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Development and validation of an expanded panel of progestins using liquid chromatography-tandem triple quadrupole mass spectrometry (LC-MS/MS) to monitor protocol compliance in hormonal contraceptive pharmacokinetic/pharmacodynamic studies

Jeffrey T. Jensen, MD MPH[a], **Bethany T. Waites, MD MCR**[a,1], **Emily R. Boniface, MPH**[a], **Sara McCrimmon, MPH**[a], **Steven Blue, BS**[b], **David W. Erikson, PhD**[b]

[a]Department of Obstetrics and Gynecology, Oregon Health & Science University, Portland, Oregon 97239

[b]Endocrine Technologies Core, Oregon National Primate Research Center, Beaverton, Oregon 97006

Abstract

We developed and validated use of ultra-high performance liquid chromatography-heated electrospray ionization-tandem triple quadrupole mass spectrometry (LC-MS/MS) to simultaneously analyze serum concentrations of ethinylestradiol (EE), dienogest (DNG), norelgestromin (NGMN), norethindrone (NET), gestodene (GSD), levonorgestrel (LNG), etonogestrel (ENG), segesterone acetate (NES), medroxyprogesterone acetate (MPA), and drospirenone (DRSP). The calibration range for all targets was 0.009 to 10 ng/ml, with lower limit of quantification of 0.009 ng/ml for all analytes except GSD (0.019 ng/ml). We used our assay to check compliance among participants in a clinical trial, confirmed use of DRSP in 11/13 study participants, and evidence of noncompliant progestins in 2 (LNG=1, NET =1). We conclude that this approach provides an accurate method to check protocol compliance in contraceptive clinical trials.

Keywords

progestins; assays; compliance; liquid chromatography

1. Introduction

Randomized trials control for unmeasured baseline confounding and provide the highest quality clinical evidence [1]. However, in small studies, randomization may not result in an even distribution of known confounders, let alone unmeasured behavioral factors. Several

Corresponding author: Jeffrey T. Jensen, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, mail code UHN-70, Portland, Oregon 97239, (503) 494-5113, jensenje@ohsu.edu.

[1]Present address: Kaiser Permanente San Francisco, 2425 Geary Boulevard, San Francisco, CA 94239

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investigators have demonstrated nonadherence to treatment regimens as a factor influencing outcomes in contraceptive clinical trials [2, 3], and epidemiological studies evaluating effects of contraceptive methods on other health outcomes have also demonstrated participant non-compliance [4, 5]. Indeed, differences in compliance likely explain discrepancies in the Pearl Indexes between European and United States studies of the same oral contraceptive formulations.

Liquid chromatography-tandem triple quadrupole mass spectrometry (LC-MS/MS) provides a platform to simultaneously evaluate serum samples for a panel of natural and contraceptive steroids [6, 7]. Recently, investigators applying this approach documented that 17% of samples from women reporting injectable use, 62% of samples from self-reported OC users, and 8% of samples from self-reported implant users had no quantifiable hormones [5].

Here, we report the development and use of an expanded LC-MS/MS platform that includes all progestins marketed in the United States.

2. Materials and Methods

We previously reported details of methods used by the Endocrine Technologies Core (ETC) at the Oregon National Primate Research Center (ONPRC, Beaverton, OR) to develop an assay for simultaneous analysis of a panel of contraceptive and ovarian steroids [6]. We modified this approach to establish a new assay for simultaneous measurement of estradiol (E2); progesterone (P4), ethinylestradiol (EE), dienogest (DNG), norelgestromin (NGMN), norethindrone (NET), gestodene (GSD), levonorgestrel (LNG), etonogestrel (ENG), segesterone acetate (NES), medroxyprogesterone acetate (MPA), and drospirenone (DRSP) using ultra-high performance liquid chromatography-heated electrospray ionization-tandem triple quadrupole mass spectrometry (LC-MS/MS) on a Shimadzu Nexera-LCMS-8050 instrument (Kyoto, Japan). We used LabSolutions Software, V5.72 (Shimadzu) for all of the processing and analysis. Briefly, 200 µl of serum were mixed with 100 µl of 10:90 (v:v) LC-MS grade methanol (Honeywell Burdick & Jackson, St Louis, MO, USA): LC-MS grade water (Honeywell Burdick & Jackson, St Louis, MO, USA) containing a mix of synthetic [EE-d7, NGMN-d6, NET-d6, GSD-d6, LNG-d6, ENG-d7, NES-C3, MPA-d6, and DRSP-C3 (Toronto Research Chemicals, Toronto, CA)] and natural [estradiol (E2-d5, Cerilliant, Round Rock, Texas), progesterone (P4-C3, IsoSciences Ambler, PA, USA] isotopic standards and added to a 400 µl SLE+ extraction plate (Biotage, Uppsala, Sweden). Isotopic standard concentrations were determined so as to yield 1,000,000 – 2,000,000 peak area for positive mode targets (P4, DNG, LNG, NET, GSD, NGMN, ENG, NES, DRSP, and MPA) and 100,000 – 200,000 peak area for negative mode targets (E2, EE). Steroids were eluted with 3 x 600 µl dichloromethane (Sigma, St. Louis, MO) into 2 mL 96-well round bottom polypropylene plates (Analytical Sales & Services, Flanders, NJ, USA) containing 100 µL of 2-propanol (Sigma-Aldrich, St. Louis, MO, USA), dried with forced air, and reconstituted in 50 µl of 25% (v:v) methanol:water. For calibration curves, we spiked charcoal-stripped human serum (Golden West Biologicals) with unlabeled P4 (IsoSciences Ambler, PA, USA), E2 and EE (Cerilliant, Round Rock, TX), and DNG, NGMN, NET, GSD, LNG, ENG, NES, MPA, and DRSP standards (Toronto Research Chemicals, Toronto, CA) in methanol, diluted serially to final concentrations

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between 0.009 and 10 ng/ml in a 12-point curve including a 0 ng/ml blank, subjected 200 μl of standard to the SLE+ extraction procedure as described above, and then used the Shimadzu SIL-30CAMP autosampler to inject 15 μl samples onto a Raptor 2.7 μm Biphenyl 50 mm X 2.1 mm column with a matching 5 mm x 2.1 mm guard column (Restek, Bellefonte, PA). Mobile phase consisted of 0.15 mM ammonium fluoride (Sigma) in water (A), and methanol (B) with a flow rate of 0.5 ml/min. Using a Shimadzu Nexera LC-30AD system (LC), the gradient elution started at 60% B, increased (0.50-3.50 min) to 65% B (held 3.50-4.25 min), further increased (4.25-7.25 min) to 95% B and then (7.25-7.35 min) where it was held at 100% B (7.35-8.05 min) before being returned (8.05-8.15 min) to 60% B and held at 60% for column re-equilibration for a total of 9.55 minutes/sample. E2 and EE were detected in negative ion mode and all other targets were detected in positive ion mode, all with multiple reaction monitoring (MRM) using a Shimadzu LCMS-8050 tandem triple-quadrupole MS with heated electrospray ionization (ESI). More details regarding method validation and assay performance are included in the Supplemental Methods.

3. Results

We achieved a calibration range for all targets of 0.009 to 10 ng/ml, with lower limit of quantification (LLOQ) of 0.009 ng/ml for E2, P4, EE, MPA, LNG, ENG, NET, NGMN, NET, DNG, and DRSP and 0.019 ng/ml for GSD. Table 1 provides all data relating to method validation including intra-and inter-assay variability, detection limits, extraction efficiency, and matrix effects. Supplemental Table 1 provides the MRM transitions and retention times for each target. The working solution concentrations used for Internal Standards are provided in Supplemental Table 2. We provide a representative chromatogram as Supplemental Figure 1

We used the assay to evaluate samples obtained from a randomized controlled double-blind pilot study (Clinical Trials #NCT03418363) designed to evaluate the effects of an oral supplement on hepatic protein levels in current users of an EE/DRSP combined oral contraceptive (COC) for compliance using the study pill. The institutional review board at Oregon Health and Science University approved this study. Among the 13 participants who completed the study, 11 showed detectable concentrations of DRSP (Table 2). We did not identify any samples above the method detection limit, but below the lower limit of quantification. We found evidence of protocol non-compliance in two participants (NET and LNG one each).

4. Discussion

We report the first validated serum assay for simultaneous evaluation of all synthetic contraceptive hormones in use in the United States. Of note, the assay includes DRSP and the recently approved DNG and NES. As the assay also includes GSD, a progestin commonly used in the United Kingdom and other countries, it provides a comprehensive platform for the evaluation of serum samples for contraceptive steroid use in clinical trials to assess protocol compliance or non-compliance. The assay has high sensitivity and reproducibility.

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This assay allowed us to detect noncompliance with reported use of a DRSP OC. Two of the 13 samples from this clinical trial did not contain DRSP, and the assay detected LNG and NET (15% noncompliance).

A major strength of our assay is its high sensitivity to detect picogram quantities of all contraceptive progestins in clinical use, along with natural ovarian hormones, in a single test. However, as the minimum concentrations of a progestin required to exert a contraceptive effect (e.g. inhibition of ovulation) has not been rigorously determined for most ligands, determining whether the limits of our assay have clinical relevance will require additional pharmacokinetic and pharmacodynamic testing.

Noncompliance with study protocols decreases study validity, particularly in small pharmacokinetic and pharmacodynamic studies that rely on subjects' use of a specific compound. Researchers and clinicians should take this into consideration when interpreting study results, and consider use of an objective measure to verify protocol compliance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Implications:

The availability of a LC-MS/MS multi-progestin analysis panel for simultaneous evaluation of the most common contraceptive steroids approved worldwide could improve monitoring of compliance and protocol adherence in clinical trials.

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Table 1.

Method validation parameters acquired during development and validation of an expanded panel of progestins using liquid chromatography-tandem triple quadrupole mass spectrometry (LC-MS/MS).

Hormone	QC	Target Concentration (ng/ml)	Measured Concentration (ng/ml)	Accuracy (%)	Intra-Assay Precision (%CV)	Inter-Assay Precision (%CV)	Extraction Efficiency (Recovery)	Matrix Effects	LLOQ (ng/ml)	ULOQ (ng/ml)	MDL (ng/ml)
E2	Low	0.09	0.089	98.9	4.9	6.7	93.9%	100.3%	0.009	10	0.007
	High	0.28	0.267	95.4	3.8	4.2					
P4	Low	0.25	0.298	119.2	2.5	4.9	88.9%	83.5%	0.009	10	0.003
	High	1.85	2.083	112.6	2.2	3.4					
EE	Low	0.07	0.071	101.4	4.2	5.2	99.7%	99.2%	0.009	10	0.003
	High	0.23	0.238	103.5	2.6	2.9					
NMGN	Low	0.08	0.086	107.5	4.6	6.1	82.0%	87.8%	0.009	10	0.002
	High	0.40	0.418	104.5	3.2	4.7					
NET	Low	0.06	0.062	103.3	3.4	5.8	91.7%	90.2%	0.009	10	0.003
	High	0.44	0.399	90.7	2.5	3.6					
GSD	Low	0.05	0.049	98.0	6.4	10.6	97.8%	99.2%	0.019	10	0.013
	High	0.30	0.313	104.3	2.2	8.3					
LNG	Low	1.60	1.658	103.6	2.3	4.4	98.9%	82.8%	0.009	10	0.002
	High	3.80	3.950	103.9	1.0	4.4					
ENG	Low	0.08	0.093	116.3	3.6	8.3	96.4%	81.1%	0.009	10	0.008
	High	0.53	0.605	114.2	1.4	5.3					
NES	Low	0.06	0.064	106.7	1.8	4.3	83.5%	93.2%	0.009	10	0.003
	High	0.48	0.485	101.0	1.2	4.6					
MPA	Low	0.06	0.067	111.7	5.2	6.3	70.3%	89.6%	0.009	10	0.002
	High	1.00	1.092	109.2	3.1	3.8					
DRSP	Low	0.09	0.097	107.8	2.4	5.1	86.0%	107.1%	0.009	10	0.002
	High	0.44	0.460	104.5	0.5	4.4					
DNG	Low	0.09	0.083	92.2	6.1	7.3	94.9%	94.0%	0.009	10	0.002
	High	0.44	0.350	80.0	1.8	5.0					

QC = quality control; LLOQ = lower limit of quantification; ULOQ = upper limit of quantification; MDL = method detection limit; E2 = estradiol; P4 = progesterone; EE = ethinyl estradiol; NGMN = norelgestromin; DNG = dienogest; NET = norethindrone; GSD = gestodene; LNG = levonorgestrel; ENG = etonogestrel; NES = segesterone acetate; MPA = medroxyprogesterone acetate (MPA); DRSP = drospirenone.

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Table 2.

Concentrations (ng/mL) of progestins detected using the LCMS/MS panel in serum from participants with reported use of an ethinyl estradiol/drospirenone combined pill.

Subject	Drospirenone	Levonorgestrel	Norethindrone
1	25.27	ND	ND
2	32.44	ND	ND
3	17.57	ND	ND
4	18.57	ND	ND
5	28.38	ND	ND
6	ND	ND	3.30
7	13.01	ND	ND
8	10.33	ND	ND
9	23.40	ND	ND
10	14.36	ND	ND
11	36.21	ND	ND
12	33.19	ND	ND
13	ND	2.40	ND

ND = not detected