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Quantification of Neurosteroids During Pregnancy Using Selective Ion Monitoring Mass Spectrometry

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Abstract

Analytical techniques used to quantify neurosteroids in biological samples are often compromised by non-specificity and limited dynamic range which can result in erroneous results. A relatively rapid and inexpensive gas chromatography-mass spectrometry (GC-MS) was developed to simultaneously measure nine neurosteroids, including allopregnanolone, estradiol, and progesterone, as well as 25-hydroxy-vitamin D3 in plasma samples collected from adult women subjects during and after pregnancy. Sample preparation involved solid-phase extraction and derivatization, followed by automated injection on a GC equipped with a mass selective detector (MSD) operated in single ion monitoring (SIM) mode to yield a run time of less than 11 minutes. Method detection limits for all neurosteroids ranged from 30 to 200 pg/mL (parts per trillion), with coefficients of variation that ranged from 3 to 5% based on intra-assay comparisons run in triplicate. Although concentrations of estradiol measured by chemiluminescent immunoassay (CIA) were consistent with values determined by GC-MS values, CIA yielded considerable higher values of progesterone, suggesting antibody cross reactions resulting from low specificity. Mean neurosteroid levels and representative time-course data demonstrate the ability of the method to quantify changes in multiple neurosteroids during pregnancy, including rapid declines in neurosteroid levels associated with delivery. This simplified GC-MS method holds particular promise for research and clinical laboratories that require simultaneous quantification of multiple neurosteroids, but lack the resources and expertise to support advanced liquid chromatography-tandem mass spectrometry facilities.

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Keywords

neurosteroids; gas chromatography; mass spectrometry; selective ion monitoring; quantification; pregnancy

1. Introduction

Steroid hormones, which are synthesized and secreted from ovarian, gonadal and adrenal glands in women, play a central role in regulation of menstrual cycles and maintenance of pregnancy. During normal pregnancy, circulating levels of steroid hormones may increase several-fold over the course of gestation, followed by a rapid decrease to preconception levels soon after delivery (e.g., Gilbert-Evans et al., 2005; Parizek et al., 2005; Soldin et al., 2005). Steroid hormones are derived from cholesterol via hydroxylation to form pregnenolone, which is then converted to progesterone via 3 β -hydroxysteroid hydrogenase (3 β HSD). Two parallel enzymatic reaction pathways act to convert pregnenolone and progesterone to other neurosteroids, including estradiol and allopregnanolone (O'Malley and Strott, 1999; Stoffel-Wagner, 2001). Estradiol, progesterone and the progesterone metabolite allopregnanolone are capable of modifying neural activity and are classified as neurosteroids because of their ability to have salient effects on neuronal function. Some steroid hormones, such as progesterone and estradiol, can impact neuronal function via both short-latency neuronal membrane-mediated effects as well as long-latency, genomic effects by binding to specific nuclear steroid receptors (e.g., progesterone receptors, (PRs) and estrogen receptors (ERs)). Other neurosteroids, such as allopregnanolone, act as direct, positive allosteric modulators of gamma-amino-butyric acid (GABA)_A receptors, augmenting the inhibitory effects of GABA by increasing the frequency and duration of chloride channel opening (Majewska et al., 1986; Rhodes et al., 2004; Harden et al., 2013; Petersen 2013; Lang 2014). As a consequence, women with epilepsy may be particularly sensitive to alterations in endogenous neurosteroid levels during various stages of the menstrual cycle, pregnancy and postpartum.

Approximately one-third of reproductive-aged women with epilepsy demonstrate a catamenial pattern, with increased seizure frequency during certain menstrual phases. The most common pattern is seizure exacerbation beginning just prior to menstrual onset, which has been attributed to declining levels of progesterone with a corresponding reduction in allopregnanolone, while the decay in estradiol levels lags behind (Herzog et al., 1997; Reddy and Rogawski, 2009). Although allopregnanolone is often cited as the primary neurosteroid responsible for reduced seizure susceptibility when progesterone is elevated. Several other neurosteroids, including 17 β -estradiol and pregnenolone sulfate (the conjugated form of pregnenolone), are considered to be proconvulsants; cyclical changes in estrogens as well as progesterone and its metabolites are likely to play a key role in catamenial epilepsy (Sharfman and MacLusky, 2006; Reddy, 2013).

During pregnancy, 16-37% of women with epilepsy experience an increase in seizure frequency (Bardy, 1987; Katz and Devinsky, 2003; Battino 2013), and seizure relapse has been reported to be the highest during the three peripartum days (Thomas 2012). The peripartum period may exhibit increased seizure frequency due to rapid changes in

neurosteroid levels, in particular the decline in progesterone and allopregnanolone. Gilbert-Evans and coworkers (2005) monitored levels of 3 α -reduced neurosteroids at five time points during pregnancy in healthy women, and found that allopregnanolone and progesterone levels increased throughout gestation, with the highest concentrations (41 ± 19 ng/mL and 164 ± 64 ng/mL, respectively) observed at 36-38 weeks. At 4-6 weeks postpartum, however, both allopregnanolone and progesterone had returned to baseline levels (0.44 ± 0.32 and 0.33 ± 0.30 ng/mL, respectively).

Given the complexity and continued uncertainty of relationships between neurosteroid levels and seizure frequency, it is important to develop accurate and reproducible analytical methods that are capable of measuring multiple steroids in a single assay. Traditional radioimmunoassay (RIA) methods, in which the target analyte is labeled with a radioactive molecule, are still widely used in clinical laboratories and animal model studies to measure a range of steroids, including cortisol, estradiol, progesterone, allopregnanolone, and 17-hydroxyprogesterone (e.g., Herzog et al., 2005; Frye and Rhodes, 2006; Munro et al., 1991; Shirtcliff et al., 2000). Although RIA is well-established, the technique is subject to low specificity due to anti-body cross reactions (Appelblad and Irgum, 2000), while the availability and cost of antibodies can be prohibitive when multiple steroids are measured. Complications arising from antibody cross reactions can be particularly acute when RIA is used to analyze serum or plasma collected from women, since steroid levels change dramatically over the course of pregnancy and the menstrual cycle. For example, Murphy (2001) noted that an allopregnanolone antibody exhibited high affinity for progesterone, and therefore, could have accounted for more than 60% of the allopregnanolone detected by RIA late in pregnancy when progesterone levels are elevated. Alternative immunoassay techniques, such as enzyme-linked immunosorbent assay (ELISA) and chemiluminescent immunoassay (CIA), have been developed to achieve greater sensitivity and avoid radioisotope exposure risks and waste disposal issues. However, direct comparisons between CIA and RIA measurements of estradiol levels in serum collected from patients receiving gonadotropins were similar, whereas values deviated by nearly a factor of four in patients treated with oral estrogen (Hershlag et al., 2000).

Advancements in mass spectrometry (MS) over the past ten years have resulted in greatly improved signal resolution and detection capabilities, to the point where multiple steroids can be quantified simultaneously at ng/mL (parts per billion), and even pg/mL (parts per trillion) levels (Abdel-Khalik et al., 2013). Recent improvements in liquid ionization techniques, such as atmospheric pressure photoionization (APPI) and atmospheric pressure chemical ionization (APCI), coupled with “tandem” mass spectrometry (MS/MS) have resulted in LC-MS/MS platforms that achieve low detection limits without derivatization (Abdel-Khalik et al., 2013; Soldin et al., 2009). Nevertheless, LC-MS/MS methods still require that samples undergo liquid-liquid extraction (LEE) and/or solid-phase extraction (SPE), and the costs associated with instrumentation and maintenance, as well as the need for highly-skilled technicians, has limited the application of LC-MS/MS for neurosteroid analysis. For this reason, we sought to develop a versatile and relatively inexpensive method for neurosteroid analysis based on a reliable and inexpensive gas chromatography-mass spectrometry (GC-MS) platform. The method employs a mass selective detector (MSD) operated in selective ion monitoring (SIM) mode to achieve high sensitivity without the

need for specialized ionization techniques or multiple reaction monitoring (MRM). Sample cleanup and preparation involves SPE followed by single-step derivatization, based partially on the method of Gilbert-Evans et al. (2005). The resulting GC-MS method was used to simultaneously monitor changes in nine neurosteroids over the course of pregnancy and postpartum in a cohort of eleven subjects (65 samples). The versatility of the method was demonstrated by the ability to subsequently include additional steroids of interest, 17-hydroxyprogesterone and 25-hydroxy-vitamin D. Intra-assay comparisons were performed on two sets of time course samples, while an inter-assay comparison was conducted on additional samples using established CIA methods for estradiol and progesterone.

2. Materials and methods

2.1. Chemicals and reagents

The following neurosteroids were purchased from Sigma-Aldrich (St. Louis, MO), with purities noted when provided: Allopregnanolone (Allo), 3 α -hydroxy-5 α -pregnan-20-one; Calcifediol (25(OH)D), 25-hydroxyvitamin D3 (HPLC grade > 98% purity); Dehydroepiandrosterone (DHEA), 3 β -hydroxy-5-androsten-17-one; 5 α -dihydroprogesterone (5 α -DHP), 5 α -pregnan-3,20-dione; Estradiol, 1,3,5-estratriene-3,17 β -diol; Estrone, 3-hydroxy-13-methyl-6,7,8,9,11,12,13,14,15,16-decahydrocyclopenta[a]phenanthrene-17-one (> 99% purity); 17-hydroxyprogesterone (17-OHP), 4-pregnen-17-ol-3,20-dione; Pregnenolone (Pregnen), 3 β -hydroxy-5-pregnen-20-one (98% purity); Pregnanolone (Pregnan), 3 α -hydroxy-5 β -pregnan-20-one; Progesterone (Prog), 4-pregnene-3,20-dione; 5 α -tetrahydrodeoxycorticosterone (5 α -THDOC), 5 α -pregnane-3 α ,21-diol-20-one (95% purity).

Four deuterated neuractive steroids, Allo-D4 (17, 21, 21, 21; 96-98% purity), 5 α -DHP-D6 (1, 2, 4, 5, 6, 7; 95% purity), Pregnan-D4 (17, 21, 21, 21; 96-98% purity), and 5 α -THDOC-D3 (17, 21, 21; 95% purity), were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). Reagent grade (ACS, 99.5% purity) dichloromethane (DCM) and acetonitrile (ACN) were obtained from Sigma Aldrich, while certified ACS grade (> 99.8% purity) methanol (MeOH) was purchased from Fisher Scientific (Fair Lawn, NJ). The derivatizing agent and reaction catalyst N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 10% trimethylsilyl (TMS) were obtained from Sigma Aldrich. All aqueous solutions were prepared with deionized (DI) water (>18 M Ω cm⁻¹) that had passed through a Nanopure[®] ultrapure purification system (Model D4741, Barnstead International, Dubuque, IA).

2.2. Human plasma samples

A total of 85 plasma samples were obtained from women enrolled in either the Specialized Center of Research (SCOR) in Women and Gender Issues program project grant study or the Clinical Research in Neurology (CRIN) registry at Emory University Hospital. The SCOR project focused on pharmacokinetic, pharmacodynamic, and pharmacogenetic modeling of psychotropic medications and antiepileptic drugs during pregnancy and lactation, while the CRIN registry includes patients with varied neurologic conditions, including epilepsy, as well as control subjects. Protocols for the collection, storage and use of human samples were approved by the Emory University Institutional Review Board. Women were enrolled in the

SCOR or CRIN study at various gestational ages (GAs), and follow-up visits occurred every 1 to 3 months during pregnancy and the first postpartum year. At each study visit, general physical and neurological examinations were performed, seizure calendars were reviewed, and intervening illnesses or obstetrical complications were recorded. Maternal blood was collected at each study visit via standard venipuncture and centrifuged at 2,750 rpm at 3°C for 10 minutes. All postpartum blood collections occurred at least 6 weeks following delivery. The plasma was stored in 300-500 μ L aliquots in polypropylene tubes and stored at -80°C until assay. The use of plasma samples for this study was predicated on sample availability, and the fact that plasma was available in sufficient quantities (300-500 μ L) to allow for neurosteroid analysis. Although serum samples are often employed for neurosteroid analysis, a number of studies have been conducted using plasma (e.g., Gilbert-Evans et al., 2005; Hill et al., 2007; Santen et al., 2007), and the ability of GC-MS methods to quantify neurosteroids in plasma has been noted in reviews of the subject (e.g., Abdel-Khalik et al., 2013).

2.3. Neurosteroid extraction and derivatization

A 300-500 μ L aliquot of plasma was transferred to a 7.5 mL amber glass vial and an equal volume of a 75:25 MeOH/water solution was added to the plasma sample. The sample was diluted to final concentration of 5% MeOH with purified DI water, and vortex mixed for 1 min. Each diluted sample (ca. 6.5 mL) was then transferred onto a C-18 solid phase extraction (SPE) column containing 1.0 g of polymer-bonded octadecyl (Discovery DSC-18, Supelco) that had been pre-equilibrated with 5 mL of MeOH followed by 5 mL of a 5:95 MeOH/water solution. Following vacuum extraction on a 12-port SPE manifold (Visiprep, Supelco), the effluent was discarded and the SPE columns were washed with 5 mL of 50:50 MeOH/water solution. The retained neurosteroids were then eluted from the SPE column with 5 mL of 100% MeOH and collected in sterile glass tubes. The resulting extract was reduced to 500 μ L under a gentle stream of high-purity nitrogen (N_2) gas at 70°C (ca. 1 hr) using a MultiVap 118 Nitrogen Evaporator (Organomotion Associates Inc., Berlin, MA), and then transferred to 1.5 mL amber glass chromatography vials. The remaining liquid was completely dried at 70°C under a gentle stream of N_2 gas (ca. 30 min) using a Reacti-Therm™ Heating Module (Pierce, Rockford, IL). Immediately after drying a mixture of 50 μ L O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 10% trimethylsilane (TMS) (Sigma-Aldrich) and 50 μ L acetonitrile was added to the dry residue and heated at 56°C for 25 minutes. Any residual derivatizing reagent was then removed by heating to 70°C under a gentle N_2 gas stream. The final derivatized residue was dissolved in 100 μ L of DCM and transferred to an amber chromatography vial equipped with a 250 μ L glass insert.

2.3. Quantification of neurosteroids by GC-MS

Quantification of neurosteroids was based on a seven-point calibration curve that was prepared by serial dilution of mixed neurosteroid stock solutions over the anticipated concentration ranges (e.g., 0.3 to 300 ng/mL, progesterone). The stock solutions underwent the same extraction, cleanup, and derivation procedure used for plasma samples described above. Derivatized neurosteroid samples were measured using an Agilent 7000 GC equipped with an Agilent 7683 autosampler and an Agilent 5795 mass selective detector (MSD). A 4 μ L aliquot of derivatized sample was introduced into the injection port, which

was heated to 300°C and operated in split-less mode. Ionization was achieved by electron impact (EI) and ultra-high-purity helium (99.999%, Grade “5”) was used as the carrier gas (1 mL/min). Separation of the neurosteroids achieved on a DB-1ms column (30 m length × 0.25 mm diameter × 0.25 µm film thickness, J&W Scientific) operated at initial oven temperature of 150°C, ramped to 250°C (2°C/min), and finally ramped to 300°C (30°C/min). Intra-assay variability was evaluated using samples collected from two different patients on four separate dates, and analyzed in triplicate.

Performance of the analytical methodology was monitored by spiking plasma samples with a 10 ng/mL mixture of deuterated neurosteroids (Allo-D4, 5α-DHP-D6, Pregnan-D4, 5α-THDOC-D3). The recovery efficiency of the method was evaluated by spiking 7.5% charcoal-stripped bovine serum albumin (BSA, Sigma-Aldrich, Inc.) with 25 ng/mL of individual and mixed neurosteroids, followed by analysis using the procedures described above. To test for potential interferences arising from the background matrix, selected standards were prepared with either water or charcoal-stripped BSA as the solvent, which yielded concentration values that varied by less than 10%.

2.4. Comparative analysis by chemiluminescence immunoassay

Concentrations of estradiol and progesterone were independently measured in 16 additional plasma samples. Samples were analyzed using commercially-available Chemiluminescence Immunoassay (CIA) kits (Beckman Coulter, Fullerton, CA) by the Brigham Research Assay Core (BRAC). The reported CIA method dynamic range for progesterone was 0.08 to 40 ng/mL, with an intra-assay variation of 6 to 11%. The reported dynamic range for the estradiol CIA method was 0.02 to 4.8 ng/mL, with an intra-assay variation of 11-21%.

2.4. Statistical Analysis

Concentrations (ng/mL) of each neurosteroid measured over the course of pregnancy and postpartum were entered into SPSS Version 21 (IBM). Mean values and their 95% confidence intervals (95 % CIs) were calculated for neurosteroids at each trimester during and after the pregnancy. The Generalized Estimating Equation (GEE) form of the Generalized Linear Model was used to assess differences between the three during pregnancy times compared to the postpartum value. Specifically, the repeated measures form of GEE was used with each subject as the unit of repeated measures. The exchangeability correlation matrix was used with robust standard error estimates. Time was modeled as a categorical variable (four time intervals corresponding to trimesters 1, 2, and 3, and post-partum) and factor effect. The Wald chi-square test was used to determine p-values for the parameter estimates of hormone difference comparing each trimester to postpartum (Time 4). Correlations between measured progesterone and estradiol concentrations determined by CIA and GC-MS methods were evaluated using a least-squares, linear regression program (SigmaPlot ver. 10, Systat Software Inc., San Jose, CA)

3. Results

3.1. Neurosteroid method validation

The spectral signature of each neurosteroid and the four deuterated analogs was initially obtained by analyzing individual samples in full scan mode (40-550 m/z), followed by confirmation against GC-EI-MS spectra available from NIST/EPA/NIH Mass Spectral Library. Individual neurosteroid standards were then prepared in either DI water or BSA, and processed following the solid phase extraction and derivatization protocols described above. The concentrated sample, which is dissolved in dichloromethane, was then analyzed in full scan mode to determine the retention time and most abundant ions (m/z). For example, based on GC-EI-MS analysis of the allopregnanolone derivative, the major ions consisted of the parent molecule (300.3 m/z, Allo), smaller fragments (285.3 m/z and 215.2 m/z), and the parent derivative molecule (375.3 m/z, Allo+TMS). For each neurosteroid, the ion that yielded the greatest response was used for quantification, while at least two secondary ions were used to confirm identity (Table 1). This information was used to program collection time windows and quantification ions for selective ion monitoring (SIM) of nine neurosteroids (DHEA, Estrone, Allo, Estradiol, Pregnan, 5 α -DHP, Prog, Pregnen, 5 α -THDOC). Subsequently, the analytical method was modified to include 17-hydroxyprogesterone (17-OHP), which increases during the third trimester due to fetal adrenal activity, and 25-hydroxyvitamin D (25(OH)D), which is typically measured in blood to monitor vitamin D levels. A representative chromatogram obtained for an 80 pg injection (4 μ L of a 20 ng/mL) of the ten neurosteroids and 25(OH)D is shown in Figure 1. The chromatogram demonstrates that eight of the neurosteroids eluted in less than 11 min, with the majority of quantification ions exhibiting high response and resolution. However, several of the higher molecular weight analytes, specifically 5 α -THDOC and 25(OH)D, yielded relatively long retention times and lower ion response.

The method recovery efficiency, based on the analysis of neurosteroid mixtures prepared in BSA and subject to the solid-phase extraction and derivatization procedure, ranged from 84.3 to 97.3% (Table 1). Limits of detection, based on the method of Hubaux and Vos (1970), ranged from 23 pg/mL for DHEA to 276 pg/mL for 5 α -THDOC. Intra-assay variability was evaluated by analyzing seven plasma samples that contained sufficient volume (e.g., 1.5 mL) to be run in triplicate. Based on this approach, intra-assay coefficients of variation (CV) were found to range from 6.54% for 5 α -DHP to 14.13% for 5 α -THDOC (Table 1). The plasma samples used for the intra-assay comparisons were collected over the course of pregnancy and postpartum, and therefore reflected the overall range of concentrations that are likely to be encountered. The observed intra-assay variability was consistent with values reported by Gilbert-Evans et al. (2005) for pregnenolone, progesterone, 5 α -DHP, allopregnanolone and pregnanolone (4.6 to 19.8%).

3.2. Inter-assay comparison of estradiol and progesterone levels

A direct comparison of estradiol levels measured in seventeen plasma samples using CIA and GC-MS yielded a slope of 0.99 with a correlation coefficient of 0.95. These findings are consistent with data of Santen et al. (2007), who reported a correlation coefficient of 0.98 for estradiol values measured in post-menopausal women using RIA and GC-MS/MS. In

contrast, the direct comparison of progesterone levels measured by CIA and GC-MS yielded a slope of 1.45 with a correlation coefficient of 0.92. In this set of samples, progesterone levels determined by GC-MS ranged 32.5 to 355.4 ng/mL, while measured CIA values ranged from 54.8 to 570.9 ng/mL. Thus, the CIA-determined progesterone levels were consistently higher than those measured by GC-MS, which was attributed to non-specificity of the antibody. The potential for antibody cross reactions is particularly problematic during pregnancy, when other steroid levels are elevated (Murphy, 2001).

3.3. Neurosteroid levels during pregnancy

Mean values of nine neurosteroids measured during each trimester and postpartum are reported in Table 2. All of the mean neurosteroid concentrations fell within mean ranges previously reported for premenopausal (PM) and pregnant (P) women based on LC-MS, GC-MS or IA analytical methods. Levels of DHEA increased slightly over the course of pregnancy, however, none of the mean values were significantly different ($p > 0.05$) between trimesters or when compared to the post-partum value. The observed stability in DHEA concentrations is consistent with the findings of Soldin et al. (2005), who reported mean DHEA levels ranging from 1.88 to 2.11 ng/mL during pregnancy, with a post-partum value of 1.92 ng/mL. With the exception of DHEA, all of the other neurosteroid levels measured during pregnancy were significantly greater ($p < 0.05$) than the post-partum values. The greatest changes in neurosteroid levels over the course of pregnancy were observed for estrone, 5 α -DHP, and progesterone, all of which exhibited statistically significant increases with each successive trimester. For example, mean concentrations of estrone, 5 α -DHP and progesterone increased by factors of 5.9, 3.8 and 2.9, respectively, from the first to third trimesters. Changes in the mean concentrations of allopregnanolone, pregnanolone, estradiol, and 5 α -THDOC increased significantly between the first and second trimesters, but remained relatively constant thereafter ($p > 0.05$). Mean concentrations of pregnenolone were not significantly different between trimesters, although the nominal value did increase from the first to second trimester. Similarly, Gilbert-Evans et al. (2005) reported that mean pregnenolone concentrations increased from the second to third trimester, but the observed changes were not statistically significant over the course of pregnancy.

Changes in the neurosteroid profile over the course of pregnancy for two representative subjects are shown in Figures 3 and 4. In the first case (Fig. 3), levels of progesterone increase rapidly from 8 to 30 weeks EGA, reaching a maximum value of approximately 110 ng/mL. Levels of 5 α -DHP, allopregnanolone, estrone, and pregnanolone followed a similar, but less pronounced trend, reaching maximum values ranging from 10 to 21 ng/mL in the third trimester. In this subject, the elevated neurosteroid levels dropped sharply at 36 weeks EGA, which immediately preceded a premature delivery at 36.4 weeks EGA. Following delivery, all neurosteroid levels, with the exception of progesterone (1.3 and 1.2 ng/mL) and pregnenolone (1.8 and 1.7 ng/mL), continued to decline to levels below 1 ng/mL.

In the second case (Fig. 4), levels of progesterone increased markedly from the second to third trimester, reaching a concentration of 167 ng/mL at 36.6 weeks EGA. Similarly, 5 α -DHP levels increased steadily in the latter stages of pregnancy, with concentrations

increasing from 16.5 to 32.4 ng/mL at 20 and 36.6 weeks EGA, respectively. Concentration of most of the other neurosteroids (i.e., pregnanolone, allopregnanolone, estrone, and estradiol) increased from the first to second trimester, and then remained relatively constant until after delivery (40 weeks EAG), when concentrations decreased sharply to baseline levels. Consistent with the mean values presented in Table 2, levels of 5 α -THDOC and pregnenolone were elevated, but relatively stable over the course of pregnancy.

4. Discussion

Quantification of neurosteroids in biological samples has traditionally relied upon on radioimmunoassay (RIA), and more recently chemiluminescence immunoassay (CIA) and enzyme-linked immunosorbent assay (ELISA). Despite recent improvements in these techniques, fundamental shortcomings related to antibody specificity and dynamic range limitations often lead to erroneous results. For example, an RIA allopregnanolone antibody (Purdy et al., 1990) was shown to cross-react with several progesterone analogs (Murphy and Allison, 2000), which could result in elevated allopregnanolone concentrations. Nonspecific antibody reactions are likely to be even more detrimental when immunoassay methods are used to monitor neurosteroid levels in pregnant women, since certain neurosteroids exhibit substantial increases while others remain relatively constant (e.g. Figure 4). The limited dynamic range (i.e., linear response) of immunoassay is also problematic when samples exhibit a wide range of concentrations (e.g., during pregnancy) and are analyzed in a randomized or “blind” manner, without prior knowledge of target neurosteroid levels. For example, the CAI analysis performed in this study had to be rerun several times to deal with the wide range of progesterone (54.8 to 570 ng/mL) and estradiol (2.6 to 14.1 ng/mL) concentrations. High variability in immunoassay methods were reported in a study that evaluate the precision and accuracy of eight estradiol immunoassays, which exhibited coefficients of variation that ranged from 7 to 43%. (Yang et al., 2004). Similar findings were obtained by Vesper et al. (2014), who evaluated eleven immunological methods and reported that the sensitivity varied widely among the methods with most detection limits well above 10 pg/mL, and the mean bias across all samples for each subject ranged from -2.4% to 235%. Other studies have reported closer reasonable agreement between neurosteroid levels measured in human serum using RIA and CIA (Hershlag et al., 2000), and in rats-exposed to atrazine, an endocrine disrupting compound, using RIA and LC-MS/MS (Riffle et al., 2013).

To overcome the limitations discussed above a number of LC- and GC-MS methods have been developed to simultaneously quantify multiple neurosteroids in a single sample injection. The molecular properties of neurosteroids, including their molecular weight, volatility and potential for ionization, fall between the ranges that are ideally suited for analysis by either LC- or GC-MS techniques. For this reason, the majority of MS analytical methods incorporate sample cleanup (e.g., solid-phase extraction) followed by derivatization (e.g., dansylation or silylation) in order to improve specificity and achieve lower detection limits (Abdel-Khalik et al., 2013). Recently, LC methods have incorporated atmospheric pressure photoionization (APPI) coupled with tandem mass spectrometry (MS/MS) and multiple reaction monitoring (MRM) to achieve low detection limits without the use of derivatization (e.g., Soldin et al., 2009). However, the instrumentation and technical

expertise required to operate these systems is greater than the current capabilities of most analytical laboratories. Thus, the GC-MS method presented herein was designed to provide similar capabilities as LC-MS/MS, but with a much less expensive analytical platform (e.g., <\$100K) and simplified sample preparation protocol that can be mastered by most laboratory technicians. The GC-MS method takes advantage of recent advances in mass selective detectors (MSD), which allows for operation in selective ion monitoring (SIM) mode over specified elution windows. In effect, this technology allows the user to select a limited number of *m/z* features that are detected over specified elution windows, which limits interference from background noise, thereby eliminating the need for more expensive tandem mass spectrometers (MS/MS) operated in MRM mode. The ability of the simplified GC-MS method to simultaneously quantify multiple neurosteroids over a wide range of concentrations was demonstrated through (i) comparisons of progesterone and estradiol measurements with a standard CIA method, (ii) quantification of nine neurosteroids during three trimesters and postpartum, and (iii) the presentation of representative neurosteroid concentration profiles obtained for two subjects over the course of pregnancy and postpartum. Thus, the GC-MSD method allows for the assessment of individual neurosteroid levels and ratios using a single analytical technique, and holds the potential to advance our understanding of diseases that are sensitive to alterations in neurosteroid levels, and inform the development of alternative therapies during pregnancy.

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Highlights

- A simplified GC-MS method was developed to simultaneously measure multiple neurosteroids in plasma.
- Versatility of method was demonstrated by inclusion of additional steroids and 25-hydroxy-vitamin D3.
- The GC-MS method was able to detect marked changes in neurosteroid profiles over the course of pregnancy and postpartum.

Figure 1.

Chromatogram illustrating separation and response to an 80 pg injection of eleven derivatized neurosteroids in dichloromethane. The spectrometer was operated in selection ion monitoring (SIM) mode to detect: DHEA (270.2 m/z), Estrone (342.2 m/z), Allo (300.3 m/z), Pregnan (300.3 m/z), Estradiol (416.3 m/z), 5α-DHP (231.2 m/z), Pregnen (298.2 m/z), Prog (272.2 m/z), 17-OHP (302.1 m/z), 5α-THDOC (463.4 m/z) and 25(OH)D (439.4 m/z) over specified elution windows.

Figure 2.

Representative chromatograms illustrating neurosteroid levels detected in serum sample collected from the same subject during (A) the first trimester, 13.7 weeks EGA and (B) the third trimester, 31.0 weeks EGA.

Figure 3.

Concentrations changes of seven neurosteroids measured over the course of a pregnancy that included early delivery (week 37), which was preceded by a sharp drop in progesterone, 5 α -DHP, allopregnanolone, pregnanolone and estrone.

Figure 4.

Concentrations changes in eight neurosteroids measured over the course of a pregnancy, which was characterized by steady increases in progesterone, 5 α -DHP, allopregnanolone, and pregnanolone until delivery, followed by rapid decline to baseline levels 8 weeks postpartum.

Table 1
Analytical parameters for nine neurosteroids measured by GC-ESI-MS operated in selective ion monitoring (SIM) mode.

Neurosteroid	Molecular Weight (g/mol)	Retention Time (min)	Quant. Ion (m/z)	Level of Detection (pg/mL)	Intra-Assay Coefficient of Variation (%) ^a	Recovery Efficiency (%)
DHEA C ₁₉ H ₂₈ O ₂	288.43	7.36	270.2	23	9.01	97.3
Estrone C ₁₈ H ₂₂ O ₂	270.37	7.76	342.2	67	13.13	93.1
Allo C ₂₁ H ₃₄ O ₂	318.49	8.72	300.3	36	7.96	83.4
Pregnan C ₂₁ H ₃₄ O ₂	318.94	8.94	300.3	94	9.93	85.5
Estradiol C ₁₈ H ₂₄ O ₂	272.38	9.07	416.3	161	10.64	88.9
5α-DHP C ₂₁ H ₃₂ O ₂	316.48	9.17	231.2	184	6.54	84.3
Pregnen C ₂₁ H ₃₂ O ₂	316.48	9.99	298.2	142	11.95	86.2
Prog C ₂₁ H ₃₀ O ₂	314.48	10.37	272.2	46	10.35	97.1
5α-THDOC C ₂₁ H ₃₀ O ₃	334.49	15.39	257.2	276	14.13	86.1

^a CV = standard error/mean; average of seven plasma samples each run in triplicate.

Table 2

Concentrations of nine neurosteroids measured during the first, second and third trimester, and postpartum. Reported neurosteroid concentrations determined by immunoassay (IA), liquid chromatography-mass spectrometry (LC), and gas chromatography-mass spectrometry (GC) are provided for comparison purposes.

Neurosteroid	TM 1 Conc. (ng/mL) n=11	TM 2 Conc. (ng/mL) n = 11	TM 3 Conc. (ng/mL) n = 18	PP 4 Conc. (ng/mL) n = 22	Reported conc. Ranges (ng/mL)
DHEA C ₁₉ H ₂₈ O ₂	4.54 (1.87-7.21)*	6.68 (3.45-9.91)	8.15 (4.42-11.89)	5.30 (3.18-7.42)	1.3-9.8 ^a (PM-RIA) 1.9-2.1 ^b (P-LC)
Estrone C ₁₈ H ₂₂ O ₂	0.67 ^{2,3,4†} (0.35-1.00)	3.13 ^{1,3,4} (1.16-5.09)	3.93 ^{1,2,4} (2.27-5.60)	0.09 ^{1,2,3} (0.07-0.11)	0.15-1.5 ^a (PM-IA) 2.6-22.6 ^c (P)
Allo C ₂₁ H ₃₄ O ₂	3.74 ^{2,3,4} (1.93-5.54)	7.41 ^{1,4} (5.03-9.80)	8.15 ^{1,4} (5.50-10.80)	0.29 ^{1,2,3} (0.22-0.97)	1.4-12.9 ^d (P-GC) 5.5-21.0 ^e (P-RIA) 6.6-18.4 ^f (P-GC)
Pregnan C ₂₁ H ₃₄ O ₂	1.39 ^{2,3,4} (0.67-2.12)	6.93 ^{1,4} (3.89-9.98)	8.37 ^{1,4} (3.68-13.05)	0.32 ^{1,2,3} (0.21-0.41)	1.2-5.9 ^d (P-GC)
Estradiol C ₁₈ H ₂₄ O ₂	2.72 ^{2,3,4} (0.56-4.88)	12.04 ^{1,4} (6.60-17.50)	14.92 ^{1,4} (8.92-20.92)	0.51 ^{1,2,3} (0.10-1.01)	0.9-6.2 ^b (P-LC) 3.5-24.2 ^f (P-GC)
5α-DHP C ₂₁ H ₃₂ O ₂	8.04 ^{2,3,4} (6.29-9.78)	18.09 ^{1,3,4} (12.73-13.44)	30.59 ^{1,2,4} (22.31-38.86)	2.40 ^{1,2,3} (1.70-3.09)	9.4-70.5 ^d (P-GC)
Pregnen C ₂₁ H ₃₂ O ₂	1.26 ⁴ (0.64-1.87)	2.41 ⁴ (1.33-3.50)	2.31 ⁴ (1.0-3.63)	0.48 ^{1,2,3} (0.25-0.72)	0.5-3.2 ^d (P-GC)
Prog C ₂₁ H ₃₀ O ₂	38.20 ^{2,3,4} (24.17-52.19)	96.25 ^{1,3,4} (52.45-140.05)	111.27 ^{1,2,4} (81.98-140.57)	2.75 ^{1,2,3} (1.65-3.85)	17.5-70.5 ^b (P-LC) 22.3-163.6 ^d (P-GC) 33.6-115.3 ^f (P-GC)
5α-THDOC C ₂₁ H ₃₀ O ₃	3.74 ^{2,3,4} (1.93-5.54)	7.41 ^{1,4} (5.02-9.80)	8.15 ^{1,4} (5.50-10.80)	0.29 ^{1,2,3} (0.21-0.36)	2.1-4.1 ^e (P-IA) 1.2-2.8 ^g (PM-IA)

* 95% confidence interval;

[†] significantly different (p < 0.05) from the time 1 (TM1), 2 (TM2), 3 (TM3) or 4 (PP).

^a Kratz et al., 2004;

^b Soldin et al., 2005;

^c Peck et al., 2002;

^d Gilbert-Evans et al., 2006;

^e Paoletti et al., 2006;

^fPariznek et al., 2005;

^gTuverti et al., 2008.

PM = premenopausal; **P** = during pregnancy, **TM 1** = trimester 1 (0 to 13 weeks), **TM 2** = trimester 2 (14-28 weeks); **TM 3** = trimester 3 (27-40 weeks), **PP 4** = postpartum, time period 4.