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Simple and rapid determination of norethindrone in human plasma by supported liquid extraction and ultra performance liquid chromatography with tandem mass spectrometry

Zhilong Gong*, Kiresha Chandler, Stephen Webster, Remy Kerley, Susan Buist, Melanie McCort-Tipton

Department of Bioanalytical Chemistry, Covance Inc., 8211 SciCor Dr., Indianapolis, IN 46214, United States

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ABSTRACT

We report for the first time an ultra performance liquid chromatographic method with tandem mass spectrometric detection (UPLC/MS/MS) for the determination of norethindrone alone in human plasma over the concentration range of 50.0–25 000 pg mL^{-1} using a sample volume of 0.250 mL. Norethindrone and its internal standard (ISTD), norethindrone-$^{13}C_2$, were extracted from human plasma by supported liquid extraction (SLE). After evaporation of the organic solvent, samples were reconstituted and analyzed on an UPLC/MS/MS system. The UPLC system used a Waters BEH C18 (100 mm × 2.1 mm, 1.7 μm) column with mobile phase A of 0.05% formic acid in water:acetonitrile (65:35, v/v) and mobile phase B of 0.05% formic acid in methanol:acetonitrile (50:50, v/v). The flow rate was 0.500 mL min^{-1}. The method was fully validated. The inter-run accuracy and precision at the lower limit of quantitation (LLOQ), low, mid and high quality control (QC) concentration levels were 99.2–108.4% with a <8.1% CV (coefficient of variation), respectively. The validated method has been successfully applied to analysis of thousands of pharmacokinetic samples.

1. Introduction

Norethindrone (or norethisterone) is a molecule used in some combined oral contraceptive pills and is also available as a stand-alone drug. It is a progestogen and can be used to treat premenstrual syndrome, painful periods, abnormal heavy bleeding, irregular periods, menopausal syndrome (in combination with estrogen), or to postpone a period. It is also commonly used to help prevent uterine hemorrhage in complicated non-surgical or pre-surgical gynecologic cases [1–3].

A number of analytical methods have been published for analysis of norethindrone alone or in combination with other compounds like ethinyl estradiol in different matrices by radioimmunoassay [4], gas chromatography coupled with mass spectrometry (GC/MS) [5,6], liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) [7–10], liquid chromatography coupled with UV detection (LC/UV) [11], etc. Radioimmunoassay has been very sensitive for norethindrone but requires handling of radioactive materials and can have issues of cross reactivity. GC/MS methods often have long run times and time-consuming sample preparation. LC/UV method often suffers from sensitivity issue. LC/MS/MS has been demonstrated to be highly selective, simple with high throughput. There are some published LC/MS/MS methods for analysis of plasma level of norethindrone combined with other compounds, primarily ethinyl estradiol [7,8]. Due to the poor ionization efficiency, norethindrone and ethinyl estradiol combined methods often have a derivatization step to improve ionization for ethinyl estradiol, which significantly extends the extraction time and also has longer chromatographic separation time due to the added matrix. These combined methods are not optimal for analysis of norethindrone alone as the derivatization step is not necessary for norethindrone only analysis. In addition, there are increasing requests in recent years from pharmaceutical companies for fast turn-around analysis of norethindrone alone in human plasma. But to the best of our knowledge, there has been no published LC/MS/MS method for analysis of norethindrone alone in human plasma. These needs led to the development of a simple, rapid and reliable analytical method for determination of norethindrone alone in human plasma.

The present work describes a fully validated UPLC/MS/MS method for determination of norethindrone in human plasma. It adopted SLE for sample preparation. SLE is a newly appeared technology that targets to substitute traditional liquid–liquid extraction. The SLE plates are packed with an optimized grade of diatomaceous earth, providing reproducible flow characteristics from sample to sample. When applied aqueous biological sample, it spreads over the surface of the support and is absorbed. The analytes remain on the surface of the support forming the

* Corresponding author. Tel.: +1 317 715 3971.
E-mail address: zhilong.gong@covance.com (Z. Gong).

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interface for the extraction, equivalent to the phase interface in liquid–liquid extraction. When the water immiscible extraction solvent is applied, analytes are efficiently desorbed, and the solvent is collected. This new technology provides a simple, rapid, convenient yet very reproducible sample preparation which is the key for the high precision and accuracy of the method. The developed method has an LLOQ of 50.0 pg mL^{-1} using a sample volume of 0.250 mL.

2. Experimental

2.1. Chemicals and reagents

Norethindrone (chemical purity 99%, $C_{20}H_{26}O_2$, MW = 298.4) was from Sigma–Aldrich (Milwaukee, WI). ISTD, norethindrone-$^{13}C_2$, was from Cambridge Isotope Laboratories, Inc. (Andover, MA). Formic acid (96%), HPLC grade acetonitrile and 1-chlorobutane were purchased from Sigma–Aldrich (Milwaukee, WI). HPLC grade methanol was from Fisher (St. Louis, MO). Barnstead type 1 water was generated in-house (Barnstead, Dubuque, IA). Human plasma and whole blood with K_2- or K_3EDTA anticoagulant were from Biochemed (Winchester, VA).

2.2. Chromatographic conditions

A Waters Acquity UPLC system and a Waters BEH C18 column (100 mm × 2.1 mm, 1.7 μm) (Milford, MA) were used for the chromatographic separation of norethindrone. The mobile phases used were 0.05% formic acid in water:acetonitrile (65:35, v/v) (A) and 0.05% formic acid in methanol:acetonitrile (50:50, v/v) (B). Optimal chromatographic separation was achieved by using a gradient from 10% B to 50% B in 2 min followed by a step gradient of mobile phase B to 99% and maintained at 99% B for 1 min to clean up the column. Mobile phase B was then returned to initial condition to equilibrate the system for 1 min. The total run time was 4 min. The column was maintained at 35 °C. The flow rate was 0.5 mL min^{-1} and all the column effluent was delivered to the mass spectrometric interface.

2.3. Mass spectrometric conditions

An API 5000 triple quadrupole mass spectrometer (ABI/MDS-Sciex, Concord, Ont., Canada) with a turbo ionspray interface operated in the positive ionization mode was used for the analysis. Multiple reaction monitoring (MRM) was used for monitoring the analyte and the ISTD. The mass spectrometer was optimized by infusing 100 ng mL^{-1} of standard solutions of norethindrone and norethindrone-$^{13}C_2$ in methanol, respectively, using a Harvard infusion pump (Harvard Apparatus, South Natick, MA) directly connected to the mass spectrometer. The optimized mass spectrometric conditions were as follows: source temperature, 600 °C; ionization voltage, 5500 V; curtain gas, 20; nebulizing gas (GS1), 45; auxiliary gas (GS2), 66; CAD gas, 11; collision energy, 28 V. The MRM transitions used were m/z 299/231 for norethindrone and m/z 301/231 for norethindrone-$^{13}C_2$ with dwell times of 100 ms. The mass spectrometer was operated at unit mass resolution.

2.4. Preparation of standards and QC samples

Stock standard solutions of norethindrone were prepared in duplicate in methanol at a concentration of 0.100 mg mL^{-1}. Stock solutions were stored in glass vials in a refrigerator set to maintain 2–8 °C. Calibration standards and QC samples were prepared from these separate stock solutions. Aliquots of the stock solutions were serially diluted with methanol to prepare working solutions at the desired concentrations for spiking calibration standards and QC samples in human plasma. The calibration standards and QC samples were prepared by spiking 25.0 μL of corresponding working solutions into 2.50 mL (final total volume after spiking) of human plasma. Eight calibration standards at 50.0, 100, 400, 1000, 5000, 10 000, 22 500, and 25 000 pg mL^{-1} and five QC samples at 50.0 (LLOQ), 150 (low QC), 2000 (mid QC), 20 000 (high QC) and 125 000 (dilution QC) pg mL^{-1} were prepared. For validation, calibration standards were prepared fresh daily. QC samples were prepared and stored at −60 to −80 °C.

ISTD stock solution of 0.100 mg mL^{-1} was prepared in methanol and stored at 2–8 °C. Working ISTD solution of 5.00 ng mL^{-1} was obtained by direct dilution of ISTD stock solution using methanol:water (50:50, v/v). Working ISTD solution was also stored at 2–8 °C.

2.5. Sample extraction procedures

Samples were briefly vortexed (Vortex Mixer, Scientific Manufacturing Industries, Bohemia, NY) at high speed for several seconds. 0.250 mL of each sample was aliquoted into a clean 2-mL Axygen 96-well plate (Axygen, Union City, CA). 0.150 mL of working ISTD solution was added to the calibration standards, QC samples, study samples and blank control zero (blank matrix with ISTD only). 0.150 mL of methanol:water (50:50, v/v) was added to blank matrix and reagent blank samples (water). Samples were vortex-mixed at high speed for approximately 1 min, then loaded to a Biotage Isolute 400 μL SLE plate (Biotage, Charlotte, NC) using a Quadra 96 (Tomtec, Hamden, CT). A pressure of 2–3 SCFH (standard cubic feet per hour) was applied for ∼5 s to initiate soaking by using a positive pressure manifold (Speedisk 96, Krackler Scientific, Albany, NY). The SLE plate was left for ∼5 min on the bench for further soaking. 0.450 mL of 1-chlorobutane was then added to each sample on the SLE plate. After ∼4 min, low pressure was applied to allow solvent to pass through. This elution step was repeated two more times to give a total 1-chlorobutane volume of 1.35 mL. The eluted organic solvent was then dried down under a nitrogen stream at 40 °C using an SPE Dry-96 manifold (Apricot Designs, Covina, CA). The total dry-down took approximately 30 min. Samples were then reconstituted using 0.150 mL of methanol:water (50:50, v/v) and vortexed at medium speed for approximately 1 min. Approximately15 μL of the reconstituted solution was injected onto the UPLC/MS/MS system for analysis.

2.6. Data analysis

Data were obtained and processed using the ABI/MDS Sciex Analyst 1.5.1 software. The calibration curve (analyte peak area/ISTD peak area versus analyte concentration) was constructed using least square linear regression and a weighing factor of $1/x^2$. Acceptance criteria for calibration curves are that the back-calculated calibrator concentration should be within ±15% of the nominal concentration (20% for LLOQ). For QC samples in precision and accuracy batches, where $n = 6$ for each QC level, the acceptance criteria for low, mid, high and dilution QCs are that the mean calculated concentration should be within ±15% of the nominal concentration and the CV should be within 15%. An accuracy within ±20% and a CV of within 20% are required for LLOQ samples.

For additional batches other than the precision and accuracy batches, $n = 2$ low, mid and high QC were required and the back-calculated concentration should be within ±15% of the nominal concentration and at least 2/3 of the QCs should pass the acceptance criteria in order for the batch to be accepted. Dilution QCs should be included in the batch if there are dilution QC samples or samples requiring dilution. They must meet the same acceptance criteria as the in-curve QCs.

2.7. Method validation

The developed method was validated for precision, accuracy, linearity, selectivity, matrix effect, dilution integrity, recovery, stability, etc. All validation steps were conducted following FDA guidelines [12]. Briefly, to evaluate selectivity, blank plasma samples from six individuals containing no analyte or ISTD were analyzed to check for the presence of interfering peaks at the elution time of norethindrone and ISTD. These same lots of human plasma were also spiked at low QC level, extracted and tested. Matrix effect was done by post spiking the extracted above six lots of blank plasma after evaporation in the extraction with a neat solution that mimicked the concentrations of both analyte and ISTD in the final extract of low QC sample. The response of both analyte and ISTD from the post spiked samples was compared with that from the neat solution. The recovery was assessed by comparing the peak area of analyte and ISTD of low, mid and high QCs with the peak area of analyte and ISTD from neat solutions that mimicked the final concentrations of analyte and ISTD in the final extracts of corresponding QC samples and were post spiked into extracted blank matrix after evaporation in the extraction. Dilution integrity was evaluated at 10-fold dilution. Matrix stability was evaluated for 24 h room temperature, 5 freeze/thaw cycles (both −20 °C and −70 °C), and 2 h whole blood collection stability at room temperature and on wet-ice. Reinjection reproducibility was evaluated for 79 h. In addition, low QC prepared in 2% hemolyzed plasma was extracted to evaluate impact of hemolysis.

3. Results and discussion

3.1. Method development

For LC/MS/MS analysis of norethindrone alone, there is no longer the need for derivatization to improve sensitivity. Since there is only one analyte, the LC condition can be then optimized to reduce the analysis time. So, the development was mainly focused on finding a convenient and simple sample extraction procedure as well as examining the LC system to obtain optimal chromatographic conditions with a short runtime for the norethindrone analysis.

Most published methods for norethindrone use liquid–liquid extraction for sample preparation. Based on this, a more convenient form of liquid–liquid extraction – Biotate Isolute SLE was adopted. SLE is simple, convenient and very reproducible. It also provides higher recovery compared with traditional liquid–liquid extraction which often has a partial organic layer transferring step that loses a portion of the analyte. In addition, traditional liquid–liquid extraction may show high well-to-well variation sometimes due to inconsistent vortex-mixing.

To obtain the desired precision and accuracy, an appropriate dilution of the plasma sample is crucial for SLE. There were two main factors determining the dilution, plasma-to-diluent ratio and the composition of the diluent. To achieve sufficient sensitivity, the plasma sample size needs to be increased to a certain volume. To achieve better soaking and smooth loading and elution, sufficient aqueous diluent needs to be added to the plasma sample. With the maximum loading volume of 0.400 mL on the SLE plate, 0.250 mL of plasma sample and 0.150 mL of aqueous diluent were found to be optimal for sufficient sensitivity as well as enough dilution for better soaking, smooth loading and elution. It was observed that when insufficient dilution was used, clogging of some wells on the plate was often encountered, causing loading and elution difficulty as well as later precision and accuracy problems. For the composition of the diluent, it was observed that addition of a small amount of methanol in the sample mixture helped sample soaking. But if methanol was added directly to the plasma sample, it caused some partial precipitation, further causing clogging of some wells on the plate. In this study, ISTD working solution was prepared in methanol:water (50:50, v/v) and was directly used as the diluent during sample extraction. This mixture provided sufficient dilution as well as needed methanol but without any precipitation. For sample elution, a number of solvents were evaluated including 1-chlorobutane, ethyl acetate, *tert*-butyl methyl ether, etc., 1-chlorobutane was found to give cleaner extract and highest recovery. Therefore, it was used as the eluent throughout the studies.

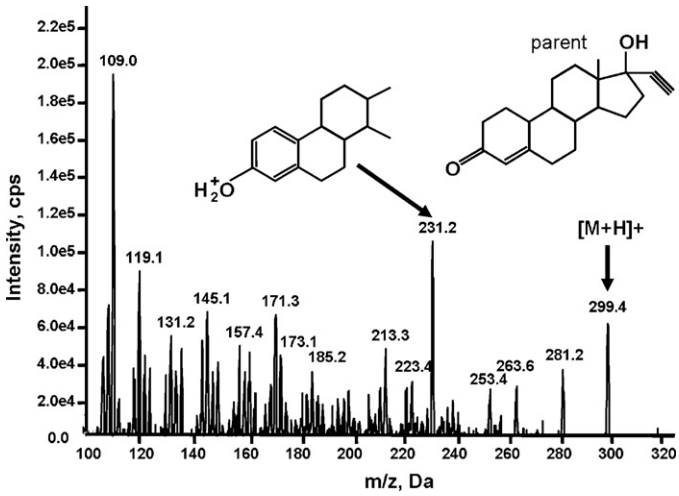

Fig. 1. Representative product ion mass spectrum of norethindrone.

For LC column selection, after evaluation of a number of reversed phase columns such as Phenomenex Gemini C18, Waters Atlantis dC18, etc., Waters BEH C18, 100 mm × 2.1 mm, 1.7 μm, column was selected for the analysis of norethindrone as it provided better peak shape and could well separate some interference peaks appeared before or after the analyte peak in some plasma lots, which benefited from the longer column and smaller particle size.

LC mobile phases were also optimized. Addition of formic acid in the mobile phase improved both sensitivity and peak shape. 0.05% formic acid was found to be sufficient. To maintain constant formic acid in the eluent, 0.05% formic acid was added to both mobile phases A and B. For organic solvent, it was observed that with only acetonitrile in the mobile phase, the above mentioned interference peaks were not well separated. Methanol alone could well separate the interference peaks, but it generated too high back pressure. A mixture of acetonitrile and methanol in the mobile phase helped with the separation of the interference peaks and generated reasonable back pressure. Therefore, a 50:50 (v/v) mixture of acetonitrile and methanol was used as the solvent in mobile phase B with some acetonitrile in mobile phase A to prevent bacteria growth. Under the optimized condition, the analysis time can be reduced to approximately 4 min.

Norethindrone was detected using positive ion electrospray in MRM mode. The MS/MS product ion spectrum of norethindrone is shown in Fig. 1. The proposed product ion used for MRM is indicated. The most abundant product ions for norethindrone were observed at *m/z* 109 and 231. The 299/109 transition showed nearly twice the sensitivity of transition 299/231, however, 299/109 transition showed much noisier background than 299/231 transition, and the signal-to-noise ratio of 299/109 transition was ~70% of that from 299/231 transition. In addition, 299/231 transition showed better analyte tracking than 299/109 transition in terms of precision and accuracy.

Considering all these factors, the *m/z* 299/231 transition was selected for the LC/MS/MS analysis.

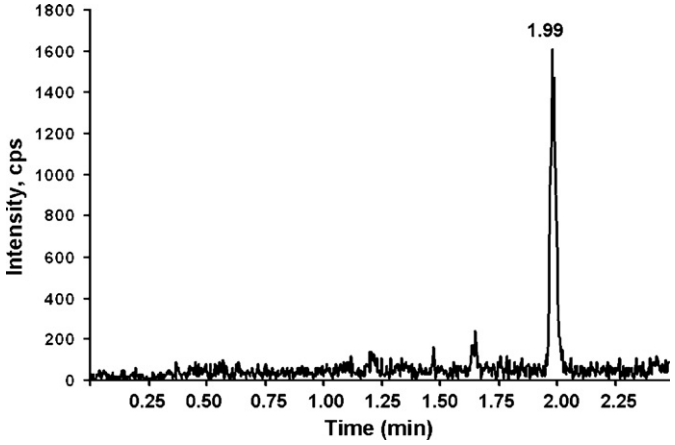

Fig. 2. Representative LC/MS/MS chromatogram of norethindrone at the LLOQ concentration (analyte only, *m/z*: 299/231).

3.2. Matrix effect

In the present study, matrix effects were observed to a varying extent for different matrix lots ranging from 2.9% to −16.6% for the analyte and from −1.7% to −21.7% for the ISTD. In this assay, stable-isotopically labeled ISTD was used and the area ratios between analyte and ISTD were not affected by observed matrix effect, and thus accuracy was not compromised.

3.3. Sensitivity

Sufficient sensitivity was achieved with an LLOQ of 50.0 pg mL^{-1} using 0.250 mL of sample size as shown in Fig. 2. Precision (expressed as CV) of <7.7% and an accuracy of 102.6% (n = 17) in three consecutive batches were obtained as shown in Table 1.

3.4. Specificity and selectivity

The developed LC conditions well separated interference peaks in the matrix from the analyte peak. Six lots of blank matrix from different individuals were evaluated and no interference peaks were observed in the chromatographic region of the analyte or the ISTD. Representative chromatograms of the blank plasma samples without ISTD are shown in Fig. 3. As there is only a 2 Da difference between norethindrone and the norethindrone-$^{13}C_2$ ISTD, cross-contribution from analyte to ISTD channel was a concern. To evaluate this, a sample containing norethindrone at the highest calibrator concentration (25 000 pg mL^{-1}) was extracted without addition of ISTD. The cross-contribution was found to be insignificant under the developed conditions and has no impact on accurate quantitation of the analyte. In addition, a CTL-0 (blank matrix with ISTD only) was also included in each batch. This sample was used to evaluate the cross-contribution of ISTD to analyte channel as well as to evaluate any potential contamination of the ISTD working solution by analyte. There was no cross-contribution observed from ISTD channel to analyte channel. These same six lots of matrix were also spiked at the low QC level (150 pg mL^{-1}), extracted and analyzed. They showed an accuracy of 102.7% and precision of 6.8%.

3.5. Linearity

In both validation and sample analysis batches, calibrators were evenly positioned across the batch to compensate for possible system fluctuation. For all validation batches, freshly prepared calibrators were used. Once storage stability was established, stored calibrators were used for sample analysis. Excellent linearity was obtained in all validation batches with correlation coefficients ranging from 0.9974 to 0.9998.

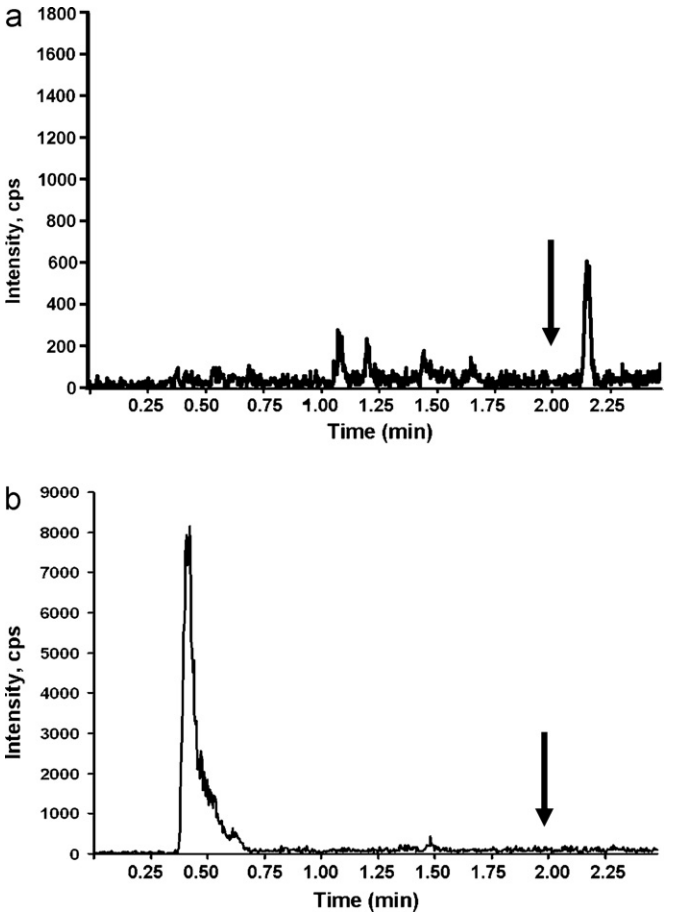

Fig. 3. Representative LC/MS/MS chromatogram of blank matrix from the selectivity evaluation (a) analyte, *m/z*: 299/231 and (b) internal standard, *m/z*: 301/231.

3.6. Precision and accuracy

Table 1 summarizes the precision and accuracy of the QC samples in three consecutive batches for norethindrone with accuracy ranging from 97.6 to 112.8% and precision ranging from 3.5 to 10.9% over the five QC concentration levels evaluated.

3.7. Dilution integrity

QC samples prepared at a concentration above the highest calibrator were diluted 10-fold into the calibration curve range with blank matrix prior to extraction to determine dilution integrity. Six replicates of dilution QCs were extracted and analyzed with an accuracy of 100.0% and precision of 6.1%, demonstrating the capability of the method to analyze samples with concentrations higher than the upper limit of quantitation.

3.8. Matrix stability of samples

Short-term matrix stability of norethindrone was evaluated under different conditions including 24 h at room temperature and 5 cycles of freeze/thaw at both −20 °C and −70 °C at low and high QC levels. It was demonstrated that the analyte was

Table 1
Precision and accuracy of quality control samples in human plasma.

Norethindrone	LLOQ 50.0 pg mL^{-1}	Low QC 150 pg mL^{-1}	Mid QC 2000 pg mL^{-1}	High QC 20 000 pg mL^{-1}	Dilution QC 12 5000 pg mL^{-1}
Day 1					
n	5	6	6	6	6
Mean	52.8	164	2260	20 000	125 000
CV%	10.9	9.8	5.4	6.6	6.1
Accuracy (%)	105.6	109.3	113.0	100.0	100.0
Day 2					
n	6	6	6	6	
Mean	51.2	151	2150	20 000	
CV%	5.4	4.8	3.6	4.4	
Accuracy (%)	102.4	100.7	107.5	100.0	
Day 3					
n	6	6	6	6	
Mean	50.1	150	2100	19 500	
CV%	6.7	6.9	4.8	6.0	
Accuracy (%)	100.3	100.0	105.0	97.5	
Inter-day					
n	17	18	18	18	
Mean	51.3	155	2170	19 800	
CV%	7.7	8.3	5.3	5.5	
Accuracy (%)	102.6	103.3	108.5	99.0	

very stable under all tested conditions with accuracy ranging from 96.7% to 101.5% and precision ranging from 1.4% to 7.1% as shown in Table 2.

Long-term matrix stability was tested after storage of low, high and dilution QCs at −20 °C and −70 °C for 78 days. All levels passed acceptance criteria with accuracy ranging from 91.2 to 102.7% and CV <9.1%. A second time point with extended storage time will be tested later.

3.9. Whole blood sample collection stability

The whole blood sample collection stability test was conducted in a validation batch. Whole blood samples with spiked norethindrone held at room temperature and also on wet ice for 2 h were evaluated with an accuracy of recovery of 100.0%, indicating the analyte is stable in blood under either condition.

3.10. Recovery

Overall recoveries of 84.9% for the analyte and 89.4% for the ISTD were obtained and three QC levels showed consistent recovery results. Less than 100% recovery was due to the liquid–liquid extraction efficiency of the SLE extraction system. Other solvents such as ethyl acetate and methyl *tert*-butyl ether were also tested but no improvement in recovery was seen.

Table 2
Freeze/thaw and room temperature stability of norethindrone in human plasma.

	Theoretical concentration (pg mL^{-1})	
	150	20 000
Freeze/thaw matrix stability at −20 °C (5 cycles)		
n	6	6
Mean	148	20 300
CV%	3.6	3.3
Accuracy (%)	98.7	101.5
Freeze/thaw matrix stability at −70 °C (5 cycles)		
n	6	6
Mean	146	19 700
CV%	7.1	1.9
Accuracy (%)	97.3	98.5
Room temperature matrix stability for 24 h		
n	6	6
Mean	145	19 600
CV%	3.5	1.4
Accuracy (%)	96.7	98.0

3.11. Reinjection reproducibility

Because of instrument availability or the reinjection of a batch that failed for some technical reasons during initial injection, an

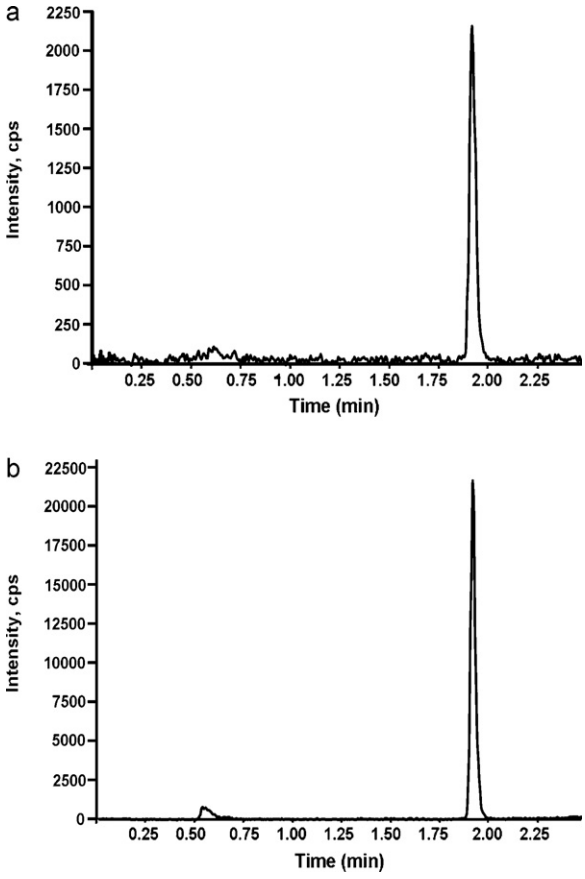

Fig. 4. Representative LC/MS/MS chromatogram of a real sample from a patient administering norethindrone ((a) analyte, *m/z*: 299/231 and (b) internal standard, *m/z*: 301/231).

extracted batch could experience storage in the autosampler or in a refrigerator for some time prior to final injection. To cover this period of storage time, an extracted batch was injected, stored at 2–8 °C for 79 h, and then re-injected. In this evaluation, only the calibration curve, six replicates of low, mid and high QC samples as well as some blank matrix, blank control zero samples were included in the reinjection. All QCs and calibrators passed acceptance criteria, demonstrating that extracted samples can be stored for extended period of time and good accuracy and precision can be still obtained.

3.12. Hemolysis evaluation

Sample hemolysis may create a matrix with components different from regular plasma samples. This matrix difference may cause suppression or enhancement to the analyte and/or ISTD. It may very often cause accuracy issues, particularly for an analog ISTD. For isotopically labeled ISTDs, even if there is significant matrix effect in some cases, the analyte-to-ISTD area ratios are not impacted, thus the accuracy will not be affected. In this study, hemolysis QCs gave a mean accuracy of 104.2% with a CV of 9.2%. In addition, the ISTD responses for hemolysis QCs were consistent with other calibrators and QC samples, indicating there was no additional matrix effect from hemolyzed samples.

4. Application

The validated method has supported multiple pharmacokinetic (PK) studies and has successfully analyzed thousands of study samples. Fig. 4 shows a typical chromatogram from a real sample from a patient administering norethindrone. Incurred sample reproducibility (ISR) evaluations in study samples were conducted following the criteria outlined in the report of the Crystal City Workshop [13]. For this method, ISR results well passed acceptance criteria, demonstrating good reproducibility of the validated method.

5. Conclusions

For the first time, a rapid and robust bioanalytical method for the determination of norethindrone alone in human plasma was developed and validated using SLE and LC/MS/MS detection. This method has a simple yet very reproducible extraction and short analysis time. It is very suitable for high-throughput PK studies.

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