HHS Public Access

Author manuscript

*Contraception*. Author manuscript; available in PMC 2019 April 01.

Published in final edited form as:

*Contraception*. 2018 April ; 97(4): 363–369. doi:10.1016/j.contraception.2018.01.015.

# Simultaneous quantitation of multiple contraceptive hormones in human serum by LC-MS/MS

**Steven W. Blue**[1], **Andrea J. Winchell**[1], **Amy V. Kaucher**[1], **Rachel A. Lieberman**[2], **Christopher T. Gilles**[2], **Maria N. Pyra**[3], **Renee Heffron**[3,4], **Xuanlin Hou**[4], **Robert W. Coombs**[5], **Kavita Nanda**[6], **Nicole L. Davis**[7], **Athena P. Kourtis**[7], **Joshua T. Herbeck**[4], **Jared M. Baeten**[3,4,5], **Jairam R. Lingappa**[4,8,9], and **David W. Erikson**[1,*]

[1]Endocrine Technologies Support Core, Oregon National Primate Research Center, Beaverton, OR

[2]Shimadzu Scientific Instruments, Columbia, MD

[3]Department of Epidemiology, University of Washington, Seattle, WA

[4]Department of Global Health, University of Washington, Seattle, WA

[5]Department of Medicine, University of Washington, Seattle, WA

[6]FHI 360, Durham, NC

[7]Division of Reproductive Health, National Center for Chronic Disease Prevention and Health Promotion, Centers for Disease Control and Prevention, Atlanta, GA

[8]Department of Medicine, University of Washington, Seattle, WA

[9]Department of Pediatrics, University of Washington, Seattle, WA

## Abstract

**Objective—**To develop a method to simultaneously quantify five commonly used hormonal contraceptives (HC) and 2 endogenous sex steroids by liquid chromatography-tandem triple quadrupole mass spectrometry (LC-MS/MS) and apply this method to human serum samples.

**Study Design—**We developed a method to simultaneously analyze ethinyl estradiol (EE2), etonogestrel (ENG), levonorgestrel (LNG), medroxyprogesterone acetate (MPA), and norethisterone (NET), along with estradiol (E2) and progesterone (P4) in human serum for a Shimadzu Nexera-LCMS-8050 LC-MS/MS platform. We analyzed serum collected from women self-reporting use of oral contraceptives, contraceptive implants or injectable contraceptives

*Corresponding author: David W. Erikson, Ph.D., Endocrine Technologies Support Core, Oregon National Primate Research Center, 505 NW 185th Ave, Beaverton, OR 97006; erikson@ohsu.edu; phone: 503-346-5078.

(n=14) and normally cycling women using no HC (n=15) as well as pooled samples from women administered various HC (ENG, n=6; LNG, n=14; MPA, n=7; NET, n=5).

**Results—**Limits of quantitation were 0.010 ng/ml for E2, EE2, and P4; 0.020 ng/ml for ENG, LNG, and MPA; and 0.040 ng/ml for NET. Precisions for all assays, as indicated by coefficient of variation (CV) were less than or equal to 12.1%. Accuracies for all assays were in the range of 95–108%. Endogenous hormone values obtained from analysis of human serum samples are in agreement with levels previously reported in the literature for normally cycling women as well as for women taking the appropriate HC.

**Conclusions—**We have developed a robust, accurate, and sensitive method for simultaneously analyzing commonly used contraceptive steroids and endogenous sex steroids in human serum.

**Implications—**This analytical method can be used for quantitating contraceptive steroid levels in women for monitoring systemic exposure to determine drug interactions, nonadherence, misreporting, and proper dosing.

### Keywords

hormonal contraceptive; LC-MS/MS; steroid; liquid chromatography; mass spectrometry

## 1. Introduction

Exogenous sex hormones are commonly provided for contraceptive and therapeutic reasons to women worldwide. These hormones can be administered as oral contraceptives, intramuscular injections, subcutaneous implants, intra-uterine devices or vaginal rings. The most common of these therapies are administration of a progestin such as etonogestrel (ENG), levonorgestrel (LNG), medroxyprogesterone acetate (MPA) or norethisterone (NET) with or without an estrogen such as ethinyl estradiol (EE2) [1].

Until recently, the predominant methods for analyzing steroid hormones were radioimmunoassay (RIA), enzyme immunoassay (EIA), and automated immunoassay (AI) [2]. RIAs can be advantageous as they are reliable when properly validated and multiple steroids can be measured in a single aliquot. However, these assays are time-consuming, can lack specificity, and often require large volumes of sample for sensitive measurements, especially for estrogens which circulate at low concentrations [2]. AI on the other hand are fast, convenient, inexpensive, and high-throughput with accurate results for most patient care [2,3]. Like RIA, AI can lack specificity, especially when the subject is treated with exogenous steroids or when the antibody may recognize a chemically similar analyte [2,3]. While commonly monitored steroid hormones such as estradiol-17β (E2) and progesterone (P4) can be assayed by AI, there are currently no AI methods to assay hormonal contraceptives and these hormones must be quantified by RIA or EIA.

The use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) has increased as the method of choice for the quantitative measurement of steroid hormones. MS assays offer dramatic improvements in specificity and automation, and often increased sensitivity of serum steroid measurements over RIA and AI [2]. In fact, because of the improvements in sensitivity and specificity, MS assays are frequently referred to as the “gold standard” of

serum steroid measurement [2]. Despite this, there are only a few methods for determining multiple progestins in a single analysis [4–6] with many devoted to a single progestin and few including simultaneous analysis of estrogens [5,7–9]. Many methods require large sample volumes to achieve adequate sensitivity [5–8] while those that use smaller sample volumes are often less sensitive [4].

When analyzing circulating levels of HC it is also useful to simultaneously measure endogenous E2 and P4 to monitor adherence or drug interactions. Here we present a newly-developed sensitive LC-MS/MS method to simultaneously quantitate five exogenous contraceptive steroids (EE2, ENG, LNG, MPA, and NET) and endogenous steroids (E2 and P4) in human serum. Simultaneous measurement of multiple HC in a single sample can be useful in validating HC use by women before and during clinical studies, to monitor drug compliance in order to evaluate associations with other health outcomes, and in developing new contraceptive protocols using existing hormones. This method uses a small sample volume, a simple sample preparation and extraction procedure, and a relatively short on-instrument run time, making this analytical method ideal for assessment of contraceptive hormone measurement in human subjects.

## 2. Materials and Methods

### 2.1 Chemicals

We purchased unlabeled standards for estradiol (E2), progesterone (P4), and ethinyl estradiol (EE2) from Cerilliant (Round Rock, TX) and unlabeled standards for etonogestrel (ENG), levonorgestrel (LNG), medroxprogesterone acetate (MPA), and norethindrone (NET) from Sigma (St. Louis, MO). We purchased internal standards E2-d5 and P4-d9 from Cerilliant and internal standards EE2-d7, ENG-d7, MPA-d6, LNG-d6, and NET-d6 from Toronto Research Chemicals (North York, ON, Canada). Ammonium fluoride ($NH_4F$) and dichloromethane (DCM) were purchased from Sigma. We purchased charcoal stripped human serum from BioChemed Services (Winchester, VA) and normal human serum from Golden West Biologicals (Temecula, CA). LC-MS grade water and methanol were purchased from Honeywell Burdick & Jackson.

### 2.2 Sample Preparation

150 μl of standard, QC, and serum samples were pipetted into 350 μl 96-well microtiter plates. 100 μl of ultrapure water containing 2.5 ng/ml E2-d5, 3.0 n g/ml P4-d9, 7.0 ng/ml EE2-d7, 6.0 ng/ml ENG-d7, 1.8 ng/ml MPA-d6, 1.8 ng/ml LNG-d6, and 4.0 ng/ml NET-d6 internal standards were added. A double blank of 0 ng/ml standard (150 μl) and 0 ng/ml deuterated hormones (100 μl) in ultrapure water was also prepared in duplicate. Plates were shaken to mix for 5 min and the contents of each well transferred to a 400 μl 96-well Isolute supported liquid extraction (SLE+) plate (Biotage, Uppsala, Sweden). We loaded the contents of each well into the SLE+ plate using a Pressure+ positive pressure manifold (Biotage) and incubated for 5 min at room temperature. Steroid hormones were eluted into a 2 ml glass-coated 96-well deep well plate (ThermoFisher, Waltham, MA) with 2 × 900 μl DCM and dried under forced air at 40°C in a TurboVap 96 automated evaporation system (Biotage). Plates were rinsed with 400 μl DCM and dried under forced air. After careful

reconstitution in 50 μl of 25:75 methanol:water (v:v) we transferred the contents of each well to a 350 μl V-bottom 96-well microtiter plate (Shimadzu) and analyzed by LC-MS/MS. Two plates with a single curve were typically prepared each day in this manner.

### 2.3 Preparation of Calibration Curve and Quality control samples

For calibration curves, we prepared a mixture of unlabeled standards for E2, P4, EE2, ENG, MPA, LNG, and NET in methanol, each at a final concentration of 10 ng/ml in charcoal-stripped human serum. Two-fold serial dilutions of this mixture were made in charcoal-stripped human serum to yield the final twelve-point calibration curve, which ranged from 0.010 ng/ml to 10 ng/ml and included calibrators at 0, 0.010, 0.020, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5, and 10 ng/ml. Serum standards were prepared daily immediately prior to sample preparation and methanolic stocks of each unlabeled standard were stored at −80°C. We prepared quality control (QC) samples by spiking a mixture of unlabeled hormone standards into normal human serum (Table 1). This QC was prepared and assayed in triplicate at the front and back of each assay. In addition, we generated QC pools of human serum samples for each of the synthetic progestins tested, by combining samples known to be positive for an individual hormone. Samples for both individual positive and negative controls, and pooled sera of defined HC characteristics were from the Partners PrEP Study, a clinical trial of HIV-1 pre-exposure prophylaxis that enrolled Kenyan and Ugandan participants from 2007–2012 [10]. We analyzed these individual pools for ENG, LNG, MPA, and NET in duplicate in each assay. A pool for EE2 was not created from subject samples in the Partners PrEP Study as there was insufficient sample volume with high enough levels of EE2 for pooling.

### 2.4 LC-MS/MS instrument parameters

Microtiter plates were prepared as described above and loaded onto a SIL-30ACMP autosampler set at 10°C (Shimadzu). 25 μl of each standard, QC or sample were injected onto a Raptor 2.7μm Biphenyl 50mm × 2.1mm column (Restek, Bellefonte, PA) with an inline guard column (2.7 μm Biphenyl 5 mm × 2.1 mm, Restek) at 40°C using reversed phase chromatography. Mobile phase A was 0.15 mM $NH_4F$ in water; mobile phase B was 100% methanol. The LC time gradient was created using two Nexera LC-30AD pumps (Shimadzu) as follows: 0.00–0.80 min, 70%–71% B; 0.80–3.10 min, 71%-71% B; 3.10–3.50 min, 73%–84% B; 3.50–3.60 min, 84%–94% B; 3.60–4.01 min, 94%–100% B; 4.01–5.55 min, hold at 100% B; 5.55–8.30 min, return to 70% B and hold for re-equilibration. The entire gradient was run at a flow rate of 0.25 ml/min. Heated electrospray injection in both positive (P4, ENG, LNG, MPA, Net) and negative (E2, EE2) modes with ultra-fast polarity switching and scheduled multiple reaction monitoring (MRM) on a Shimadzu LCMS-8050 was used for detection of steroids. The interface temperature was 300°C, desolvation line temperature was 150°C, and heat block temperature was 500°C. Gas was supplied by a Peak Genius 1051 nitrogen and air generator (Peak Scientific, Inchinnan, UK). Nitrogen gas was used for nebulizing and drying gases, while air was used for heating gas. Nebulizing gas flow was 3 L/min, heating gas flow was 10 L/min, and drying gas flow was 10 L/min. We used a capillary B needle in the interface and needle protrusion was set to 1.0 mm. Argon (Airgas, Radnor, PA) was used for the collision induced dissociation at 270 kPa. The MS/MS conditions for each target were optimized using the automated MRM optimization

procedure in LabSolutions (Shimadzu). The MRM transitions for all steroids analyzed in this method can be found in Table 2.

### 2.5 Recovery and matrix effects

We determined analyte recovery (extraction efficiency) by spiking the target hormone into separate aliquots of charcoal-stripped serum both before and after extraction, but before the dry-down step. Samples were analyzed in triplicate. We evaluated matrix effects by comparing blank serum spiked with target hormone after extraction with reference standards spiked at the same concentration into 25:75 methanol:water. These samples were analyzed in quadruplicate.

### 2.6 Specificity

We analyzed the specificity of each assay in the method by comparing extracted blank serum (analyte-free matrix) against unlabeled standards spiked into extracted blank serum samples. For each compound at least two MRMs were monitored to ensure that no interference was present at the retention time for each analyte.

### 2.7 Precision and accuracy

We tested assay precision by analyzing multiple replicates of QCs within each assay (intra-assay precision) and over multiple assays (inter-assay precision) (n=14). Precisions were reported as coefficients of variation (CV). We evaluated accuracy by comparing the analyzed concentration against a nominally spiked concentration of each analyte in normal human serum.

### 2.8 Stability

Stability after preparation was tested during the validation procedure. This was done to cover the anticipated run time for a typical analytical batch (approximately 25 h), during which prepared samples would be resident in the autosampler at 10°C. Prepared samples were kept at autosampler temperature for up to 72 h and analyzed with fresh samples for reference.

### 2.9 Data analysis

Data were analyzed using LabSolutions version 5.72 (Shimadzu). Target reference ion ratios were set according to the reference ion ratio of the highest standard. Default ion allowance for peak identification was 30% relative to this target ratio. Linear regression with 1/C weighting was used for analysis of calibration curves.

## 3. Results

### 3.1 LC-MS/MS analysis

We performed the simultaneous quantitation of contraceptive steroids EE2, ENG, LNG, MPA, and NET, and endogenous steroids E2 and P4 in a single analysis using LC-MS/MS (Figure 1). Excellent linearity was observed for each target hormone within the calibration range ($R>0.99$). Limits of quantitation for each target can be found in Table 3 and were determined by the lowest concentration calibrator that was successfully integrated in

LabSolutions with accuracy between 80–120% [11]. This resulted in lower limits of quantitation (LLOQ) for E2, EE2, and P4 at 0.010 ng/ml, for ENG, LNG, and MPA at 0.020 ng/ml, and for NET at 0.040 ng/ml. LLOQs for ENG, LNG, MPA, and NET were set more conservatively because of matrix-associated peaks that were recognized by MRMs which potentially interfered with integration at lower concentration calibrators. These interfering peaks were at different retention times than found for the hormones, but adjacent to the hormone peaks (Figure 1). The affected MRMs were 313.20→109.25, 313.20→245.25, and 313.20→79.00 for LNG; 387.20→123.10 for MPA; 325.20→147.00 for ENG; and 299.20→109.25 for NET (Figure 1). We included these MRMs in the method as they resulted in robust quantitation of the target compounds at acceptable levels, and optimized chromatographic conditions allowed for sufficient resolution and separation of these compounds from the adjacent peaks (Figure 1). In total, 36 transitions were required for all seven analytes and their internal standards over approximately 5.2 min of analysis time. An additional 3 min of equilibration for instrument stabilization after each injection was required before the next injection was initiated.

We determined the method detection limit (MDL) for each compound using a previously published method [12]. The limit of detection (LOD) and LOQ are typically indicated as the concentration at which the signal to noise ratio for a particular analyte is 1:3 (LOD) or 1:10 (LOQ). However, many sources of noise in modern MS/MS instrumentation have been eliminated, making signal to noise comparisons difficult to determine if not meaningless to LOD and LOQ determinations [13]. The MDL is defined as the minimum concentration of an analyte that can be measured and reported with 99% confidence that the analyte concentration is greater than zero. The MDL is determined from analysis of replicate standard injections in matrix at concentrations near the LOQ to evaluate the uncertainty in the system [13]. For determination of MDLs in this method we performed five replicate injections on two separate days of the lowest concentration calibrator for each analyte and calculated each MDL using the formula described by the Environmental Protection Agency [12]. MDLs for the analytes in this method can be found in Table 3. An amount of analyte equal to or greater than the MDL is both detectable and distinguishable from the background with 99% confidence [13]. We calculated specific MDLs in lieu of determining the LOD for each compound in the method.

We selected chromatographic conditions to provide good peak shapes and optimal separation for all compounds, with a focus on the need for adequate sensitivity of the estrogen compounds E2 and EE2. These requirements were met by using a biphenyl column with mobile phase consisting of 0.15 mM $NH_4F$ in water (A) and methanol (B) paired with the gradient program described in 2.4 above. In this method, separation of the progestins from each other and potential interfering matrix peaks were critical to obtain acceptable levels of sensitivity for these compounds. We selected ammonium fluoride as an additive, as it is known to give up to 11 times more sensitivity in negative mode and 2.4 times more sensitivity in positive mode than other commonly used additives such as ammonium hydroxide [14,15], thus eliminating the need for derivatization during sample preparation to achieve appropriate sensitivity.

### 3.2 Method validation

We performed validation of the analytical method largely according to FDA guidelines [11], by assessing the following parameters: specificity; precision, accuracy, and recovery; calibration curve; sensitivity; and reproducibility.

**3.2.1 Specificity—**The specificity of each assay in the method was confirmed by ensuring that no interfering peaks or signals at expected retention times for each analyte were detectable in blank matrix for all MRMs used within the method. We assessed specificity with eight different lots of charcoal stripped serum from two different vendors (Golden West Biologicals and BioChemed Services). In addition, we confirmed specificity of hormonal contraceptive (EE2, ENG, LNG, MPA, NET) assays utilizing several in-house prepared pools of nonhuman primate serum that were not spiked with any of the five HC.

**3.2.2 Precision, accuracy, and recovery—**Intra- and inter-assay precisions and accuracies are reported in Table 1. Precisions are reported as coefficients of variation (CV), the standard deviation of the replicates tested divided by the average value of the replicates. Precisions for all assays were less than or equal to 12.1%. Accuracies for all assays were in the range of 95–108%. Recoveries are reported in Table 1. We assessed matrix effects by analysis of unlabeled standards spiked into matrix after extraction at the same time as into 25:75 water:methanol. We found a decrease of signal intensity ranging from 15–66% depending on the analyte (Table 1). We were, however, able to control for this ion suppression by using appropriate deuterated internal standards. Matrix effects did not vary by different concentrations of analyte and were stable across replicates in each analysis.

**3.2.3 Calibration curve—**We ran a 12-point calibration curve, including 0 ng/ml, for each analyte in the method. The lowest concentration calibrator for ENG, LNG, and MPA, and the two lowest concentration calibrators for NET were not used because of potential interfering matrix peaks as described above. Calibration curves were linear within the calibration range ($R>0.99$) for all analytes. Calibration ranges were 0.010 – 10 ng/ml for E2, EE2, and P4; 0.020 – 10 ng/ml for ENG, LNG, and MPA; and 0.040 – 10 ng/ml for NET. The double blank was run immediately after the highest standard and confirmed the absence of carryover. Acceptable accuracies for calibration points were within the range of 85–115% for all calibrators but those at the LLOQ and upper LOQ (ULOQ). The acceptable accuracy range for these calibrators was 80–120%.

**3.2.4 Sensitivity—**We determined the sensitivities of each assay within the method by the lowest and highest concentration calibrators in the calibration curve. The LLOQ and ULOQ for each assay were accurate within the range of 80–120%. We determined the MDL for each assay as described above. The LOQ and MDL for each assay are listed in Table 3.

**3.2.5 Reproducibility—**Reproducibility of the assay was determined largely by observation of the intra- and inter-assay CVs of the assay QCs. These data are in Table 3. In addition, we compared values obtained from 14 women with known hormonal contraceptive use, and 15 women with no reported hormonal contraceptive use, as well as pooled human

serum samples containing known amounts of the HC analyzed herein, to values found in the literature and found good agreement between our method and the literature (see section 3.3).

### 3.3 Method application to human serum samples

We used our method to analyze serum samples obtained from 29 women with self-reported use of implant (ENG or LNG; n=5), injectable (MPA; n=5) or oral HC (EE2, LNG; n=4), or women self-reporting no HC use (n=15, Table 4). We also generated sample pools from women known to be administered ENG (n=6 women), LNG (n=14 women), MPA (n=7 women) or NET (n=5 women). We applied the method to these pools (4 pools, 1 pool for each of the hormones, Table 5). Our results were similar to levels previously reported in the literature for normally cycling women or women taking the appropriate HC [7,16–19].

## 4. Discussion

The method described allows for simultaneous analysis of five commonly used contraceptive hormones (EE2, ENG, LNG, MPA, and NET), with concurrent monitoring of endogenous sex hormones (E2 and P4). Advantages of this method over previously published HC analysis methods include smaller sample volume, simple sample preparation, and high sensitivity for all hormones evaluated. While other methods for analyzing these hormones do exist, they often require larger sample volumes [5–8], a more involved sample preparation procedure including derivatization [17,20] or ability to monitor only a select number of hormones [4–8].

While MS assays are now considered the “gold standard” for steroid hormone analysis, sensitive simultaneous analysis of these hormones is challenging. Steroids typically have low ionization efficiency in the MS due to their lack of polarity [20], and the concentration of many endogenous steroids, including E2, is in the low pg/ml range in human serum. Several aspects of our method were effective in addressing these. We achieved high levels of hormone recovery using the SLE procedure and dramatically improved the signal for all compounds, especially estrogens, using $NH_4F$ in the aqueous mobile phase. Ionization efficiency is improved on the Shimadzu LCMS-8050 when compared to other instrumentation because of the presence of heated ESI. Heated ESI also increases desolvation efficiency, which facilitates ionization and improves the quantitation of compounds found in low concentrations. The LCMS-8050 also features short pause times (the time needed between each measurement for the MS to reset voltages and allow for the collision cell to be emptied), which allows increased time for recording signal intensity (dwell time), thus increasing sensitivity. On this system it is possible to prepare and analyze 2 × 96 samples in a 24 hr period.

The analysis of HC levels for the purpose of validating HC use by women has recently been gaining attention [21]. Epidemiologic studies often evaluate associations between hormonal contraceptives and health outcomes, such as cardiovascular disease, cancer or HIV; proper classification of exposure is critical. However, when our method was applied to a cohort of African women who selfreported HC use, a large number of discordant responses was evident [22]. This included 17% of women using injectable HC (MPA, NET), 62% of oral HC (EE2, LNG) and 8% of implantable HC users (ENG, LNG). In addition, 14% of self-

reported non-users had at least one exogenous HC present. A recent similar study found that 27% of women had detectable exogenous hormones when self-reporting non-use, mostly related to oral or injectable use [21]. The introduction of this method, which allows for simultaneous testing of five commonly-used HC, will allow researchers to corroborate self-reporting by study participants, in order to determine HC use amongst subjects or estimate the degree to which self-reporting may be inaccurate. The small volume of serum required by our method is another advantage, particularly for studies where stored samples are valuable and used for many assays.

Finally, we believe that our method can be used for studies evaluating new contraceptive protocols using the hormones targeted in this method. The small sample volumes needed, simple sample preparation, and short run times would be particularly advantageous for contraceptive protocol development.

One potential area of concern is the use of deuterated standards for normalization of steroid hormones during quantification. Several recent papers report increased accuracy when using C-13 labeled internal standards rather than deuterium-labeled internal standards, particularly for estrogens [23–25]. However, we did not feel the increased cost of C-13 labeled standards would have sufficient benefits on assay performance, given that intra- and inter-assay precisions for all hormones in the method were less than 8.1% and 12.1%, respectively, and that accuracies for all targets ranged from 95%–108% (Table 1). In addition, availability from reputable commercial sources of C-13 labeled standards for the hormones targeted in this method is limited. We were able to control for the variability in ion suppression due to matrix effects (Table 3) by using deuterated internal standards.

In summary, we have developed a robust, accurate, and sensitive method for simultaneously quantitating five commonly used contraceptive steroids and the endogenous steroids E2 and P4. Because of its wide dynamic range and high sensitivity this method can serve as a powerful tool for monitoring contraceptive use.

## Acknowledgments

The authors would like to thank the staff of the Endocrine Technologies Support Core at the Oregon National Primate Research Center. We are also grateful to Shimadzu Scientific for equipment support and troubleshooting expertise.

Funding Sources: This work was supported by the National Institutes of Health (grant numbers P51 OD011092 and R01 AI111738); and the Centers for Disease Control and Prevention (grant number U48DP005013).

## References

1. Allen C, Evans G, Sutton EL. Pharmacologic Therapies in Women's Health: Contraception and Menopause Treatment. Med Clin North Am. 2016; 100:763–89. [PubMed: 27235614]
2. Stanczyk FZ, Clarke NJ. Advantages and challenges of mass spectrometry assays for steroid hormones. J Steroid Biochem Mol Biol. 2010; 121:491–5. [PubMed: 20470886]
3. Bock JL. The new era of automated immunoassay. Am J Clin Pathol. 2000; 113:628–46. [PubMed: 10800395]
4. Moser C, Zoderer D, Luef G, Rauchenzauner M, Wildt L, Griesmacher A, et al. Simultaneous online SPE-LC-MS/MS quantification of six widely used synthetic progestins in human plasma. Anal Bioanal Chem. 2012; 403:961–72. [PubMed: 22160205]

5. Licea-Perez H, Wang S, Bowen CL, Yang E. 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. J Chromatogr B Analyt Technol Biomed Life Sci. 2007; 852:69–76.
6. S Augustine M. Medroxyprogesterone Acetate and Progesterone Measurement in Human Serum: Assessments of Contraceptive Efficacy. J Anal Bioanal Tech. 2014:s5.
7. Thomas T, Petrie K, Shim J, Abildskov KM, Westhoff CL, Cremers S. A UPLC-MS/MS method for therapeutic drug monitoring of etonogestrel. Ther Drug Monit. 2013; 35:844–8. [PubMed: 24081205]
8. Li W, Li Y-H, Li AC, Zhou S, Naidong W. 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. J Chromatogr B Analyt Technol Biomed Life Sci. 2005; 825:223–32.
9. Wang R, Tian Y, Zhang L, Zhang Z. Simultaneous determination of levonorgestrel and two endogenous sex hormones in human plasma based on LC-MS/MS. Bioanalysis. 2016; 8:1133–44. [PubMed: 27211854]
10. Baeten JM, Donnell D, Ndase P, Mugo NR, Campbell JD, Wangisi J, et al. Antiretroviral prophylaxis for HIV prevention in heterosexual men and women. N Engl J Med. 2012; 367:399–410. [PubMed: 22784037]
11. Food and Drug Administration guidance for industry - Bioanalytical method validation. 2013
12. U.S. EPA - Title 40: Protection of the Environment; Part 136 - Guidelines establishing test procedures for the analysis of pollutants; Appendix B to Part 136 - Definition and procedure for the determination of the method detection limit - revision 1.11 2011.
13. Wells G, Prest H, Russ CW IV. Why use signal-to-noise as a measure of MS performance when it is often meaningless? Agil Technol Tech Note. 2011:5990–8341EN.
14. EPA Method 539 - Determination of hormones in drinking water by solid phase extraction (SPE) and liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), EPA document no. 815-B-10-001 2010.
15. Hindle R. Improved analysis of trace hormones in drinking water by LC/MS/MS (EPA 539) using the Agilent 6460 triple quadrupole LC/MS. Agil Technol Tech Note. 2013:5991–2473EN.
16. Edelman AB, Cherala G, Stanczyk FZ. Metabolism and pharmacokinetics of contraceptive steroids in obese women: a review. Contraception. 2010; 82:314–23. [PubMed: 20851224]
17. Kumar P. A Rapid and Sensitive UPLC–ESI-MS/MS Method for Determination of Levonorgestrel by Chemical Derivatization in Human Plasma and its Application to Pharmacokinetic Study. J Anal Bioanal Tech. 2014:S6.
18. Alvarez F, Brache V, Tejada AS, Cochon L, Faundes A. Sex hormone binding globulin and free levonorgestrel index in the first week after insertion of Norplant implants. Contraception. 1998; 58:211–4. [PubMed: 9866001]
19. Vieira CS, Bahamondes MV, de Souza RM, Brito MB, Rocha Prandini TR, Amaral E, et al. Effect of antiretroviral therapy including lopinavir/ritonavir or efavirenz on etonogestrel-releasing implant pharmacokinetics in HIV-positive women. J Acquir Immune Defic Syndr 1999. 2014; 66:378–85.
20. Ke Y, Bertin J, Gonthier R, Simard J-N, Labrie F. A sensitive, simple and robust LC-MS/MS method for the simultaneous quantification of seven androgen- and estrogen-related steroids in postmenopausal serum. J Steroid Biochem Mol Biol. 2014; 144(Pt B):523–34. [PubMed: 25158021]
21. Achilles SL, Mhlanga FG, Musara P, Poloyac SM, Chirenje ZM, Hillier SL. Misreporting of contraceptive hormone use in clinical research participants. Contraception. 2017; doi: 10.1016/j.contraception.2017.09.013
22. Pyra MN, Baeten JM, Blue SW, Davis NL, Erikson DW, Heffron R, et al. Misreporting of hormonal contraceptive use among African women with and at risk for HIV. Contraception. 2017
23. Szarka S, Nguyen V, Prokai L, Prokai-Tatrai K. Separation of dansylated 17β-estradiol, 17α-estradiol, and estrone on a single HPLC column for simultaneous quantitation by LC-MS/MS. Anal Bioanal Chem. 2013; 405:3399–406. [PubMed: 23371528]

24. Szarka S, Prokai-Tatrai K, Prokai L. Application of screening experimental designs to assess chromatographic isotope effect upon isotope-coded derivatization for quantitative liquid chromatography-mass spectrometry. Anal Chem. 2014; 86:7033–40. [PubMed: 24922593]

25. Bussy U, Chung-Davidson Y-W, Buchinger TJ, Li K, Li W. High-sensitivity determination of estrogens in fish plasma using chemical derivatization upstream UHPLC-MSMS. Steroids. 2017; 123:13–9. [PubMed: 28456451]

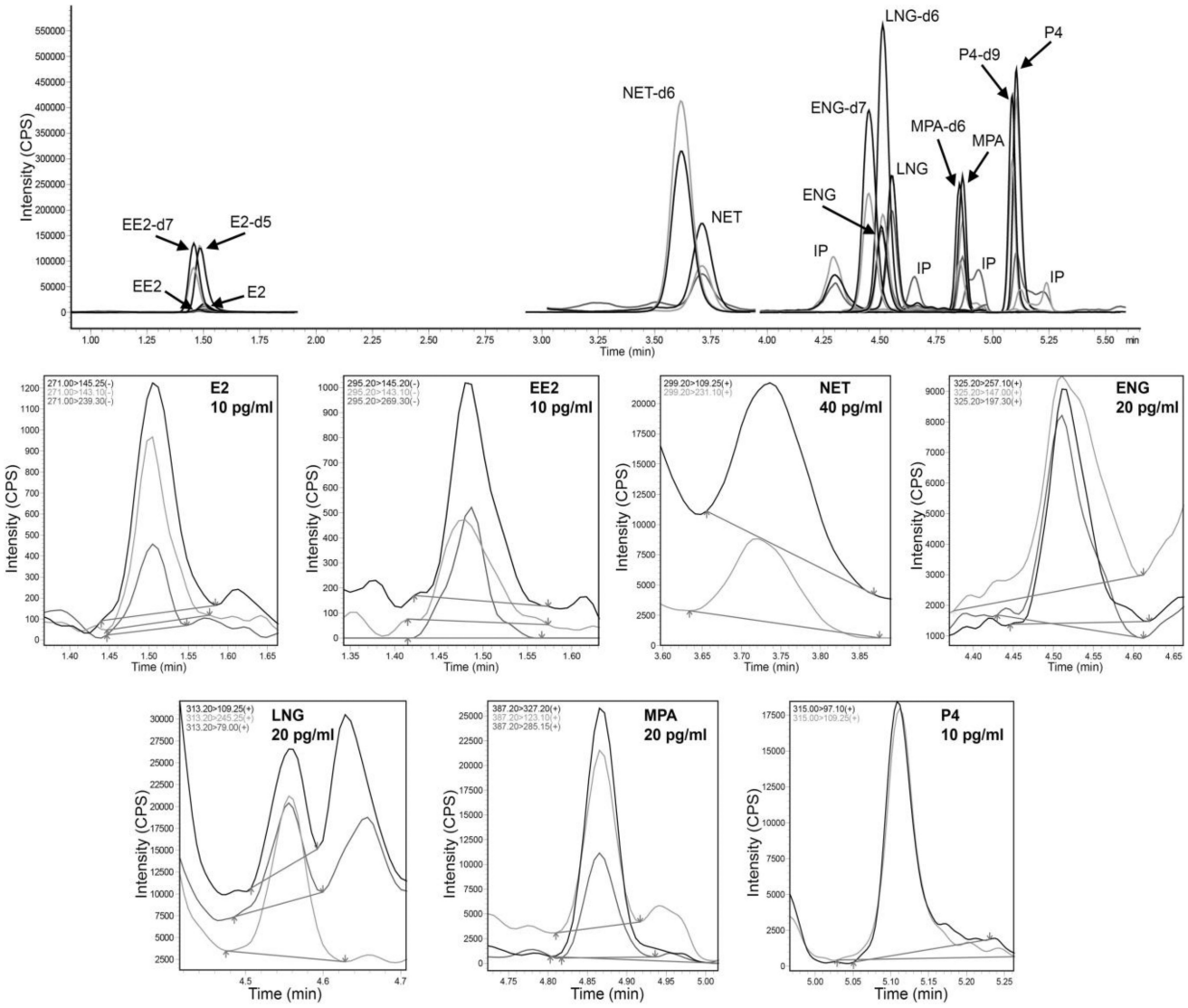


**Figure 1.**

Representative multiple reaction monitoring chromatogram of unlabeled and labeled hormonal contraceptives and endogenous sex steroids. The top chromatogram is a representative chromatogram of simultaneous analysis of E2, EE2, ENG, LNG, MPA, NET, and P4 (1 ng/ml each). Below are representative chromatograms for each individual analyte at the lower limit of quantitation (LLOQ) for each hormone, with LLOQ indicated in each respective panel. E2, estradiol; EE2, ethinyl estradiol; NET, norethisterone; ENG, etonogestrel; LNG, levonorgestrel; MPA, medroxyprogesterone acetate; P4, progesterone; IP, interference peak; d, deuterium; CPS, counts per second; min, minutes.

**Table 1**

Accuracies and precisions (expressed as the coefficients of variation, CV) for the simultaneous quantification of hormonal contraceptives and endogenous sex steroids (n=14).

| Analyte | Spiked Value (ng/ml) | Mean Assayed Value (ng/ml) | Accuracy | Intra-assay CV | Inter-assay CV |
|---|---|---|---|---|---|
| Estradiol | 0.300 | 0.285 * | 95% | 4.8% | 5.3% |
| Ethinyl Estradiol | 0.125 | 0.126 | 101% | 6.5% | 7.2% |
| Etonogestrel | 0.800 | 0.840 | 105% | 3.7% | 6.9% |
| Levonorgestrel | 0.500 | 0.473 | 95% | 4.8% | 6.4% |
| Medroxyprogesterone acetate | 1.000 | 1.082 | 108% | 6.8% | 8.7% |
| Norethisterone | 0.500 | 0.530 | 106% | 5.5% | 12.1% |
| Progesterone | 1.500 | 1.580 * | 105% | 8.1% | 9.1% |

* adjusted for endogenous E2 and P4 found in normal human serum.

**Table 2**

MS parameters used for simultaneous quantification of hormonal contraceptives and endogenous sex steroids.

| Compound | Precursor m/z | Product m/z | Type | Dwell Time (msec) | Interface Voltage (kV) | Q1 Pre Bias (V) | CE (V) | Q3 Pre Bias (V) | Retention Time (min) |
|---|---|---|---|---|---|---|---|---|---|
| E2 | **271.00** | **145.25** | Target | 40.0 | −2.0 | 13.0 | 40.0 | 27.0 | 1.516 |
| | 271.00 | 183.20 | | 40.0 | | 13.0 | 41.0 | 17.0 | |
| | 271.00 | 143.10 | | 40.0 | | 13.0 | 50.0 | 25.0 | |
| | 271.00 | 239.30 | | 40.0 | | 13.0 | 39.0 | 25.0 | |
| E2-d5 | 276.20 | 147.10 | ISTD | 30.0 | −2.0 | 20.0 | 44.0 | 29.0 | 1.499 |
| | 276.20 | 187.30 | | 30.0 | | 20.0 | 45.0 | 18.0 | |
| EE2 | **295.20** | **145.20** | Target | 40.0 | −2.0 | 21.0 | 41.0 | 26.0 | 1.487 |
| | 295.20 | 143.10 | | 40.0 | | 21.0 | 54.0 | 26.0 | |
| | 295.20 | 269.30 | | 40.0 | | 21.0 | 33.0 | 27.0 | |
| EE2-d7 | 302.20 | 150.20 | ISTD | 30.0 | −2.0 | 22.0 | 45.0 | 27.0 | 1.467 |
| | 302.20 | 272.20 | | 30.0 | | 22.0 | 29.0 | 27.0 | |
| NET | **299.20** | **109.25** | Target | 70.0 | 2.0 | −13.0 | −26.0 | −19.0 | 3.746 |
| | 299.20 | 231.10 | | 70.0 | | −13.0 | −18.0 | −24.0 | |
| | 299.20 | 83.20 | | 70.0 | | −13.0 | −27.0 | −15.0 | |
| NET-d6 | 305.05 | 113.20 | ISTD | 107.0 | 2.0 | −14.0 | −28.0 | −20.0 | 3.660 |
| | 305.05 | 237.25 | | 107.0 | | −14.0 | −19.0 | −25.0 | |
| ENG | **325.20** | **257.10** | Target | 21.0 | 2.0 | −15.0 | −16.0 | −26.0 | 4.519 |
| | 325.20 | 147.00 | | 21.0 | | −15.0 | −22.0 | −15.0 | |
| | 325.20 | 197.30 | | 21.0 | | −15.0 | −20.0 | −20.0 | |
| ENG-d7 | 332.20 | 263.25 | ISTD | 15.0 | 2.0 | −15.0 | −17.0 | −29.0 | 4.472 |
| | 332.20 | 115.10 | | 15.0 | | −15.0 | −30.0 | −21.0 | |
| LNG | **313.20** | **109.25** | Target | 21.0 | 2.0 | −14.0 | −26.0 | −20.0 | 4.563 |
| | 313.20 | 245.25 | | 21.0 | | −14.0 | −18.0 | −26.0 | |
| | 313.20 | 79.00 | | 21.0 | | −14.0 | −48.0 | −30.0 | |
| LNG-d6 | 319.20 | 251.30 | ISTD | 15.0 | 2.0 | −14.0 | −19.0 | −27.0 | 4.526 |
| | 319.20 | 114.10 | | 15.0 | | −14.0 | −28.0 | −21.0 | |
| MPA | **387.20** | **327.20** | Target | 21.0 | 0.5 | −14.0 | −14.0 | −16.0 | 4.868 |

| Compound | Precursor m/z | Product m/z | Type | Dwell Time (msec) | Interface Voltage (kV) | Q1 Pre Bias (V) | CE (V) | Q3 Pre Bias (V) | Retention Time (min) |
|---|---|---|---|---|---|---|---|---|---|
| | 387.20 | 123.10 | | 21.0 | | −14.0 | −27.0 | −23.0 | |
| | 387.20 | 285.15 | | 21.0 | | −14.0 | −18.0 | −29.0 | |
| MPA-d6 | 393.10 | 330.20 | ISTD | 15.0 | 0.5 | −11.0 | −15.0 | −23.0 | 4.856 |
| | 393.10 | 288.15 | | 15.0 | | −11.0 | −19.0 | −30.0 | |
| P4 | **315.00** | **97.10** | Target | 21.0 | 0.5 | −16.0 | −21.0 | −17.0 | 5.114 |
| | 315.00 | 109.25 | | 21.0 | | −16.0 | −22.0 | −20.0 | |
| | 315.00 | 79.10 | | 21.0 | | −16.0 | −49.0 | −29.0 | |
| P4-d9 | 324.25 | 100.20 | ISTD | 15.0 | 0.5 | −15.0 | −23.0 | −19.0 | 5.089 |
| | 324.25 | 113.10 | | 15.0 | | −15.0 | −26.0 | −20.0 | |

The mass transition for each unlabeled analyte in bold was used as the quantifying ion; all other ions were used as qualifying ions. E2, estradiol; EE2, ethinyl estradiol; NET, norethisterone; ENG, etonogestrel; LNG, levonorgestrel; MPA, medroxyprogesterone acetate; P4, progesterone; kV, kilovolts; Q, quadrupole; CE, collision energy; V, volts; ISTD, internal standard.

**Table 3**

Method lower (LLOQ) and upper (ULOQ) limits of quantification, extraction efficiencies, and matrix effects for simultaneous analysis of hormonal contraceptives and endogenous sex steroids (n=14).

| Target | LLOQ (ng/ml) | ULOQ (ng/ml) | MDL (ng/ml) | Extraction Efficiency (%) | Matrix Effect (%) |
|---|---|---|---|---|---|
| Estradiol | 0.010 | 10.000 | 0.005 | 94.6 | 81.3 |
| Ethinyl estradiol | 0.010 | 10.000 | 0.007 | 99.9 | 85.2 |
| Etonogestrel | 0.020 | 10.000 | 0.006 | 99.4 | 58.9 |
| Levonorgestrel | 0.020 | 10.000 | 0.007 | 96.8 | 77.1 |
| Medroxyprogesterone acetate | 0.020 | 10.000 | 0.003 | 94.5 | 50.8 |
| Norethisterone | 0.040 | 10.000 | 0.004 | 93.2 | 33.8 |
| Progesterone | 0.010 | 10.000 | 0.003 | 92.4 | 54.5 |

**Table 4**

Application of the method to human serum samples. Range of quantifiable hormone levels is indicated for each method (n=14).

| Delivery Method | Number of Subjects | E2 ng/ml | EE2 ng/ml | P4 ng/ml | LNG ng/ml | ENG ng/ml | MPA ng/ml |
|---|---|---|---|---|---|---|---|
| Control | 15 | <0.010–0.367 | <0.010 | <0.010–>10 | <0.020 | <0.020 | <0.020 |
| Injection | 5 | <0.010–0.046 | <0.010 | <0.010–0.017 | <0.020 | <0.020 | 0.157–2.423 |
| Implant | 5 | <0.010–0.288 | <0.010 | <0.010–0.035 | <0.020–1.830 | 0.232–0.476 | <0.020 |
| Oral | 4 | <0.010–0.230 | <0.010–0.030 | <0.010–2.370 | <0.020–6.721 | <0.020 | <0.020 |

Injection, MPA; implant, ENG or LNG; oral, EE2 or LNG.

**Table 5**

Accuracies and precisions (expressed as coefficients of variation, CV) for target-specific sample pools.

| Target Hormone | Target Value (ng/ml) | Mean Assayed Value (ng/ml) | Accuracy | Intra-assay CV | Inter-assay CV |
|---|---|---|---|---|---|
| ENG | 0.392 | 0.393 | 100% | 4.4% | 13.7% |
| LNG | 2.975 | 2.871 | 96.50% | 3.3% | 5.0% |
| MPA | 2.040 | 1.915 | 93.90% | 2.9% | 6.2% |
| NET | 6.525 | 6.035 | 92.50% | 2.0% | 4.9% |