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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 min. 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.

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., [6,22,23]. Steroid hormones are derived from cholesterol via hydroxylation to form pregnenolone, which is then converted to progesterone via 3β-hydroxysteroid hydrogenase (3βHSD). Two parallel enzymatic reaction pathways act to convert pregnenolone and progesterone to other neurosteroids, including estradiol and allopregnanolone [20,35]. 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 ([16,28,7,24,13]). 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.

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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 [9,26]. Although allopregnanolone is often cited as the primary neurosteroid responsible for reduced seizure susceptibility when progesterone is elevated, several other neurosteroids, including 17β-estradiol and pregnenolone sulfate (the conjugated form of pregnenolone), are considered to be proconvulsants. Therefore, cyclical changes in estrogens as well as progesterone and its metabolites are likely to play a key role in catamenial epilepsy [31,27].

During pregnancy, 16–37% of women with epilepsy experience an increase in seizure frequency ([3,14,4]), and seizure relapse has been reported to be the highest during the three peripartum days [36]. 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 [6] monitored levels of 3α-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 post-partum, 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., [10,5,17,32]). Although RIA is well-established, the technique is subject to low specificity due to anti-body cross reactions [2], 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 [18] 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 [8].

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 [1]. 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 [1,34]). 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. [6]. 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), 3α-hydroxy-5α-pregnan-20-one; Calcifediol (25(OH)D), 25-hydroxyvitamin D3 (HPLC grade > 98% purity); Dehydroepiandrosterone (DHEA), 3β-hydroxy-5-androsten-17-one; 5α-dihydroprogesterone (5α-DHP), 5α-pregnan-3,20-dione; Estradiol, 1,3,5-estratriene-3,17β-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), 3β-hydroxy-5-pregnen-20-one (98% purity); Pregnanolone (Pregnan), 3α-hydroxy-5β-pregnan-20-one; Progesterone (Prog), 4-pregnene-3,20-dione; 5α-tetrahydrodeoxycorticosterone (5α-THDOC), 5α-pregnane-3α,21-diol-20-one (95% purity).

Four deuterated neuroactive steroids, Allo-D4 (17, 21, 21, 21; 96–98% purity), 5α-DHP-D6 (1, 2, 4, 5, 6, 7; 95% purity), Pregnan-D4 (17, 21, 21, 21; 96–98% purity), and 5α-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 MΩ 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