Studies on Neurosteroids XX. Liquid Chromatography–Tandem Mass Spectrometric Method for Simultaneous Determination of Testosterone and 5α-Dihydrotestosterone in Rat Brain and Serum

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Abstract

Testosterone (T) and 5α-dihydrotestosterone (DHT) are now referred to not only as androgenic steroid hormones, but also as neuroactive steroids, because they elicit anesthetic and anxiolytic effects. Methods using liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS–MS) coupled with derivatization are developed and validated to examine rat brain and serum levels of T and DHT and their stress-induced changes. The steroids are extracted with methanol–acetic acid from the brain tissue or serum, purified using solid-phase extraction cartridges, derivatized with a permanently charged reagent, 2-hydrazino-1-methylpyridine, and subjected to LC–MS–MS. [19,19,19-$^2H_3$]-T is used as the internal standard. The intra- and inter-assay coefficients of variation are below 10.0%, and the analytical recoveries are 98.1–103.0%. The developed methods are applied to the animal study and it was found that a fair amount of DHT is continuously and locally synthesized in the brain, and its level is not changed by the immobilization stress and depends on the brain T level.

Introduction

Recently, several papers have described that testosterone (T) and its metabolite, 5α-dihydrotestosterone (DHT), elicit anesthetic and anxiolytic effects in animal models (1,2), and they are now referred to not only as androgenic steroid hormones, but also as neuroactive steroids. It has now been demonstrated that steroids possessing depressant properties for the central nervous system may act through membrane receptors, mostly the γ-aminobutyric acid type A ($GABA_A$) receptor complex (3). For example, allopregnanolone (AP; 3α-hydroxy-5α-pregn-20-one) positively modulates the action of GABA at these receptors to raise the threshold of brain excitability during the response to stressful stimuli. It is also known that the endogenous AP level in the animal brain is rapidly elevated from a trace level (practically none) to a ng/g tissue level by several acute stress paradigms (4,5). Contrary to this, the brain levels of the androgenic neuroactive steroids and their stress-induced changes have been poorly examined. A specific method for the determination of the brain and blood levels of the androgenic neuroactive steroids can contribute to the elucidation of their effects on brain function.

Numerous methods have been described for the characterization and determination of neuroactive steroids, such as immunoassay and gas chromatography (GC)–mass spectrometry (MS) (6). Recently, liquid chromatography (LC) coupled with atmospheric pressure ionization, such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI)-MS, has been used for these purposes due to its specificity, versatility, and simultaneous multi-analyte quantitation capability (5,7–10). Steroids with the 3-oxo-4-ene-structure, such as T, show relatively high responses in the positive ESI-MS, because they are more basic than most neutral steroids due to the charge delocalization in the protonated form and can provide some characteristic A-ring product ions in tandem MS (MS–MS) (11). We have developed an LC–ESI-MS–MS method for profiling the rat brain 3-oxo-4-ene-neuroactive steroids including T and found that T is always present at a ng/g tissue concentration in the male rat brains regardless of the stress stimulus using the method (12). However, 5α-reduced steroids, such as DHT, show extremely poor responses in both ESI- and APCI-MS due to their low proton affinity. Although the introduction of a newly developed API technique, atmospheric pressure photoionization (APPI), has also been for steroid analysis, that technique has limitations on its sensitivity and is susceptible to the matrix effect (13). The lack of a specific method for the brain DHT analysis is a major obstacle for further characterization of the physiological function of this steroid and the mechanism by which it affects the brain function. The determination of the blood level of DHT is also helpful for identifying its origin (i.e., whether it is locally synthesized in the brain or synthesized in the peripheral organs and then transported into the brain).

To enhance the assay sensitivity in ESI-MS, derivatization (i.e., tagging a poorly ionizable steroid with a permanently charged moiety) is extremely useful (14,15). We have developed a

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permanently charged derivatization reagent, 2-hydrazino-1-methylpyridine (HMP), for the LC–ESI-MS of oxosteroids (16) and also successfully used this reagent for the determination of trace amounts of various steroids in biological samples (5,17). With this background information, we first developed and validated methods for the simultaneous determination of T and DHT in the rat brain and serum using LC–MS–MS coupled with derivatization. In the next step, the stress-induced changes in their rat brain and serum levels were analyzed using the developed methods.

Experimental

Materials and reagents

T and DHT were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Stock solutions of T and DHT were prepared as 100 µg/mL solutions in ethanol. Subsequent dilutions were carried out with ethanol to prepare 0.5, 1, 2, 5, 10, and 20 ng/mL solutions. [19,19,19-$^2H_3$]-T (d_3-T) (18) was dissolved in and diluted with ethanol and used as the internal standard (IS) for the quantitative analyses of both T and DHT. The isotopic purity of d_3-T was extremely high (over 99%) and, therefore, $^2H_0$-T was not detected. [16,16,17α-$^2H_3$]-DHT (d_3-DHT) (19) was used for the determination of the recovery rate of DHT during the pretreatment procedure. HMP was synthesized in our laboratories as previously reported (16). Strata-X cartridges (60 mg adsorbent; Phenomenex, Torrance, CA) were successively washed with ethyl acetate (2 mL), methanol (2 mL), and water (2 mL) prior to use. Bond Elut Si cartridges (500 mg adsorbent; Varian, Harbor, CA) were successively washed with ethyl acetate (4 mL) and hexane (4 mL) prior to use. All other reagents and solvents were of analytical grade.

LC–MS–MS

LC–MS–MS was performed using an Applied Biosystems API 2000 triple quadrupole-mass spectrometer (Foster City, CA) connected with a Shimadzu LC-10AT chromatograph (Kyoto, Japan). The receiver (N_2 storage tank with the capacity of 36 L, Anest Iwata SAT-36-100, Yokohama, Japan) was placed between the nitrogen generator and the mass spectrometer for the large and stable supply of nitrogen. All the compounds were analyzed by ESI-MS–MS in the positive-ion mode and the conditions were as follows: declustering potential, 80 V; focusing potential, 200 V; entrance potential, 10 V; ion spray voltage, 5 kV; curtain gas (nitrogen), 45 psi; ion source gas 1 (nitrogen), 80 psi; ion source gas 2 (nitrogen), 80 psi; turbo gas temperature, 500°C; and interface heater, on. Nitrogen was used as the collision gas in the selected reaction monitoring (SRM) mode with a collision energy of 60 eV and a collision cell exit potential of 10 V. The precursor and product ions used were as follows: T-HMP, *m/z* 394.3 $[M]^+$ and 108.2 [*N*-methylpyridine+NH]$^+$; DHT-HMP, *m/z* 396.3 $[M]^+$ and 108.2; and IS-HMP, *m/z* 397.3 $[M]^+$ and 108.2. A J'sphere ODS-H80 column (4 µm, 150 × 2.0 mm i.d.; YMC, Kyoto, Japan) was used at 40°C. Acetonitrile–methanol–10mM ammonium formate (8:3:7, v/v/v) was used at the flow rate of 0.2 mL/min. The data were collected and quantified using Applied Biosystems Analyst software (version 1.3.1).

Animals

Male Wistar strain rats (7-weeks old, 190–200 g) obtained from Japan S.L.C. (Hamamatsu, Japan) were assigned either to an unstressed group (n = 10) or a group subjected to immobilization stress (n = 10). All the animals were unrestrained in a quiet place for 2 h before the experiments. The unstressed rats were then immediately euthanized by decapitation. The rats of the second group were immobilized on their backs on a board for 20 min. After immobilization, the rats were unrestrained for 30 min and then euthanized. Blood was collected from the cut end immediately after the decapitation, stored at 4°C for 3 h, then centrifuged at 1500 × g (4°C, 15 min). The serum was separated and stored at –20°C. An animal study was performed between 13:00 h and 15:00 h. All animal care and use were approved by the Institutional Animal Care and Use Committee of Kanazawa University. The statistical comparisons were performed using the Welch test in Microsoft Excel 2003 (Redmond, WA).

Pretreatment procedures

T and DHT were extracted from the rat brain tissue with methanol–acetic acid (99:1, v/v) by a previously reported method (5,12). The brain extract was diluted to adjust the concentrations of the 100 mg brain tissue/mL with methanol–acetic acid (99:1, v/v) and stored at –20°C prior to use. After the addition of an ethanolic solution of IS (200 pg in 10 µL) and water (1 mL), 1 mL of the brain extract (corresponding to 100 mg of brain tissue)

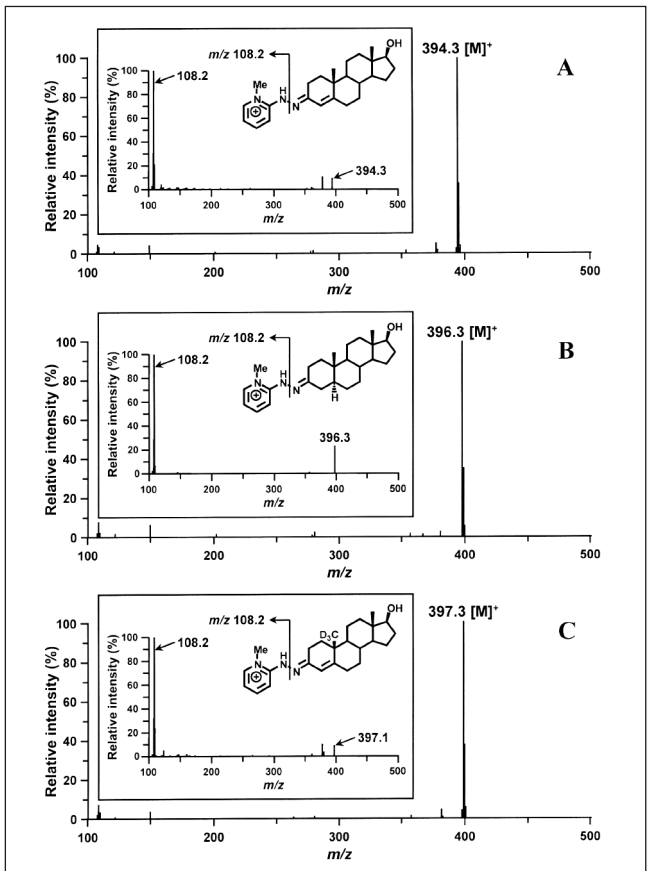

Figure 1. ESI-MS and ESI-MS–MS spectra of the HMP derivatives of T (A), DHT (B), and IS (C). MS–MS spectra (insets) were recorded by the collisional activation of the respective $[M]^+$ with a 60 eV of collision energy. Other conditions were described in the experimental section.

was passed through a Strata-X cartridge. After washing with water (2 mL) and methanol–water (7:3, v/v, 2 mL), the steroids were eluted with ethyl acetate (1 mL). The eluent from the cartridge was evaporated, dissolved in hexane–ethyl acetate (2:1, v/v, 0.2 mL), and applied to a Bond Elut Si cartridge. After washing with hexane (3 mL) and hexane–ethyl acetate (2:1, v/v, 3 mL), the steroids were eluted with ethyl acetate–hexane (3:1, v/v, 3 mL). After evaporation, the residue was subjected to derivatization with HMP as described.

Rat serum (50 µL) was added to methanol–acetic acid (99:1, v/v, 1 mL) containing IS (200 pg), vortex-mixed for 30 s, and centrifuged at 1500 × g (4°C, 5 min). The supernatant was diluted with water (1 mL) and purified using Strata-X and Bond Elut Si cartridges in the same way as already described. The eluent from the cartridge was evaporated and then the residue was subjected to derivatization with HMP.

Derivatization

To a solution of the standard steroids, brain or serum samples in ethanol (30 µL), a freshly prepared solution of HMP (10 µL) in ethanol (50 µL) containing 25 µg of trifluoroacetic acid was added, and the mixture was kept at 60°C for 1 h. After removal of solvents, the product was dissolved in the mobile phase, an aliquot of which was subjected to LC–MS–MS.

Calibration curves

Standard solutions of T and DHT (5, 10, 20, 50, 100, or 200 pg each/10 µL) and IS (200 pg/10 µL) placed in a tube were derivatized with HMP and subjected to LC–MS–MS. The calibration curves were constructed by plotting the peak area ratios (T/IS or DHT/IS) versus the amounts of T or DHT (pg/30 µL/tube).

Method validation

Absolute recoveries of T and DHT during pretreatment

Since T is present at a detectable level even in the female rat brain and serum, the absolute recovery rates of T and DHT during pretreatment were determined using their deuterium labeled forms (d_3-T and d_3-DHT). d_3-T (50 pg in 10 µL of ethanol, spiked sample for T), d_3-DHT (50 pg in 10 µL of ethanol, spiked sample for DHT), or ethanol (10 µL, control sample) was added to the brain extract (1 mL) or serum (50 µL), and the resulting samples were pretreated. d_3-T and d_3-DHT (50 pg each) were then added to the control sample. d_3-DHT (50 pg) or d_3-T (50 pg) was added to the spiked samples for T or DHT, respectively. The samples were derivatized and subjected to LC–MS–MS. The absolute recoveries of T and DHT during pretreatment were calculated from the peak area ratios (d_3-T/d_3-DHT and d_3-DHT/d_3-T, respectively) of the spiked and control samples.

Matrix effects

The matrix effects of the brain extract and serum for quantitation of T and DHT were examined by comparing the slopes of the calibration curves constructed as described earlier and those of curves prepared by adding T and DHT (10, 20, 50, 100, and 200 pg each) to the brain extract (1 mL) or serum (50 µL) (matrix samples). The matrix samples were prepared using 5 different brain extracts or sera.

Assay precision

The intra-assay precision was assessed by determining 2 brain and serum samples obtained from different rats (n = 5 for each sample) on a day. The inter-assay precision was assessed by determining these samples over 5 days. The precision was determined as the coefficient of variation (CV, %).

Analytical recoveries

The analytical recoveries of T and DHT in the brain assay were determined as follows. Ethanol (10 µL, unspiked sample) or the mixture of T and DHT (50 pg each in 10 µL of ethanol, spiked sample) was added to the brain extract (1 mL) (the spiked concentrations of T and DHT were both 0.5 ng/g tissue, n = 2). After the addition of IS (200 pg), each of the resulting samples was pretreated, derivatized and analyzed by LC–MS–MS. The analytical recoveries of T and DHT were defined as $F/(F_0 + 0.5) \times 100$ (%), where F is the concentration of T or DHT in the spiked sample and F_0 is the concentration of T or DHT in the unspiked sample. The analytical recovery of T in the serum assay was also determined using the serum (50 µL) that had been spiked with 25 pg of T (the spiked concentration was 0.5 ng/mL, n = 2), as the spiked sample in a similar manner as already described.

Results and Discussion

LC–ESI-MS–MS of HMP derivatives of T and DHT

For the ESI-MS operating in the positive-ion mode, the HMP derivatives of T, DHT, and IS provided only their molecular cations, $[M]^+$ (Figure 1). A characteristic product ion, [*N*-methylpyridine+NH]$^+$ (16), was also obtained at *m/z* 108.2 by collision-induced dissociation (collision energy, 60 eV) of $[M]^+$ (Figure 1, insets). Based on these results, the SRM mode using their $[M]^+$ (T; *m/z* 394.3, DHT; *m/z* 396.3 and IS; *m/z* 397.3) and [*N*-methylpyridine+NH]$^+$ (T, DHT and IS; *m/z* 108.2) as the precursor and product ions, respectively, was employed in the following quantitative analysis. The amounts of the HMP derivatives of T and DHT giving a signal to noise ratio (S/N) of 5 were 2.8 and 3.4 fmol (equivalent to 0.8 and 1.0 pg of intact steroids) on column, respectively; the derivatization increased the detectabilities 4- and 200-fold over the intact T and DHT, respectively.

Due to the formation of the *E*- and *Z*-isomers during the derivatization of T and DHT with HMP (16), the derivatives produced twin peaks (Figure 2). When acetonitrile–methanol–1mM ammonium acetate (8:3:7, v/v/v) was used as the mobile phase, a satisfactory chromatographic separation of the slower-eluting isomers (colored in black in Figure 2) of the T and DHT derivatives (retention time 9.0 and 7.7 min, respectively) was achieved with a relatively short chromatographic run time (11 min/assay).

Pretreatment procedures

The brain sample was homogenized with methanol containing acetic acid according to the method of Liere et al., by which steroids are quantitatively extracted from the brain tissue (20). The brain extract or serum sample was purified using two solid-phase extraction cartridges. The recovery rates [mean ± standard

deviation (SD) from 5 different samples] of T and DHT during the pretreatment were 81.1 ± 2.0% and 79.4 ± 5.2%, respectively, in the brain assay and 83.8 ± 1.6% and 84.2 ± 2.2%, respectively, in the serum assay. Thus, the reproducibility of the recovery rates was satisfactory and there was no significant difference between the analytes.

Typical chromatograms of the brain and serum samples are shown in Figures 2A and 2B, respectively. The peaks corresponding to the derivatized T and DHT were clearly observed in the brain sample. In the serum sample, only T was detected. Based on these results, validation studies were performed for both T and DHT in the brain assay, while only for T in the serum assay.

Specificity

The retention times of the derivatized other androgens were 4.8 min for dehydroepiandrosterone, 7.3 and 8.4 min (*E*- and *Z*-isomers) for 5β-dihydrotestosterone, 8.1 min for androsterone, and 5.4 min for epiandrosterone. Thus, 5β-dihydrotestosterone and androsterone were closely eluted DHT (retention time 7.7 min). Therefore, the peak purity of the brain sample was confirmed by a different mobile phase system; the retention time of the derivatized DHT (12.5 min) of the brain sample completely agreed with that of the standard sample under the LC condition using acetonitrile–methanol–10mM ammonium formate (4:1:5, v/v/v) as the mobile phase, where the derivatized androsterone (single peak, retention time 13.9 min) and 5β-dihydro - testosterone (twin peaks, retention times 11.2 and 13.9 min) showed different retention. We further examined the occurrence of 5β-dihydrotestosterone in the brain and serum samples obtained from 5 rats, and it was not detected at all. These data demonstrate that the other endogenous steroids do not interfere with the present assays.

Calibration curves and matrix effects

The regression lines obtained from the combination of 5 standard curves are summarized in Table I. The CV values of the slopes of the T and DHT calibration curves were 1.9% and 1.4%, respectively.

To determine the extent to which the brain and serum matrices affect the quantitation, the slopes of the described calibration curves were compared to those of the curves prepared with the matrix samples. As a result, the slopes of the matrix samples were practically identical to those of the curves constructed with standard solutions (Table I). This result clearly revealed that the brain and serum matrices did not affect the calibration curves. Based on this result and the fact that it is very difficult to prepare the steroid-free brain extract and serum, the calibration curves were constructed using the standard solutions in the following studies. The applicability of these curves to the brain and serum assays was also examined in the analytical recovery test that will be discussed later.

Table I. Summary of Calibration Curves and Matrix Effects of Brain and Serum

	Slope (mean ± SD and CV, n = 5)	Intercept (mean ± SD)	Correlation coefficient (r)
T*	$(5.269 \pm 0.098) \times 10^{-3}$ and 1.9%	$(-1.009 \pm 0.4377) \times 10^{-3}$	0.999 (10–200 pg per tube)
T†	$(5.365 \pm 0.165) \times 10^{-3}$ and 3.1%	–	0.999 (10–200 pg per tube)
T‡	$(5.230 \pm 0.053) \times 10^{-3}$ and 1.0%	–	0.999 (10–200 pg per tube)
DHT*	$(4.289 \pm 0.060) \times 10^{-3}$ and 1.4%	$(5.758 \pm 4.650) \times 10^{-3}$	0.999 (10–200 pg per tube)
DHT†	$(4.254 \pm 0.095) \times 10^{-3}$ and 2.2%	–	0.999 (10–200 pg per tube)

* = Standard sample.
† = Brain matrix sample.
‡ = Serum matrix sample

Table II. Assay Precision

		Brain 1		Brain 2	
Variation		T	DHT	T	DHT
Intra-assay	Mean ± SD*	0.27 ± 0.010	0.16 ± 0.015	0.80 ± 0.021	0.40 ± 0.031
	CV (%)	3.7	9.4	2.6	7.8
Inter-assay	Mean ± SD*	0.28 ± 0.011	0.16 ± 0.016	0.80 ± 0.21	0.38 ± 0.020
	CV (%)	3.9	10.0		2.6 5.3
		Serum 1		**Serum 2**	
Variation		**T**	**DHT**	**T**	**DHT**
Intra-assay	Mean ± SD†	0.48 ± 0.023	ND‡	1.06 ± 0.029	ND
	CV (%)	4.8	–	2.7	–
Inter-assay	Mean ± SD	0.49 ± 0.037	ND	1.11± 0.086	ND
	CV (%)	7.6	–	7.7	–

* = ng/g tissue, n = 5.
† = ng/mL, n = 5.
‡ = not detected.

Assay precision, limits of quantitation, and analytical recoveries

The intra-assay (n = 5) CV values for the brain T and DHT were less than 3.7% and 9.4%, respectively, and good inter-assay (n = 5) CV values (less than 3.9% and 10.0%, respectively) were also obtained, as shown in Table II. The reproducibility of the intra- and inter-assays in the serum T assay was also satisfactory.

Because the steroid-free brain and serum matrices were not available, the accurate limits of quantitation (LOQs) of the assays could not be determined. However, as shown in Table II, the concentrations of T (0.27 ng/g tissue) and DHT (0.16 ng/g tissue) in the Brain 1, which were minimum levels among the brain samples examined in this study, were precisely measured. Furthermore, the peaks of the derivatized T and DHT in this sample had 11- and 6-times the responses compared to the background responses near the peaks. Considering from these data, the LOQs in the brain assay were inferred to be around 0.15

ng/g tissue for both T and DHT, when a 100-mg of tissue sample was used. From the similar reasoning, the LOQ of the serum T was determined to be around 0.3 ng/mL, when a 50-µL of sample was used.

Next, the brain extract or serum to which known amounts of the T and DHT had been added were pretreated and analyzed in order to examine the analytical recoveries. Satisfactory recovery rates ranging from 98.1 to 103.0% were obtained (Table III). Although the calibration curves were constructed using the standard solutions of T and DHT in the present study as mentioned above, this result demonstrates that the brain T and DHT and the serum T can be accurately determined using the curves. These data indicate that the present methods are highly reproducible and accurate.

Applicability of the developed methods; changes in brain and serum T and DHT levels by immobilization stress

In order to examine the applicability of the proposed method for a pharmacological study, changes in the steroid levels in the brain and serum due to immobilization stress, a representative physical stress, were analyzed using the developed method. Table IV summarizes the measured values of the brain and serum T and DHT in the unstressed and stressed rats. The brain and serum T of the stressed rats had the same levels as those of the unstressed rats (no statistical difference). The serum T level was always higher than the brain level in all the rats, which is one of the data supporting that most of the brain T is derived from the peripheral organs. Regarding the T levels, the results obtained in the present study agreed well with those in the previous studies (9,12).

DHT was detected in all the brains, but not detected at all in the serum. This result clearly demonstrates that DHT found in the brain is locally synthesized by the catalysis of 5α-reductase in the brain, but not derived from the blood circulation. Table IV shows that there is no significant difference in the brain DHT levels between the unstressed and stressed rats. When the brain DHT concentration was plotted as the ordinate and the brain T concentration as the abscissa, it was found that they lay almost on a straight line (Figure 3). These data clearly show that the brain DHT level is not influenced by the stress and depends on the brain T level.

The stress-induced level changes in the brain pregnane-type neuroactive steroids, such as progesterone (PROG) and AP, have been well documented (3). The brain levels of these steroids increased by the acute physical stress paradigms, such as forced swimming (4) and immobilization (5,8), and by the din stress (rats were exposed to the screams of the immobilization stressed rats through an amplifier), which is the short-term mental stress (8). The brain levels of PROG (12) and AP (5) in the rats exposed to immobilization, which had been obtained in the previous studies, are also demonstrated in Table IV. The present study confirmed that the stress-induced level changes in the brain androgenic neuroactive steroids were much lower (undetectable magnitude) than those of the pregnane-type neuroactive steroids. AP is surely able to counteract the inhibitory effect of stress on the $GABA_A$ receptor function (3). Therefore, the brain accelerates the synthesis of AP under the stressful conditions, and with this synthesis, the brain levels of its precursors, such as PROG, also increase. If DHT is involved in the defensive response against the stress, just as AP, the brain level of DHT should be increased by the stress and at the same time, the brain level of T should be also increased as a source of DHT supply, in the same manner as the relation between AP and PROG. However, these phenomena were not observed in this study. Based on this result, the endogenous androgenic steroids may be little involved in the defensive response against the stress, though several reports

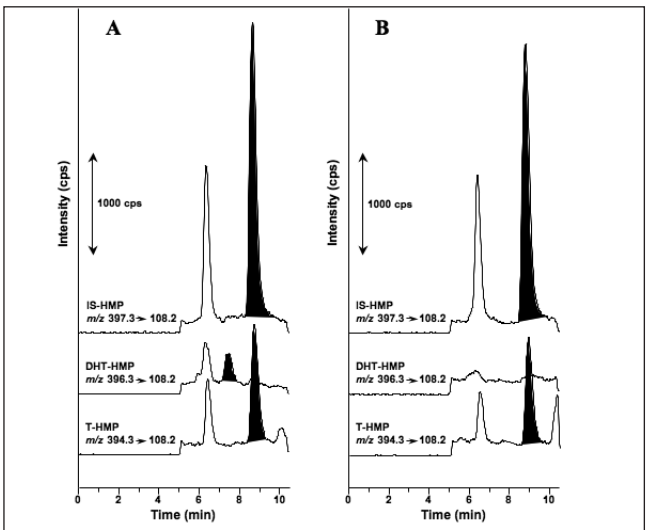

Figure 2. Typical LC–ESI-MS–MS chromatograms of derivatized T, DHT and IS in the brain (measured values; T, 1.20 ng/g tissue and DHT, 0.54 ng/g tissue) (A) and serum (measured values; T, 2.18 ng/mL and DHT, not detected) (B) of an unstressed rat.

Table III. Analytical Recoveries

	Brain 1: T		DHT		Brain 2: T		DHT	
Added*	Measured*	Recovery (%)	Measured	Recovery (%)	Measured	Recovery (%)	Measured	Recovery (%)
0	0.28	–	0.16	–	0.80	–	0.38	–
0.5	0.79	101.3	0.68	103.0	1.28	98.5	0.87	98.9

	Serum 1: T		Serum 2: T	
Added†	Measured†	Recovery (%)	Measured	Recovery (%)
0	0.49	–	1.11	–
0.5	1.00	101.0	1.58	98.1

* = Unit: ng/g tissue.
† = Unit: ng/mL.

Table IV. Brain and Serum Neuroactive Steroid Levels

	Unstressed (mean ± SD)	Stressed (mean ± SD)
Brain T (ng/g tissue, *n* = 10)	1.31 ± 1.00	1.25 ± 0.82
Brain DHT (ng/g tissue, *n* = 10)	0.70 ± 0.60	0.59 ± 0.40
Brain PROG (ng/g tissue, *n* = 12)*	0.14 ± 0.10	11.2 ± 6.21‡
Brain AP (ng/g tissue, *n* = 10)†	Not detected	1.74 ± 0.71‡
Serum T (ng/mL, *n* = 10)	2.43 ± 1.92	1.77 ± 1.08
Serum DHT (ng/mL, *n* = 10)	Not detected	Not detected

* Data from the reference 5.
† Data from the reference 12.
‡ $P < 0.05$ (Welch test), compared with the level of the unstressed rat.

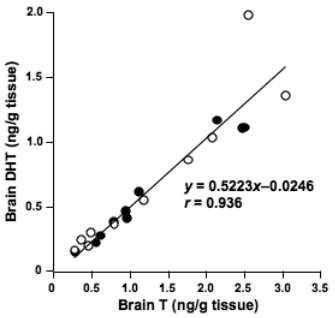

Figure 3. Scatter diagram to examine correlation between brain T and DHT levels. Open and closed symbols represent the unstressed and stressed rats, respectively.

described that the pharmacological dose of the steroids elicit the anesthetic and anxiolytic effects in animal models (1,2).

Conclusion

We developed LC–MS–MS methods for the determination of the brain and serum T and DHT after converting them to highly detectable derivatives in positive ESI-MS. The methods were specific and reproducible, and enabled the analysis of trace amounts of T and DHT using a small amount of sample. The animal study using the developed methods revealed that a fair amount of DHT is continuously synthesized in the brain and its level is not influenced by the immobilization stress. It is expected that the developed methods are useful for the further characterization of the physiological functions of androgenic neuroactive steroids, especially DHT, on the central nervous system.

Acknowledgments

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