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Simultaneous determination of norethindrone and ethinyl estradiol in human plasma by high performance liquid chromatography with tandem mass spectrometry—experiences on developing a highly selective method using derivatization reagent for enhancing sensitivity

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Abstract

In the present work, for the first time, a liquid chromatographic method with tandem mass spectrometric detection (LC–MS/MS) for the simultaneous analysis of norethindrone, and ethinyl estradiol, was developed and validated over the concentration range of 50–10000 pg/ml and 2.5–500 pg/ml, respectively, using 0.5 ml of plasma sample. Norethindrone, ethinyl estradiol, and their internal standards norethindrone-$^{13}C_2$, and ethinyl estradiol-d_4, were extracted from human plasma matrix with *n*-butyl chloride. After evaporation of the organic solvent, the extract was derivatized with dansyl chloride and the mixture was injected onto the LC–MS/MS system. The gradient chromatographic elution was achieved on a Genesis RP-18 (50 mm × 4.6 mm, 3 μm) column with mobile phase consisted of acetonitrile, water and formic acid. The flow rate was 1.0 ml/min and the total run time was 5.0 min. Important parameters such as sensitivity, linearity, matrix effect, reproducibility, stability, carry-over and recovery were investigated during the validation. The inter-day precision and accuracy of the quality control samples at low, medium and high concentration levels were <6.8% relative standard deviation (RSD) and 4.4% relative error (RE) for norethindrone, and 4.2% RSD and 5.9% RE for ethinyl estradiol, respectively. Chromatographic conditions were optimized to separate analytes of interest from the potential interference peaks, arising from the derivatization. This method could be used for pharmacokinetic and drug–drug interaction studies in human subjects.
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1. Introduction

Norethindrone or norethindrone acetate (0.5–1.5 mg), a progestin, in combination with ethinyl estradiol (10–50 μg), an estrogen, have been used worldwide in oral contraceptive preparations for many years with sales over billions in the United States each year [1]. This combination is believed to provide a reliable, reversible and easy to use method of contraception. Many brand and generic combinations of these compounds have been introduced to pharmaceutical market [2]. New formulations and/or new applications of this combination have been investigated for other therapeutic indications, including hormonal replacement therapy (HRT), acne, vulgaris, osteoporosis and antiaging, etc., and the non-contraceptive use has dramatically increased since mid 1980s [2,3]. Recently, low dose of norethindrone (0.2–1.0 mg) with even lower dose of ethinyl estradiol (1–20 μg) has been investigated for the treatment of intact uterus in postmenopausal women [4,5], and androgenic markers and acne in young women [6].

Pharmacokinetics and bioavailability of norethindrone and ethinyl estradiol in human subjects have been characterized in many cases with the absolute bioavailability reported at ∼64 and 55% for norethindrone and ethinyl estradiol, respectively [7,8]. With the introduction of low dose

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combination of these two compounds, there is a growing concern about the possible interaction from co-administrated drugs, and a potential failure of contraception in women taking oral contraceptives or failure in women using this combination for non-contraceptive indications due to the altered circulating levels of norethindrone and ethinyl estradiol. Many well-documented studies have been done to investigate the interaction between co-administrated drugs and contraceptives [9–11]. For example, cytochrome P450 3A4 (CYP3A4) inhibitors, such as grapefruit juice, have been shown to decrease the pre-systemic elimination of many drugs, including ethinyl estradiol [12], by inhibiting metabolism. Cytochrome P450 CYP3A4 inducers, such as nevirapine [13] and troglitazone [14], have been reported to cause a moderate reduction in $AUC_{infinity}$, C_{max}, mean residence time (MRT) and $t_{1/2}$ for both norethindrone and ethinyl estradiol in human subjects. It is well known that human cytochrome P450 isozyme CYP3A4 is involved in the biotransformation and clearance of over 50% of the current drugs [15]. Thus, it is very important to investigate potential interaction of new drug candidates with norethindrone and ethinyl estradiol in the course of drug development and clinical trials. To address this issue, a highly sensitive and selective bioanalytical method will be needed to accurately determine the low levels of norethindrone and ethinyl estradiol in human matrices.

For many years, radioimmunoassay methods (RIAs) have been the most sensitive analytical techniques available for the determination of norethindrone and ethinyl estradiol in biological matrices [16–18]. But these methods require handling of radioactive materials and prolonged incubation, and are prone to cross reactivity by endogenous steroids, co-administrated steroids and their metabolites. RIAs are also susceptible to artifacts caused by non-specific binding or radioactivity. Therefore, pre-assay separation should be performed through chromatography after extraction [17–19]. Methods based on gas chromatography coupled with mass spectrometry (GC–MS) or tandem mass spectrometry (GC–MS/MS) typically employed a liquid–liquid extraction or solid phase extraction, and one or multiple steps of derivatization. GC–MS or GC–MS/MS is more specific and selective than RIAs, and they have allowed greater routine use [1,20–23]. However, the GC–MS run times may be longer than 20 min per sample, thus limiting throughput. More importantly, time-consuming sample preparation made GC/MS less suitable for the high-throughput analysis of large number of samples.

Recently, liquid chromatography coupled with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI)-tandem mass spectrometry has been applied for the quantitative analysis of norethindrone in river sediments [24], and ethinyl estradiol and its metabolites in environmental and biological samples [25–27]. Liquid chromatographic method with tandem mass spectrometric detection (LC–MS/MS) was demonstrated to be superior to RIAs and GC–MS in terms of selectivity, sensitivity, simplicity and analysis throughput [24–27]. However, one possible drawback to LC–MS/MS analysis of norethindrone and ethinyl estradiol is their low ionization efficiency in ESI or APCI under acidic or basic condition. Therefore, conventional LC–MS/MS might not have the required sensitivity for the quantification of ethinyl estradiol, which was reported to be up to 200 pg/ml for steady-state plasma concentration [28], much lower than that of up to 10,000 pg/ml for norethindrone [20,29]. So far, separate methods have been required to analyze norethindrone and ethinyl estradiol in the same sample, thus limiting the sample analysis throughput. Another issue associated with this approach is the relative large sample volume required for both methods, often resulting in insufficient sample volume for re-assay. The objective of the current work is to develop and validate the first LC–MS/MS bioanalytical method for the simultaneous determination of norethindrone and ethinyl estradiol in human plasma with LLOQ of 50 and 2.5 pg/ml, respectively, using a 0.500 ml volume of plasma. To achieve this, we employed a chemical derivatization procedure with dansyl chloride to increase the detection sensitivity of ethinyl estradiol in LC–MS/MS [30–32].

2. Experimental

2.1. Chemicals and reagents

Norethindrone (chemical purity 98.6%, $C_{20}H_{26}O_2$, MW = 298.4) and ethinyl estradiol (chemical purity 100%, $C_{20}H_{24}O_2$, MW = 296.4) were obtained from Aldrich (Milwaukee, WI, USA) and USP, respectively. Internal standard, norethindrone-$^{13}C_2$ (chemical purity 98% and isotopic purity 100%) and ethinyl estradiol-d_4 (chemical purity 98% and isotopic purity 100%) were purchased from Cambridge Isotope Laboratories, Inc (Andover, MA, USA) and Steraloids, Inc (Newport, RI, USA), respectively. HPLC grade solvents, acetonitrile, acetone, hexane, ethyl acetate, methyl *tert*-butyl ether (MTBE) and *n*-butyl chloride were Fisher products (St. Louis, MO, USA). Sodium bicarbonate (Na_2CO_3) and sodium hydroxide were obtained from Sigma (St. Louis, MO, USA). Dansyl chloride was purchased from Aldrich. Formic acid was from Acros (New Jersey, USA). Deionized water (Barnstead, Dubuque, IA, USA) was produced in-house. Human plasma with K_3-EDTA as the anticoagulant was obtained from Biochemed (Winchester, VA, USA).

2.2. Chromatographic condition

A Shimadzu liquid chromatograph model 10ADVP integrated system, consisting of an autosampler, a multichannel mobile phase degasser, a column heater, two pumps (Shimadzu, Columbia, MA, USA), and a Genesis RP-18 (50 mm × 4.6 mm, 3 μm particle size) column (Jones Chromatography, Lakewood, CO, USA) was used for the chromatographic separation of norethindrone, dansylated ethinyl estradiol and internal standards. The mobile phases used

were water containing 0.1% formic acid (A), and acetonitrile containing 0.1% formic acid (B). The optimal separation of norethindrone and dansylated ethinyl estradiol were achieved by running 55% B for 1.75 min isocratically, from 55 to 85% B over next 0.05 min in a sharp gradient elution and then 85% B isocratically for the next 3.2 min. The column was maintained at room temperature. The flow rate was 1.0 ml/min and all the column effluent was delivered to the mass spectrometer interface.

2.3. Mass spectrometric conditions

An API 4000 triple quadrupole mass spectrometer (AB/MDS-Sciex, Concord, Ontario, Canada) with a turboionspray (TIS) interface operated in the positive ionization mode was used for the multiple reaction monitoring (MRM) LC–MS/MS analyses. The mass spectrometric conditions were optimized for norethindrone, norethindrone-$^{13}C_2$, dansylated ethinyl estradiol and dansylated ethinyl estradiol-d_4 by infusing a 100 ng/ml standard solution in acetonitrile–water–formic acid (50:50:0.1, v/v) at 10 μl/min using a Harvard infusion pump (Harvard Apparatus, South Natick, MA, USA) directly connected to the mass spectrometer. To prepare dansylated ethinyl estradiol and dansylated ethinyl estradiol-d_4 tuning solutions, ethinyl estradiol and ethinyl estradiol-d_4 neat solution (1 μg/ml in methanol) was evaporated to dryness under a stream of nitrogen. The resulting residue was derivatized with dansyl chloride as indicated in Section 2.5. The reaction mixture was re-extracted with MTBE. After dryness, the obtained residue was dissolved in acetonitrile–water–formic acid (50:50:0.1, v/v). The optimized instrument conditions were as follows: TIS source temperature, 550 °C; TIS voltage, 5000 V; curtain gas, 10; nebulizing (GS1), 50; TIS (GS2) gas, 60; CID gas, 6; collision energy, 39 eV for norethindrone and norethindrone-$^{13}C_2$ and 52 eV for dansylated ethinyl estradiol and dansylated ethinyl estradiol-d_4. The following precursor to product ion transitions were used for the multiple reaction monitoring: norethindrone, m/z 299 → 109; dansylated ethinyl estradiol, m/z 530 → 171; norethindrone-$^{13}C_2$, m/z 301 → 109; and dansylated ethinyl estradiol-d_4, m/z 534 → 171, with dwell time of 200 ms for analytes and 100 ms for internal standards. The mass spectrometer was operated at unit mass resolution (half-height peak width set at 0.7 Da) for both the first quadrupole and the third quadrupole.

2.4. Preparations of standards and quality control (QC) samples

Two separate primary stock solutions for norethindrone and ethinyl estradiol (each) were prepared in methanol at concentration of 0.1 mg/ml, respectively, in 10 ml volumetric flasks. The stock solutions were stored in glass vials and kept refrigerated (2–8 °C). For validation purposes, the stock solutions from the two weighings must have less than a 5% difference in the LC–MS/MS responses. The stock solutions were serially diluted with methanol to prepare standard or QC working solutions at the desired concentrations. The calibration standards were freshly prepared by spiking 25 μl of appropriate amount of the standard working solutions into 0.500 ml pooled human plasma. Eight calibration standards were at 50.0, 100, 400, 1000, 2000, 4000, 8000 and 10,000 pg/ml for norethindrone, and at 2.50, 5.00, 20.0, 50.0, 100, 200, 400 and 500 pg/ml for ethinyl estradiol, respectively. Quality control samples were prepared by spiking appropriate amount of QC working solutions into human plasma with non-matrix composition less than 2% of the final volume. Low, medium and high level QC samples were prepared for norethindrone (150, 1600 and 7600 pg/ml) and ethinyl estradiol (7.50, 80 and 380 pg/ml), respectively. Dilution QC and lower limit of quantification (LLOQ) QC were also prepared at concentrations of 50,000 and 50.0 pg/ml for norethindrone, and 2500 and 2.50 pg/ml for ethinyl estradiol, respectively. QC samples were aliquoted into 2 ml polypropylene vials and stored at −70 °C.

2.5. Sample preparation

Samples were briefly vortex-mixed and aliquots of 0.500 ml of samples were then transferred from the vials into 16 × 125 mm glass test tubes with screw caps. Twenty five microliter of methanol was added to all samples except standards. Calibration standards were prepared by fortifying 25.0 μl of the appropriate standard working solutions to 0.500 ml of blank plasma as indicated above. Internal standard working solution in methanol (25.0 μl) was then added to all samples except blank. The final concentrations of norethindrone-$^{13}C_2$ and ethinyl estradiol-d_4 are 2000 and 100 pg/ml, respectively. To all samples, 4.0 ml of n-butyl chloride were added, and the tubes were capped and vortexed at high speed for 3 min. The samples were then centrifuged at 4000 rpm for 10 min. The aqueous layer was frozen in a dry ice-acetone bath and the organic layer was decanted into pre-labeled 13 × 100 mm glass tube. The organic layer was evaporated to dryness under a stream of nitrogen in a Turbo-Vap solvent evaporator (Zymark, Hopkinton, MA, USA) set at 40 °C. To the residue, 100 μl of sodium bicarbonate buffer (pH 11) was added, followed by vortexing at high speed for 3 min and then by adding100 μl of dansyl chloride in acetone (1.00 mg/ml). The tubes were vortexed for 1 min and kept in a water bath set at 60 °C for 6 min to facilitate derivatization. Tubes were then placed into another water bath at room temperature. After vortexing for 1 min, the samples were transferred into a 0.7 ml plastic HPLC vial and 30.0 μl was injected onto the LC–MS/MS system for analysis.

2.6. Data analysis

Data were processed using the AB/MDS-Sciex Analyst 1.3 software. The calibration curves (analyte peak area/IS peak area versus analyte concentration) were constructed using the least square linear regression fit ($y = a + bx$), and a

weighing factor of $1/x^2$ was applied to the data. Acceptance criteria were established to be >0.98 for the calibration curve coefficient of correlation (r^2), and within ±15% of the nominal concentration and <15% RSD for accuracy and precision for low, medium and high QC samples in the inter-day and intra-day assay, and within ±20% of the nominal concentration and <20% RSD for the inter-day and intra-day assay accuracy and precision for LLOQ samples.

2.7. Matrix effects

The assessment of matrix effect and assay reliability is critical when highly sensitive assay method is needed. The matrix ion suppression effect on the sensitivity of the current method was evaluated by the post-column infusion of the analytes. Standard working solution containing 100 ng/ml of norethindrone and dansylated ethinyl estradiol was infused at a flow rate of 10 μl/min and mixed with mobile phase (1 ml/min) in "T" before entering the mass spectrometer interface. Aliquots of 30.0 μl of extracted blank plasma were then injected onto the Genesis HPLC column, and the MRM LC–MS/MS chromatogram was acquired for each analyte. Effluent from the HPLC analytical column was mixed with the infused test compounds and entered the ESI interface. Undetected co-eluting endogenous impurities may affect the ionization efficiencies of the analytes. Any significant decrease of the LC–MS/MS response in the retention time range of norethindrone and dansylated ethinyl estradiol was used as an indication of matrix ionization suppression.

2.8. Validation

The current LC–MS/MS assay was validated for specificity, sensitivity, linearity, recovery, dilution integrity and stability. The method specificity was evaluated by screening six lots of blank plasma prior to the main validation batches. In this screening batch, six lots of plasma were fortified, individually, with norethindrone and ethinyl estradiol at medium QC level, extracted and analyzed along with a calibration curve prepared in one of the six lots of plasma. The reproducibility of these six spiked samples was used to evaluate the presence or absence of interference, and the lot-to-lot variation.

Three validation batches were assayed to assess the precision and accuracy of the method and each batch was processed on a separate day and contained one set of calibration standards and six replicates of QC samples at low, medium and high concentration levels. Among the three validation batches, one batch included six replicates of the dilution QC samples treated with 10-fold dilution by blank plasma prior to extraction. The short-term stability was included in one of the three validation batches, in which the QC samples at low, medium and high concentration levels experiencing three cycles of freeze-thaw (free-thaw stability) or sitting on lab-bench at room temperature for approximately 24 h (bench-top stability) were processed together with one set of calibration standards and regular QC samples. One of the three validation batches has 96 injections in order to simulate a routine analysis run size. Among the three batches, one batch of extracted samples was stored in the auto-sampler for approximately 51 h before re-injection onto the LC–MS/MS system to determine the storage and re-injection reproducibility of the processed samples. The recovery or extraction efficiency of the method was determined by extracting blank plasma samples and spiking analyte neat solution with the concentration the same as low, medium and high QC samples. Samples were derivatized and analyzed. Recovery was calculated by comparison of the analyte peak areas of extracted QC samples with those of post-extracted plasma blanks fortified with the known amount of analyte neat solutions.

3. Results and discussion

3.1. Method development

The proposed LLOQ of the current method is 50 and 2.5 pg/ml for norethindrone and ethinyl estradiol, respectively, with a 0.5 ml plasma sample. This LLOQ was easily achievable for norethindrone using a simple liquid–liquid extraction method with LC–MS/MS, adopted from a previously unpublished method from our laboratory. Unfortunately, use of LC–MS/MS method without derivatization resulted in poor sensitivity for the quantification of ethinyl estradiol. Thus, dansylation of ethinyl estradiol was employed due to its reported selectivity and simplicity [30,31]. Dansyl chloride is known to react with phenolic hydroxyls, primary and secondary amines, but not with alkyl hydroxyl function groups [33]. By introducing a dansyl functional group that bears a function group containing basic nitrogen, the ionization efficiency of dansylated ethinyl estradiol should be significantly enhanced when compared with that of underivatized ethinyl estradiol under acidified mobile phase condition [30–32]. The derivatization step is very simple and easy to handle. As illustrated in Section 2, after liquid–liquid extraction and evaporation of the extraction solvent (*n*-butyl chloride), the obtained extract was vortexed with 100 μl of sodium bicarbonate buffer (pH 11), followed by the addition of 100 μl of dansyl chloride (1.00 mg/ml) in acetone. The incubation was completed within 6 min at 60 °C and the resulting mixture was directly injected onto LC–MS/MS system after a simple step of vortexing without any additional treatment or pre-analytical sample preparation, which was usually needed for GC–MS analysis. In comparison with other commonly used extraction solvents such as MTBE, ethyl acetate, hexane and ethyl ether, *n*-butyl chloride as the extraction solvent yields a cleaner extract with lower matrix suppression.

Positive ion electrospray MS/MS product-ion spectra of norethindrone and dansylated ethinyl estradiol are shown in Fig. 1. The proposed product ions used in multiple reaction

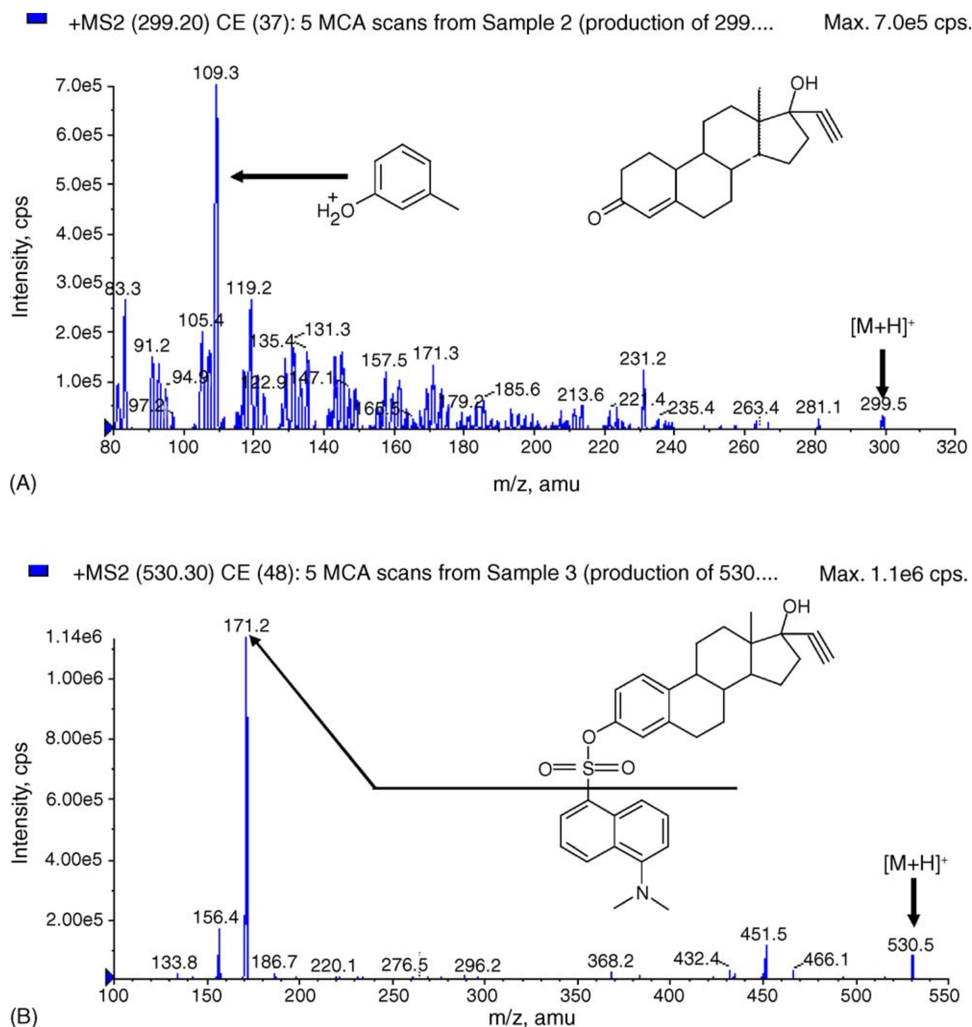

Fig. 1. Representative product ion mass spectra of norethindrone (A) and dansylated ethinyl estradiol (B).

monitoring are inserted in the figure. For norethindrone, the most abundant product ions were observed at *m*/*z* 109, 119 and 231 (Fig. 1A). The formation of the fragment ion at *m*/*z* 109 was due to the cleavage of the B ring (b_2') of the protonated molecule of norethnidrone (*m*/*z* 299) with the transfer of hydrogen. In the product ion mass spectrum of dansylated ethinyl estradiol, the only predominant peak was seen at *m*/*z* 171 and was used in MRM in the current method. The formation of the radical of *m*/*z* 171 is through a characteristic cleavage of sulfonyl function group and it is resonance-stabilized thought the aromatic skeleton. It should be noted that the product ion (*m*/*z* 171) used for MRM is from the derivatization reagent and is relatively non-selective since other compounds with the phenol or amine groups could also produce the same product ion. Attempts of using the less sensitive but more selective product ion failed to achieve adequate sensitivity. For the present study, the samples could be substantially cleaned-up by using a back-extraction procedure as we previously described [31]. However, an additional evaporation/reconstitution step was required, thus limiting the sample analysis throughput. Here, we chose to use chromatographic separation to achieve the desired selectivity.

The progress in the degree of dansyl chloride derivatization of ethinyl estradiol in extracted plasma sample was monitored through the formation of corresponding dansylated ethinyl estradiol at different time intervals, ranging from 3 to 8 min. At each time interval three replicate LLOQ samples containing 2.5 pg/ml of ethinyl estradiol were processed and analyzed. The relative progress of the derivatization was found to be rapid and steady. A reaction time of 6 min was chosen.

3.2. Matrix effects

It was reported that the suppression or enhancement effects of matrix might be caused by polar, non-retained matrix components (solvent front, salts, etc.) and also depend on the nature of individual biological matrix, ionization source used, and source design [34]. In the present study, the determination of norethindrone and ethinyl estradiol is not affected

Table 1
Precision and accuracy of quality control samples

Norethindrone	LLOQ, 50.0 pg/ml	LQC, 150 pg/ml	MQC, 1600 pg/ml	HQC, 7600 pg/ml	Dilution QC, 50,000 pg/ml
Day 1					
n	6	6	6	6	
Mean	46.0	158	1710	8060	
RSD%	4.9	8.1	7.0	2.9	
RE%	−8.0	5.3	6.9	6.1	
Day 2					
n	6	6	6	6	6
Mean	50.7	155	1680	7580	48300
RSD%	3.8	5.6	5.7	4.2	5.8
RE%	1.4	3.3	5.0	−0.3	−3.3
Day 3					
n		6	6	6	
Mean		153	1610	7450	
RSD%		7.1	4.3	7.2	
RE%		2.0	0.6	−2.0	
Inter-day					
n		18	18	18	
Overall mean		155	1670	7690	
RSD%		6.8	6.0	5.9	
RE%		3.3	4.4	1.2	

by co-extracted matrix components under the LC–MS/MS conditions used.

3.3. Sensitivity

The current assay has a LLOQ of 50 and 2.50 pg/ml for norethindrone and ethinyl estradiol, respectively, based on a 0.500 ml plasma volume. Reliable precision (RSD% <7.7%) and accuracy (RE% <8.0%) was obtained by analyzing two sets of six replicate LLOQ samples (Tables 1 and 2) with a standard curve and QCs at the concentration of low, medium and high levels in each set. A typical LC–MS/MS chromatogram of the LLOQ sample is shown in Fig. 2. During the validation, it was observed that two aspects needed to be emphasized in order to continuously achieve the desired sensitivity at such low pg/ml concentration levels. It was primarily important to clean the LC–MS interface at least every 200 injections. Vortexing the mixture after the addition of sodium bicarbonate buffer before the derivatization step was also critical.

Table 2
Precision and accuracy of quality control samples

Ethinyl estradiol	LLOQ, 2.50 pg/ml	LQC, 7.50 pg/ml	MQC, 80.0 pg/ml	HQC, 380 pg/ml	Dilution QC, 2500 pg/ml
Day 1					
n	6	6	6	6	
Mean	2.47	7.71	84.6	394	
RSD%	7.7	3.7	1.7	1.1	
RE%	−1.2	2.8	5.7	3.7	
Day 2					
n	6	6	6	6	6
Mean	2.52	7.77	82.7	391	2370
RSD%	3.2	4.1	5.5	2.5	2.4
RE%	0.8	3.6	3.4	2.9	−5.3
Day 3					
n		6	6	6	
Mean		8.10	86.7	416	
RSD%		3.5	1.1	2.2	
RE%		8.0	8.4	9.5	
Inter-day					
n		18	18	18	
Overall Mean		7.86	84.7	400	
RSD%		4.2	3.7	3.4	
RE%		4.8	5.9	5.3	

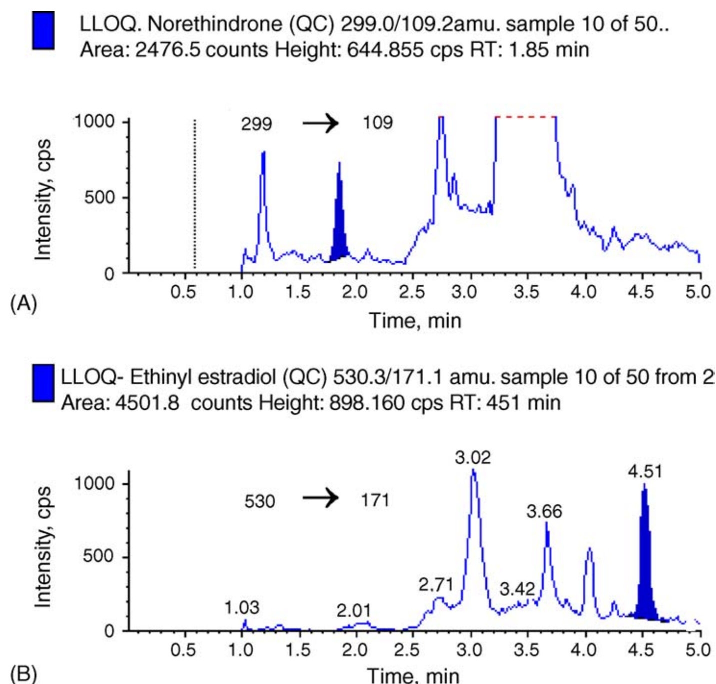

Fig. 2. Representative LC–MS/MS chromatograms of norethindrone (m/z 299 → 109) and dansylated ethinyl estradiol (m/z 530 → 171) at LLOQ concentration levels.

3.4. Specificity and selectively

Under the current LC–MS/MS conditions, norethindrone and dansylated ethinyl estradiol were well separated from interferences in the matrix blank. LC–MS/MS chromatograms of six lots of blank plasma were found to contain no endogenous peak co-eluted with any of the analytes and internal standards. Representative chromatograms of blank plasma samples without (blank) or with internal standards (QC0) were shown in Figs. 3 and 4, respectively. Injection of norethindrone at the highest concentration (10,000 pg/ml) did not show significant interference (<2% of the internal standard response) at the norethindrone-$^{13}C_2$ channel, even though norethindrone-$^{13}C_2$ is only 2 Da different from norethindrone. These six lots blank plasma fortified with norethindrone and ethinyl estradiol at medium QC concentration (1600 pg/ml for norethindrone and 80 pg/ml for ethinyl estradiol) were quantified with RSD% and RE% less than 2.9 and 3.7, respectively.

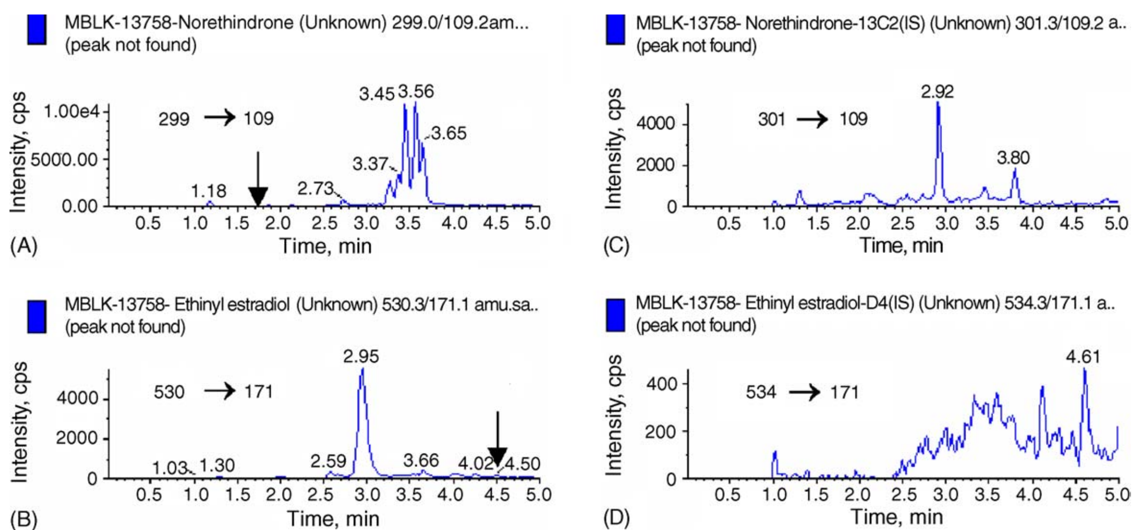

Fig. 3. Representative LC–MS/MS chromatograms of matrix blank samples (MBLK): (A) norethindrone (m/z 299 → 109), (B) dansylated ethinyl estradiol (m/z 530 → 171), (C) norethindrone-$^{13}C_2$ (m/z 301 → 109) and (D) ethinyl estradiol-d_4 (m/z 534 → 171). The arrows indicate the retention time of the analytes.

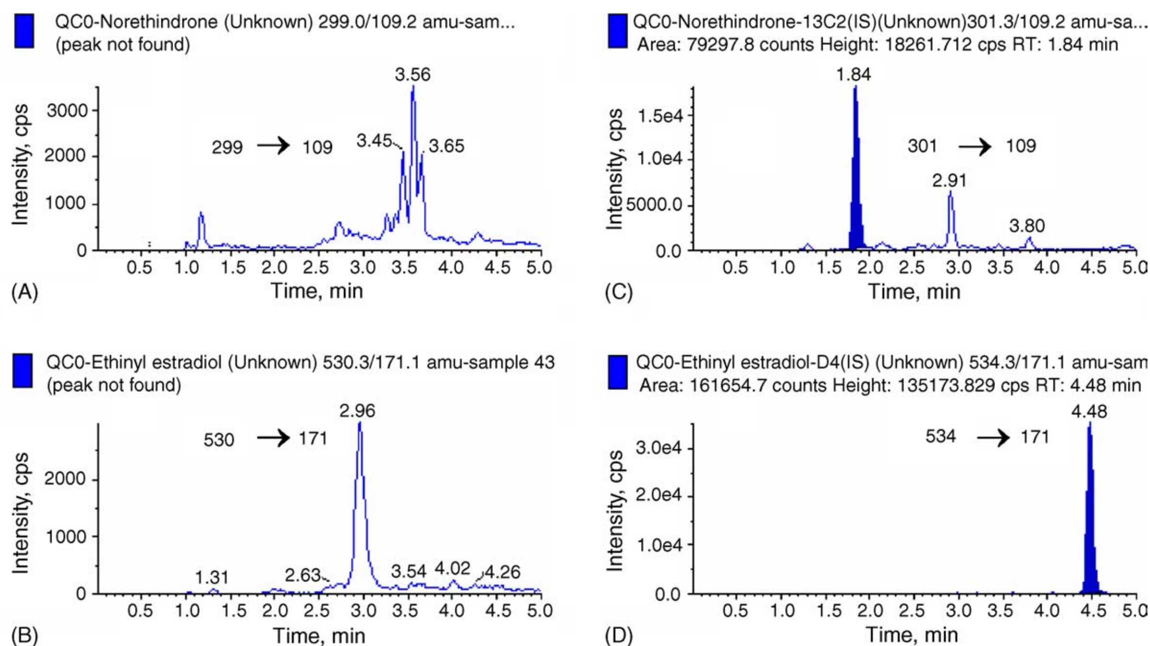

Fig. 4. Representative LC–MS/MS chromatograms of zero control samples (QC0): (A) norethindrone (m/z 299 → 109), (B) dansylated ethinyl estradiol (m/z 530 → 171), (C) norethindrone-$^{13}C_2$ (m/z 301 → 109) and (D), ethinyl estradiol-d_4 (m/z 534 → 171).

3.5. Linearity

The standard curve range was 50–10,000 pg/ml for norethindrone and 2.50–500 pg/ml for ethinyl estradiol when 0.500 ml of plasma was used for the assay. Eight none-zero calibration standards for the analytes were obtained by plotting the peak area ratio of the analytes and their corresponding internal standards against the corresponding concentrations of the analytes in the freshly prepared plasma calibrators. Excellent linearity was achieved in these specified concentration ranges with the correlation coefficients greater than 0.9988 for all validation batches with linear regression (weighing of 1/concentration^2). The calibration curves obtained as described above were suitable for the quantification of norethindrone and ethinyl estradiol in the samples during the intra- and inter-day validations and stability tests.

3.6. Precision and accuracy

The intra-assay precision and accuracy of the method were determined by analyzing six QC replicates at 150, 1600 and 7600 pg/ml for norethindrone, and 7.50, 80.0 and 380 pg/ml for ethinyl estradiol, respectively, in each validation batch. The accuracy of the method was determined by calculating RE and the precision by calculating RSD. Tables 1 and 2 summarized the precision and accuracy on each of three assays for norethindrone and ethinyl estradiol in human plasma with accuracy ranging from −2.0 to 9.5% RE of nominal values and the precision ranging from 1.1 to 8.1% RSD over the three concentration levels evaluated.

3.7. Dilution integrity

A 10-fold dilution of the dilution QC samples by blank matrix prior to extraction was used to determine dilution integrity. Six replicates of partial volume of dilution QC samples were extracted and analyzed in one of the validation batches, with accuracy of 96.7 and 94.7%, and RSD% of 5.8 and 2.4 for norethindrone and ethinyl estradiol, respectively (Tables 1 and 2), demonstrating that samples with concentrations greater than the upper limit of the standard curve could be analyzed to obtain acceptable data after dilution with blank matrix.

3.8. Stability of plasma sample during storage

The bench-top stability of norethindrone and ethinyl estradiol in human plasma was evaluated at ambient temperature (∼22 °C) over 24 h using QC samples at low, medium and high QC level. The measured concentrations of norethindrone and ethinyl estradiol in these QC samples sitting at room temperature for 24 h were compared to the nominal values, with RE ranging from −8.1 to +2.8% for norethindrone and +7.7 to 8.3% for ethinyl estradiol. (Table 3), indicating that norethindrone and ethinyl estradiol were stable for at least 24 h in human plasma when stored at ambient temperature. Freeze-thaw stability of QC samples at the low, medium and high concentration levels experiencing

Table 3
Freeze/thaw and room temperature stability as well as re-injection reproducibility of norethindrone and ethinyl estradiol

	Theoretical concentration (pg/ml)					
	Norethindrone			Ethinyl estradiol		
	150	1600	7600	7.5	80	380
(A) Stability after three freeze-thaw cycles, $N = 6$						
Mean	145	1580	7430	8.06	87.7	417
RSD%	4.1	6.1	5.7	4.1	2.9	1.3
RE%	−3.4	−1.1	−2.2	7.4	9.6	9.6
(B) Room temperature stability for 24 h, $N = 6$						
Mean	154	1640	6990	8.08	86.3	412
RSD%	4.0	7.0	4.1	4.1	2.3	1.9
RE%	2.8	2.5	−8.1	7.7	7.8	8.3
(C) Re-injection reproducibility after 51 h, $N = 6$						
Mean	152	1660	7810	8.02	82.3	381
RSD%	2.2	4.2	3.6	3.0	6.4	3.4
RE%	1.1	7.5	2.8	2.2	6.4	0.13

three cycles of freeze-thaw were analyzed together with one set of calibration standards and regular QC samples. The RE is −3.4 to −1.1% for norethindrone and +7.4 to 9.6% for ethinyl estradiol, respectively (Table 3).

3.9. Reinjection reproducibility

During the validation, one of the validation batches was stored in the HPLC autosampler for over 51 h and then re-analyzed and quantified. The precision (RSD%) and accuracy (RE%) for norethindrone and ethinyl estradiol from these processed samples were less than 7.5 and 6.4% (Table 3), respectively, for the QC samples at low, medium and high concentration level, demonstrating that extracted samples could be analyzed after standing in the HPLC autosampler (10 °C) for at least 51 h.

3.10. Recovery

The extraction recovery was estimated by analyzing low, medium and high QC samples ($n = 6$). The extracted samples were compared with post-extracted ones as illustrated in Section 2. Results were calculated by comparing the mean peak areas of norethindrone, ethinyl estradiol and the corresponding internal standards in the extracted samples with those of corresponding post-extraction spiked samples. The overall recovery was 93.9 and 58.2% for norethindrone and ethinyl estradiol, respectively. Although the recovery of ethinyl

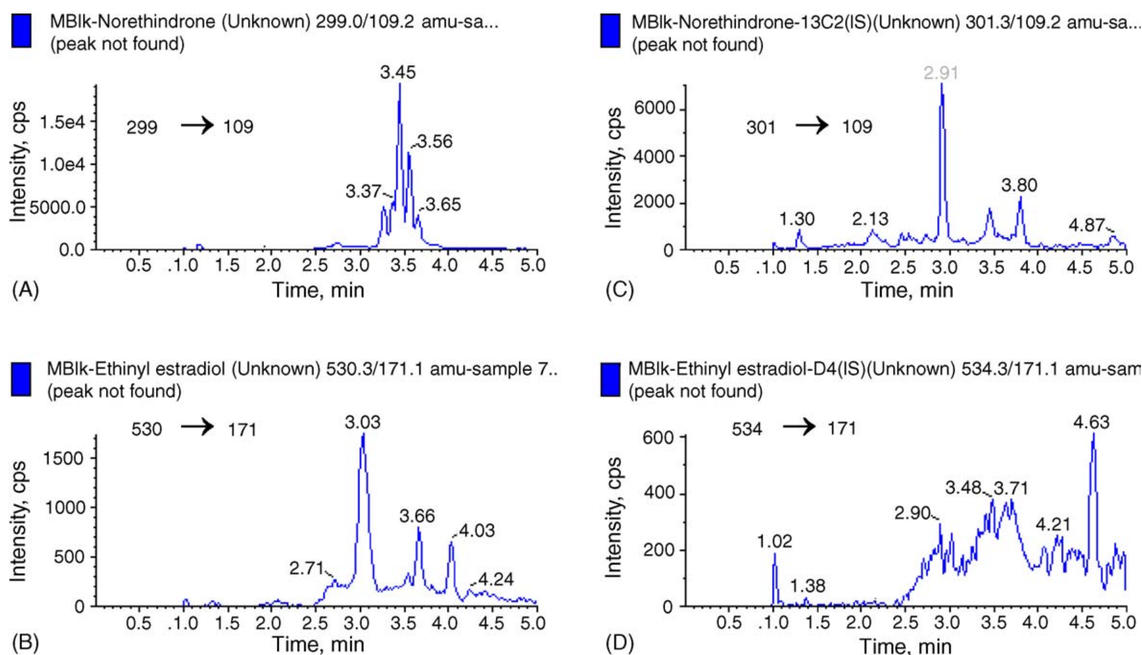

Fig. 5. Representative LC–MS/MS chromatograms of matrix blank samples (MBLK) injected right after the upper limit of quantification (ULOQ) samples.

estradiol was relatively low, it was consistent at all concentration levels. Due to the use of deuterated analyte as the internal standard, the precision and accuracy of the method were not adversely compromised. The sensitivity of ethinyl estradiol was also adequate. Attempts of increasing the recovery by using different extraction solvents such as ethyl acetate and MTBE failed to increase recovery. Additional clean-up steps such as back extraction using MTBE or hexane resulted in cleaner samples but did not improve the sensitivity.

3.11. Carryover

In the current assay, the mobile phase B (0.1% formic acid in acetonitrile) was used as the autosampler needle wash solution with rinse volume of 500 μl and needle stroke of 52 mm. No significant carryover was observed (Fig. 5).

4. Conclusions

For the first time, a highly sensitive bioanalytical method for the simultaneous determination of norethindrone and ethinyl estradiol in human plasma was developed and validated using liquid–liquid extraction, derivatization and tandem mass spectrometric detection. The lower limit of quantitation is 50 pg/ml for norethindrone and 2.5 pg/ml for ethinyl estradiol, using 0.500 ml of plasma sample. The use of chemical derivatization of ethinyl estradiol with dansyl chloride significantly enhanced the detection sensitivity of the analyte in electrospray ionization. Potential pitfalls of using derivatization reagents to enhance sensitivity were demonstrated and chromatographic separation of analytes of interest and interference peaks was highlighted. This validated method can be applied for their bioavailability in clinical pharmacokinetic and drug–drug interaction studies.

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