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A Sensitive and Robust UPLC-MS/MS Method for Quantitation of Estrogens and Progestogens in Human Serum

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Abstract

Objective: With the widespread use of sex-steroid hormones in contraceptives and hormone replacement therapy, there is an increasing need for reliable analytical methods. We report the development of a sensitive and robust UPLC-MS/MS method for quantitation of both endogenous and synthetic sex-steroid hormones in human serum.

Study Design: We developed and validated a UPLC-MS/MS method to quantify progestogens (etonogestrel, levonorgestrel, medroxyprogesterone acetate, norethindrone, progesterone) and estrogens (estradiol and ethinyl estradiol) with good accuracy, high sensitivity, and excellent robustness. We then applied the method to the analysis of sex-steroid hormones in serum from 451 clinical research participants.

Results: Each UPLC-MS/MS analysis was 6.5 min. The lower limits of quantitation (LLOQs) were 25 pg/ml for the progestogens, and 2.5 and 5.0 pg/ml for estradiol and ethinyl estradiol, respectively. When estradiol was analyzed without assessment of progestogens, the LLOQ was reduced to 1 pg/ml. The calibration curves were linear from 25-50,000, 2.5-2,000 (1-2,000 for estrogens-only analysis) and 5-2,000 pg/ml, respectively. Both the accuracy and precision were below $\pm 15\%$ not only for routine validation (intraday and interday), but for long-term (> two years) assay robustness with external controls, thereby, demonstrating the utility of this method for

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Declaration of Interest

The authors declare that there is no conflict of interest that could affect the impartiality of the research reported.

Supplemental Information

The mass spectrometric conditions for quantitation of the sex-steroid hormones are listed in Supplemental Table S1, which is freely available online.

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multi-year clinical trial assessments of progestogens and estrogens. We applied the method to quantify sex-steroid levels in 1804 clinical samples.

Conclusions: We successfully developed a UPLC-MS/MS method, and overcame the matrix suppression to allow sensitive quantitation of both synthetic and endogenous sex-steroid hormones in human serum.

Keywords

Sex-steroid hormones; UPLC-MS/MS; reproducibility; quantitation; clinical sample analysis

1. Introduction

Commonly used in modern contraceptives (1, 2) and hormone replacement therapy (3), sex-steroid hormones play important roles in human health and disease. Sensitive, accurate and robust methods are needed for their quantitation to support reproductive health research and clinical practice for diagnosis, treatment and disease prevention.

Steroid immunoassays such as conventional radioimmunoassay (RIA) (4) and competitive enzyme immunoassay (EIA) (sometimes referred to as enzyme-linked immunosorbent assay (ELISA) (5)) have been widely used in clinical and environmental laboratories yet they are prone to interferences, particularly at low levels of quantification (6-8). Since the first report in 1960 (9), hyphenated methods coupling chromatographic separations (i.e. gas chromatography (GC) and liquid chromatography (LC)) and tandem mass spectrometry (MS/MS) detection have gained popularity with improved selectivity, specificity, sensitivity, speed (afforded by ultra-performance liquid chromatography (UPLC)), and multiplexing capabilities (6, 7, 10-12). In the past decade, LC-MS/MS has been increasingly becoming a method of choice while limiting the time-consuming water-free derivatization and intense analyte cleanup and concentration steps required by GC-MS/MS (7, 11).

One complication of steroid hormone analysis, particularly with estradiol, is the reduced degree of ion formation during electrospray ionization, limiting the sensitivity without chemical derivatization prior to LC-MS/MS (13). Another barrier to accurate quantification is the existence of a significant known matrix effect in plasma/serum and the identification of an estrogen-free matrix source. The question of matrix reproducibility is particularly prominent in long-term and ongoing clinical studies in which samples are to be evaluated over years of acquisition, yet such long-term assessment of assay reproducibility has not been previously reported.

We aimed to develop a sensitive and robust UPLC-MS/MS method for quantitation of both synthetic and endogenous sex-steroid hormones in human serum. Five progestogens (etonogestrel, levonorgestrel, medroxyprogesterone acetate, norethindrone, progesterone) and two estrogens (estradiol and ethinyl estradiol) (Fig. 1) were included in this study to meet the requirement of our clinical applications. Our method was designed to overcome ionization and matrix interference barriers, as well as, to provide long-term assay reproducibility for clinical sample analysis.

2. Materials and methods

2.1 Materials

We purchased etonogestrel, levonorgestrel, medroxyprogesterone acetate, norethindrone, progesterone, testosterone-d3, estradiol, ethinyl estradiol, dansyl chloride, sodium bicarbonate, ammonium acetate and formic acid from Sigma-Aldrich. Estradiol-d5 was purchased from CDN Isotopes. Progesterone and estradiol stock solutions were purchased from Abbott Laboratories. Water, methanol, acetonitrile, N-butylchloride, and sodium hydroxide were purchased from Fisher Scientific. We purchased double-stripped human serum from Golden West Biologicals.

2.2 Calibration and quality control standards

The progestogen panel: We prepared calibration stocks (1 mg/ml) in methanol for each of the five progestogens purchased from Sigma (etonogestrel, levonorgestrel, progesterone, norethindrone and medroxyprogesterone acetate). Calibration standards were prepared at 25, 50, 100, 250, 500, 1,000, 2,000, 10,000 and 20,000 pg/ml in double-stripped human serum. Quality control (QC) standards were prepared at 35, 175, 875, 10,000, 25,000 and 50,000 pg/ml from separate stock solutions. We diluted a progesterone stock solution purchased from Abbott Laboratories to make a 35 or 100 pg/ml external QC standard. A testosterone-d3 stock solution (100 µg/ml) was diluted in methanol to 20 ng/ml as an internal standard.

The estrogen panel: We prepared calibration stock solutions of estrogens (estradiol and ethinyl estradiol) from Sigma in a similar way. The calibration standards were made at 1, 2.5, 5, 10, 25, 50, 75, 100, 200, 500, 1000 and 2,000 pg/ml. QC standards were prepared at 3, 30 (or 50), 90 pg/ml for the low range or 35, 175, and 875 pg/ml for the high range samples. We prepared a 25 pg/ml external QC standard of estradiol by dilution of a stock solution purchased from Abbott Laboratories. An estradiol-d5 stock solution (1 µg/ml) was diluted to 0.5 ng/ml as an internal standard. It should be mentioned that extreme care must be taken when weighing powdered estradiol to avoid contamination of reagents and equipment near the balance. A significant portion of the powder becomes airborne. We have addressed this problem by never weighing estradiol powder in the same sample preparation room.

The progestogen and estrogen panels: We prepared a single series of calibration standards with the desired progestogen and estrogen concentrations with both internal standards present. At least eight calibration standards and three QC standards were included per sample batch, and their concentrations were chosen according to the sample source (pre- or post-menopausal women, men, or children) and expected steroid levels (low range for endogenous only or high range with hormone treatment) within the batch.

2.3 Precision, accuracy, linearity, limits of detection and quantitation

The linearity and sensitivity was evaluated by analyzing sex-steroid hormones over a broad concentration range (8 calibration solutions) in duplicate and spanning three days. Calibration curves were calculated based on weighted (1/Y) linear regression of peak area ratios plotted against steroid hormone concentrations in pg/ml. Intraday and interday

accuracy and precision were determined by analyzing three QCs (low, medium, high) in six replicates on two different days followed by twelve replicates on a third day.

2.4 Processing efficiency, matrix effect, sample stability and specificity

Progestogens were spiked into different stages of the sample extraction procedures. All the samples were run in six replicates under each condition to determine the matrix effect and processing efficiency. In addition, we spiked the sex-steroid hormones at different concentrations into Lyphochek (prepared from human serum by BioRad Laboratories) and human plasma from central blood bank to check specificity. The sample stability was verified online by cycling the introduction of calibration solutions and QCs with each batch of clinical samples.

2.5 Robustness and reliability

To further validate our UPLC-MS/MS method and test its robustness and reliability, we purchased external quality control samples from a separate vendor (Abbott Laboratories) and analyzed them multiple times over a two-year period.

2.6 Sample preparation

Serum samples of 0.5 ml each were spiked with 10 μ l of 20 ng/ml testosterone-d3 and/or 25 μ l of 0.5 ng/ml estradiol-d5 as internal standard(s). The sample preparation procedures were adapted from previous work (14, 15) with slight modifications. Briefly, the samples were mixed, followed by addition of 3 ml N-butylchloride. After vortexing for 2 min and centrifugation for 10 min at full speed (Thermo Scientific CL2), approximately 2.5 ml of the organic layer was transferred to new tubes and then vaporized under a gentle nitrogen stream at 40 °C. The residue was reconstituted in 50 μ l of 50:50 methanol: water. Half of the reconstituted sample (25 μ l) was saved for progestogen analysis, and the remaining half (25 μ l) was evaporated under nitrogen. For derivatization of estrogens, 50 μ l of 50 mM bicarbonate buffer (pH adjusted to 10.5) and 50 μ l of 1 mg/ml dansyl chloride in acetonitrile were added. The mixture was then heated to 60 °C for 3 min.

2.7 UPLC-MS/MS

A Waters Acquity UPLC system was used to separate the sex-steroid hormones at 55 °C with a UPLC BEH C18, 1.7 μ m (2.1 mm \times 150 mm) reversed phase column (Waters) protected by a guard (2.1 mm \times 5 mm) (Waters). The injection volume was 7.5 μ l. The flow rate was 0.3 ml/min. Mass spectrometric detection was conducted via a TSQ Quantum Ultra (Thermo Fisher Scientific) using a heated electrospray ionization (HESI) source in the positive mode. The collision gas pressure was kept at 1.5 mTorr and the scan time set at 0.010 s. Selected reaction monitoring (SRM) was used for quantitation (Supplemental Table S1). Two separate injections were made under different LC conditions for the progestogen and estrogen panels to optimize sensitivity.

The progestogen panel: Mobile phase A consisted of 2 mM ammonium acetate, 0.1% formic acid in water and mobile phase B contained 100% methanol. The mobile phase composition was held at 50% B for 0.5 min and then increased to 85% B in a linear fashion over 3 min.

After maintaining at 85% B for 1 min, the mobile phase was returned to the initial composition in 2 min.

The estrogen panel: Mobile phases consisted of 0.1% formic acid in water (A) and 100% acetonitrile (B). The mobile phase composition was held at 50% B for 1 min and then increased to 85% B in a linear fashion over 3 min. After maintaining at 85% for 1 min, the mobile phase was returned to the initial composition in 1.5 min.

2.8 Clinical samples

We performed a Zim CHIC parallel cohort study ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02038335) number: NCT02038335) approved by The University of Pittsburgh Institutional Review Board and The Medical Research Council of Zimbabwe (16). Our UPLC-MS/MS assay was used to quantify progestogens and estrogens in a total of 1804 serum samples collected from 451 women.

Results

3.1 Method development

To achieve optimal chromatographic separation and adequate MS sensitivity, we found it necessary to use slightly different UPLC-MS/MS conditions for progestogens as compared to estrogens. Sample preparation included liquid-liquid extraction with N-butylchloride followed by derivatization of estrogens by dansyl chloride.

3.2 Method validation

We largely followed the 2013 FDA guidelines on bioanalytical method validation (17).

3.2.1 Precision and accuracy—Accuracy is the relative deviation in the calculated value of a standard from its true value as defined by relative error (RE). Precision is the coefficient of variation (CV) of the mean concentrations. Both intraday and interday accuracy and precision fell within $\pm 15\%$ for all the steroids (Table 1).

3.2.2 Linearity and sensitivity—The linear ranges were 25-50,000 pg/ml (8 calibration standards between 25-2,000 pg/ml and validated with additional QC standards at 10,000, 25,000 and 50,000 pg/ml) for progestogens, 2.5-2,000 and 5-2,000 pg/ml for estradiol and ethinyl estradiol, respectively. It should be noted that a wider linear range from 1-2,000 pg/ml was achieved when estradiol was analyzed without progestogen assessment. All the calibration standards fell within 15% RE of back-calculated amounts from nominal spiked amounts for all analytes.

Lower limit of quantitation (LLOQ) was defined as the lowest concentration that could be measured with RE < 20% and CV < 20%. LLOQs were 25 pg/ml for progestogens, 2.5 pg/ml (1 pg/ml without progestogen assessment) and 5.0 pg/ml for estradiol and ethinyl estradiol, respectively (Fig. 2, Table 1).

3.2.3 Processing efficiency, matrix effect, sample stability and specificity—Matrix effect was the relative difference in measured concentrations of sex-steroid hormones

between samples spiked into the blank matrix after extraction and those spiked into solvents. We found matrix effect to be between -8% and 37% at 35 pg/ml, and between -15% and -8% at 875 pg/ml, respectively.

Processing efficiency was defined as the ratio of measured sex-steroid hormone concentrations between samples spiked into the blank matrix before extraction and those spiked into solvents. Therefore, processing efficiency accounted for both the recovery and matrix effect. We found processing efficiency to be between 65% - 83% at 35 pg/ml, and 53% - 63% at 875 pg/ml, respectively. Similarly, the matrix effect and processing efficiency for estradiol at 100 pg/ml was 48% and 64%, respectively. All the REs were between -1.2% and 13.7%. Double-stripped human serum was used as the matrix for all the calibration standards and QCs to compensate for the matrix effect and processing efficiency.

For the steroids spiked into Lyphochek and human plasma, all the measured amounts were found to agree with the spiked amounts with REs 15%, demonstrating the good recovery, selectivity and specificity of our method. The latter two characteristics were also made possible by the inherent specificity of the triple quadrupole mass spectrometer, which allowed us to monitor the specific transitions for the analytes of interest. Though estradiol and ethinyl estradiol share the same fragment ion at m/z 171, their precursor ions have different m/z values (506 and 530, respectively). Therefore, two distinct transitions (506 \rightarrow 171, 530 \rightarrow 171) could be monitored to distinguish the two estrogens.

Fresh standards were constantly compared with those having undergone up to four freeze-thaw cycles. No noticeable change in stability was observed.

3.3 Robustness and reliability

For the external QCs assayed over a two-years, the differences between the measured and true values were very small and the REs were all below 4%. The CVs fell below 13% for both progesterone and estradiol (Table 2).

3.4 Quantitation of sex-steroid hormones in clinical samples

The sex-steroid hormones were quantified for all the 1804 serum samples collected from the Zim CHIC parallel cohort study. All samples that were positive for a sex-steroid hormone are reported here for each hormone as median with interquartile range (IQR) (Table 3). The progestogen and estrogen levels in these clinical samples spread over three and two orders of magnitude, respectively, yet all were within our linear dynamic ranges.

4. Discussion

We developed a fast, sensitive and selective UPLC-MS/MS method for the detection of five progestogens and two estrogens (Fig. 1). Good accuracy and precision (Table 1) were achieved with the utilization of a single deuterated internal standard per panel, making our method cost effective. The specific sex-steroid hormones included in the panels were selected to cover the full spectrum of regionally available contraceptives at the time of the Zim CHIC parallel cohort study for which these panels were developed. The 25 and 1 pg/ml LLOQs were among the lowest compared to what has been reported by others for

progesterones (15, 18-22) and estrogens (11, 15, 18, 20, 23), respectively. It should be noted that the higher sensitivity we achieved in estrogen LLOQs compared to what Blue et al. recently reported (24) might be attributable to the derivatization we have used to enhance the ionization efficiency of estradiol and ethinyl estradiol (13).

Among the numerous reports on UPLC-MS/MS analysis of steroids in circulation, many quantified only one or two analytes and/or multiple analytes within the same class. Endogenous steroids were often the sole focus even for multi-panel multiple steroid analysis (11, 18, 25-35), while only a few included both endogenous and synthetic compounds (19, 22). One notable exception is a paper published by Blue et al. (24) (see discussions above), otherwise, we found no previous publications that include sensitive assessment of both synthetic and endogenous steroids in both progesterone and estrogen panels.

The robustness and reproducibility of our assay was demonstrated with the measured accuracy (REs < 4%) and precision (CVs <13%) for our external quality control samples over a period of two years (Table 2). This is an objective assessment of our UPLC-MS/MS method, demonstrating the robustness and reliability with good accuracy, precision and reproducibility over time. This is critical for clinical research sample analysis that often extends multiple years with combined sample analysis for interpretation. Such long-term assessment of assay reproducibility has not been previously reported.

The double-stripped human serum (Golden West Biologicals) we used as matrix was deprived of endogenous estrogens. It still contains trace amount of progesterone (Fig. 2), which didn't interfere with sex-steroid hormone analysis when the actual level of progesterone was much higher for clinical sample analysis. Furthermore, our method was fully validated with 0.2% cyclodextrin as a true blank.

Our UPLC-MS/MS method has been optimized in response to clinical and research needs and thus has evolved over time. It has allowed us to accurately and precisely quantify estrogen and/or progesterone levels in pre- peri- and post-menopausal women (16, 36-39), men (36), children and young female rats (40). It is also notable that our method is not limited to the sex-steroid hormones reported in this manuscript. It is likely to be extended to other steroid metabolites as well since we have also adapted this method for the analysis of estrone (36).

Clinical studies have largely relied on participant self-report for important variables including last menstrual period (LMP) and contraceptive use to critically classify them into analysis cohorts. This self-reported status has likely often resulted in mixed analysis cohorts and published outcome data from studies accessing use of hormonal contraception and HIV acquisition risk have been similarly mixed and difficult to interpret (41). To this point, our recent Zim CHIC parallel cohort study demonstrated that only 64% of the overall samples were associated with accurate reporting of contraceptive use (16). This work is considered broadly applicable to contraceptive researchers as studies that rely on self-reporting to identify contraceptive hormone exposure could suffer from significant misclassification. Thus, the methodology described here is likely widely applicable to studies that include women using contraceptive hormones.

In conclusion, a sensitive and robust UPLC-MS/MS method for quantitation of serum progestogens and estrogens was developed and validated. The sensitivity, specificity and longterm assay reproducibility of our method makes it possible to verify and closely monitor sex-steroid hormone concentrations of participants in ongoing clinical studies and patients on hormonal contraception or hormone replacement therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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IMPLICATIONS

We developed a sensitive and robust UPLC-MS/MS method to accurately measure the levels of sex-steroid hormones in serum. The method overcame matrix interference barriers and achieved excellent long-term stability and reproducibility (96.9% accuracy; 13.0% relative variability measured with external controls over 2 years), demonstrating its utility in clinical sample analysis.

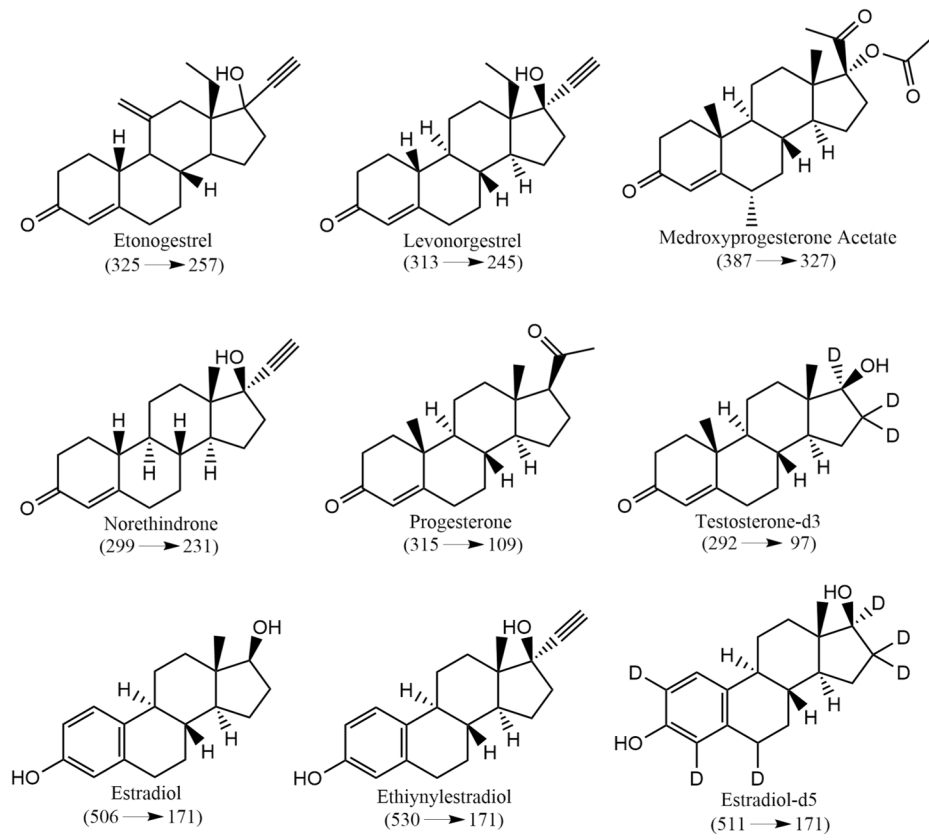


Figure 1. Sex-steroid hormones studied in this work. The SRM transitions used for quantitation are listed in parenthesis.

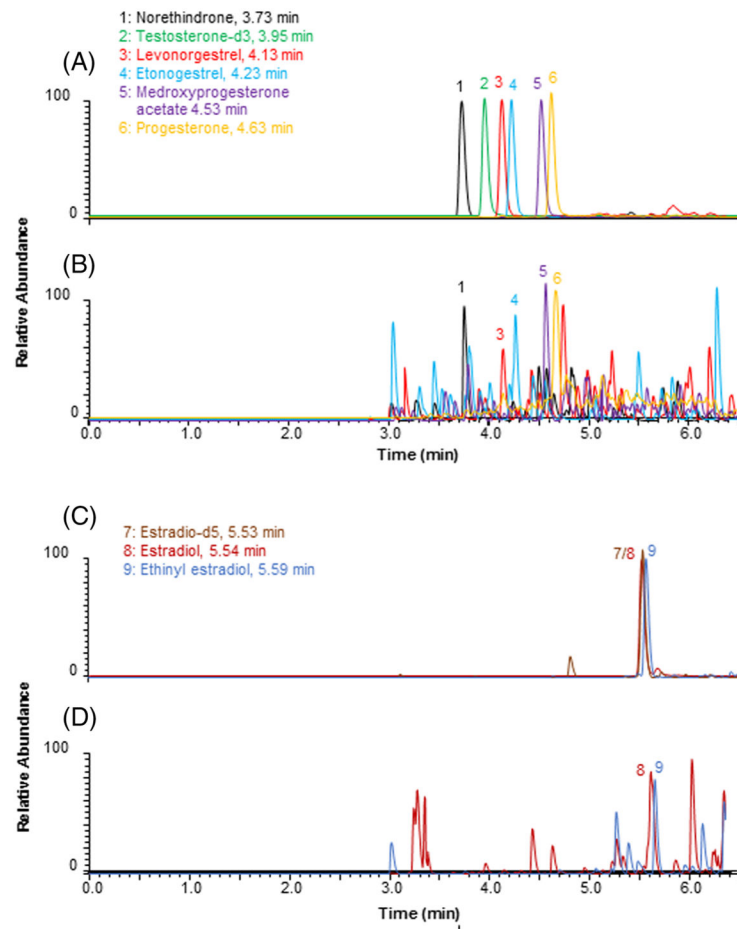


Figure 2. Representative UPLC-MS/MS chromatograms of sex-steroid hormones spiked to double stripped human serum. (A) progesterogens at 1,000 pg/ml and internal standard testosterone-d3 at 400 pg/ml, (B) progesterogens at LLOQs (25 pg/ml), (C) estrogens at 100 pg/ml and internal standard estradiol-d5 at 25 pg/ml, (D) estrogens at LLOQs (2.5 and 5.0 pg/ml for estradiol and ethinyl estradiol, respectively).

Table 1.

Accuracy and precision of our UPLC-MS/MS method.

Sex-Steroid Hormone	Experiment	Spiked (pg/ml)	Intraday Measured (mean±SD) (pg/ml)	RE (%) ^a	CV (%) ^b	Interday Measured (mean±SD) (pg/ml)	RE (%) ^a	CV (%) ^b
Etonogestrel	LLOQ	25	25.0±1.4	0.0	5.7	27.0±2.0	8.0	7.4
		35	34.0±4.1	-2.9	11.9	36.6±3.4	4.6	9.2
	QC	175	163±11.4	-6.9	7.0	170±13.8	-2.9	8.1
		875	776±17.8	-11.4	2.3	827±68.4	-5.5	8.3
Levonorgestrel	LLOQ	25	27.5±0.7	10.0	2.6	26.7±0.8	6.7	3.1
		35	35.7±3.9	2.0	11.0	35.7±3.6	2.0	10.0
	QC	175	185±9.7	5.9	5.2	176±14.4	0.5	8.2
		875	972±15.8	11.1	1.6	888±80.0	1.4	9.0
Medroxyprogesterone Acetate	LLOQ	25	28.5±0.7	14.0	2.5	25.7±2.7	2.7	10.4
		35	33.0±2.6	-5.7	7.7	34.6±3.0	-1.1	8.8
	QC	175	154±6.4	-12.0	4.2	170±16.3	-3.0	9.6
		875	784±39.6	-10.4	5.1	862±72.3	-1.4	8.4
Norethindrone	LLOQ	25	25.0±2.8	0.0	11.3	25.2±2.6	0.8	10.3
		35	33.8±2.8	-3.4	8.3	35.0±3.3	0.0	9.3
	QC	175	154±2.6	-12.0	1.7	164±12.2	-6.5	7.5
		875	783±35.3	-10.5	4.5	843±65.9	-3.7	7.8
Progesterone	LLOQ	25	23.0±1.4	-8.0	6.1	24.2±3.6	0.0	14.9
		35	31.8±1.8	-9.1	5.6	34.9±3.1	-0.3	9.0
	QC	175	160±8.9	-8.7	5.6	169±12.0	-3.6	7.1
		875	756±29.2	-13.7	3.9	819±69.2	-6.4	8.5
Estradiol	LLOQ	2.5	2.62±0.2	4.6	5.7	2.71±0.1	8.4	4.4
		35	36.9±2.1	5.4	5.8	36.4±3.1	4.0	8.4
	QC	175	180±16.4	2.7	9.1	175±19.3	0.1	11.0
		875	969±43.4	10.7	4.5	902±81.6	3.1	9.1
EthinyI Estradiol	LLOQ	5	4.75±0.4	-5.0	8.0	5.00±0.5	0.1	9.7
		35	31.8±1.9	-9.1	6.1	35.8±3.6	2.3	10.1
	QC	175	189±10.2	8.0	5.4	170±18.9	-3.0	11.1
		875	992±33.1	13.4	3.3	840±97.5	-4.0	11.6

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$RE = \frac{\text{relative error} = (\text{calculated value} - \text{true value}) / \text{true value} * 100}{p}$

$CV = \frac{\text{relative standard deviation} = \text{sample standard deviation} / \text{sample mean} * 100}{q}$

Intraday RE ranged from -13.7% to 14.0%, interday RE ranged from -6.5% to 8.4%.

Intraday CV ranged from 1.6 to 11.9%, interday CV ranged from 3.1 to 14.9%.

Table 2.

Robustness and reliability of our UPLC-MS/MS method.

Sex-Steroid Hormone	External QC (pg/ml)	Measured (mean±SD) (pg/ml)	# of Measurements	Time Period	RE (%) ^a	CV (%) ^b
Progesterone	35	34.4±3.8	46	02/25/2015 - 07/07/2016	1.7	11.1
	100	103±7.3	18	07/14/2016 - 02/22/2017	3.1	7.1
Estradiol	25	24.5±3.2	61	01/13/2015 - 02/22/2017	2.0	13.0

^aRE=relative error=(calculated value-true value)/true value * 100.

^bCV=relative standard deviation=sample standard deviation/sample mean* 100.

Summary of sex-steroid hormone levels (pg/ml) in clinical serum samples for the Zim CHIC parallel cohort study.

Table 3.

Sex-Steroid Hormone	Etonogestrel	Levonorgestrel	Medroxyprogesterone Acetate		Norethindrone	Progesterone	Estradiol	Ethinyl Estradiol
# of Samples	186	391	467	211	1239	1475	147	
Minimum Value	26	25	26	29	25	10	10	
Maximum Value	2249	23839	3358	4952	36956	833	1245	
IQR ^a 25th Percentile	241	440	182	771	33	29	20	
Median	309	722	278	1156	46	48	39	
IQR ^a 75th Percentile	389	2620	553	1636	87	94	97	

^aIQR=Interquartile range.