Studies on Neurosteroids X. Determination of Pregnenolone and Dehydroepiandrosterone in Rat Brains Using Gas Chromatography–Mass Spectrometry–Mass Spectrometry

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Abstract

An assay method for pregnenolone and dehydroepiandrosterone in rat brains is developed using gas chromatography (GC)–electron ionization-mass spectrometry (MS)–MS. The extract of the rat brain homogenate containing deuterated internal standard with organic solvent is purified by silica gel minicolumn chromatography. The obtained fraction is derivatized into methyloxime, treated with dimethylisopropylsilylimidazole, and then subjected to GC–MS–MS. The method is applied to the determination of these steroids in the gray matter and olfactory bulbs of rat brains, which are divided into control and acute stressed specimens. Although pregnenolone in both regions of the rat brains increases more than three times after stress, dehydroepiandrosterone in both regions is not so clearly influenced by stress.

Introduction

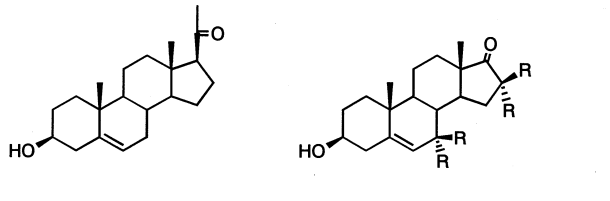
Since the discovery of dehydroepiandrosterone in rat brains, several 17- and 20-oxosteroids (including pregnenolone) called "neurosteroids" or "neuroactive steroids" have been elucidated in mammalian brains (1). Significant interest has thus been focused on their biological properties in this organ (2). In a previous paper of this series (3), we developed a determination method for pregnenolone and its 3-stearate in rat brains using high-performance liquid chromatography (HPLC)–atmospheric pressure chemical ionization (APCI)-mass spectrometry (MS). The method clarified that pregnenolone increased more than five times after stress; its 3-stearate decreased after stress. However, the quantitative limit of the method is 7.5 ng/g tissue, which is not sensitive enough to determine dehydroepiandrosterone (approximately 0.42 ng/g tissue) (4) and a lower amount of pregnenolone in rat brains. Although a very sensitive determination method using gas chromatography (GC)–MS has been developed, complicated pretreatment methods using preparative HPLC or preparative thin-layer chromatography (TLC) are necessary (2,5). Radioimmunoassay has also been used for the determination, but a radioactive compound is not dispensable (1).

In this report, we have developed a determination method for pregnenolone and dehydroepiandrosterone in rat brains using GC–electron ionization (EI)-MS–MS, and the application of the method is also reported (Figure 1).

Experimental

Materials, reagents, and animals

Pregnenolone, dehydroepiandrosterone, dimethylisopropylsilylimidazole, and NH_2OCH_3•HCl were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). [7,7,16,16-$^2H_4$]-Dehydroepiandrosterone ([$^2H_4$]-dehydroepiandrosterone) was prepared in the laboratory (6). Its MS spectrum was measured by GC–MS as described later: *m/z* 292 $[M]^+$ (100%), 291 (6.16%), 290 (6.65%), 289 (0.75%), 288 (0%). Minicolumn chromatographies were performed with silica gel 60 (70–230 mesh; E. Merck, Darmstadt, Germany) and Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden). ISOLUTE C_{18} (EC) cartridges (500 mg, Internal Sorbent Tech., Hengoed, U.K.) were obtained from Uniflex (Tokyo, Japan). All other reagents were of analytical grade and



pregnenolone

dehydroepiandrosterone : R=H
[$^2H_4$]-dehydroepiandrosterone (IS) : R=^{2}H

Figure 1. Structures of pregnenolone, dehydroepiandrosterone, and IS.

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commercially available. Wistar strain rats (male, 7 weeks old) were obtained from Japan S.L.C. (Hamamatsu, Japan). Some of these rats were stressed after fixation of four legs on a plate with strings for 1 h before decapitation.

Apparatus

A Finnigan MAT GCQ GC–ion trap MS (Austin, TX) equipped with a Restex (Bellefonte, PA) 5% diphenyl–95% dimethylpolysiloxane capillary column Rtx-5MS (30 m × 0.25-mm i.d., 0.25-µm d_f) was used under the following conditions: EI, 70 eV, positive ion mode; carrier gas, helium; linear flow rate, 40 cm/s, transfer line temperature, 275°C; and ion source temperature, 200°C. The column oven temperature was set at 60°C for 4 min and ramped to 290°C at 40°C/min. The temperature programmable injector was set at 60°C for 1 min and ramped to 290°C at 180°C/min. The applied collision energy used for the MS–MS analysis was 0.85 V (for the derivative of pregnenolone) or 0.8 V (for derivatives of dehydroepiandrosterone and [$^2H_4$]-dehydroepiandrosterone), which provided satisfactory results in its dissociation and sensitivity.

Procedure for the determination of pregnenolone and dehydroepiandrosterone in rat brains

A Wistar strain rat (130–155 g) was decapitated, and its gray matter (100 mg) or olfactory bulbs (30–50 mg) were homogenized in isotonic saline (2 mL) under ice cooling. After the addition of the internal standard (IS), 5 ng [$^2H_4$]-dehydroepiandrosterone/50 µL MeOH, the entire mixture was extracted with EtOH (3 mL) and centrifuged at 1500 × *g* for 15 min. The precipitate was further extracted with 3 mL EtOH and centrifuged (1500 × *g*, 15 min). All of the supernatants were combined, centrifuged at 1500 × *g* for 15 min, and then diluted with water (approximately 10 times). The whole was subjected to an ISOLUTE C18 cartridge and washed with water (6 mL) and then 50% MeOH (6 mL) and eluted with 90% MeOH (6 mL). After evaporation of the solvent, the obtained residue was subjected to silica gel minicolumn chromatography (3 × 0.6-cm i.d.) and washed with 10 mL hexane, 5 mL hexane–AcOEt (10:1), and 5 mL hexane-AcOEt (7:1). The fraction containing the desired compounds and IS was eluted with 5 mL hexane–AcOEt (1:1), and the solvent was evaporated under a N_2 gas stream. The residue was treated with 1% NH_2OCH_3•HCl–pyridine (0.1 mL) at 70°C for 1 h. The entire solution was extracted with AcOEt, and the organic layer was successively washed with chilled H_2O, 5% HCl, and H_2O. After evaporation of the solvent, the residue was subjected to silica gel minicolumn chromatography (6 × 0.6-cm i.d.) and washed with 10 mL hexane and 10 mL hexane– AcOEt (10:1). The fraction eluted with 10 mL hexane–AcOEt (5:2) was evaporated under a N_2 gas stream, and the obtained residue was treated with 50 µL dimethylisopropylsilylimidazole–pyridine (1:4) at 60°C for 30 min. The reaction mixture was diluted with 0.5 mL hexane–$CHCl_3$–MeOH (10:10:1) and subjected to Sephadex LH-20 minicolumn chromatography (6 × 0.6-cm i.d.). The fraction eluted with additional solvent (1 mL) of the same system was evaporated under a N_2 gas stream. The obtained residue was dissolved in hexane (50 µL), and an aliquot of this (10 µL) was subjected to GC–MS–MS: pregnenolone retention time (t_R), 12.5 min; precursor ion, *m/z* 402 ([M-C_3H_7]$^+$); product ions, *m/z* 326, 255, 159; dehydroepiandrosterone t_R, 11.5 min; precursor ion, *m/z* 374 ([M-C_3H_7]$^+$); product ions, *m/z* 342, 298, 268; IS t_R, 11.5 min; precursor ion, *m/z* 378 ([M-C_3H_7]$^+$); product ions, *m/z* 346, 302, 272. The sum of these three product ions was used for the determination.

Recoveries of pregnenolone, dehydroepiandrosterone, and IS before methyloxime derivatization

The entire brains (without gray matter and olfactory bulbs, 600 mg tissue) of two Wistar strain rats (135 and 150 g) were homogenized in isotonic saline (6 mL), and 1 mL of this was used as the blank sample (3 samples). Pregnenolone, dehydroepiandrosterone, and IS (2.5 ng each) in MeOH (50 µL each) were added to the homogenate (1 mL, 3 samples) and treated as previously described. After derivatization with NH_2OCH_3•HCl, 2.5 ng of [17,21,21,21-$^2H_4$] pregnenolone 20–methyloxime (3) in MeOH (50 µL) was added as IS_2.

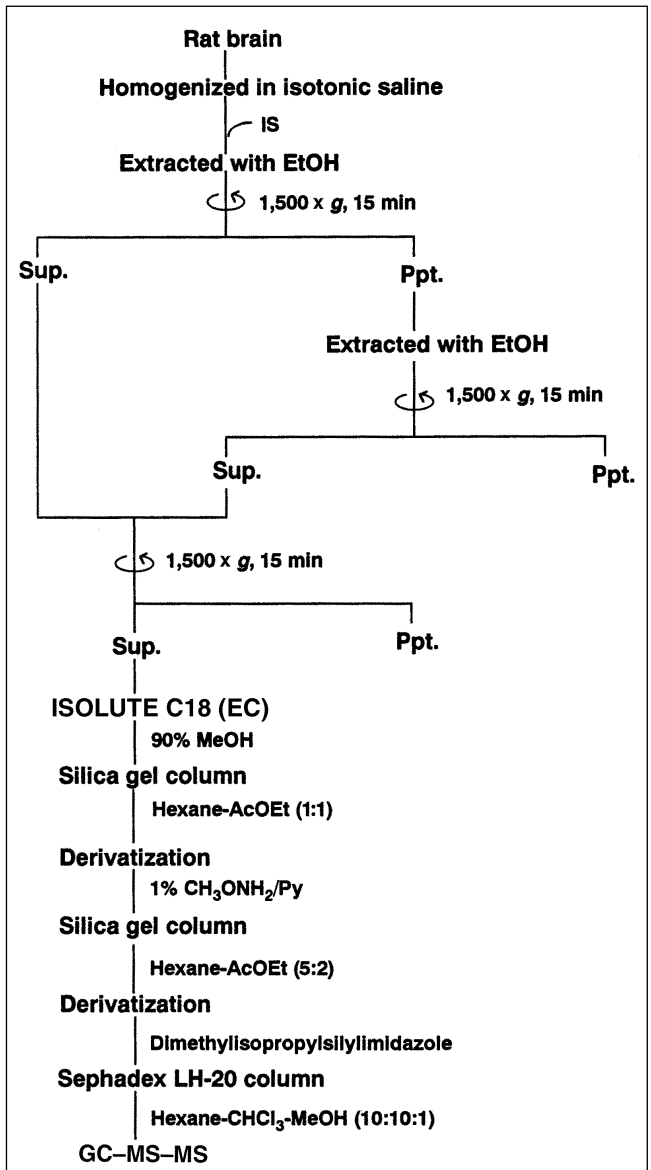

Figure 2. Procedure for the determination of pregnenolone and dehydroepiandrosterone in rat brains.

Calibration graphs for pregnenolone and dehydroepiandrosterone

Each tube containing pregnenolone and dehydroepiandrosterone (0.1, 0.4, 1.0, 5 ng) and IS (5 ng) was treated as previously described and then subjected to GC–MS–MS. The calibration graphs were constructed by the peak area ratio method using the sum of the three product ions, and the obtained linear relationships were as follows: pregnenolone, $y = 0.210x - 0.019$ (correlation coefficient $r = 1.000$), and dehydroepiandrosterone, $y = 0.224x + 0.003$ ($r = 0.999$).

Validation of the method for the determination of pregnenolone and dehydroepiandrosterone

The known amounts of pregnenolone and dehydroepiandrosterone were added to the rat brain homogenate (without gray matter and olfactory bulbs, 100 mg tissue), and each of the resulting solutions was assayed with the proposed method.

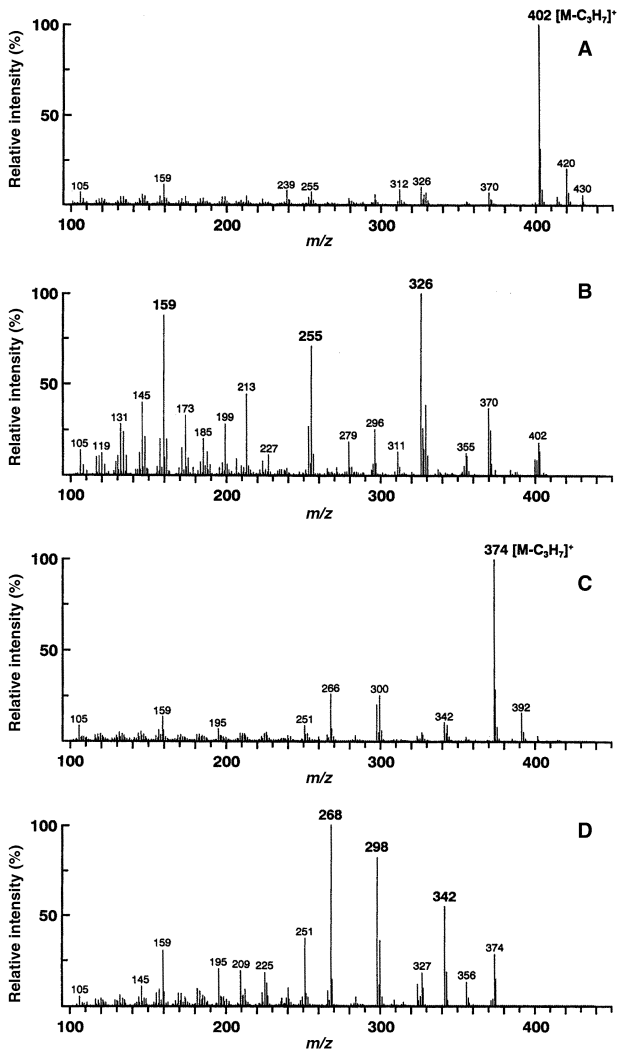

Figure 3. MS and product ion MS spectra of dimethylisopropylsilyl-methyloxime derivatives of pregnenolone and dehydroepiandrosterone: MS spectra of pregnenolone (A) and dehydroepiandrosterone derivatives (C) and product ion MS spectra of pregnenolone (B) and dehydroepiandrosterone derivatives (D). Conditions: precursor ion; *m/z* 402 (B) and *m/z* 374 (D); collision energy; 0.85 V (B) and 0.8 V (D).

Application of the method

The developed method was applied to the determination of pregnenolone and dehydroepiandrosterone in the gray matter and olfactory bulbs of Wistar strain rat brains. The rats were divided into two groups: the control rats under normal conditions and the stressed rats after fixation for 1 h.

Results and Discussion

Determination of pregnenolone and dehydroepiandrosterone in rat brains

A method for the determination of pregnenolone and dehydroepiandrosterone in rat brains using GC–EI-MS–MS was developed. [$^2H_4$]-Dehydroepiandrosterone was used as the IS. In a previous paper of this series, it was clarified that the derivatization of oxosteroids into methyloxime followed by silica gel minicolumn chromatography was effective for the cleanup procedure in LC–APCI-MS (3). These data prompted us to use the derivatization method for the determination of pregnenolone and dehydroepiandrostreone in rat brains using GC–MS–MS.

The brains of adult Wistar strain rats were homogenized in isotonic saline; IS was added to the homogenate and then extracted with EtOH. The extract was subjected to solid-phase extraction and then purified by silica gel minicolumn chromatography to get the desired compounds including IS. The fraction was derivatized with *O*-methylhydroxylamine, purified by silica gel minicolumn chromatography, and then derivatized into dimethylisopropylsilyl ether. The derivatives were purified by Sephadex LH-20 minicolumn chromatography and then subjected to GC–MS–MS (Figures 2 and 3).

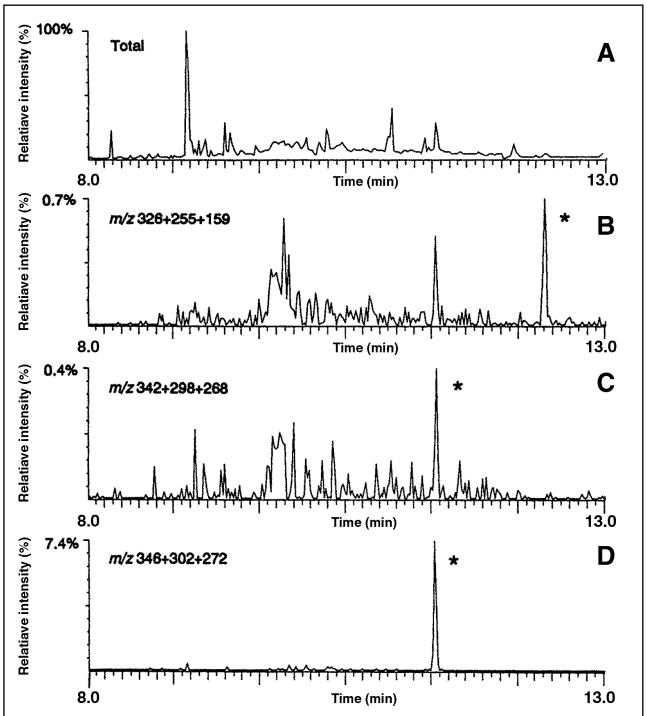

Figure 4. Product ion MS chromatograms of pregnenolone (B), dehydroepiandrosterone (C), and IS (D) obtained from rat brains.

The chromatogram of oxime usually shows two peaks, corresponding to the *E*- and *Z*-isomers, but the methyloximes of pregnenolone, dehydroepiandrosterone, and IS showed respective single peaks (Figure 4).

Recoveries before methyloxime derivatization

The recoveries of pregnenolone, dehydroepiandrosterone, and IS before the methyloxime derivatization were examined according to the cleanup procedure, and the results are summarized in Table I. A significant difference has not been observed based on the results obtained from the determining targets and IS.

Method validation

After spiking the authentic samples into the rat brain homogenate, the concentrations of pregnenolone and dehydroepiandrosterone were determined using the proposed method. The obtained data are summarized in Table II. Satisfactory recoveries were obtained with a relative standard deviation (RSD) of less than 13.0%.

The quantitative limits (signal-to-noise ratio *S/N* =10) of pregnenolone and dehydroepiandrosterone using the authentic samples were 8 and 5 pg on-column, respectively. However, the chromatogram obtained from biological fluids sometimes showed high background; therefore, *S/N* = 10 for each chromatogram was used as the guide to the determination of each compound in rat brains.

Table I. Recovery of Pregnenolone, Dehydroepiandrosterone, and IS

Compound	Blank* (ng/tube)	Added (ng/tube)	Found† (ng/tube)	Recovery (%)‡	RSD (%)
Pregnenolone	0.65	2.50	2.60	78.0 ± 1.4	1.8
Dehydroepiandrosterone	n.d.§	2.50	1.97	78.8 ± 7.5	9.5
IS	n.d.§	2.50	2.08	83.2 ± 6.3	7.6

* Mean (n = 3), 100 mg tissue/tube.
† Mean (n = 3).
‡ $(F - F_0)/A \times 100$, where A is the added steroid, F is the steroid in a supplemented brain, and F_0 is the steroid in an unsupplemented brain (mean ± SD, n = 3).
§ n.d., not detectable.

Table II. Accuracy and Precision of the Determination of Pregnenolone and Dehydroepiandrosterone in Rat Brains

Compound	Blank* (ng/tube)	Added (ng/tube)	Found† (ng/tube)	Recovery (%)‡	RSD (%)
Pregnenolone	0.84	2.50	3.33	99.6 ± 1.9	1.9
	0.60	5.00	5.35	95 ± 5.5	5.8
Dehydroepiandrosterone	n.d.§	0.20	0.21	105 ± 13.5	12.9
	n.d.§	1.25	1.21	96.8 ± 8.1	8.4

* Mean (n = 5), 100 mg tissue/tube.
† Mean (n = 5).
‡ Relative recovery using IS (%) = $(F - F_0)/A \times 100$, where A is the added steroid, F is the steroid in a supplemented brain, and F_0 is the steroid in an unsupplemented brain (mean ± SD, n = 5).
§ n.d., not detectable.

Application of the method

The developed method was applied to the determination of pregnenolone and dehydroepiandrosterone in the gray matter and olfactory bulbs of Wistar strain rat brains. Although pregnenolone in both regions of the rat brains increased more than three times after stress, dehydroepiandrosterone in those regions was not influenced by the stress (Figure 5).

Further discussion

In a previous paper of this series, a method for the determination of pregnenolone and its 3-stearate in rat brains using LC-APCI-MS was developed (3). However, the quantitative limit of the method was not sensitive enough to determine dehydroepiandrosterone and a lower amount of pregnenolone in rat brains. The developed method using GC–MS–MS is more than 10 times more sensitive (quantitative limit using 100 mg tissue: pregnenolone, approximately 0.4 ng/g tissue; dehydroepiandrosterone, approximately 0.25 ng/g tissue) than the previous one (approximately 7.5 ng/g tissue)(3). The method using the sum of the three product ions in MS–MS is sensitive and satisfactory in its accuracy and precision (Figure 4, Table II). The derivatizations with *O*-methylhydroxylamine and dimethylisopropylsilylimidazole are suitable for the cleanup procedure; that is, the derivative is stable and can be treated by gel filtration. Furthermore, these derivatives of pregnenolone and dehydroepiandrosterone showed strong base ion peaks in their MS spectra corresponding to $[M\text{-}C_3H_7]^+$, which is convenient for further MS analysis (Figure 3). In contrast, methyloxime and trimethylsilyl ether derivatives of these steroids gave complicated MS spectra that were not suitable for further MS analysis because of MS sensitivity. Uzunov et al. (5) used *O*-(pentafluorobenzyl)hydroxylamine as a derivatization reagent for the analysis of neurosteroid by negative-ion MS, and the present authors also attempted this (9). Despite using longer and varied temperature conditions, it was not possible to improve the yield of derivative. The strong electron capturing properties of pentafluorobenzyl moiety compensated the poor yield of the derivative. Although the proposed method needs the series of extraction– purification procedures (Figure 2), it is more convenient than the previously reported methods using GC–MS with preparative HPLC and/or preparative TLC (2,5).

It has been reported that a high concentration of pregnenolone is observed in the gray matter (1) and olfactory bulbs of rat brains (5) and that the synthetic enzyme of the steroid from cholesterol has been found in the olfactory bulbs (7). It was also demonstrated that pregnenolone increases in rat brains after acute stress (3), but that dehydroepiandrosterone is not influenced by stress (8). These data prompted us to apply the developed method to the determination of pregnenolone and dehydroepiandrosterone in the gray matter and olfactory bulbs of Wistar strain rat brains. The rats were divided into the control

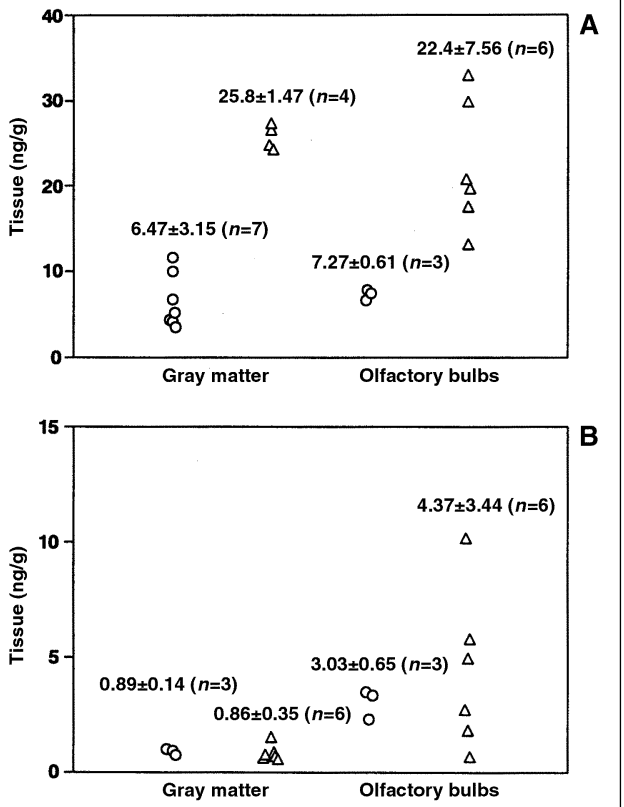

Figure 5. Pregnenolone (A) and dehydroepiandrosterone (B) in rat brains (mean ± SD). Control, O; stress-induced, Δ.

and acute stressed groups. The data of pregnenolone in normal and post-stressed rat brains are compatible with those previously obtained in our laboratories (3). Although the reported amount of dehydroepiandrosterone in rat brains varied from a low value (0.42 ng/g tissue)(4) to high value (12 ng/g tissue)(2), the obtained data are compatible with the former data. Although one high value (10.2 ng/g tissue) was observed in the olfactory bulbs with stress, dehydroepiandrosterone in both regions has not been so clearly influenced by stress (Figure 5).

The physiological significance of these steroids is under investigation in our laboratory, and the results will be reported in a future study.

Conclusion

Sensitive GC–MS–MS has been established for the quantitative determination of pregnenolone and dehydroepiandrosterone in rat brains. Application of the method to the analysis of these compounds in the gray matter and olfactory bulbs in rat brains proved satisfactory. The method could be used for the investigation of the physiological significance of these neurosteroids.

Acknowledgments

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