ANALYTICAL SCIENCES AUGUST 2007, VOL. 23



Notes

# Studies on Neurosteroids XXI: An Improved Liquid Chromatography–Tandem Mass Spectrometric Method for Determination of 5α-Androstane-3α,17β-diol in Rat Brains

**Tatsuya HIGASHI,† Tadashi NISHIO, Hiroyuki YOKOI, Yasuaki NINOMIYA, and Kazutake SHIMADA**

*Division of Pharmaceutical Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920–1192, Japan*

A sensitive liquid chromatography–electrospray ionization–tandem mass spectrometric (LC-ESI-MS/MS) method for the determination of the rat brain 5α-androstane-3α,17β-diol (3α,5α-Adiol) has been developed and validated. The brain extract was purified using solid-phase extraction cartridges, derivatized with isonicotinoyl azide, and subjected to LC-MS/MS. The method was accurate and reproducible, and the limit of quantitation was 0.1 ng/g tissue when a 100-mg tissue sample was used. The change in the brain 3α,5α-Adiol level by immobilization stress was also analyzed using the developed method.

**(Received April 16, 2007; Accepted May 28, 2007; Published August 10, 2007)**

## Introduction

Neurosteroids affect the neurotransmission through action at the membrane ion-gated receptors and at other neurotransmitter receptors. For example, 3α-hydroxy-5α-pregnan-20-one (allopregnanolone, AP) binds to the γ-aminobutyric acid type A ($GABA_A$) receptors with a high affinity, positively modulates the action of GABA at these receptors and thus elicits marked anesthetic, sedative and anxiolytic effects.[1] It has been also demonstrated that the endogenous AP in the animal brain is rapidly elevated from a trace level (practically none) to a ng/g tissue level by several acute stress paradigms.[2,3]

Recently, several reports have described that the pharmacological doses of testosterone (T) and its metabolite, 5α-androstane-3α,17β-diol (3α,5α-Adiol), elicit anesthetic and anxiolytic effects in animal models.[4,5] 3α,5α-Adiol is structurally similar to AP (both are 3α-hydroxy-5α-reduced steroids), so it also has an allosteric activity at the $GABA_A$ receptors.[5] Thus, 3α,5α-Adiol can play a key physiological role in mediating the effect of T on the central nervous system.

AP and related neurosteroids in the brain have been recently measured by liquid chromatography (LC)–mass spectrometry (MS) coupled with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) due to its selectivity, versatility and simultaneous multi-analyte quantification capability.[3,6,7] We have reported a validated LC–electron capture APCI (ECAPCI)-MS assay for the determination of the brain 3α,5α-Adiol after converting it to an electron-affinitive derivative.[8] Although ECAPCI-MS was a highly sensitive technique, this ionization occurred only when the ThermoQuest LCQ mass spectrometer was used, and it was unusable for other mass spectrometers, such as the Applied Biosystems API series instruments; the method has limitations on its versatility. Reddy *et al*. reported an LC-APCI-MS assay combined with the standard addition method for the rat plasma 3α,5α-Adiol, but its sensitivity was limited [the limit of quantitation (LOQ): 10 ng/ml][9] and therefore, this method is not applicable to the brain 3α,5α-Adiol. Although the introduction of atmospheric pressure photoionization has also been examined for steroid analyses, it has limitations on its sensitivity and susceptibility to the matrix effect.[10] The lack of a specific method for the brain 3α,5α-Adiol 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. Quite recently, Meffre *et al*. reported the steroid profiling in the brain and serum of the rats after traumatic brain injury using gas chromatography–MS.[11] However, this method required a high-performance LC for the sample purification, which is time consuming and causes a low recovery of the steroid.

Based on this background information, a more practical LC-MS method, *i.e*., one coupled with an universally applicable ionization, such as ESI, is now required for the analysis of the brain 3α,5α-Adiol. The biggest problem encountered in the measurement of 5α-reduced steroids including 3α,5α-Adiol using ESI-MS is the lack of sensitivity; the amount of 3α,5α-Adiol giving a signal to noise ratio (*S*/*N*) of 5 was 220 pg on column. To overcome this problem, derivatization, *i.e*., tagging a poorly ionizable steroid with an ESI-active moiety, is extremely useful.[12] In this study, we developed and validated an LC-ESI–tandem MS (MS/MS) method employing derivatization for the determination of 3α,5α-Adiol in the rat brain. The application of the method to the analysis of changes in the steroid levels by immobilization stress is also reported.

## Experimental

*Materials and chemicals*

3α,5α-Adiol was purchased from Steraloids (Newport, RI, USA) and its stock solution was prepared as a 100 μg/ml

† To whom correspondence should be addressed.
E-mail: higashi@p.kanazawa-u.ac.jp

solution in ethanol. Subsequent dilutions were carried out with ethanol to prepare 0.5, 1, 2, 5, 10 and 20 ng/ml solutions. [16,16,17$\alpha$-$^2H_3$]-3$\alpha$,5$\alpha$-Adiol[13] used as the internal standard (IS) was synthesized in our laboratories, then dissolved in and diluted with ethanol. The isomers of 3$\alpha$,5$\alpha$-Adiol, (3$\beta$,5$\alpha$-Adiol, 3$\alpha$,5$\beta$-Adiol and 3$\beta$,5$\beta$-Adiol) were synthesized by reducing epiandrosterone, etiocholanolone and epietiocholanolone (Steraloids) with $NaBH_4$. Isonicotinoyl azide (NA) was prepared from isonicotinic acid (Tokyo Kasei Kogyo, Tokyo, Japan) by the known method.[14] Strata-X cartridges (60 mg adsorbent; Phenomenex, Torrance, CA, USA) 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 chloroform–methanol (30:1, v/v, 2 ml) and chloroform (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-stage quadrupole-mass spectrometer (Foster City, CA, USA) connected to a Shimadzu LC-10AT chromatograph (Kyoto, Japan). The receiver (Anest Iwata SAT-36-100, Yokohama, Japan) was placed between the nitrogen generator and the mass spectrometer for a large and stable supply of nitrogen. The NA derivatives of the steroids were analyzed by ESI-MS in the positive-ion mode. The conditions were as follows: declustering potential, 30 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 35 eV. The precursor and monitoring ions of the NA derivatives were as follows: 3$\alpha$,5$\alpha$-Adiol-NA, *m/z* 533.3 and 139.0 and IS-NA, *m/z* 536.3 and 139.0. A YMC-Pack Pro C18 RS (5 µm, 150 × 2.0 mm i.d.; YMC, Kyoto) was used at 40˚C. Methanol–acetonitrile–10 mM ammonium formate (2:1:1, v/v/v) was used at the flow rate of 0.2 ml/min as the mobile phase. The data were collected and quantified using Applied Biosystems Analyst software (Ver. 1.3.1).

*Treatment of rats*

Wistar strain rats (7-weeks old, male, 190 – 200 g) obtained from Japan S.L.C. (Hamamatsu, Japan) were assigned either to an unstressed group ($n$ = 11) or to a group subjected to immobilization stress ($n$ = 11). The rats in the second group were immobilized on their backs on a board for 20 min.[3] All the animal experiments were carried out between 13:30 and 15:00. 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, USA).

*Pretreatment procedure*

3$\alpha$,5$\alpha$-Adiol was extracted from the rat brain tissue with methanol–acetic acid (99:1, v/v) by a previously reported method.[3] 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 (250 pg in 10 µl) and water (1 ml), 1 ml of the brain extract (corresponding to 100 mg of brain tissue) was passed through a Strata-X cartridge. After being washed 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 chloroform (0.4 ml) and applied to a Bond Elut Si cartridge. After being washed with chloroform (3 ml), the steroids were eluted with chloroform–ethyl acetate (20:1, v/v, 3 ml). After evaporation, the residue was subjected to derivatization with NA as described below.

*Derivatization reaction*

To the pretreated samples (calibration and test samples), a freshly prepared solution of NA (200 µg) in benzene (100 µL) was added, and the mixture was maintained at 80˚C for 30 min. After the solvents were evaporated, the residue was then dissolved in methanol–10 mM ammonium formate (1:1, v/v, 30 µl), 10 µl of which was subjected to LC-MS/MS.

*Calibration curve*

Because 3$\alpha$,5$\alpha$-Adiol was not detected in the brain extract obtained from a female rat (Wistar strain, 7-weeks old), this was used as the blank brain extract for the construction of the calibration curve and validation studies. The blank brain extract (1.0 ml) was spiked with 3$\alpha$,5$\alpha$-Adiol (10, 20, 50, 100 or 200 pg each; corresponding to 0.1, 0.2, 0.5, 1.0 or 2.0 ng/g tissue) and IS (250 pg), which was then pretreated, derivatized and subjected to LC-MS/MS. The calibration curve was constructed by plotting the peak area ratios (3$\alpha$,5$\alpha$-Adiol/IS) *versus* the concentration of 3$\alpha$,5$\alpha$-Adiol (ng/g tissue).

*Recoveries of 3$\alpha$,5$\alpha$-Adiol and IS during pretreatment*

An ethanolic solution of 3$\alpha$,5$\alpha$-Adiol (50 pg in 10 µl, sample A), IS (250 pg in 10 µl, sample B) or ethanol (10 µl, sample C) was added to the blank brain extract (1.0 ml) obtained from a female rat and the resulting samples were pretreated. IS (250 pg) or 3$\alpha$,5$\alpha$-Adiol (50 pg) was then added to samples A or B, respectively, and both IS (250 pg) and 3$\alpha$,5$\alpha$-Adiol (50 pg) were added to sample C. After derivatization, the samples were subjected to LC-MS/MS. The recovery of 3$\alpha$,5$\alpha$-Adiol during pretreatment was calculated from the peak area ratios (3$\alpha$,5$\alpha$-Adiol/IS) of samples A and C, and that of IS was calculated from the peak area ratios (IS/3$\alpha$,5$\alpha$-Adiol) of samples B and C.

*Influence of the endogenous components on the derivatization*

The influence of the endogenous components on the derivatization was examined by comparison of the peak area ratio (3$\alpha$,5$\alpha$-Adiol/IS) in samples A and B as described below. Sample A: The blank brain extract (1.0 ml) was pretreated as described above without addition of IS. 3$\alpha$,5$\alpha$-Adiol (50 pg) was added to the residue and then derivatized with NA. After the addition of IS-NA (100 pg), which had been prepared beforehand, the sample was subjected to LC-MS/MS. Sample B: The standard 3$\alpha$,5$\alpha$-Adiol (50 pg) was derivatized. After the addition of IS-NA (100 pg), the sample was subjected to LC-MS/MS.

*Assay precision and accuracy*

Quality control (QC) samples with concentrations of 0.3 and 1.5 ng/g tissue were prepared by adding the standard 3$\alpha$,5$\alpha$-Adiol to the blank brain extract. The intra-assay precision and accuracy were evaluated by analyzing multiple replicates ($n$ = 5) of the QC samples on the same day. The inter-assay precision and accuracy were evaluated by analyzing the QC samples on five different days. The inter-assay ($n$ = 5) reproducibility and intra-assay ($n$ = 5) repeatability of the assay were also evaluated by analyzing the brain sample of a male rat that contained endogenous 3$\alpha$,5$\alpha$-Adiol in a significant amount.

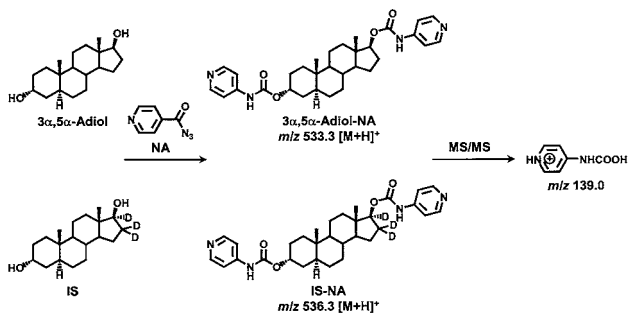


Fig. 1 Chemical structures of NA derivatives of 3$\alpha$,5$\alpha$-Adiol and IS (D = deuterium, $^2$H) and product ions formed during MS/MS. The detailed data regarding the MS/MS of the derivatives are presented in Ref. 15.

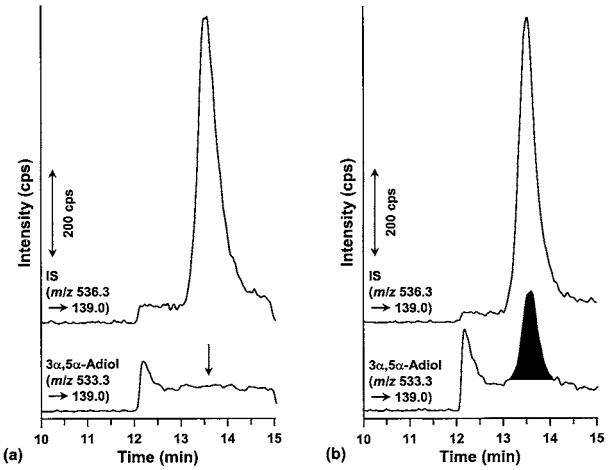


Fig. 2 Chromatograms of derivatized 3$\alpha$,5$\alpha$-Adiol and IS in the brain of (a) female and (b) male rats. The LC-ESI-MS/MS conditions were described in the Experimental section. The LC eluent entered the mass spectrometer 12 to 15 min after injection through a diversion valve. The arrow indicates the elution position of the 3$\alpha$,5$\alpha$-Adiol derivative. The measured concentrations of 3$\alpha$,5$\alpha$-Adiol in the male rat was 0.72 ng/g tissue.

*Measurement of brain T concentration*

The brain T concentration was measured by a method that had been developed and validated in our laboratories.[8]

## Results and Discussion

*ESI-MS/MS of NA derivatives*

We have reported that the derivatization with NA is very effective to enhance the detection response of dihydroxysteroids, such as 3$\alpha$,5$\alpha$-Adiol, in ESI-MS operating in the positive-ion mode.[15] The NA derivatives of 3$\alpha$,5$\alpha$-Adiol and IS provided their protonated molecules, $[M+H]^+$ as the base peak ions. The product ion mass spectrum of the NA derivatives employing the respective $[M+H]^+$ as the precursor ions and a 35-eV collision energy showed that a base peak ion was observed at *m/z* 139.0, which was assigned to the protonated pyridyl carbamic acid (Fig. 1).[15] Based on these results, the SRM mode using the $[M+H]^+$ and the ion at *m/z* 139.0 as the precursor and product ions, respectively, was employed in the following studies. When using this mode, the amount of the standard 3$\alpha$,5$\alpha$-Adiol derivative giving an *S/N* of 5 was 2.4 fmol (equivalent to 0.7 pg of intact 3$\alpha$,5$\alpha$-Adiol) on column, which was not significantly inferior to that using ECAPCI-MS[8] (1.7 fmol on column).

Recently, Yamashita *et al.*[16] examined the LC-ESI-MS(/MS) behavior of picolinoyl, nicotinoyl and isonicotinoyl derivatives of hydroxysteroids and found the picolinoyl derivative was superior to other two derivatives in the ESI-response characteristic. Based on this information, picolinoyl azide might be also useful for the analysis of the brain 3$\alpha$,5$\alpha$-Adiol, but we did not examine this reagent, because the NA-derivatization that we had developed provided sufficient sensitivity in the present study.

*Pretreatment procedure*

The brain tissue was homogenized in methanol containing acetic acid, and the brain extract was then purified using two disposable cartridges. The steroid fraction was then treated with NA. The recovery rates [mean ± standard deviation (SD), three different rats] of 3$\alpha$,5$\alpha$-Adiol and IS during the pretreatment were 73.7 ± 1.3 and 74.1 ± 0.7%, respectively. The reproducibility of the recovery rates was satisfactory, and there was no significant difference between 3$\alpha$,5$\alpha$-Adiol and IS.

The NA-derivatization has been proved to be effective in increasing the detectability of 3$\alpha$,5$\alpha$-Adiol even when picogram amounts of the steroid are handled.[15] Furthermore, we examined the influence of the endogenous components on the derivatization according to the procedure described in the Experimental section. The derivatization rate of 3$\alpha$,5$\alpha$-Adiol in the brain sample was 89.1 ± 1.9% ($n$ = 5) of that of standard sample. Although there was some lowering in the derivatization rate in the brain sample, the rate was good enough for the brain 3$\alpha$,5$\alpha$-Adiol determination.

*Specificity*

The chromatograms shown in Fig. 2a were obtained from a female rat brain in which 3$\alpha$,5$\alpha$-Adiol was not detected; these revealed that there was no interfering peak derived from the endogenous components or from the derivatization reagent at the elution position of the derivatized 3$\alpha$,5$\alpha$-Adiol. Typical chromatograms of the brain sample obtained from a male rat are shown in Fig. 2b. The peak corresponding to the derivatized 3$\alpha$,5$\alpha$-Adiol was clearly observed at 13.6 min. The NA derivatives of the isomers, 3$\beta$,5$\alpha$-Adiol [retention time ($t_R$) 12.4 min], 3$\alpha$,5$\beta$-Adiol ($t_R$ 15.2 min) and 3$\beta$,5$\beta$-Adiol ($t_R$ 10.6 min), were completely separated from the derivatized 3$\alpha$,5$\alpha$-Adiol. These data demonstrate that the other endogenous steroids do not interfere with the present assay.

*Calibration curve and LOQ*

The regression line obtained from the combination of five different curves for 3$\alpha$,5$\alpha$-Adiol was $y = 0.3806x + 0.0071$. The coefficient of variation (CV) value of the slope of five curves was 0.7%. The CV value and relative error (RE) of the back-calculated concentration at the minimum point (0.1 ng/g tissue) was 9.2 and 3.0%, respectively. The peak of the derivatized 3$\alpha$,5$\alpha$-Adiol at this concentration was clearly observed with an *S/N* of more than 5 (Fig. 3). The LOQ was defined as the lowest concentration on the calibration curve of the analyte measured with an acceptable precision and accuracy (*i.e.*, CV and RE < 15%) and with at least an *S/N* of 5. On the basis of this criterion and the above result, the LOQ of 3$\alpha$,5$\alpha$-Adiol was determined to be 0.1 ng/g tissue when a 100-mg tissue sample was used. This LOQ value was one-half of that of the LC-ECAPCI-MS assay[8] (0.2 ng/g tissue), which proves that

Table 1 Intra- and inter-assay precision and accuracy for determination of 3$\alpha$,5$\alpha$-Adiol in rat brain

| | | QC sample (0.3 ng/g tissue) | QC sample (1.5 ng/g tissue) | Male rat brain sample |
|---|---|---|---|---|
| Intra-assay | Measured concentration[a] | 0.302 ± 0.013 | 1.495 ± 0.052 | 0.662 ± 0.032 |
| | CV, % | 4.3 | 3.5 | 4.8 |
| | Accuracy, % | 100.7 | 99.7 | — |
| Inter-assay | Measured concentration[a] | 0.301 ± 0.013 | 1.498 ± 0.050 | 0.691 ± 0.019 |
| | CV, % | 4.3 | 3.3 | 2.7 |
| | Accuracy, % | 100.3 | 99.9 | — |

a. Mean ± SD (ng/g tissue, $n$ = 5).

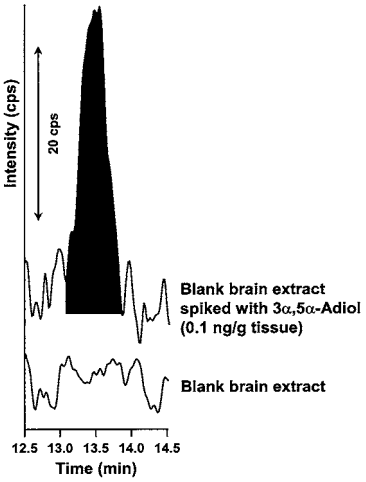


Fig. 3 Chromatograms obtained from the blank brain extract spiked with 3$\alpha$,5$\alpha$-Adiol (0.1 ng/g tissue, upper) and without spiking 3$\alpha$,5$\alpha$-Adiol (lower). These chromatograms reveal that the peak height of the derivatized 3$\alpha$,5$\alpha$-Adiol is more than 5 times of the blank response at the concentration of 0.1 ng/g tissue.

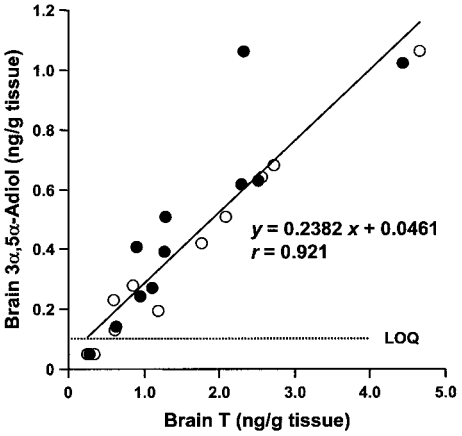


Fig. 4 Scatter diagram to examine the correlation between the brain T and 3$\alpha$,5$\alpha$-Adiol levels. The open and closed symbols represent the unstressed and stressed rats, respectively.

the present method is superior to the previous one in the sensitivity.

*Assay precision and accuracy*

The assay precision and accuracy were examined using QC samples with 2 different concentrations. The intra-assay ($n$ = 5) CV values were less than 4.3%, and good inter-assay ($n$ = 5) CV values (less than 4.3%) were also obtained, as shown in Table 1. Moreover, the reproducibility and repeatability in the analysis of a male rat brain sample, which contained endogenous 3$\alpha$,5$\alpha$-Adiol in a significant amount, are also good (CV < 4.8%). Satisfactory % accuracy values ranging from 99.7 to 100.7% were also obtained. These data indicate that the present method is highly reproducible and accurate.

*Applicability of the developed method; change in the brain 3$\alpha$,5$\alpha$-Adiol level by immobilization stress*

In order to examine the applicability of the proposed method for a pharmacological study, we analyzed the change in the brain 3$\alpha$,5$\alpha$-Adiol level due to immobilization stress, a representative physical stress, using the developed method. The brain 3$\alpha$,5$\alpha$-Adiol levels of the unstressed and stressed rats (mean ± SD, $n$ = 11) were 0.38 ± 0.31 (range: < LOQ – 1.06) and 0.48 ± 0.33 (range: < LOQ – 1.06) ng/g tissue, respectively (the mean and SD values were calculated by substituting 0.05 ng/g tissue in "< LOQ", although the brain 3$\alpha$,5$\alpha$-Adiol levels of two rats in the unstressed group and one rat in the stressed group were "< LOQ"). There was no significant difference in the brain 3$\alpha$,5$\alpha$-Adiol levels between the unstressed and stressed rats ($P$ = 0.48, two-sided test). These results demonstrate that 3$\alpha$,5$\alpha$-Adiol is always present in the brain and its level is not influenced by the stress. These are significantly different from the change in the brain AP level; it increased from < 0.15 (unstressed rats) to 1.74 ± 0.71 (stressed rats) ng/g tissue (mean ± SD, $n$ = 10) by the immobilization.[3] Figure 4 shows the correlation between the testosterone concentration and 3$\alpha$,5$\alpha$-Adiol concentration in the brain; a linear relationship was found to be positive. This result shows that the brain 3$\alpha$,5$\alpha$-Adiol level depends on the brain T level.

## Conclusion

In this study, we developed the LC-MS/MS method for the determination of the brain 3$\alpha$,5$\alpha$-Adiol after converting it to a highly detectable derivative in the positive ESI-MS. This method was sensitive, specific and reproducible, and applicable to pharmacological studies. The animal study demonstrated that the brain 3$\alpha$,5$\alpha$-Adiol level is not influenced by the immobilization stress and depends on the brain T level. It is expected that the developed method will be useful for the further characterization of the physiological function of 3$\alpha$,5$\alpha$-Adiol on the central nervous system.

### Acknowledgements

Parts of this study were supported by grants from Japan Society for the Promotion of Science (JSPS).

## References


1. M. L. Barbaccia, M. Serra, R. H. Purdy, and G. Biggio, *Int. Rev. Neurobiol*., **2001**, *46*, 243.
2. M. Vallée, J. D. Rivera, G. F. Koob, R. H. Purdy, and R. L. Fitzgerald, *Anal. Biochem*., **2000**, *287*, 153.
3. T. Higashi, A. Nagahama, N. Otomi, and K. Shimada, *J. Chromatogr., B*, **2007**, *848*, 188.
4. C. A. Frye and A. M. Seliga, *Cogn. Affect. Behav. Neurosci*., **2001**, *1*, 371.
5. K. L. Edinger and C. A. Frye, *Psychoneuroendocrinology*, **2005**, *30*, 418.
6. S. Liu, J. Sjövall, and W. J. Griffiths, *Anal. Chem*., **2003**, *75*, 5835.
7. T. Higashi, N. Takido, and K. Shimada, *Steroids*, **2005**, *70*, 1.
8. T. Higashi, Y. Ninomiya, N. Iwaki, A. Yamauchi, N. Takayama, and K. Shimada, *Steroids*, **2006**, *71*, 609.
9. D. S. Reddy, L. Venkatarangan, B. Chien, and K. Ramu, *Steroids*, **2005**, *70*, 879.
10. A. Leinonen, T. Kuuranne, and R. Kostiainen, *J. Mass Spectrom*., **2002**, *37*, 693.
11. D. Meffre, A. Pianos, P. Liere, B. Eychenne, A. Cambourg, M. Schumacher, D. G. Stein, and R. Guennoun, *Endocrinology*, **2007**, *148*, 2505.
12. T. Higashi and K. Shimada, *Anal. Bioanal. Chem*., **2004**, *378*, 875.
13. L. Muller and G. Phillipou, *Clin. Chem*., **1987**, *33*, 256.
14. J. Holt, T. Andereassen, J. M. Bakke, and A. Fiksdahl, *J. Heterocycl. Chem*., **2005**, *42*, 259.
15. T. Higashi, T. Nishio, N. Hayashi, and K. Shimada, *Chem. Pharm. Bull*., **2007**, *55*, 662.
16. K. Yamashita, S. Kobayashi, S. Tsukamoto, and M. Numazawa, *Steroids*, **2007**, *72*, 50.