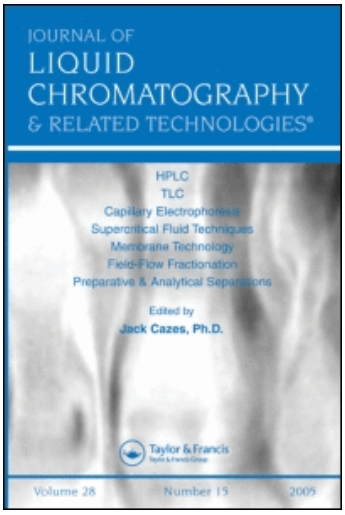

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Studies on Neurosteroids. VII. Characterization of Pregnenolone, Its Sulfate and Dehydroepiandro-Sterone in Rat Brains Using Liquid Chromatography/Mass Spectrometry

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STUDIES ON NEUROSTEROIDS. VII. CHARACTERIZATION OF PREGNENOLONE, ITS SULFATE AND DEHYDROEPIANDROSTERONE IN RAT BRAINS USING LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY

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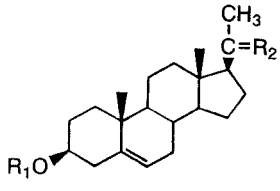
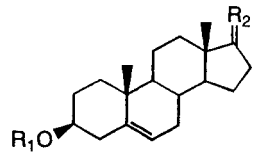
ABSTRACT

The separation and characterization of pregnenolone, its 3-sulfate and dehydroepiandrosterone in rat brains (neurosteroids) were carried out using liquid chromatography/mass spectrometry. The genins obtained from rat brains were acetylated and then derivatized with *O*-methylhydroxylamine to give the respective acetate-methyloximes, which were identified by comparison of their chromatographic behavior with authentic samples during liquid chromatography/ atmospheric pressure chemical ionization-mass spectrometry. Pregnenolone 3-sulfate in rat brains was also identified with an authentic sample using liquid chromatography / electrospray ionization - mass spectrometry.



P : R_1 = H, R_2 = O
PS : R_1 = SO_3H, R_2 = O
P acetate : R_1 = Ac, R_2 =O
P methyloxime : R_1 = H, R_2 = NOMe
P acetate-methyloxime : R_1 = Ac, R_2 = NOMe



D : R_1 = H, R_2 = O
D acetate : R_1 = Ac, R_2 =O
D methyloxime : R_1 = H, R_2 = NOMe
D acetate-methyloxime : R_1 = Ac, R_2 = NOMe

Figure 1. Structures of P, PS, D and their derivatives.

INTRODUCTION

In previous papers of this series, we clarified the existence of pregnenolone (P) and dehydroepiandrosterone (D) in rat brains (neurosteroids) using fluorometric derivatization followed by high-performance liquid chromatography (HPLC).[1,2] We also separated and characterized the fatty acid esters (stearate, palmitate) of P and D in rat brains using liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry (LC/APCI-MS) operating in the positive-ion mode.[3] The derivatization of these esters with *O*-methylhydroxylamine gave the respective methyloximes which showed the quasi-molecular ion [QM] in its analysis using LC/APCI-MS and is helpful in identifying the compound. This paper deals with the separation and characterization of P and D in rat brains using LC/APCI-MS, in which the derivatization is important to obtain satisfactory information. Pregnenolone 3-sulfate (PS) in rat brains was also identified by comparison with authentic sample during the course of LC/electrospray ionization (ESI)-MS (Fig. 1).

EXPERIMENTAL

Materials and Methods

Melting points were measured on a Yanagimoto melting point apparatus (Kyoto, Japan) without correction. Proton nuclear magnetic resonance spectra

(^{1}H-NMR) were measured with a Jeol JNM-EX270 (270 MHz) (Tokyo, Japan) using tetramethylsilane as an internal standard and the following abbreviations were used: s=singlet, d=doublet and m=multiplet.

Silica gel column chromatography and preparative (prep.) thin layer chromatography (TLC) were performed on silica gel 60 (70-230 mesh, E. Merck, Darmstadt, Germany) and pre-coated silica gel HF_{254} (0.25 mm; 20 x 20 cm)(E. Merck), respectively. The Isolute C_{18} (EC) cartridges (International Solvent Technology, Hengoed, UK) were obtained from Uniflex (Tokyo) and piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) was prepared in this laboratory.[4] P and D were obtained from Tokyo Kasei Kogyo (Tokyo). The other reagents and materials were commercially available and of analytical reagent grade.

Preparative HPLC was carried out using a JASCO TRI ROTAR chromatograph (JASCO, Tokyo) equipped with a UVIDEC-100-II UV (223 nm) detector (JASCO) at a flow rate of 1 mL/min. The reversed-phase column [YMCPack ODS-AM (5μm, 15 x 0.46 cm i.d.; YMC)] was used at 30.8°C.

Preparation of pregnenolone 3-sulfate

P (100 mg) was dissolved in pyridine (2 mL), freshly prepared chlorosulfonic acid-pyridine complex (*ca.* 100 mg) was added to the solution under ice-cooling, and the mixture was kept at room temperature for 10 min and then 50°C for 1 hr. After the addition of H_2O (30 mL), the mixture was neutralized with 5% $NaHCO_3$ (w/v) and applied to an Amberlite XAD-2 (Organo, Tokyo) column (12 x 1.8 cm i.d.). After washing with H_2O, the steroid was eluted with MeOH, which was evaporated *in vacuo*. The obtained residue was subjected to prep. TLC using $CHCl_3$-MeOH-H_2O (80:20:2.5, v/v) as a developing solvent. The zone corresponding to *Rf* 0.30 was extracted with the same solvent. After evaporation of the solvent, the obtained residue was recrystallized from MeOH-ethyl ether to give colorless amorphous substances (52.3 mg). mp 143-145°C. ^{1}H-NMR ($CDCl_3$/CD_3OD) δ : 0.61 (3H, s, H-18), 0.84 (3H, s, H-19), 2.13 (3H, s, H-21), 4.31 (1H, m, H-3 α), 5.42 (1H, d, *J*=5.3 Hz, H-6).

LC/MS

LC/MS was performed using a Hitachi M-1000H (Tokyo, Japan) connected to a Hitachi L-6200 chromatograph. The following conditions were used for the analysis, including flow injection, unless otherwise specified. The multiplier voltage was set at 2.5 kV and focus voltages were set at 120 V (positive-ion mode) and -120 V (negative-ion mode), respectively. The

desolvator temperature was set at 399°C. The vaporizer and nebulizer temperatures were set at 300°C (APCI) and 200°C (ESI), respectively. The drift voltages were set at 50 V (positive-ion mode) and -80 V (negative-ion mode), respectively. The reversed-phase LC/APCI-MS was performed on a J'sphere ODS-H80 or -L80 column (4μm, 15 x 0.46 cm i.d.) (YMC, Kyoto, Japan) at a flow rate of 1 mL/min, under ambient conditions. The reversed-phase LC/ESI-MS was performed on a Develosil ODS-HG-5 column (5 μm, 15 x 0.1 cm i.d.) (Nomura, Seto, Japan) at a flow rate of 50 μL/min, under ambient conditions.

Animals

Female Wistar strain rats (120-155 g, Japan SLC Co., Hamamatsu, Japan) were decapitated and the entire brains were kept at -10°C until use.

Characterization of Pregnenolone and Dehydroepiandrosterone in Rat Brain

The P and D in the entire rat brain or half of one (0.85-1.10 g; each case at least 2 specimens) were extracted with AcOEt-$CHCl_3$ (6:1, v/v)(5 mL) and subjected to silica gel column chromatography as previously reported.[2] The obtained fraction [hexane-AcOEt (1:1, v/v)(5 mL)] was evaporated under an N_2 gas stream, and the obtained residue was treated with pyridine-Ac_2O (2:1, v/v)(0.3 mL) at 70°C for 30 min. After evaporation of the solvent under an N_2 gas stream, the residue was treated with a solution of *O*-methylhydroxylamine · HCl in pyridine (2%, w/v) (0.3 mL) at 70°C for 60 min. The mixture was diluted with AcOEt, and the organic layer was washed with 5% HCl (v/v), H_2O and evaporated under an N_2 gas stream. The obtained acetate-methyloxime was subjected to silica gel column chromatography (3 x 0.6 cm i.d.), the obtained fraction [hexane-AcOEt (10:1, v/v)(5 mL)] was evaporated under an N_2 gas stream, and the obtained residue was again subjected to the same silica gel column chromatography. The obtained residue was redissolved in MeOH-$CHCl_3$ (1:1, v/v) and subjected to LC/APCI-MS operating in the positive-ion mode: P {J'sphere ODS-H80; t_R 14.8, 18.8 (main) min [MeOH-H_2O (11:1, v/v) containing $AcONH_4$ (20 mM)]: J'sphere ODS-L80; t_R 9.2, 10.5 (main) min [MeCN-H_2O (4:1, v/v) containing $AcONH_4$ (20 mM)]} ; D {J'sphere ODS-H80; t_R 10.1 (main), 11.0 min [MeOH-H_2O (8:1, v/v) containing $AcONH_4$ (20 mM)]: t_R 11.4 (main), 13.8 min [MeCN-H_2O (5:1, v/v) containing $AcONH_4$ (20 mM)]}.

Characterization of Pregnenolone 3-Sulfate in Rat Brain

Two entire rat brains (3.2-3.6 g; 3 experiments were done) were homogenized in isotonic saline (6 mL) under ice-cooling. EtOH (5 mL) was added to the homogenate and the mixture was centrifuged at 2,800 rpm for 30 min. The precipitate was again suspended in EtOH (5 mL) and centrifuged as described above. Both supernatants were combined and centrifuged again at 2,800 rpm for 30 min. The supernatant was diluted with H_2O (90 mL) and the whole solution was applied to an Isolute C_{18} (EC) cartridge, washed with H_2O (5 mL), eluted with 90% EtOH (v/v)(5 mL), and applied to a PHP-LH-20 column (2 x 0.6 cm i.d.). After washing with 90% EtOH (v/v)(10 mL) and 0.1 M AcOH/90% EtOH (v/v)(10 mL), the sulfate was eluted with 0.3 M $AcONH_4$/90% EtOH (v/v)(pH 9.5) (6 mL), diluted with H_2O (40 mL) and then applied to an Isolute C_{18} (EC) cartridge as described above to remove $AcONH_4$. The eluate with EtOH was evaporated under an N_2 gas stream, and the obtained residue was subjected to the following successive preparative HPLC [MeCN-52 mM $(NH_4)_2CO_3$ (3:5.5, v/v); t_R 8.34 min: MeOH-52 mM $(NH_4)_2CO_3$ (9:5, v/v); t_R 7.05 min]. Part of the obtained residue was redissolved in MeOH and applied to LC/ESI-MS operating in the negative-ion mode: PS {t_R 4.80 min [MeOH-H_2O (9:4, v/v) containing $AcONH_4$ (0.65 mM)]}.

The other part of the residue was redissolved in AcOEt (1 mL) containing 1 μL of 0.5 M H_2SO_4, the mixture was kept at room temperature 1 hr and then washed with H_2O. After evaporation of the solvent, the residue was redissolved in MeOH and applied to LC/APCI-MS (drift voltage 30V) operating in positive-ion mode: P {J'sphere ODS-L80; t_R 8.47 min [MeOH-H_2O (9:2, v/v)]}.

RESULTS

LC/MS Data of Neurosteroids and Their Derivatives

The LC/APCI-MS of P, D and their derivatives were scanned using flow injection analysis and the data are summarized in Tables 1 and 2.

The data showed that genin itself and its acetate exhibited $[QM-H_2O]^+$ and $[QM-AcOH]^+$ as a base peak, respectively (Table 1). On the contrary, methyloxime and acetate-methyloxime gave $[QM]^+$ as a base peak. The two step derivatization of the steroid to acetate-methyloxime gave $[QM]^+$ as a base peak with a higher mass number, which is more reliable for identifying the compound. The addition of $AcONH_4$ to the mobile phase increased the relative intensity of $[QM]^+$ in almost every case.

Table 1

LC/APCI-MS Data of Neurosteroids and Their Derivatives

Compound	Mw	Ion	
P	316	317$[M+H]^+$ (18%)[1](22%)[2]	299$[M+H-H_2O]^+$ (100%)(100%)
P acetate	358	359$[M+H]^+$ (3%)(3%)	299$[M+H-AcOH]^+$ (100%)(100%)
P methyloxime	345	346$[M+H]^+$ (100%)(100%)	328$[M+H-H_2O]^+$ (60%)(9%)
P acetate-methyloxime	387	388$[M+H]^+$ (100%)(100%)	328$[M+H-AcOH]^+$ (87%)(26%)
D	288	289$[M+H]^+$ (21%)(15%)	271$[M+H-H_2O)^+$ (100%)(100%)
D acetate	330	331$[M+H]^+$ (1%)(1%)	271$[M+H-AcOH]^+$ (100%)(100%)
D methyloxime	317	318$[M+H]^+$ (100%)(100%)	300$[M+H-H_2O]^+$ (65%)(11%)
D acetate-	359	360$[M+H]^+$ (100%)(100%)	300$[M+H-AcOH]^+$ (73%)(11%)

1,2) Relative intensity. Mobile phase: 1) MeOH, 2) MeOH containing $AcONH_4$ (20 mM).

Table 2

Comparison of Relative Sensitivity in Neurosteroids and Their Derivatives

Compound	Mw	Base Peak	Relative Sensitivity[1]
P	316	299$[M+H-H_2O]^+$	1
P acetate	358	299$[M+H-AcOH]^+$	2.9
P methyloxime	345	346$[M+H]^+$	43.9
P acetate-methyloxime	387	388$[M+H]^+$	43.3
D	288	271$[M+H-H_2O]^+$	1
D acetate	330	271$[M+H-AcOH]^+$	3.3
D methyloxime	317	318$[M+H]^+$	48.9
D acetate-methyloxime	359	360$[M+H]^+$	61.8

[1] Each sample [*ca.* 0.32 nmol/MeOH-$CHCl_3$ (1:1)(5 μL) was applied to APCI-MS [SIM (base peak) mode; mobile phase, MeOH containing $AcONH_4$ (20 mM)] by flow injection. The sensitivity was comparisoned in molar base.

The comparison of the relative sensitivity between genins (neurosteroids) and its derivatives is summarized in Table 2. The data showed that the detection responses of the derivatives (acetate, methyloxime, acetate-methyloxime) were increased by 2.9 to 61.8 fold over their intact forms (genin).

In particular, the methyloxime-derivatization is an effective method of increasing response on APCI-MS for these compounds having oxo group. These data prompted us to derivatize the neurosteroids to acetate-methyloxime and then subjected this to LC/APCI-MS in the positive-ion mode using $AcONH_4$ as a mobile phase additive.

Although the quantification of serum D 3-sulfate using LC/APCI-MS in the negative-ion mode has been reported, the post-column addition of acetone is necessary to detect the compound (detection limit 10 ng/injection).[5] In order to avoid the tedious post-column addition technique, we used LC/ESI-MS in the negative-ion mode for the detection of PS in rat brain and the $[M-H]^-$ (*m/z* 395) has been detected with reasonable sensitivity (1 ng/injection, signal to noise=5).

Characterization of Neurosteroids in Rat Brain

The P and D in rat brain were extracted with AcOEt-$CHCl_3$ and subjected to silica gel column chromatography as previously reported.[2] The fraction containing the steroids was treated with pyridine-Ac_2O and then *O*-methylhydroxylamine to give the acetate-methyloxime of the respective steroids, which showed a $[QM]^+$ as a base peak in its LC/APCI-MS. P and D were identified by LC/APCI-MS with selected-ion monitoring (SIM; P acetate-methyloxime: *m/z* 388 $[M+H]^+$; D acetate-methyloxime: *m/z* 360 $[M+H]^+$) by comparison of their chromatographic behavior with those of authentic samples, in which *E*- and *Z*-isomers of the methyloxime are clearly separated and helpful in identifying the compound. MS spectra with total-ion monitoring (TIM; *m/z* 200-400) were also identical with those of the authentic samples, and a typical example is shown in Fig. 2.

The separation of PS from rat brain was carried out as shown in Fig. 3. After the solid phase extraction using the cartridge, the fraction containing steroids was separated with anion-exchanger (PHP-LH-20) to give the fraction containing PS, which was identified by LC/ESI-MS with SIM (*m/z* 395 $[M-H]^-$) by comparison of its chromatographic behavior with that of an authentic sample (Fig. 4).

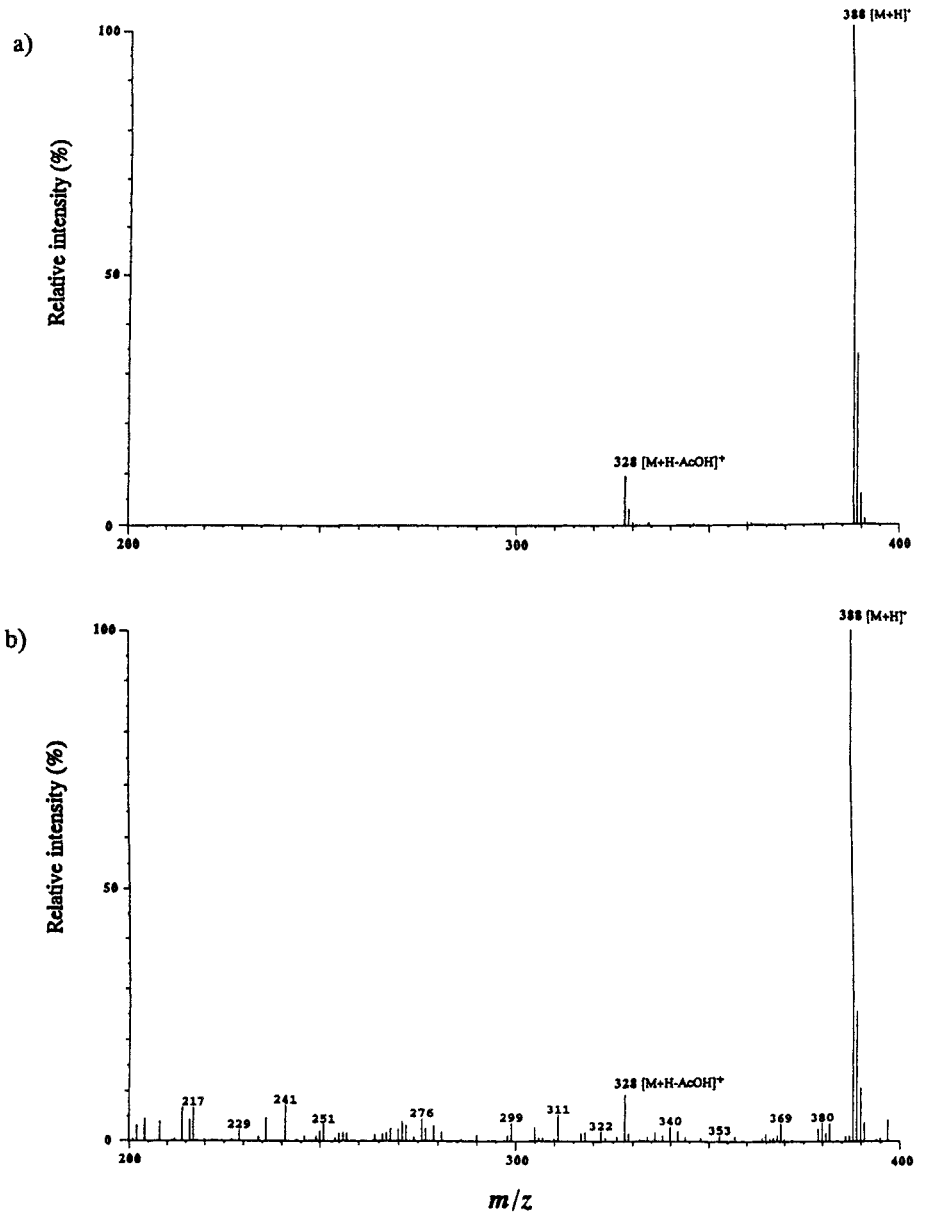


Figure 2. TIM of P derivative. Conditions: LC/APCI-MS (positive-ion mode); column, J'sphere ODS-H80; mobile phase, MeOH-H_2O (11:1, v/v) containing $AcONH_4$ (20 mM); flow rate 1 mL/min; *m/z* 200-400. a) authentic sample b) from rat brain.

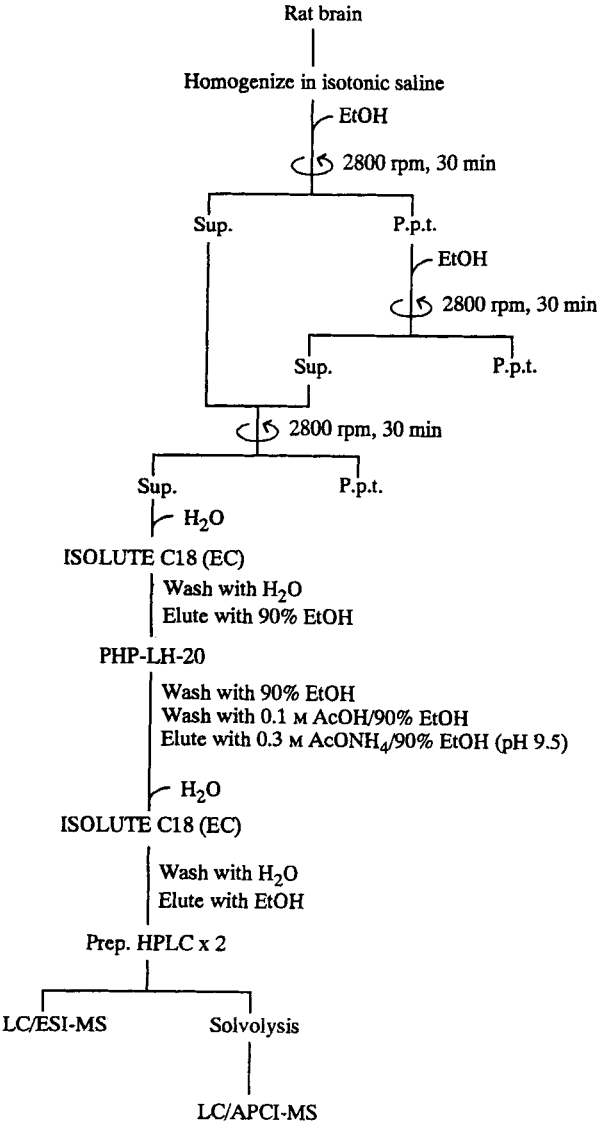


Figure 3. Separation of PS from rat brain.

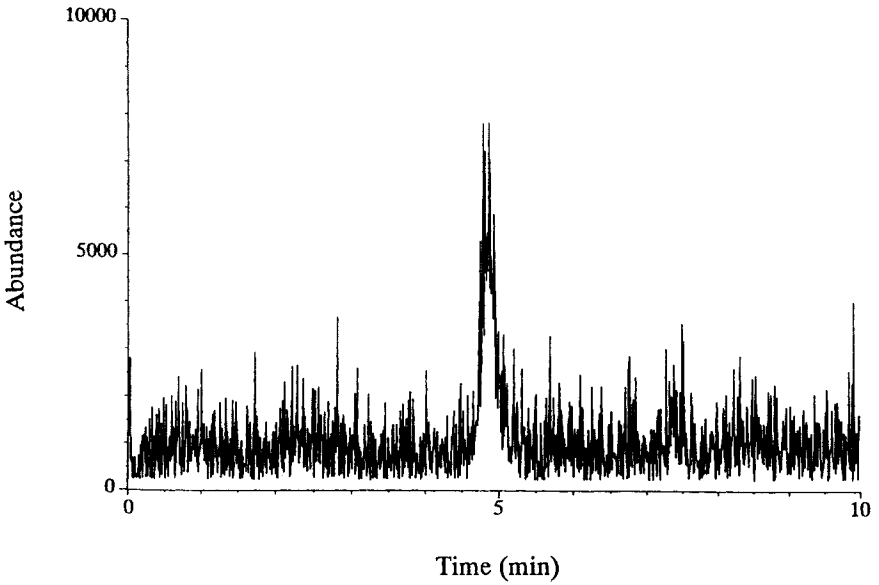


Figure 4. Selected ion recording of PS from rat brain. Conditions: LC/ESI-MS (negative-ion mode); column, Develosil ODS-HG-5; mobile phase, MeOH-H_2O (9:4, v/v) containing $AcONH_4$ (0.65 mM); flow rate 50 μL/min; SIM (*m/z* 395 $[QM]^-$).

In order to confirm the structure of the genin part, the fraction was subjected to solvolysis and the obtained P was identified by LC/APCI-MS using *m/z* 299 $[M+H-H_2O]^+$ as a monitoring ion. All of the above experiments were done more than twice, and the existence of these compounds were confirmed in every case.

DISCUSSION

Although the existence of P and D in rat brain has been reported, these were identified with HPLC,[1,2] radioimmunoassay or gas chromatography/mass spectrometry (GC/MS).[6] All of the above data with LC/APCI-MS showed the existence of the above steroids in rat brains, and the acetate-methyloxime of the above steroids showed a $[QM]^+$ as a base peak in its APCI-MS. LC showed two peaks corresponding to the *E*- and *Z*-isomers of the methyloxime of P or D, which is helpful in identifying the compound in the brain. The existence of PS in rat brains has been already done with GC/MS, but the method is an indirect one which determined the genin before and after the solvolysis.[6] The above

described LC/ESI-MS clarified the existence of PS in rat brain, which was also confirmed by solvolysis to give P determined by LC/APCI-MS. The quantitative determination of these steroids using the hyphenated technique including LC/MS are now in progress in our laboratories.

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