mixing nor centrifugation was required. However, it was found, perhaps due to slightly lower recovery in the Combilute plate, that a plasma volume of at least 500 μL was required to achieve the desired LLQ for the OCs. Unfortunately, this has exceeded the capacity limit of these plates and plasma breakthrough was observed in some cases. As an alternative, the ArcticWhite 96-well plates with 2 mL well size and ArctiSeal mats from ArcticWhile LLC were tested. These plates can handle large sample volumes which allowed the extraction of 300 μL plasma with 1 mL MTBE. The ArctiSeal mats also provided excellent sealing, preventing leaking during mixing and centrifugation. However, two problems were noted with these plates: plastic residue was observed in the plates after vortexing with MTBE, and a minor interfering peak was detected in the dansyl-EE SRM trace at a similar retention time. Both problems were later eliminated by washing the plate and seal mat with MTBE prior to extraction. Using this approach, it was possible to achieve the desired LLQ for the OCs while maintaining the automation capability in 96-well plate format. Moreover, using the proposed semi-automated extraction method relatively clean samples were obtained which greatly reduced the frequency of instrument cleaning.

Optimum conditions for the EE derivatization have been investigated before [17,18,20] and was only slightly modified for our experiment. However, it was found that sodium bicarbonate (pH = 11) found in the final derivatization solution strongly suppressed the ionization of NE and LN, and therefore needed to be removed prior to LC/MS analysis. This was performed by loading the derivitization mixture onto the LLE hydromatrix plate followed by elution with hexane. The hexane was collected into a 2 mL well size 96-well plate with glass inserts. The hexane was evaporated and then reconstituted with 50/50 acetonitrile/water and stored in the autosampler at 10 °C during sample analysis. The use of deactivated (silanized) glass inserts was crucial due to the complete loss of dansyl-EE and dansyl-EE-d4 to tube absorption when the extracts were stored in polypropylene or other non-deactivated glass vials.

Using the semi-automated sample extraction method in 96-well plate as described above, one analyst is capable of easily processing four plates of samples in a day. However, development of an LC method for OC to match with the sample preparation throughput has proven to be difficult. Dansyl-EE was significantly more hydrophobic than NE or LN and eluted much later on the reversed phase column, resulting in a long LC runtime. Moreover, despite cleaning up samples with LLE, endogenous steroids in plasma often cause interference and also lead to variability in the MS baseline from sample to sample. Therefore, a good separation of OCs from the interferences is critical for the accurate determination of analyte concentrations in the low concentration level samples. Prior to the arrival of UPLC, a LC/MS method using the conventional HPLC with a run time of 6 min was validated in our lab using Genesis C18 (50 × 2 mm) column packed with 3 μm particles. The HPLC-MS/MS chromatograms for LN and derivatized EE in blank and LLQ samples are shown in Fig. 1A and B. Reasonably good separations between the analytes of interest and endogenous peaks were observed in the LLQ samples, and the signal-to-noise ratios were also acceptable for both analytes at the LLQ levels. We have employed this method to support one clinical study with 767 samples successfully. However, the long LC run time has limited our sample analysis throughput to around two 96-well plates daily.

The implementation of the UPLC technology in our lab has greatly increased the speed of sample analysis for the OC assay. The sub-2 μm particles have generated higher resolution power, while allowing us to increase the linear velocity and shorten run time without sacrificing LC resolution. In our experiment, the run time was reduced from 6.0 to 2.7 min and the throughput was improved by a factor of two, allowing us to run the samples from four 96-well plates (∼300 samples plus calibration standards, blanks and quality controls) in an overnight run (details to be discussed below).

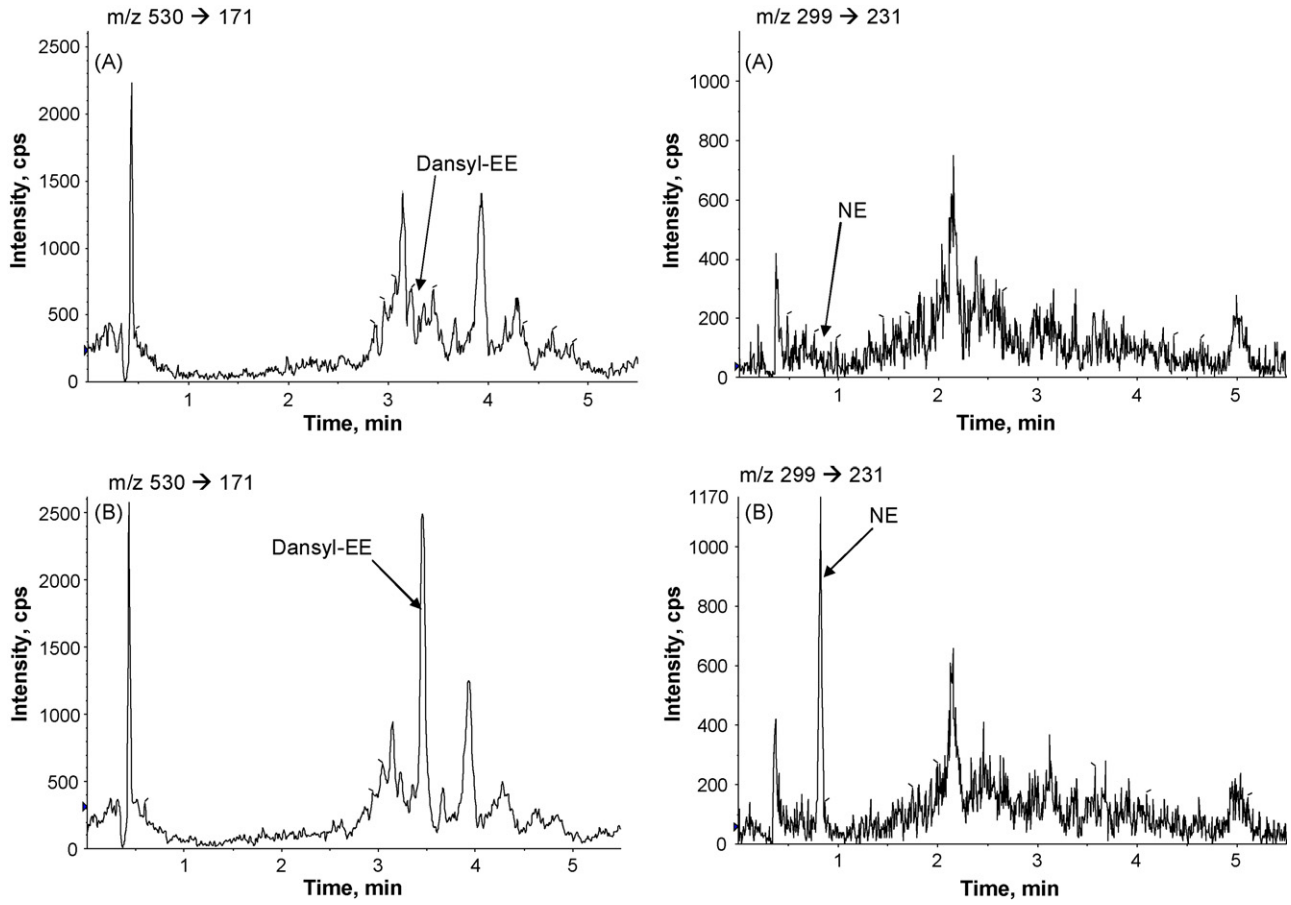

Fig. 1. Representative HPLC–MS/MS chromatograms for the derivatized EE and NE from a blank sample (A) and LLQ at 0.01 and 0.1 ng/mL for EE and NE respectively (B).

3.2. Selectivity and linearity

The characteristic precursor $[M+H]^+$ to product ions transitions, *m/z* 530 to 171, *m/z* 299 to 231, and *m/z* 313 to 245 are consistent with the structures of dansyl-EE, NE, and LN, respectively. The single MS and product ion mass spectra of dansyl-EE, LN, and NE were already shown in the literature and are not illustrated in this paper [17,20]. The selectivity of the method was established by the analysis of samples of control human plasma from 6 individual female volunteers. Matrix effect tests were performed by spiking the six lots of plasma with EE at 0.04 and 1.6 ng/mL and NE (or LN) at 0.4 and 16 ng/mL in triplicates. The bias and precision for the all of the back-calculated concentrations were found to be within the acceptance criteria, indicating of absence of unacceptable matrix effect for this method. UPLC–MS/MS chromatograms of the blanks and validation samples were visually examined and compared for chromatographic integrity and potential interferences. Representative UPLC–MS/MS chromatograms of blank sample LLQ, a clinical sample, and internal standards are shown in Figs. 2–4, respectively. No unacceptable interferences at the retention times of dansyl-EE, NE, LN, and their internal standards were observed. When compared to the HPLC–MS/MS (chromatograms shown in Fig. 1A and B), the LC run time using UPLC was reduced by more than half while the analytes of interest are better resolved from the interfering endogenous plasma components. The signal-to-noise ratio was also increased by a factor of two for the dansyl-EE but remained unchanged for NE and LN. In overall, the signal-to-noise ratios at LLQ were >10 for all the analytes (Figs. 2B, 3B and 4B). The base peak widths for the analytes were approximately 5 s which correspond to 16 data points across each peak.

The linearity of the method was evaluated by analyzing eight calibration standards in duplicates over the nominal concentration range of 0.01–2 ng/mL for EE and 0.1–20 ng/mL for NE/LN. The correlation coefficients obtained using $1/x^2$ weighted linear regressions were better than 0.9993 and 0.9988 for EE and NE method (or 0.9993 and 0.9994 for the EE and LN method), respectively.

3.3. Bias and precision

At all validation sample concentrations examined, the bias is less than 15%. The summary results of the validated method are presented in Tables 1 and 2. The maximum bias observed was 5.6% for EE and 4.2% for NE in EE and NE combo method, or −6.4% for EE and 5.3% for LN in EE and LN combo method.

At all validation sample concentrations examined, the within- and between-run precision values are less than or equal to 15%. The maximum within-run precision value observed was 10.7%

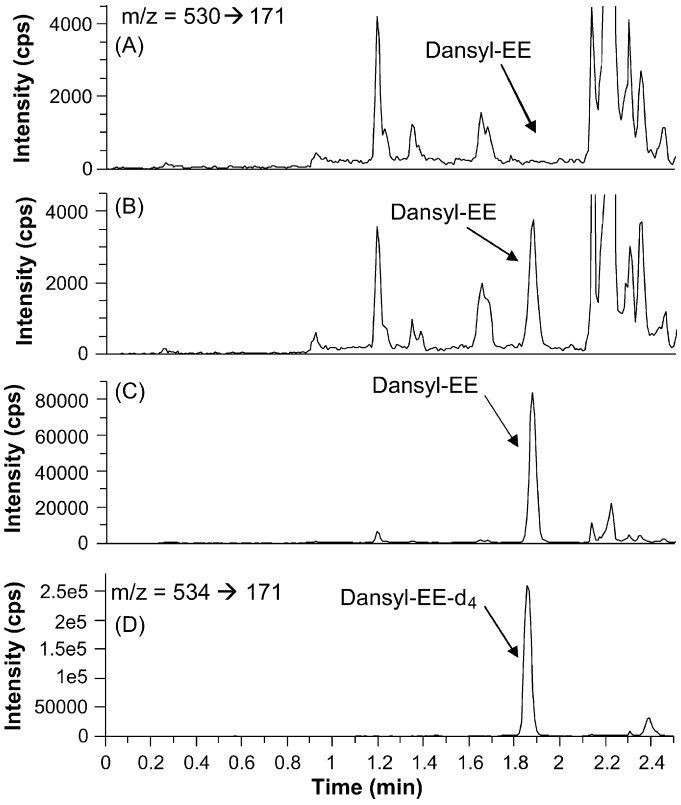

Fig. 2. Representative UPLC–MS/MS dansyl-EE chromatograms of blank sample (A), LLQ at 0.01 ng/mL (B), a clinical sample at 0.21 ng/mL (C), and internal standards (D).

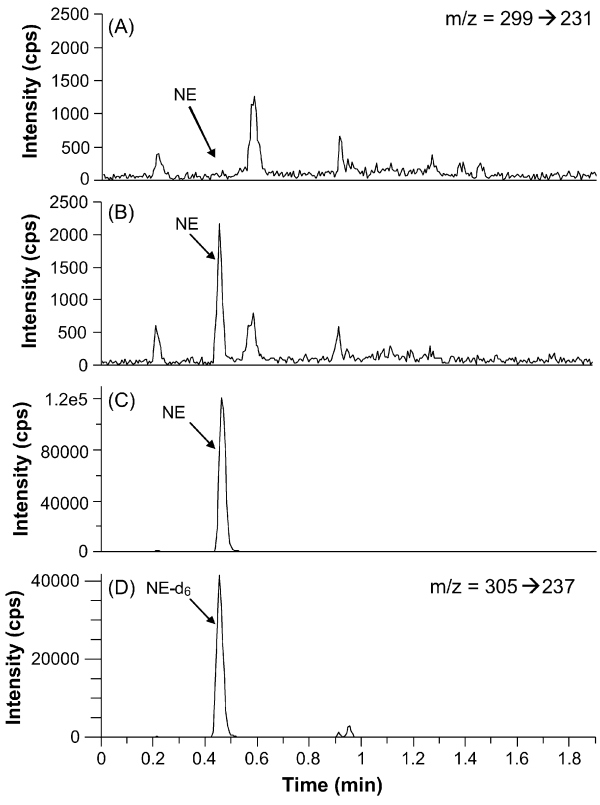

Fig. 3. Representative UPLC–MS/MS NE chromatograms of a blank sample (A) LLQ at 0.1 ng/mL (B), a clinical sample at 19 ng/mL (C), and the internal standards (D).

for EE and 13.4% for NE in EE and NE combo method, or 7.1% for EE and 5.3% for LN in EE and LN combo method. The maximum between-run precision values observed were 8.5% for EE and 8.9% for NE in EE and NE combo method, or 3.5% for EE and 5.4% for LN in EE and LN combo method.

As defined by the lower and upper validation sample concentrations possessing acceptable accuracy and precision, the validated range of this method based on 300 μL of EDTA human plasma is 0.01–2 ng/mL for EE and 0.1–20 ng/mL for NE or LN.

Table 1
Bias, precision and mean validation sample concentrations for EE and NE in EDTA human plasma

Concentration (ng/mL)	EE					NE				
	0.01	0.04	0.1	1.6	2	0.1	0.4	1	16	20
Run 1	$n=6$					$n=6$				
Mean	0.01	0.04	0.10	1.63	1.98	0.1	0.4	1.0	16.2	19.9
CV (%)	5.7	2.4	1.6	1.9	1.1	4.8	3.7	2.3	2.1	1.8
Bias (%)	−4	2.4	−1.1	1.9	−0.8	2.9	1.8	−0.5	1.1	−0.3
Run 2	$n=6$					$n=6$				
Mean	0.01	0.04	0.10	1.60	1.99	0.1	0.4	1.0	16.1	20.0
CV (%)	5.2	2.5	2.2	2.1	0.9	7.6	3.3	3.2	3.0	2.6
Bias (%)	4.5	2.1	1.7	−0.1	−0.6	0.5	2.7	−0.5	0.6	0.1
Run 3	$n=6$					$n=6$				
Mean	0.01	0.04	0.10	1.59	1.96	0.1	0.4	1.0	16.0	19.5
CV (%)	10.7	2.8	2.7	2.1	2.3	13.4	2.9	3.4	2.1	2.7
Bias (%)	5.6	0	−2.3	−0.8	−2.2	4.2	0.2	−1.6	−0.1	−2.5
Overall totals										
Mean	0.01	0.04	0.10	1.60	1.98	0.1	0.4	1.0	16.1	19.8
Bias (%)	2.1	1.5	−0.6	0.3	−1.2	2.5	1.6	−0.9	0.6	−0.9
n					18					
Between-run CV (%)	4.0	0.7	1.8	1.1	0.6	Negligible	1.0			

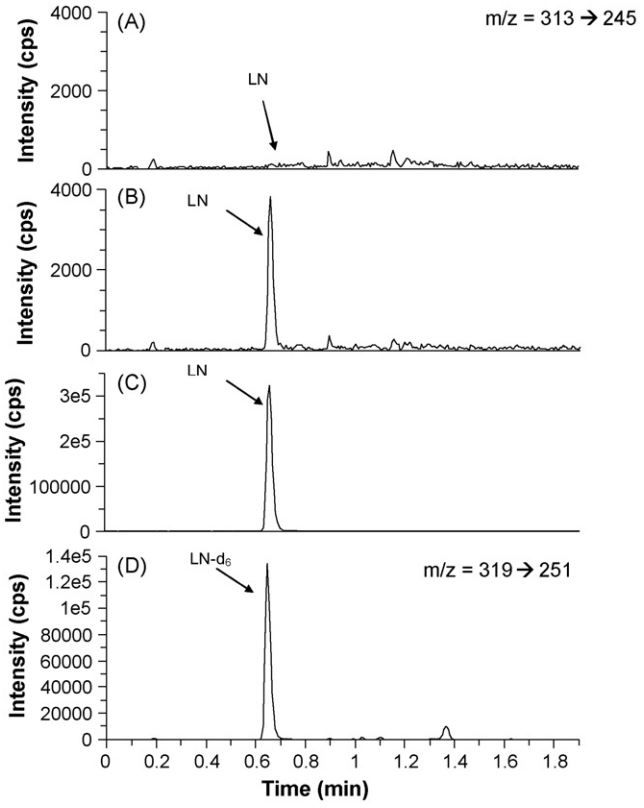

Fig. 4. Representative UPLC–MSMS LN chromatograms of a double blank (A), LLQ at 0.1 ng/mL (B), a clinical sample at 8.7 ng/mL (C), and the internal standards (D).

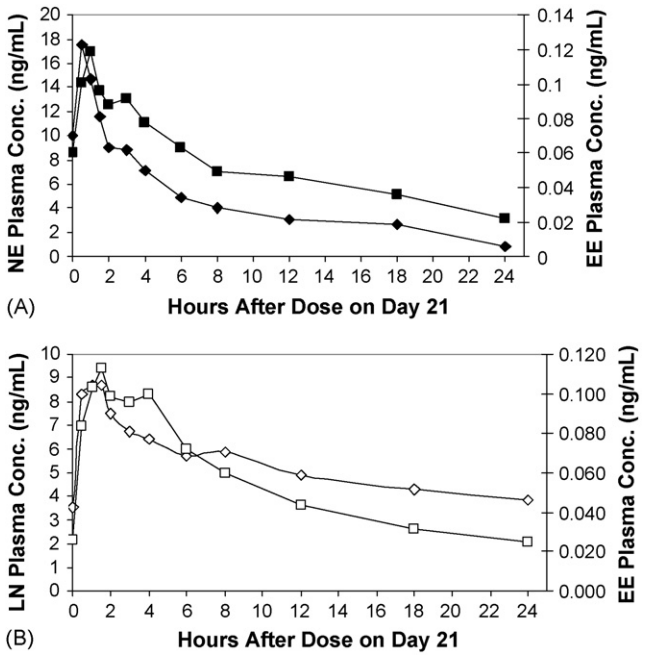

Fig. 5. The pharmacokinetic profile of EE [■] and NE [♦] (A) EE [□] and LN [◇] (B) from a healthy volunteer after daily administration of Brevicon® Tablet (A) or Microgynon30® (B).

3.4. Application to pharmacokinetic study

Following validation, the assay was used for pharmacokinetic evaluation of EE combined with either NE or LN in drug–drug interaction studies. After a single oral administration of Brevicon® 500 and 35 μg/tablet/day for NE and EE, respectively, to a healthy female volunteer, the plasma concentrations EE and NE were determined as described in the method. Blood samples were drawn pre-dose and at intervals from 0.5 to 24 h post-dose. The maximum concentration (C_{max}) of 0.1 and 17.5 ng/mL for this healthy volunteer was reached rapidly at 1 h and 0.5 h (T_{max}) for EE and NE, respectively, (Fig. 5A). In another pharmacokinetic study, after single oral administration of Microgynon30®; 150 and 30 μg/tablet/day for LN and EE,

Table 2
Bias, precision and mean validation sample concentrations for EE LN in EDTA human plasma

Concentration (ng/mL)	EE					LN				
	0.01	0.04	0.1	1.6	2	0.1	0.4	1	16	20
Run 1	$n=6$					$n=6$				
Mean	0.01	0.04	0.10	1.61	1.99	0.1	0.4	1.0	16.0	19.9
CV (%)	4.8	1.6	1.8	1.5	0.8	3.7	1.6	2.2	2.7	2.2
Bias (%)	3.3	1.1	4.1	0.4	−0.5	5.3	0.8	2.3	0.1	−0.5
Run 2	$n=6$					$n=6$				
Mean	0.01	0.04	0.10	1.53	1.88	0.1	0.4	1.0	16.1	19.5
CV (%)	7	1.5	1.7	1.1	1.3	5.3	2.3	2.3	2.4	1.7
Bias (%)	−2.4	−3.1	−1.3	−4.1	−6.2	−4.3	−1.5	2.8	0.3	−2.6
Run 3	$n=6$					$n=6$				
Mean	0.01	0.04	0.10	1.54	1.87	0.1	0.4	1.0	15.7	18.9
CV (%)	6.2	7.1	1.7	2	1.4	5.3	1.1	2.6	1.6	2.3
Bias (%)	3.1	0.2	−0.6	−3.6	−6.4	−4.7	−0.9	−0.4	−1.9	−5.5
Overall totals										
Mean	0.01	0.04	0.10	1.56	1.91	0.1	0.4	1.0	16.0	19.4
Average bias (%)	1.3	−0.6	0.7	−2.4	−4.4	−1.2	−0.5	1.6	−0.5	−2.8
n	18									
Between run CV (%)	2.1	1.4	2.8	2.5	3.5	5.4	0.9	1.3	0.9	2.4

respectively, to a healthy female volunteer the maximum concentration (C_{max}) of 0.1 and 8.7 ng/mL were reached at 1 to 1.5 h and 1 h (T_{max}) for EE and LN, respectively, (Fig. 5B). The LLQ of 0.01 and 0.1 ng/mL for EE and NE or LN was proven adequate to provide concentration data for all the time points for this given subject. To date, approximately 1700 (EE and NE) and 156 (EE and LN) samples after oral administration of OC to healthy females in three clinical projects have been analyzed using the methods described here.

4. Conclusion

For the first time, a semi-automated sample preparation method in 96-well plate format for the determination of EE and NE/LN concentrations in human plasma was developed and validated over the range of 0.01–2 and 0.1–20 ng/mL, respectively. UPLC and small particle size columns have greatly increased sample analysis speed and assay selectivity over conventional HPLC, proving very useful for the OC methods. The speed, robustness, sensitivity, and selectivity of the method make it suitable for high-throughput quantitative analysis of EE and NE/LN in drug–drug interaction clinical studies.

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