

Novel neurosteroid panel quantitation method: Role of placental factors in neonatal brain development.

Karolina M. Krasinska^a, Florian Ermini^b, Theresa McLaughlin^a, Anna Penn^b, Allis S. Chien^a

^aVincent Coates Foundation Mass Spectrometry Laboratory, Stanford University; ^bDepartment of Pediatrics, Stanford School of Medicine, Stanford, CA

Overview

- Simultaneous, quantitative analysis of 8 steroids in serum and tissue from different brain regions (cortex, cerebellum and hippocampus) of male and female mice
- Multistep extraction procedure was employed for optimal recovery of analytes
- Low- to sub-nanomolar limits of detection for progesterone (PROG), allopregnanolone (ALLO), estrogen (EST), pregnenolone (PREG), corticosterone (CORT), testosterone (TES), DHT and DHEA were observed

Introduction

During pregnancy, progesterone and estrogen levels rise 100-fold in maternal plasma, and increasing evidence suggests that these maternal steroid hormones and many of their derivatives have stimulating and neuroprotective effects on the fetal brain. Prematurely born infants suffer from early withdrawal of these important neurodevelopmental factors; this lack may contribute to the prevalence of later cognitive impairment in these infants.

To investigate the effect of this early steroid withdrawal on the development of the mouse brain, LC-MS/MS is used to quantify steroids extracted from plasma and brain tissue. Developing a highly efficient extraction procedure and a sufficiently sensitive and selective detection method were significant challenges in this study. The resultant method enables the concurrent quantitation of eight steroids at subnanomolar levels, in extremely small samples.

FIGURE 1. Biosynthetic pathway of neurosteroids in brain. Progesterone, estrogen and their close derivatives were selected for the LC-MS/MS assay.

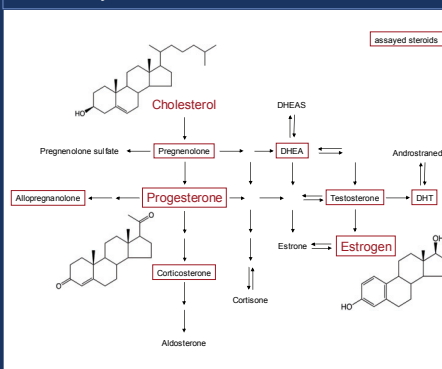


FIGURE 2. Progesterone and estrogen levels in human pregnancy. Preterm infants miss up to 15 weeks of exposure to high hormone levels.

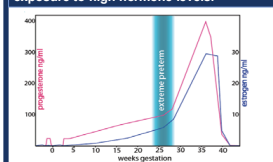
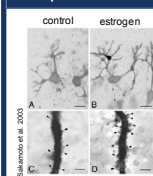


FIGURE 3. Estrogen stimulates dendrite development in rats.¹

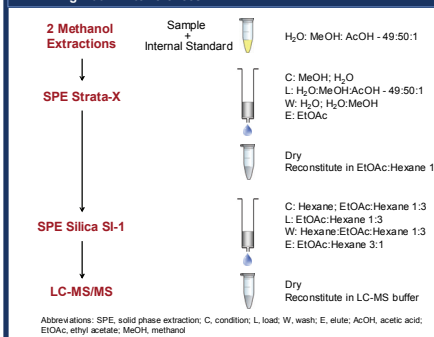


Methods

Sample preparation: 11 day old CD1 mice were separated by gender, and brains were dissected in PBS. Cortices, cerebella and hippocampi were collected and sonicated in PBS; protein content was measured by Bradford assay and sample sizes were adjusted to 1 mg protein each. After addition of stable isotope labeled internal standards, steroids were extracted twice with methanol/1% acetic acid. The supernatants were combined and cleaned up via two stages of solid phase extraction (SPE), utilizing first Strata-X reverse phase cartridges and then Strata Si-1 silica cartridges². Dried samples were reconstituted in 50% methanol/1% acetic acid and analyzed by LC-MS/MS.

Instrumentation: Analysis was carried out by ESI LC-MS/MS using an Agilent 1100 HPLC and Waters Quattro Premier triple quadrupole MS. HPLC conditions: Luna C18, 50 x 1 mm column, 3 μm particle size (Phenomenex, CA); gradient 20%-100% B in 5.7 min., hold at 100% for 3 min., total run time 15 min.; A: 50% MeOH/1% acetic acid, B: MeOH; 150 μl/min flow rate.

SCHEME 1. Sample preparation protocol. Employment of two methanol extractions followed by two stages of solid phase extraction (SPE) was essential in obtaining satisfactory recovery of analytes and minimizing matrix interferences.



Results

FIGURE 4. SRM transitions. Unique precursor ion – fragment ion pairs were established for each analyte and corresponding internal standard. LC gradient optimization was necessary in order to achieve satisfactory separation between analytes and their isomers.

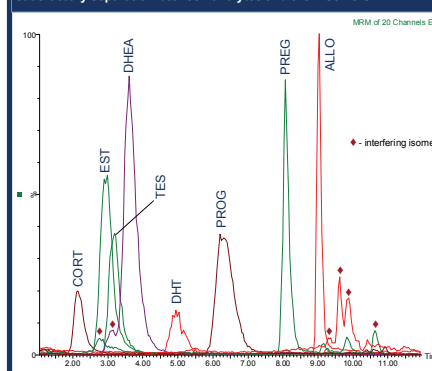


FIGURE 5. Steroid levels in brain tissue. In these samples, six steroids were quantified with our method. Surprisingly, we found no major differences among regions of increased growth (cerebellum, hippocampus) vs. normal growth (cortex). We detected only small gender specific differences: notably, estrogen and testosterone appear elevated in the female hippocampus and DHEA is elevated in the female cortex.

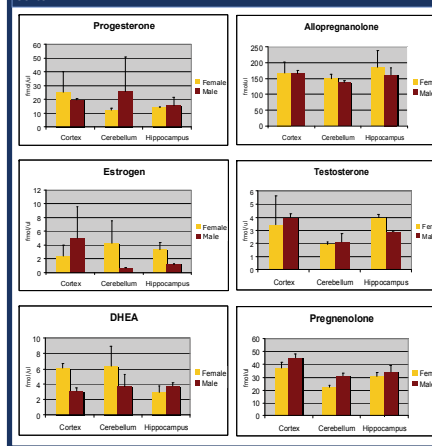


FIGURE 6. Method validation. Limited stability of DHT, DHEA and PREG was observed which resulted in low r^2 values. Use of an isotopically labeled internal standard specific to each analyte rather than a chemical analogue would negate this effect. Analyte recovery varied significantly among steroids, and CORT is lost altogether, demonstrating the need for further improvement in extraction procedures.

Steroid name	IS	SRM transition	RT (min)	Linear range (fmol)	LLOD* (fmol on column)	r^2	Analyte recovery (%)
Corticosterone (CORT)	EST IS	347.4 > 120.9	2.15	3-10 ⁴	3	0.982	0
Estrogen (EST)	EST IS	255.3 > 158.8	2.95	20-5x10 ⁴	20	0.994	68.2
Testosterone (TES)	EST IS	289.3 > 96.86	3.21	1-5x10 ⁴	1	0.984	20.3
DHEA	EST IS	271.3 > 253.1	3.60	30-10 ⁵	30	0.912	44.0
DHT	EST IS	291.4 > 252.2	4.95	5-10 ⁵	5	0.851	24.8
Progesterone (PROG)	PROG IS	315.3 > 96.73	6.3	0.1-10 ⁴	0.1	0.997	102
Pregnenolone (PREG)	EST IS	299.4 > 281.3	8.1	10-10 ⁵	10	0.967	112
Allopregnanolone (ALLO)	ALLO IS	301.2 > 134.8	9.0	20-10 ⁴	20	0.996	74.1

* LLOD defined as S/N ratio of 3:1

Conclusions

- Developed method enables the concurrent monitoring of multiple steroids without derivatization
- Steroid extracts from brain tissue can be efficiently cleaned up with SPE for subsequent highly sensitive LC-MS/MS; the analytes' recoveries vary, however, and the extraction procedure requires further modifications.
- There are minimal differences in steroid levels in the analyzed brain regions and between genders

Future work

- Optimize the extraction method in order to increase the number of steroids included in the assay
- Study the effect of maternal high steroid levels on steroid levels in the embryonic brain
- Utilize in vivo models for steroid treatment: Effect of peripheral application on steroid levels in brain
- Apply to clinical research: Steroids in the cerebrospinal fluid of neonates as biomarkers for brain injury and brain development

References

- Sakamoto, H.; Mezaki, Y.; Tsutsui T., et al. *Endocrinology* **2003**, 144, 4466-4477
- Higashi, T., et al. *Biomedical Chromatography* **2008**, 22, 34-43

Acknowledgements

Thanks to the Vincent and Stella Coates Foundation, John Merck Fund for Disabilities, Susman and Asher Foundation and Packard Children's Health Initiative Funds (A.A.P.)

This poster may be downloaded from the Stanford University Mass Spectrometry website at <http://mass-spec.stanford.edu/Publications.html>

