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Determination of endogenous testosterone in rat tissues following fetal alcohol exposure using HPLC with UV detection

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Abstract

A novel method for quantitation of brain neurosteroid levels using HPLC with UV detection is described. In this simple and reliable method, testosterone from the brain and whole blood, and the internal standard, 17α-methyl testosterone, were extracted in 20% acetonitrile–phosphate buffer (pH 2.8), followed by solid phase extraction (SPE). The calibration curve was linear in concentration ranges from 0.1 to 10 ng from 0.2 g of tissue. We successfully applied this method to the analysis of endogenous testosterone in the male offspring of rats exposed to alcohol in utero. The concentration of testosterone at 21 post delivery in fetal alcohol exposure (FAE) group was significantly greater than the concentrations in either pair-fed or the ad libitum controls. These results support the usefulness of this method as a means of quantitating neurosteroids, and illustrate its applicability to fetal alcohol exposure.

Keywords: Endogenous testosterone; Fetal alcohol exposure; HPLC; UV

1. Introduction

A possible consequence of fetal alcohol exposure (FAE) is the development of neuropsychiatric disturbances such as attention deficit hyperactivity disorder [1] and/or cognitive difficulties associated with fetal alcohol spectrum disorders [2]. Neurosteroids, synthesized within neurons independently from peripheral endocrine glands, are thought to be involved in the development of these disorders [3]. It is known that fetal alcohol exposure alters the normal surge pattern of plasma testosterone [4,5]. It is possible that exposure to alcohol could modulate the action on neural steroids, as well as plasma. A challenge in investigating this hypothesis, however, has been the lack of a simple and reliable method for quantitating neurosteroids.

The current methods for measuring sex steroids include radioimmunoassay (RIA), gas chromatography–mass spectrometry (GC–MS), gas chromatography with electron capture negative chemical ionization mass spectrometry (GC–ECNCI–MS), and liquid chromatography–electron capture atmospheric pressure chemical ionisation–mass spectrometry (LC–ECAPCI–MS). Each of these methods has several limitations when applied to the quantitation of neurosteroids. RIA requires a large amount of sample, often more than what can be obtained from rat neonates [6]. Additionally, RIA entails elaborate sample preparation [7], and cross reactivity with unidentified compound even when performed correctly [8]. More sensitive techniques include GC–MS, GC–ECNCI–MS, and LC–ECAPCI–MS. GC–MS in particular, offers the advantage of being able to simultaneously quantitate various neurosteroids. However, it too has elaborate sample preparation requirements including HPLC fractionation [9] or multiple purification steps [10]. LC–ECAPCI–MS offers the advantage of high sensitivity with simplified sample preparation [11]. The high cost of the detectors for these methods [11–13], however, prevents their utilization by many laboratories. Recently, a simplified LC method using a UV detector has been developed for plasma testosterone measurement [14]. This assay, however, is not optimal for analysis of neuro-testosterone because of chemical dissimilarities between the internal standard and testosterone, and the inappropriateness of the extraction method for brain matrix.

We developed a simple and reliable analytical method that combines HPLC with an inexpensive UV detector to quantify endogenous testosterone in rat tissues. We then successfully applied this method to the analysis of testosterone in the brain

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and whole blood of male rats chronically exposed to alcohol in utero.

2. Materials and methods

2.1. Materials and reagents

Testosterone was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). All other reagents were purchased from Sigma–Aldrich Co. (St. Louis, MO). All chemicals used in this study were of analytical grade. Phosphate buffer (50 mM, pH 2.8) for solid phase extraction (SPE) was prepared by adding 40 mmol of sodium dihydrogenphosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$) and 10 mmol of phosphoric acid (85%, 14.7 mol/L) to 1 L of distilled water. Phosphate buffer (10 mM, pH 6.9) for mobile phase was prepared by adding 5 mmol of sodium dihydrogenphosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$) and 5 mmol of disodium hydrogenphosphate 12-water ($Na_2HPO_4 \cdot 12H_2O$) to 1 L of distilled water. Mobile phase consisted of acetonitrile:10 mM phosphate buffer (pH 6.9) = 50:50 (v/v).

2.2. Analytical method validation

Testosterone and the internal standard (IS), 17α-methyl testosterone, were each dissolved in mobile phase to make up a concentration of 1 mg/ml. Standard solutions of testosterone were diluted at 10, 1 and 0.1 μg/ml and the IS solution at 1 μg/ml. Calibration curves for brain samples were prepared by using non-alcohol treated tissues and those homogenates were diluted with methanol to prevent interference by endogenous testosterone (Fig. 1C). Preliminary tests of a 1:20 sample dilution showed the peak of endogenous testosterone under the limit of detection. Six percent albumin (Fraction V) in a saline solution was used for the calibration curves for blood [12]. Samples for the calibration curves and tests of intra- and inter-assay accuracy and precision were prepared by spiking the standard solutions to these preparations at final concentrations of 0.05–5 ng/ml. Extraction recovery was obtained by spiking standard solutions to the matrices, which were prepared in the same manner as the tested samples, before or after the extraction steps. The calculation was made by comparing the ratio of the peak area of testosterone to that of the IS, which was added after the SPE extraction.

2.3. Sample preparation

A 0.2 g tissue or 2 ml of blood sample along with 10 ng of IS were added to 2 ml of methanol and sonicated for 3 min. The preparation was centrifuged at 16,600 × *g* at 4 °C for 10 min, and the supernatant was removed and evaporated to dryness under a stream of nitrogen at 60 °C. The extraction step was repeated once more on the residue with 1 ml of methanol.

2.4. Solid phase extraction procedure

Crude extraction residues after evaporation were dissolved in 2 ml of 20% acetonitrile diluted with 50 mM phosphate buffer (pH 2.8) and centrifuged at 17,600 × *g* at 4 °C for 10 min. The supernatants were applied to 1 cc Oasis HLB cartridges (Waters, MA, USA) previously equilibrated with 1 ml of acetonitrile and 1 ml of phosphate buffer (pH 2.8) in succession. The cartridges were then washed with 2 ml of 5% acetonitrile–50 mM phosphate buffer (pH 2.8). Testosterone was eluted with 1 ml of acetonitrile and then evaporated to dryness under a stream of nitrogen at 60 °C. The residue was dissolved in 100 μl of mobile phase and 10 μl was applied to the HPLC apparatus.

2.5. HPLC analysis

Separation and quantitation of testosterone was carried out on a SHIMADZU LC-10AD VP liquid chromatograph (Shimadzu Co., Kyoto, Japan) equipped with DGU-12A degasser, a CTO-10AS VP column oven and an SPD-10A VP UV spectrophotometric detector. A 250 mm × 3.0 mm I.D., 5 μm CAPCELL PAK C18 MG analytical column (Shiseido Co. Ltd., Tokyo,

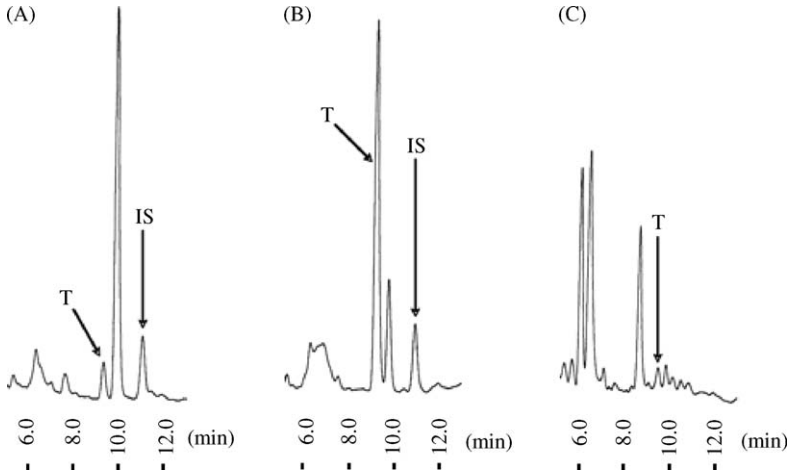

Fig. 1. (A) Chromatogram of brain extract from the intact rat neonatal tissue diluted with 20-fold methanol, spiked with 0.5 ng of T and 1 ng of IS (17α-methyltestosterone). (B) Chromatogram of 6% albumin spiked with 5 ng of T and 1 ng of IS. (C) Blank chromatogram of the intact rat neonatal tissue without methanol dilution. T, testosterone; IS, internal standard.

Japan) was used for the chromatographic separation. The flow-rate was 0.4 ml/min and the column temperature was set at 13 °C. The wavelength was 240 nm with a sensitivity range of 0.005 a.u.f.s. Data were recorded on a SHIMADZU chromatopak C-R8A. The concentration of testosterone in each sample was determined by calculating the ratio of the peak area of testosterone to that of the IS and comparing it to the calibration curve for each HPLC run.

2.6. Biological application of the method

The experimental procedure was reviewed by the Committee of the Ethics on Animal Experiment in Yamaguchi University School of Medicine and conformed to the Guideline for Animal Experiment in Yamaguchi University School of Medicine and The Law (no. 105) and Notification (no. 6) of the Government.

Female Sprague–Dawley rats weighing 180–230 g were paired nightly with male rats. On the following morning, vaginal smears were evaluated and those positive for sperm were identified as Day 1 of gestation. Each pregnant dam was randomly assigned to the ad libitum control, pair-feeding control, or FAE groups. The pair-fed and FAE groups were fed a liquid diet based on the Lieber–DeCarli formation [15] at 7:00 p.m. each day. The FAE group received the liquid diet containing no ethanol for the first 2 days, 2% ethanol for Days 3 and 4, 3% ethanol for Days 5 and 6, and 5% ethanol for the remainder of gestation. The pair-feeding control group received the same liquid diet containing no ethanol throughout gestation. The ad libitum control group had free access to breeder block chow and water. At birth, pups from the liquid diet groups were cross fostered onto surrogate untreated dams. Blood and brain samples of neonates from postnatal Days 14 and 21 were collected and stored at −80 °C until analysis. Specimens were prepared as described above.

2.7. Statistical analysis

All values are expressed as mean ± S.D. Statistical comparisons between the FAE group and controls were carried out using a one-way ANOVA. A P-value of 0.05 was considered as the threshold for a significant difference.

3. Results and discussion

3.1. Sample preparation

Our method using methanol plus a 20% acetonitrile–phosphate buffer resulted in superior sample preparation as compared to other methods. Methanol containing 1% acetic acid has been used for effective extraction of steroids from brain matrices. However, an excess of 1.5 g wet weight of sample was required for this method, rendering it unusable for 1 g neonate rat brains [9,11]. Additionally, we observed that the organic acid used in this extraction method resulted in several interference peaks on the chromatogram, making it unsuitable for LC. A method using 75% methanol is capable of extracting steroids from as little as 0.15 g of brain tissue [12]. To improve the steroid yield, we increased the percentage of methanol up to 100%. This modification, however, resulted in insufficient deproteinization to produce a clear supernatant, necessitating the deproteinization by 20% acetonitrile–phosphate buffer (pH 2.8) to complete the protein removal.

Further purification was achieved with a simple solid phase extraction. For brain matrices, Amini et al. [6] used C2 cartridges for the extraction of neurosteroids with nonpolar solvents. Oasis HLB cartridges have also been used for the extraction of neurosteroids with a methanol mixture; however, an additional SPE procedure by normal phase extraction is required [11]. By using acetonitrile instead of methanol, and lowering the solvent of pH, we were able to develop a single step SPE protocol using the Oasis HLB cartridges with a superior recovery of testosterone and the IS, and no interference peaks.

3.2. Chromatographic method

Fig. 1A shows chromatogram of brain extract from the intact neonatal tissue diluted with 20-fold methanol, spiked with 0.5 ng of testosterone and 1 ng of IS. Fig. 1B show chromatogram of 6% albumin spiked with 5 ng of testosterone and 1 ng of the IS. The retention times of both matrices were about 9.8 min for testosterone and 11.7 min for the IS. Calibration curves for HPLC chromatograms have been prepared by either subtracting the peak height of the endogenous testosterone [14] or by analyzing dilutions of an intact matrix [16]. Valleé et al. [12] used intact brain homogenates for the preparation of calibration curves; however, we observed a peak of endogenous testosterone with the potential to interfere with assay validation (Fig. 1C). Dilution of the matrix 20-fold in methanol, however, abolished this peak on the LC chromatograms.

Intra-assay accuracy and precision results, as shown in Table 1, were determined by adding 0.1 to 10 ng of testosterone to each matrix, which being the assay range of both tissues. The correlation coefficient for both the diluted brain homogenates and albumin exceeded 0.999. The limit of detection and of quantitation in both the diluted brain homogenates and albumin was

Table 1
Intra-assay accuracy and precision of HPLC analysis of testosterone (n = 3–5)

	Amount of testosterone (ng)				
	0.1	0.5	1	5	10
Standard					
Mean	0.10	0.57	1.08	5.56	10.7
S.D.	0.011	0.019	0.053	0.356	1.021
R.S.D. (%)	11.2	3.4	4.9	6.4	9.6
Brain					
Mean	0.11	0.53	1.05	5.01	11.0
S.D.	0.013	0.051	0.067	0.238	0.626
R.S.D. (%)	11.8	9.6	6.4	4.8	5.7
Albumin					
Mean	0.13	0.66	1.17	7.13	13.2
S.D.	0.015	0.064	0.90	0.903	1.047
R.S.D. (%)	12.0	9.7	9.4	12.7	7.9

Values are expressed as mean ± S.D. R.S.D., relative standard deviation.

Table 2
Inter-assay accuracy and precision of HPLC analysis of testosterone ($n = 3$–5)

	Amount of testosterone (ng)				
	0.1	0.5	1	5	10
Standard					
Mean	0.11	0.57	1.06	5.69	10.7
S.D.	0.018	0.014	0.030	0.326	0.548
R.S.D. (%)	15.9	2.4	2.9	5.7	5.1
Brain					
Mean	0.12	0.48	0.93	5.03	10.6
S.D.	0.010	0.014	0.013	0.065	0.046
R.S.D. (%)	8.5	2.9	1.4	1.3	0.4
Albumin					
Mean	0.16	0.56	1.41	7.83	14.1
S.D.	0.009	0.019	0.021	0.279	0.177
R.S.D. (%)	5.9	3.4	1.5	3.6	1.3

Values are expressed as mean ± S.D. R.S.D., relative standard deviation.

50 pg and 0.1 ng, respectively. The relative standard deviation (R.S.D.) was less than 11.8% in diluted brain homogenates and 12.7% in albumin. Measurement of inter-assay accuracy and precision are shown in Table 2. The R.S.D. was less than 8.5% in diluted brain homogenates and 5.9% in albumin. Extraction recovery of testosterone from HPLC analysis was shown in Table 3. The extraction efficiency was about 80–90% in both tissues.

3.3. Application

We applied our new method to the determination of testosterone levels of postnatal Days 14 and 21 in the FAE group and the non-FAE control groups. Chromatograms of tissue extracts from rats exposed to alcohol in utero are shown in Fig. 2. We observed much higher level of testosterone in the whole blood than that in the brain. This may be due to systemic delivery of testosterone, which is secreted from peripheral endocrine glands and reaches target cells to respond. Fig. 3 shows the concentration of testosterone in the brain of male neonates. At 21 days of age, the level of testosterone was significantly higher in the FAE group, compared to that in the pair-fed and the ad libitum controls groups ($P < 0.05$). No significant differences were observed at postnatal Day 14. Chronic exposure of alcohol in utero increases the level of pregnenolone sulfate from gestational Day 14 to postnatal Day 5 [17]. Pregnenolone sulfate, an excitatory neurosteroid, is a precursor of testosterone. Therefore, it is plausible that testosterone biosynthesis may be upregulated in infants exposed to alcohol in utero.

Table 3
Extraction recovery of testosterone from HPLC analysis (%, $n = 3$–5)

Amount of testosterone (ng)	Brain	Albumin
0.1	89.5	84.0
0.5	88.4	87.1
1	81.2	86.2
5	88.9	91.4
10	91.8	87.2

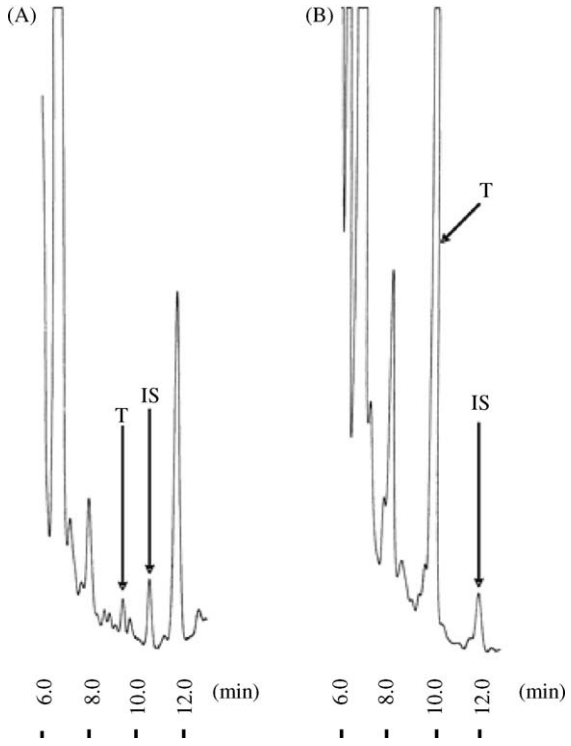

Fig. 2. Chromatograms of extracts from rat neonatal tissues exposed to alcohol in utero: (A) brain; (B) whole blood. T, testosterone; IS, internal standard.

Fig. 4 shows the concentration of testosterone in whole blood. At postnatal Day 21, the level of testosterone was significantly higher in the FAE group compared to the non-FAE controls groups ($P < 0.01$). Our measurement was higher than expected compared to the existing literature, which range from 1.5 to 3.0 ng/ml [4,18–20]. This may be the result of our use of whole blood rather than the more commonly tested plasma. An alternate explanation is the duration of ethanol exposure in this study. It is well known that in intact rats, plasma testosterone abruptly rises at gestational Day 18 [21]. This testosterone surge (T surge) is modulated by the duration of alcohol exposure, and is increased when alcohol exposure is begun at gestational Day 10 [4,18]. In contrast, exposure to alcohol from gestational Day

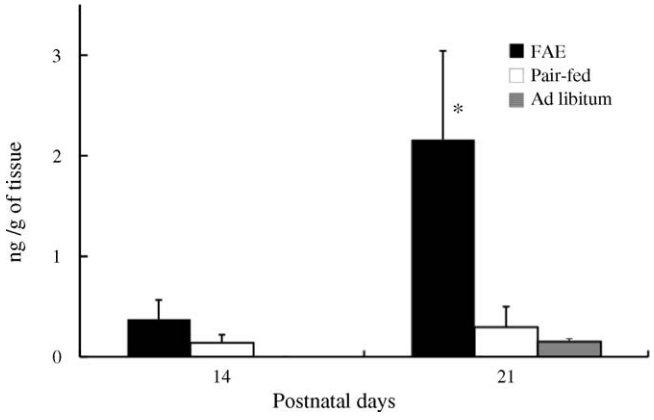

Fig. 3. The concentration of testosterone in rat neonatal brain tissue at 14 and 21 postnatal days of conception ($n = 3$–5). Values are expressed as mean ± S.D. (*) $P < 0.05$ versus pair-fed and ad libitum controls.

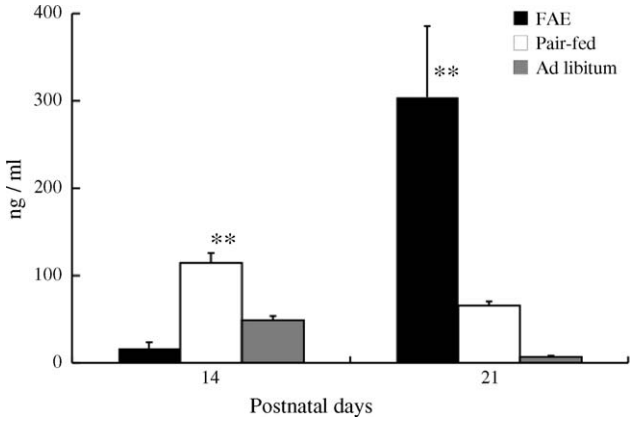

Fig. 4. The concentration of testosterone in rat neonatal whole blood at 14 and 21 postnatal days of conception ($n = 3$–8). Values are expressed as mean ± S.D. (**) $P < 0.01$ versus pair-fed and ad libitum controls.

14 significantly suppresses the T surge in both the fetus [19] and newborn [5,20]. We began the ethanol exposure at gestational Day 7 so it is likely that this early and long-standing exposure augmented the level of testosterone in the neonate. At postnatal Day 14 the concentration of testosterone was the highest in the pair-fed group ($P < 0.01$, Fig. 4 left). The level of allopregnenolone, a neurosteroid that modulates stress responses, is easily induced by pair-feeding [22]. Allopregnanolone is the metabolite of pregnenolone, suggesting that alteration of stress responses by pair-feeding might upregulate testosterone biosynthesis in the neonate.

Numerous studies have been performed to examine the effect of in utero alcohol exposure on changes in plasma testosterone levels, in the fetus and newborn. We applied our new quantitation method to measure testosterone levels in the brain of juvenile rats. To fully elucidate the effects of FAE on neural steroids and the behaviors modulated by them, additional studies should also be elucidated.

4. Conclusion

We developed a simple and sensitive method to accurately quantify the level of endogenous testosterone in rat brain and whole blood. This method is an improvement on several existing methods of quantitation. Using this assay, we demonstrated that the concentration of testosterone in both the brain and whole blood of FAE rats at postnatal Day 21 was significantly higher than that of controls.

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