SUPPLEMENTAL DATA

Liquid chromatography/ tandem mass spectrometry (LC/MS/MS)

In a parallel experiment, we attempted to measure tissue steroid levels using liquid chromatography and tandem mass spectrometry. The methods and results are presented below.

METHODS

Multiple sets of tissue samples for PN0-PN10 animals were sent to the Center for Human Toxicology, University of Utah for analysis by LC/MS/MS. Initially, individual tissue samples were homogenized with 1 mL of 50 mM sodium phosphate and methanol (90:10). For the analysis of testosterone and dihydrotestosterone a 0.4-mL volume of each homogenate was hydrolyzed with a solution of beta-glucuronidase. The hydrolyzed sample homogenates were then extracted with methyl *t*-butyl ether (MTBE). The extracts were evaporated to dryness and then reconstituted with 0.1 % formic acid:methanol (60:40). The derivatized extracts from samples prepared in this manner were analyzed by two approaches, the first analysis was done using a ThermoFinnigan TS Quantum LC/MS/MS and the second set of samples were analyzed with an Applied Biosystems Qstar XL mass spectrometer (LC/MS-TOF). Calibration standards containing known amounts of testosterone or dihydrotestosterone were also prepared and analyzed by each method

For analysis of estradiol, a 0.4-mL volume of each homogenate was hydrolyzed with a solution of beta-glucuronidase in order to release conjugated estradiol in an attempt to increase the amount of steroid available for quantification. The hydrolyzed sample homogenates were then extracted with MTBE. The extracts were derivatized with Dansyl chloride and then extracted with 1-chlorobutane:acetonitrile (4:1). These derivatized extracts were evaporated to dryness and then reconstituted with 0.1 % formic acid and acetonitrile (60:40). The extracts were analyzed by the same LC/MS/MS and LC/MS-TOF instruments as used for testosterone and DHT. Calibration standards containing known amounts of 17α-estradiol and 17β-estradiol in 50 mM sodium phosphate and methanol (90:10) were also prepared and analyzed.

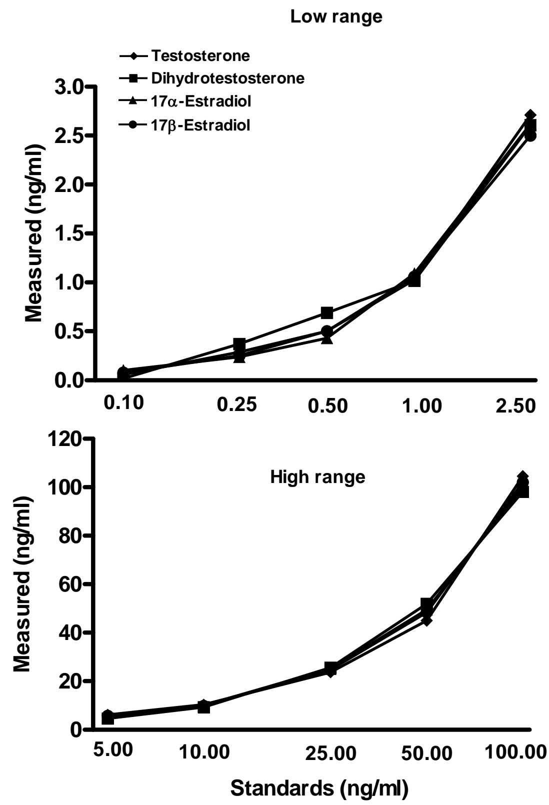
When it became apparent that the amount of steroid in individual samples was too low to be detected an additional set of samples was prepared and tissue from multiple animals pooled (12-32 individual samples) and analyzed by LC/MS-TOF

RESULTS

The calibration curves presented in the Supplemental Figure indicate the precision of LS/MS/MS in detecting all four steroids at concentrations ranging from 0.1ng/ml to 100 ng/ml. Quality control samples indicated that the variance in measurement between samples was on average 4%. All four steroids were undetectable in individual samples. This prompted us to pool large numbers of samples (12 -32). Of the 20 pooled samples analyzed, testosterone was detected in one (32 pooled samples of female cortex and hippocampus at PN4-6, the peak period for testosterone) and DHT was detected in five samples but there was no clear pattern or relationship to those measures obtained by RIA of individual samples. Estradiol was not detected in any sample. It was concluded that the limits of sensitivity of LC/MS are above the threshold for detection of the very low quantities of steroid present in the developing brain.

Figure Legend

Supplemental Figure. Calibration curves for four steroids assessed by LC/MS/MS. Testosterone, dihydrotestosterone, 17a-estradiol and 17b-estradiol were quantified by LC/MS/MS in standardized samples to generate a calibration curve for tissue analyses. The close agreement between measured and actual amounts of steroids attests to the high degree of specificity of this technique for measuring individual steroids. However, attempts to quantify steroids in tissue samples from the neonatal brain were unsuccessful because of low sensitivity.



Low range
Testosterone
Dihydrotestosterone
17α-Estradiol
17β-Estradiol
Measured (ng/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.10
0.25
0.50
1.00
2.50
High range
Measured (ng/ml)
120
100
80
60
40
20
0
5.00
10.00
25.00
50.00
100.00
Standards (ng/ml)