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RESEARCH PAPER

Development and validation of an LC-MS/MS assay for the quantification of allopregnanolone and its progesterone-derived isomers, precursors, and cortisol/cortisone in pregnancy

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Abstract

Neuroactive steroids are potent neuromodulators that play a critical role in both maternal and fetal health during pregnancy. These stress-responsive compounds are reportedly low in women with perinatal depression and may be associated with poor pregnancy outcomes in animal models. Chronic stress is a risk factor for adverse birth outcomes. Simultaneous quantification of neuroactive steroids, in combination with stress hormones cortisol/cortisone, provides an opportunity to investigate the synergistic relationship of these analytes within the convenience of one assay. A simple, reliable, and sensitive method for quantifying these endogenous compounds is necessary for further research with the potential to advance clinical diagnostic tools during pregnancy. Analytes were extracted from serum with a simple protein precipitation using methanol and then separated and quantified using high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS). After online extraction, analytes were separated using an Agilent Poroschell 120, 50 × 4.6 mm, 2.7 μm particle size, EC-C18 analytical column. The reliable quantification range was from 0.78 to 1000 ng/mL. QC sample inter- and intraday trueness was between 90 and 110% while inter- and intraday imprecision was less than 10%. Extracted samples were stable up to 7 days at 4 °C and extraction recovery was above 95%. Serum samples from 54 women in pregnancy were analyzed using this method. Here, we provide a validated, fast, and specific assay with sufficient sensitivity that allows for simultaneous quantification of blood serum concentrations of allopregnanolone (3α-hydroxy-5α-pregnan-20-one), pregnanolone (3α-hydroxy-5β-pregnan-20-one), epipregnanolone (3β-hydroxy-5β-pregnan-20-one), pregnenolone, progesterone, cortisol, and cortisone in pregnancy for clinical study samples and clinical diagnostics.

Keywords Allopregnanolone · Neurosteroids · Liquid chromatography–tandem mass spectrometry (LC-MS/MS) · Biomarker · Pregnancy · Diagnostic

Introduction

Neuroactive steroids rapidly alter neuronal excitability and are synthesized from cholesterol de novo in the brain and in the common steroidogenic organs [1]. These neuroprotective compounds modulate many CNS functions such as memory and cognition in addition to behavioral and emotional effects including anxiety and depression [2–4]. Neuroactive steroids generally mediate their actions at neurotransmitter receptor sites such as γ-aminobutyric acid type A receptor ($GABA_A$), N-methyl-D-aspartate receptor (NMDA), and sigma-1 receptor, instead of at classic steroid hormone receptor sites [5]. Allopregnanolone, the predominant progesterone metabolite and most studied neuroactive steroid, is a potent,

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positive allosteric $GABA_A$ modulator, inhibiting neuronal excitability resulting in anxiolytic, sedative, and anticonvulsant effects on the body [6–9].

Neuroactive steroids are "stress-responsive," and low levels of these endogenous compounds have been implicated in a number of conditions related to dysregulated stress response [10–15]. Maternal stress is a key variable affecting maternal–placental–fetal–neuroendocrine system plasticity [16]. Investigation of the combination of neuroactive steroids and stress hormones may yield a greater understanding of the synergistic relationship these compounds share in relation to both maternal and fetal health during pregnancy [17]. Low allopregnanolone levels during the perinatal (pregnancy, birth, and postpartum) period have been linked directly to mood disturbances [17–21]. Notably, in March 2019, a synthetic version of allopregnanolone (brexanolone IV) was approved for the first ever treatment of postpartum depression [22]. Animal models demonstrate low levels of allopregnanolone late in pregnancy result in adverse birth outcomes, including decreased gestational length, asphyxia-induced brain injury to the fetus, impaired cognitive and neuroendocrine function, and developmental issues for the offspring later in life [23–28].

Pregnancy and the perinatal period are promising areas for further research of neuroactive steroids with the potential to advance clinical diagnostic tools [29]. Neuroactive steroids circulate in low concentrations in the body; however, in pregnancy, they increase significantly, reaching the highest serum concentrations at term (between 37 and 42 weeks' gestation) with maximum mean maternal serum concentrations of approximately 75 nmol/L (23.6 ng/mL) [30–33]. These compounds are readily obtained in blood—making procedures for diagnostic quantification no more invasive than routine blood work already incorporated into perinatal care [34]. A sensitive and accurate method for detecting and quantifying endogenous concentrations of neuroactive steroids is essential for advancing our understanding of these compounds during pregnancy.

High-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS) provides a reliable and accurate multi-analyte technology for quantification of neuroactive steroids in blood for clinical use [21, 35–37]. Initially, most neuroactive steroid analyses were performed using radioimmunoassay (RIA) while gas chromatography–mass spectrometry (GC-MS) has become more common [38]. Both methodologies, while successful, have limitations for clinical use. RIA lacks the specificity of both LC-MS/MS and GC-MS [39]. While GC-MS is highly sensitive, it is labor intensive and costly for routine clinical use [40]. LC-MS/MS is the preferred assay for clinical applications including many endocrine laboratories [39, 41].

We used LC-MS/MS to simultaneously quantify blood serum concentrations of allopregnanolone (3α-hydroxy-5α-pregnan-20-one), pregnanolone (3α-hydroxy-5β-pregnan-20-one), and epipregnanolone (3β-hydroxy-5β-pregnan-20-one), as well as their precursors pregnenolone and progesterone, in addition to cortisol and cortisone as part of an exploratory study investigating the role of these analytes in preterm birth (birth prior to 37 weeks' gestation). Please see Fig. 1 for a biochemical sketch of the synthesis pathway for all analytes [42].

The present assay was used to analyze blood serum samples, taken at two time points during pregnancy, from 54 women (27 women whose pregnancies were carried to term and 27 women whose pregnancies resulted in preterm birth).

Materials and methods

Chemicals, reagents, and human blood serum

Allopregnanolone-d5, progesterone-d9, and cortisol-d4 were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Epipregnanolone and cortisone-d8 were from Toronto Research Chemicals (Toronto, ON, Canada) and deuterated pregnenolone-d4 and pregnanolone from Sigma-Aldrich (Miamisburg, OH, USA). Based on the manufacturers' respective certificates of analysis, purities of the reference materials were as follows: allopregnanolone-d6 > 98%, allopregnanolone > 95%, progesterone-d9 > 98%, progesterone > 98%, cortisol-d4 > 99%, cortisol > 98%, epipregnanolone 98%, cortisone-d8 98%, cortisone 98%, pregnenolone-d4 > 98%, pregnenolone > 98%, and pregnanolone 98%. Methanol, LC-MS-grade water, and formic acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Charcoal-stripped steroid-free human serum as well as regular human serum were purchased from BioIVT (Frederick, MD, USA) and used for calibrators and quality controls (QC) for assay development and validation.

The study samples were obtained from the Colorado School of Public Health's Lifecourse Epidemiology of Adiposity and Diabetes (LEAD) Center, University of Colorado Anschutz Medical Campus (Aurora, CO, USA) as part of a nested case–control study using banked serum samples. A total of 108 samples, taken from 54 women at two time points during pregnancy, were analyzed. Samples were originally collected as part of the LEAD Center's Healthy Start Study (Colorado Multiple-institutional Review Board, Aurora, CO, USA, COMIRB #09-0563). Samples were collected as fasting venous blood samples between December 2009 and May 2014 [43]. Serum samples had been stored at − 80 °C, and some samples had undergone multiple freeze–thaw cycles prior to the analysis as detailed herein (see "Discussion" for further information regarding sample stability). The COMIRB granted exempt status to de-identified blood samples from volunteers used for the development,

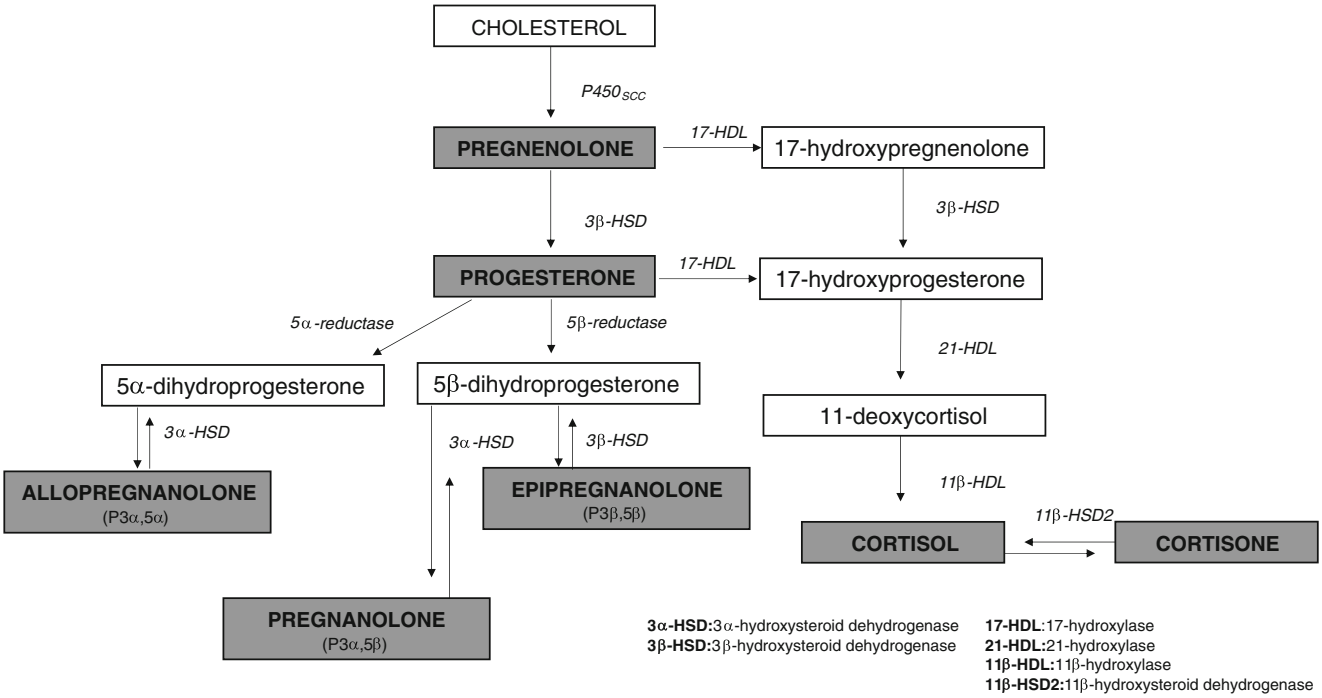

Fig. 1 Biosynthesis pathway of assay analytes. Assay analytes are highlighted in gray. Adapted from Mellon et al. 2001 [42]

validation, and quality control of analytical assays as well as de-identified blood samples used for secondary research.

Calibrators, quality control (QC) samples, and internal standards

Stock solutions for all compounds were prepared in 100% methanol at a concentration of 1 mg/mL. Calibrators for allopregnanolone, pregnanolone, and epipregnanolone ranged from 100 to 0.39 ng/mL. Calibrators for pregnenolone, progesterone, and cortisone ranged from 400 to 1.56 ng/mL. Calibrators for cortisol ranged from 1000 to 3.91 ng/mL. The final concentrations of calibrators in serum were 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100 ng/mL for allopregnanolone, pregnanolone, and epipregnanolone. For pregnenolone, progesterone, and cortisone, the calibrator concentrations in serum were 1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200, and 400 ng/mL. Calibrator concentrations in matrices for cortisol were 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, and 1000 ng/mL. QC working solutions yielded final concentrations of 1, 5, 25, and 75 ng/mL for allopregnanolone, pregnanolone, and epipregnanolone. QC concentrations of 4, 20, 100, and 300 ng/mL were used for pregnenolone, progesterone, and cortisone and of 10, 50, 250, and 750 ng/mL for cortisol. Calibrators and QC samples were obtained by adding ten milliliters (10 mL) working solution to one hundred milliliters (100 mL) 1:5 diluted charcoal stripped blank human serum in Dulbecco phosphate-buffered saline (PBS). All stock and standard solutions were stored in amber glass vials at − 80 °C. Calibration standards and QC samples were prepared from separate stock solutions (each prepared from three independent weighings) by serial dilution.

Sample preparation

Frozen serum samples were thawed and aliquoted at two hundred microliters (200 μL). Eight hundred microliters (800 μL) of pure methanol containing 50 ng/mL of the deuterated internal standards was then added to each sample to precipitate proteins. Samples were vortexed for 5 min and centrifuged at 25,000×g and 4 °C for 10 min. The supernatant was transferred into HPLC vials and placed into the autosampler of the LC-MS/MS system.

LC-MS/MS

LC-MS/MS analysis was performed using a Sciex API5000 triple quadrupole mass spectrometer in positive electrospray ionization mode (Sciex, Concord, ON, Canada). The LC system included Agilent 1100 series components including two binary pumps, a column thermostat, and an Agilent 1290 Infinity LC Injector/ HTS autosampler kept at 4 °C (Agilent Technologies, Santa Clara, CA, USA).

For online extraction, one hundred microliters (100 μL) of the extracted samples, calibrators, and QC samples was injected and loaded onto an Agilent Zorbax XDB-C18, 50 × 2.1 mm (5 μm particle size) HPLC extraction column at 85% LC-MS grade water with 0.1% formic acid and 15% LC-MS grade methanol with 0.1% formic acid at a flow rate increasing from

0.8 to 1.5 mL/min within 1 min [44]. The sample was then backflushed using a six-port switching valve onto the analytical column (Agilent Poroschell 120, 50 × 4.6 mm, 2.7 µm particle size, EC-C18 material, Agilent Technologies, Santa Clara, CA, USA). The connections and positions of the 6-port switching valve (Rheodyne, Cotati, CA, USA) are shown in Fig. S1. The analytical mobile phase consisted of LC-MS-grade water and methanol, both supplemented with 0.1% formic acid. The gradient began at 70% methanol for 1.2 min, moving to 80% methanol in 6.8 min, moving to 100% methanol within 2.5 min, then returning to 70% methanol within 0.1 min, and remaining there for 1.4 min for re-equilibration. The flow rate was kept at 0.8 mL/min and the HPLC column at 45 °C for the duration of the run.

The API5000 tandem mass spectrometer was used in the positive electrospray ionization mode (+ESI) at an ionization voltage of 5500 V in combination with multiple reaction monitoring (for more details, please see Table S1). Representative MS/MS spectra showing the fragments used for quantification are shown in Fig. S2.

Data analysis

Calibration curves were constructed with peak area ratios (analyte/internal standard) plotted against the amount of each analyte with 1/x weighting and quadratic curve fit.

Method validation

This method was developed and validated in accordance with the applicable guidelines from the Clinical Laboratory Standards Institute (CLSI) and the Food and Drug Administration (FDA) for bioanalytical assays [45, 46]. Validation procedures were conducted to establish lower limit of quantification (LLOQ), range of reliable response, selectivity and sensitivity, trueness, imprecision, extraction of recovery, extent of carry-over, matrix effect, dilution integrity, and stability. Method development, validation procedures, and experimental setups are described in detail in the Supplementary information.

Statistics

Analyst 1.6.2 was used for control of the LC-MS/MS system and data acquisition; Sciex OS-MQ 1.6.1 for data analysis (Sciex, Concord, ON, Canada). Statistical analyses for patient samples were performed with IBM SPSS Statistics for Mac version 27.0 (IBM, Armonk, NY, USA).

Results

The present HPLC assays achieved baseline separation of allopregnanolone, pregnanolone, epipregnanolone, progesterone, pregnenolone, cortisol, and cortisone on an Agilent Poroschell analytical column after using a simple protein precipitation extraction procedure and online extraction from human serum. Epi-allopregnanolone could not be quantified due to interference from unknown co-eluting peaks. No significant interference in blank or zero samples (less than 20% of the signal at the LLOQ) was detected for any of the analytes. The LLOQ was 0.78 ng/mL for allopregnanolone, pregnanolone, and epipregnanolone, while the LLOQ for pregnenolone, cortisone and progesterone was 1.56 ng/mL. The upper limits of quantification (ULOQ) for progesterone and cortisone were 400 ng/mL. For cortisol, the LLOQ was 3.91 ng/mL and the ULOQ was 1000 ng/mL. For the remaining analytes, allopregnanolone, pregnanolone, and epipregnanolone, the ULOQ was 100 ng/mL. All calibration curve correlation coefficients exceeded 0.99. Trueness of 3/4 of the calibration samples (= 75%) was better than ± 15% from nominal, except for the LLOQ sample, which was better than ± 20% demonstrating the calibration range met the acceptance criteria.

Interday and intraday analytical trueness and imprecision were within the acceptance limits (trueness at LLOQ: ± 20%, at all other concentrations: ± 15%; imprecision at LLOQ: ≤ 20%, at all other concentrations: ≤ 15%). Specifically, intraday trueness ranged from ± 0.3 to ± 13.6% while imprecision ranged from ± 4.6 to ± 14.5%. Interday trueness ranged from ± 0.1 to ± 13.6% and imprecision ranged from ± 0.8 to ± 13.9%; please see Table 1 for further details. The results for matrix effect did not show significant ion suppression or enhancement at the expected retention times for the analytes (Table 2). Extraction recovery was 96.7% for all analytes and both absolute and relative matrix effect were within ± 20%. For further details, please see Table 2. Consistently, less than 0.25% carryover was found for all analytes after pure methanol was injected following the highest calibrator. Negative specimens did not contain any of the analytes above the limit of detection ($n = 4$). Dilution integrity was established, and results are shown in Table S2.

Analytes remained stable after refrigerating up to 7 days at 4 °C with trueness within ± 20% of the nominal. Additionally, all analyte concentrations were within ± 15% of the reference after 3 months at − 80 °C. After three freeze–thaw cycles, the concentrations of all the analytes were within ± 20% of the reference, and the stability in the autosampler at 4 °C was within ± 10% or better for all the analytes up to 72 h.

Representative ion chromatograms of the serum study samples are shown in Fig. 2. Please see Figs. S3-S4 for representative blanks and ion chromatograms at the LLOQ.

Table 1 Analytical trueness and imprecision for analytes in human serum

Analyte	Concentration (ng/mL)	Trueness (% expected concentrations) intraday (n=6)	% Imprecision (% coefficient of variation) intraday (n=6)	Trueness (% expected concentrations) interday (n=18)	% Imprecision (% coefficient of variation) interday (n=18)
Pregnenolone					
QC3	5	99.3	11.3	99.9	1.6
QC2	25	95.5	5.4	95.1	8.9
QC1	75	96.1	7.2	95.9	6.7
Allopregnanolone					
QC4	1	93.3	13.4	88.3	4.5
QC3	5	105.4	8.0	106.7	10.7
QC2	25	100.5	7.2	100.5	1.5
QC1	75	97.9	8.8	97.8	8.0
Pregnanolone					
QC4	1	100.3	12.3	101.3	4.5
QC3	5	93.4	6.3	92.7	3.1
QC2	25	99.7	5.2	99.5	4.8
QC1	75	97.5	9.3	97.8	4.1
Epipregnanolone					
QC4	1	111.1	14.5	112.8	13.9
QC3	5	102.2	14.2	103.7	2.0
QC2	25	103.9	8.8	104.5	4.3
QC1	75	103.7	13.6	104.3	1.3
Progesterone					
QC4	4	98.1	12.0	98.1	13.0
QC3	20	113.6	5.7	113.6	2.4
QC2	100	107.1	5.3	107.1	1.6
QC1	300	103.9	6.3	103.9	5.4
Cortisone					
QC4	4	96.9	4.6	105.0	5.7
QC3	20	98.6	6.3	103.4	0.8
QC2	100	95.7	7.1	97.2	3.5
QC1	300	96.7	8.4	97.8	3.9
Cortisol					
QC4	10	93.2	8.2	93.2	6.5
QC3	50	95.9	10.3	95.9	4.6
QC2	250	97.6	10.4	97.6	6.6
QC1	750	96.7	8.0	96.7	5.2

Allopregnanolone (3α-hydroxy-5α-pregnan-20-one), pregnanolone (3α-hydroxy-5β-pregnan-20-one), epipregnanolone (3β-hydroxy-5β-pregnan-20-one)

For proof of concept, one hundred eight samples (n = 108) from 54 individuals, taken twice during pregnancy, were analyzed with the present assay. Blanks and zero samples did not show any interfering peaks exceeding 20% of the analyte signals at the LLOQ, for all the analytes. All calibrators and quality control samples met acceptance criteria. Mean concentrations of analytes in the study samples are provided in Table 3. Analyte concentrations were comparable to concentrations reported in other studies for the same time periods during pregnancy using GC-MS for quantification [17, 30–32, 47].

Discussion

Neuroactive steroid detection and their quantification is challenging because both the molecular structure and mass/charge (m/z) of these compounds are very similar. In addition, quantifying both the progesterone metabolites and cortisol requires a large dynamic range of the assay.

Sample stability

The study serum samples were originally collected between December 2009 and May of 2014 as part of a longitudinal study on diabetes named The Healthy Start Study [43]. Research using maternal blood serum collected during pregnancy offers a unique ability to follow the offspring of these pregnancies longitudinally into adulthood and assess potential underlying mechanisms of health and disease. Long-term sample stability is of paramount importance for this research. Multiple studies have addressed long-term steroid sample stability specifically for progesterone and cortisol, in addition to other sex steroids, and found steroid concentrations were consistent with fresh samples in some cases for up to 40 years [48, 49]. Although there do not

Table 2 Mean recovery, absolute, and relative matrix effect for analytes

Analyte	Linear range (ng/mL)	Recovery mean (%±SD)	Absolute matrix effect (%±SD)	Relative matrix effect (%±SD)
Pregnenolone	**1.56–100**			
1 ng/mL		107.3 ± 25	74.1 ± 28.9	92 ± 27.7
5 ng/mL		75.3 ± 11	43.5 ± 20.7	81.1 ± 7.9
25 ng/mL		98.8 ± 22.7	40.6 ± 18.2	102.9 ± 21.2
75 ng/mL		94.9 ± 18.8	40 ± 21.6	97.2 ± 20.7
Allopregnanolone	**0.78–100**			
1 ng/mL		102.2 ± 26.7	55.5 ± 24.6	95 ± 29.5
5 ng/mL		95.7 ± 19.1	44.3 ± 19.3	99.6 ± 10.7
25 ng/mL		84.1 ± 14.3	42.4 ± 20.1	94.3 ± 20.1
75 ng/mL		97.5 ± 23.5	46.6 ± 22.5	103.5 ± 16
Pregnanolone	**0.78–100**			
1 ng/mL		100.7 ± 25.6	53.9 ± 14.1	106.8 ± 28.2
5 ng/mL		90.6 ± 25.6	44.7 ± 21	93.4 ± 14.2
25 ng/mL		86.5 ± 17.2	41.6 ± 19.6	95.3 ± 11.3
75 ng/mL		97.6 ± 20.1	48.9 ± 21.1	104.2 ± 15.4
Epipregnanolone	**0.78–100**			
1 ng/mL		127.9 ± 21.8	73.6 ± 17	117.9 ± 18.7
5 ng/mL		87.6 ± 19.7	43.4 ± 16	91.5 ± 22.5
25 ng/mL		94.5 ± 23.3	45.9 ± 21.1	102.6 ± 6.5
75 ng/mL		93.5 ± 21.7	45.9 ± 25.6	99.8 ± 17.9
Progesterone	**1.56–400**			
4 ng/mL		97.4 ± 11.3	64.8 ± 26.8	91.1 ± 19.4
20 ng/mL		82.2 ± 8.6	59.3 ± 28	86.8 ± 14.6
100 ng/mL		92.7 ± 17.3	62.7 ± 25.2	102.2 ± 10.9
300 ng/mL		97.7 ± 24.1	67.1 ± 27.6	107.2 ± 31.3
Cortisone	**1.56–400**			
4 ng/mL		119.9 ± 8.3	85.7 ± 28.5	96.7 ± 20.6
20 ng/mL		95.4 ± 13.8	60.8 ± 13.4	101.6 ± 22.8
100 ng/mL		91.5 ± 22.3	45.4 ± 12.5	98.6 ± 20
300 ng/mL		91.6 ± 15	59.3 ± 17.4	94.5 ± 14
Cortisol	**3.91–1000**			
10 ng/mL		113.7 ± 18.8	83.9 ± 26.2	67.5 ± 11.1
50 ng/mL		99.4 ± 13.5	54.9 ± 11.9	92 ± 25.9
250 ng/mL		99.7 ± 13.7	62.1 ± 11.8	110 ± 17.2
750 ng/mL		86.6 ± 14.2	59.9 ± 20.6	87.4 ± 18.1

Allopregnanolone (3α-hydroxy-5α-pregnan-20-one), pregnanolone (3α-hydroxy-5β-pregnan-20-one), epipregnanolone (3β-hydroxy-5β-pregnan-20-one)

appear to be any studies directly investigating the stability of the progesterone precursor pregnenolone or the progesterone metabolites allopregnanolone, pregnanolone, and epipregnanolone, due to the similar molecular structure of these isomers, it is reasonable to believe they would behave comparably. Further, mean concentrations of all analytes were consistent with other studies assessing the concentrations of these same compounds during pregnancy; see Table 3 [17, 21, 30–33, 47, 50–53].

The present assay was specifically developed for quantification of these analytes in pregnancy. Online extraction with back-flushing of the analytes has several advantages including, but not limited to, cleaner extracts with a reduction of matrix effects, analyte concentration on the top of the online extraction column, and a virtually "volume-less injection" onto the analytical column resulting in sharper peaks, better separation, and more reliable automated integration especially at lower concentrations. For more details, please see reference

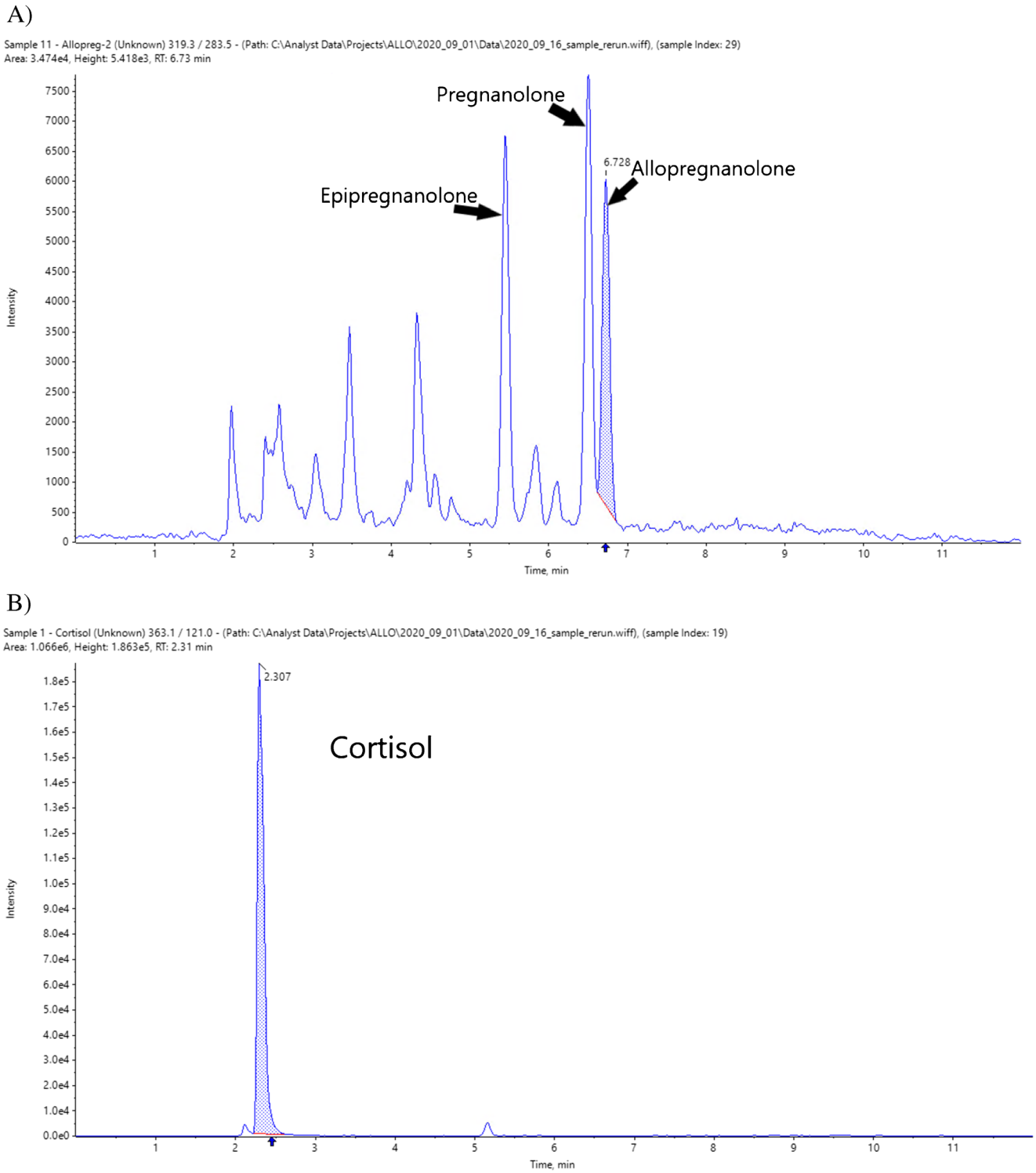

Fig. 2 Representative ion chromatograms of patient samples. **A** Representative chromatogram ion transitions of patient sample for allopregnanolone, pregnanolone, and epipregnanolone. Analyte indicated in figure, **B** representative ion chromatogram of patient sample for cortisol, **C** representative ion chromatogram of patient sample for cortisone, **D** representative ion chromatogram of patient sample for pregnenolone, **E** representative ion chromatogram of patient sample for progesterone

[54]. As aforementioned, the column switching setup and procedure was taken from an assay previously published by our group [44]. Based on our extensive experience with this and similar online extraction procedures used in our laboratory, we know that the online extraction columns last for more than 2000 injections; however, these are routinely exchanged after 1500 injections.

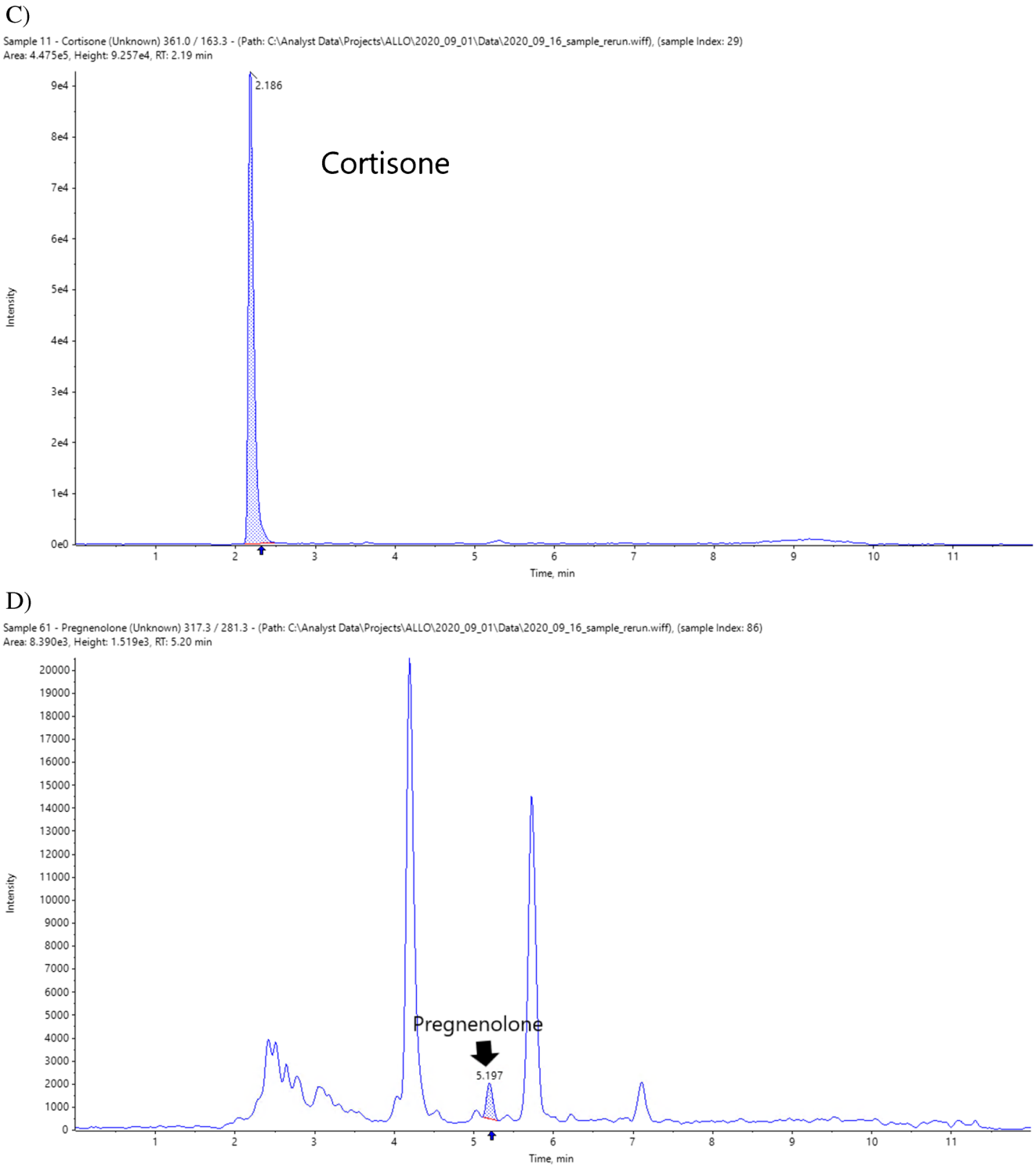

Fig. 2 (continued)

Concentrations of neuroactive steroids are lower in non-pregnant women compared with pregnant women. The linear range of this assay covers both pregnant and non-pregnant concentrations for cortisol and cortisone in addition to non-luteal levels of progesterone. Please see Table 3 for further details. If a more sensitive assay for non-pregnant levels of neuroactive steroids is needed, then derivatization will be required [35, 36, 55]. Six (6) of the one hundred and eight (108) study samples fell below the LLOQ for the analyte pregnanolone (0.78 ng/mL). Three (3) of the one hundred and eight (108) study samples fell below the

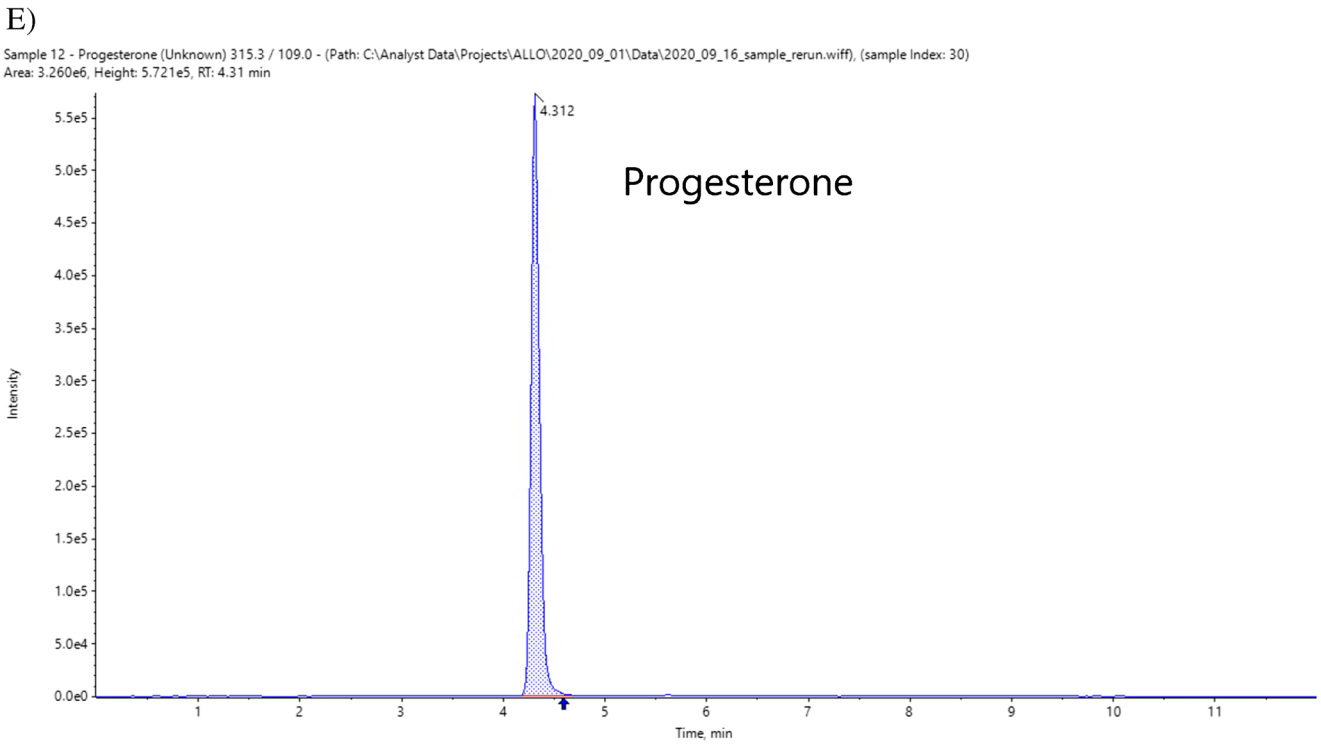

Fig. 2 (continued)

LLOQ for the analyte epipregnanolone (0.78 ng/mL). Deligiannidis et al. quantified neurosteroids during pregnancy using LC-MS/MS and did so using a two-step solid-phase extraction method [56]. Additionally, Jin et al. validated their assay for allopregnanolone and pregnanolone in pregnant and non-pregnant women using derivatization and achieved a linear range of 40 to 25,000 pg/mL [35]. Their range covered the dramatic increase of neurosteroids from non-pregnant to pregnant concentrations; however, the addition of derivatization adds complexity and cost to the assay, making it less feasible for routine clinical use.

Table 3 Mean concentrations of analytes from study samples with a comparison of pregnant and non-pregnant ranges

Analyte	1st serum sample[a] (*n*=54) (mean±SD, ng/mL)	2nd serum sample[b] (n=54) (mean±SD, ng/mL)	P-value[c]	Linear range for assay (ng/mL)	Range of analyte concentrations during pregnancy (ng/mL[d])	Range of analyte concentrations outside of pregnancy (ng/mL[d])
Allopregnanolone	4.42±1.69	7.64±3.39	**<.0001**	0.78–100	1.4–31.5 [14, 15, 17, 33, 53, 61, 63]	0.01–1.2 [14, 42–44, 63, 64]
Pregnanolone	2.10±1.36	5.35±2.63	**<.0001**	0.78–100	1.1–15 [14, 15, 53, 61]	0.01–0.42 [14, 43, 61]
Epipregnanolone	1.62±0.83	3.23±1.84	**<.0001**	0.78–100	0.1 –1.3* [15, 53]	0.03–0.07* [63, 65]
Pregnenolone	n/a	n/a	n/a	1.56–400	2.4–17.9 [14, 61, 63]	0.03–3.27 [44, 64]
Progesterone	57.49±18.13	103.02±36.45	**<.0001**	1.56–400	8–342 [58]	0.4**–36 [66]
Cortisone	47.49±8.77	59.70±10.77	**<.0001**	1.56–400	23.2–81.6 [56, 57, 59]	3.6–36 [66]
Cortisol	289.11±118.41	448.45±137.38	**<.0001**	3.91–1000	70–500 [58]	36–360 [66]

[a] 1st serum sample had a mean gestational age of 16.85 weeks, range 12.43–25 weeks

[b] 2nd serum sample 2 mean GA 26.51 weeks, range 22.3–32 weeks

[c] Paired t test was used to calculate the significance in the increase between the 1st and the 2nd blood samples for each analyte. All analytes significantly increased from the 1st to the 2nd blood samples. All serum concentrations were log transformed prior to analysis

[d] All unit conversions were done using the AMA Manual of Style 11th Edition SI Conversion Calculator at https://www.amamanualofstyle.com/page/si-conversion-calculator. Cortisol was used for the conversion of cortisol and cortisone. Progesterone was used for the conversion of progesterone, pregnenolone, and all metabolites

*There is very little published data on epipregnanolone levels in pregnancy (and non-pregnancy). Our samples show that epipregnanolone is higher in pregnancy than the two studies cited. Only 3 of our samples fell below the LLOQ

**This value goes under our assay range only for follicular levels; normal luteal levels start at 3.6 ng/mL

This appears to be the only LC-MS/MS assay, validated in accordance with the CLSI and FDA, to simultaneously quantify neuroactive steroids, along with their precursors, in addition to cortisol and cortisone in human serum during pregnancy. As such, this assay allows for analysis of the synergistic relationship between neuroactive steroids and stress hormones. Park et al. used HPLC/MS to achieve simultaneous quantification of various sex steroids and neurosteroid sulfates in rat brain tissue and plasma [15]. Their assay is validated in accordance with applicable FDA guidelines and sought to look at the stress-induced responses of these compounds in a similar fashion to our goal of understanding the relationship between these analytes within the convenience of one assay [15]. Park et al. achieved a linear range of between .05 and 500 ng/mL which adequately covers the dramatic increase between non-pregnant and pregnant neurosteroid concentrations in addition to stress hormones during pregnancy [15]. However, their methodology also required derivatization, which adds significant time and cost to the assay and as a result sacrifices the simplicity of extraction, which can be very important for clinical research and effective monitoring. Additionally, because they quantified sulfates, certain extraction procedures were added to the method to avoid protein binding and as a result their methodologies further varied from our own [15].

In conclusion, here, we present a straightforward, simple, specific, and sufficiently sensitive multi-analyte LC-MS/MS assay for simultaneous quantification of the 3α-reduced progesterone metabolites, allopregnanolone and pregnanolone, as well as the 3β-reduced metabolite epipregnanolone, their precursors pregnenolone and progesterone, as well as cortisol and cortisone in human serum during pregnancy. The simplicity of this validated assay makes its use for clinical research attractive, and the assay has potential use for diagnostic applications in pregnancy.

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Data availability All data and material are available upon request to the corresponding author.

Code availability Not applicable.

Declarations

Ethics approval The Colorado Multiple-institutional Review Board considered this study "exempt" (COMIRB #19-2419).

Consent to participate Not applicable.

Consent for publication All authors consent to the publication of this work.

Conflict of interest The authors declare no competing interests.

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