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著者	Mitamura Kuniko, Yatera Misako, Shimada Kazutake
journal or publication title	Analytical Sciences
volume	15
number	10
page range	951-955
year	1999-01-01
URL	http://hdl.handle.net/2297/3899

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Quantitative Determination of Pregnenolone 3-Sulfate in Rat Brains Using Liquid Chromatography/Electrospray Ionization-Mass Spectrometry

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A method for the quantitative determination of pregnenolone 3-sulfate (PS) in rat brains has been developed using liquid chromatography/electrospray ionization–mass spectrometry (LC/ESI-MS). The PS fraction was obtained from the rat brain homogenate by solid-phase extraction and ion-exchange chromatography. After the derivatization with 4-(*N,N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole, PS was determined using LC/ESI-tandem MS along with the standard addition method. This method was applied to the quantitative determination of this steroid in the brains of Wistar strain rats, most of which showed a much lower amount than that previously reported.

Keywords Neurosteroid, pregnenolone 3-sulfate, rat brain, LC/MS, derivatization

Since the discovery of dehydroepiandrosterone in rat brains, several 17- and 20-oxosteroids including pregnenolone (P), called "neurosteroids", have been elucidated in mammalian brains.[1] The neurosteroids that exist in rat brains are reported as the free form, sulfates, lipoidal esters and sulfolipids.[2] Although significant interest has thus been focused on their biological properties in this organ, the characterization and quantitative determination of these conjugated neurosteroids relied on gas chromatography/mass spectrometry (GC/MS) or RIA analysis of genin obtained by solvolysis.[2,3] In a previous paper of this series, we reported the identification and quantitative determination of P and its 3-stearate in rat brains using derivatization into acetate and/or methyloxime followed by liquid chromatography/atmospheric pressure chemical ionization (LC/APCI)-MS without deconjugation.[4] We also clarified the existence of pregnenolone 3-sulfate (PS) in rat brains using LC/electrospray ionization (ESI)-MS.[5] In this paper, we developed a quantitative determination method of PS in rat brains using LC/ESI-tandem MS (LC/ESI-MS/MS).

Experimental

Apparatus

LC/ESI-MS was performed on a Finnigan MAT LCQ liquid chromatograph-ion trap mass spectrometer (ThermoQuest, Tokyo, Japan) connected to a JASCO PU-980 (Tokyo) chromatograph. A YMC-Pack ProC18 (5 μm, 150×3.0 mm i.d.) (YMC, Kyoto, Japan) column or a Develosil ODS-HG-5 (5 μm, 150×2.0 mm i.d.) (Nomura Chem., Seto, Japan) column was used at a flow rate of 0.4 or 0.1 ml/min, respectively, at 30°C. The detection conditions were as follows: the source voltage and current were 5 kV and 20 μA, respectively. The heated capillary temperature, sheath gas flow rate and auxiliary gas flow rate were set at 270°C, 70 units and 20 units, respectively. The capillary voltage was –2 V, and the tube lens offset was –25 V.

Materials, reagents and animals

PS[5] and [17,21,21,21-$^2H_4$]PS=internal standard (IS) were synthesized from P and [17,21,21,21-$^2H_4$]P[6], respectively, along with chlorosulfonic acid-pyridine complex in our laboratories. *O*-Methylhydroxylamine and 4-(*N,N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H) were obtained from Tokyo Kasei (Tokyo). *O*-(2,3,4,5,6-Pentafluorobenzyl)-hydroxylamine (PFB-H) was purchased from Wako (Osaka, Japan). The ISOLUTE C18 (EC) cartridges (500 mg or 200 mg) (International Sorbent Technology, Hengoed, UK) were obtained from Uniflex (Tokyo) and piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) was prepared in our laboratories.[7] All other reagents and solvents were commercially available and of analytical grade. Wistar strain rats (7 weeks old, male) were obtained from Japan S.L.C. (Hamamatsu, Japan).

Cleanup of PS in rat brains

A Wistar strain rat was decapitated; its whole brain was homogenized in EtOH (7 ml) and sonicated for 10 min. The suspension was shaken at 39°C overnight and centrifuged at 1500*g* for 10 min. The supernatant was saved, and the precipitate was extracted with EtOH (5 ml) with continuous shaking at room temperature for 10 min. The extract was centrifuged at 1500*g* for 10 min,

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all the supernatants were combined and diluted with H_2O, in which the concentration of EtOH was adjusted to 10%. The resulting solution was applied on an ISOLUTE C18 (EC) (500 mg) cartridge. After washing with H_2O (5 ml) and then with 20% EtOH (5 ml), the eluate with 90% EtOH (6 ml) was applied on a hydrophobic ion-exchange column [PHP-LH-20 (2×0.6 cm i.d.)]. After washing with 90% EtOH (5 ml) and 0.1 M AcOH/90% EtOH (5 ml) to remove any neutral compounds, the sulfate was eluted with 0.3 M $AcONH_4$/90% EtOH (pH 9.5; adjusted with NH_4OH)(5 ml). The fraction was diluted with H_2O (40 ml) and applied on an ISOLUTE C18 (EC) (200 mg) cartridge to remove $AcONH_4$. After washing with H_2O (5 ml), the eluate with EtOH (2 ml) was evaporated to dryness. DBD-H (100 µg in 100 µl of EtOH containing 0.05% trifluoroacetic acid) was added to the obtained residue and the resulting solution was kept at room temperature overnight. After dilution with H_2O (0.9 ml), the solution was applied on an ISOLUTE C18 (EC) (200 mg) cartridge and washed with 20% EtOH (3 ml) to remove the excess reagent. The eluate with EtOH (2 ml) was evaporated to dryness, the residue was redissolved in MeOH (50 µl) and then an aliquot was subjected to LC/ESI-MS.

Identification of PS in rat brains

A whole brain (*ca.* 1 g tissue) was subjected to the pretreatment procedure as described above, and the desired fraction was applied on an LC/ESI-MS [column, YMC-Pack ProC18; mobile phase, MeOH-4 mM $AcONH_4$ (3:1); t_R 10.4 min; total ion monitoring (*m*/*z* 300 - 700)] or LC/ESI-MS/MS [relative collision energy, 30%; precursor ion, *m*/*z* 634; product ion monitoring (*m*/*z* 300 - 450)].

Calibration graph

PS (2, 4, 8, 16, 20 pmol/tube) and IS (200 pmol/tube) were derivatized with DBD-H followed by purification in the manner described above, and the obtained fraction was assayed using the proposed method. The calibration graph was constructed using the peak area ratio method (Y=0.0067X+0.0003, r^2=0.9997; Y=peak area ratio, X=PS value).

Method validation

PS (4, 8, 12 pmol/tube) and IS (200 pmol/tube) were added to the rat brain (0.4 g tissue) homogenate, and the resulting solution was assayed using the proposed method. The concentration of PS was quantified by the calibration graph. The analytical recovery was calculated from the PS concentration found in the added sample (8 or 12 pmol/tube minus 4 pmol/tube).

Quantitative determination of PS in rat brains by standard addition method

The DBD-IS showed the satisfactory ESI-MS spectrum {*m*/*z* 637 (30%), 638 $[M-H]^-$ (100%), 639 (30%)} in which the ion corresponding to $[M-H]^-$ (*m*/*z* 634) of DBD-PS was not observed as previously reported[4] and $[^2H_4]$PS is usable as an IS of isotope dilution LC/ESI-MS. A whole brain was homogenized and extracted with EtOH as previously described and the obtained supernatant was divided into several parts so that the content of brain in each tube is approximately 0.4 g tissue. PS (4, 6, 8, 10 pmol) and IS (200 pmol) were added to the tube, and the resulting solution was treated using the same method as described above. The desired fraction was applied on an LC/ESI-MS/MS with selected reaction monitoring (SRM) mode [column, Develosil ODS-HG-5; mobile phase, MeOH-4 mM $AcONH_4$ (2:1); t_R 18.2 min (PS), 17.9 min (IS); relative collision energy, 10%; precursor ion, *m*/*z* 634 (PS) and *m*/*z* 638 (IS); monitoring ion, *m*/*z* 634 (PS) and *m*/*z* 638 (IS)]. The concentration of the PS in the brain was obtained using the regression line (r^2>0.99), which was constructed by the peak area ratio method.

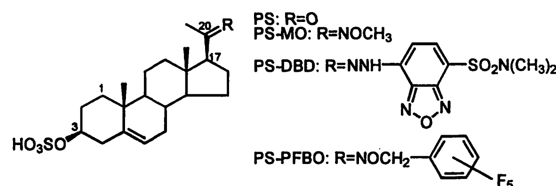

Fig. 1 Structures of PS and its derivatives.

Results

LC/MS of PS and its derivatives

In a previous paper of this series, we reported that the derivatization of oxosteroids into methyloxime strongly increased their sensitivity in LC/MS.[5] These results prompted us to use the derivatization method for the quantitative determination of PS using LC/MS. The commercially available *O*-methylhydroxylamine, DBD-H and PFB-H were selected as the derivatization reagents; these reacted with the carbonyl group of PS to give PS-methyloxime (MO), -DBD and -pentafluorobenzyloxime (PFBO), respectively (Fig. 1).

Although the mass spectrum of PS using flow injection analysis showed the $[M-H]^-$ ion as the base ion using ESI with negative-ion mode, the characteristic molecular related ions were not obtained in APCI and ESI with positive-ion mode. Several attempts including ESI-MS/MS did not give satisfactory sensitivity to establish the determination method of PS in rat brains. Therefore, the usefulness of the derivatization was examined using ESI with negative-ion mode. The obtained ions and the relative sensitivity between PS and its derivatives using the peak area of the base ion measured by selected ion monitoring (SIM) are summarized in Table 1. The data showed that PS and its derivatives exhibited the $[M-H]^-$ ion as the base ion, and the detection responses of the derivatives were increased by 8 to 11 fold of PS. Despite using longer

Table 1 Comparison of relative sensitivity in PS and its derivatives[a]

Compound	MW	Base ion	Relative sensitivity[b]
PS	396	$395[M-H]^-$	1
PS-MO	425	$424[M-H]^-$	9
PS-DBD	635	$634[M-H]^-$	8
PS-PFBO	591	$590[M-H]^-$	11

a. The characteristic molecular related ions were not obtained in APCI (heated capillary temperature, 150°C; vaporizer temperature, 400°C; capillary voltage, +5 V or −5 V), and ESI-MS by flow injection (mobile phase, MeOH–4 mM $AcONH_4$ (3:1); flow rate, 0.4 ml/min) was employed.
b. The relative sensitivity of each derivative against PS was calculated using the peak area of the base ion measured by SIM.

and various temperature conditions, it was not possible to improve the yield of PS-PFBO reported earlier.[8] The strong electron capturing properties of the PFBO moiety compensated the poor yield of the derivative. Although *O*-methylhydroxylamine gave PS-MO in a satisfactory yield, the excess reagent interferes with the chromatographic behavior of the sample of rat brains. All these results showed that DBD-H is the most useful in its reactivity with PS and the chromatographic behavior of the resulting derivative.

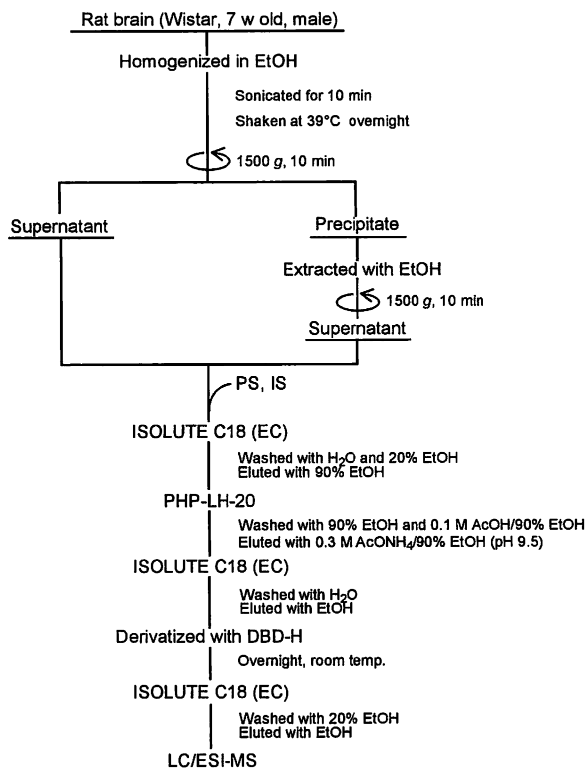

Fig. 2 Procedure for quantitative determination of PS in rat brains.

Identification and quantitative determination of PS in rat brains

The brains of adult Wistar strain rats were homogenized and extracted with EtOH, and the extract was subsequently purified by solid-phase extraction and ion-exchange chromatography. The fraction containing PS was derivatized with DBD-H, followed by purification with solid-phase extraction, and then the obtained residue was applied on an LC/ESI-MS with total ion monitoring mode (Fig. 2). PS was identified by comparison with authentic PS-DBD based on its mass chromatographic behavior, as shown in Fig. 3a. LC/ESI-MS/MS (precursor ion, $[M-H]^-$, relative collision energy, 30%) also gave the characteristic product ion $[M-H-282]^-$, which was produced by dissociation of the C_{17}-C_{20} bond (Fig. 3b). However, the obtained peak intensity was not high enough to determine PS in smaller amount of brain, so the collision energy was reduced to 10% and the residual $[M-H]^-$ ion was selected as the monitoring ion. In this system, the desired peak was detected without interfering peaks, and the detection limit of PS was 0.2 pmol (signal/noise=5).

After the addition of the authentic PS to the rat brain homogenate, the concentration of PS was determined using the proposed calibration graph method. The obtained results are summarized in Table 2. Satisfactory recoveries (91.0 - 117.3%) were obtained, and the relative standard deviation (RSD) of the intra- and inter-assays were less than 13.3% and 11.2%, respectively. The absolute recoveries of PS (14 pmol/tube) and IS (80 pmol/tube) before the derivatization reaction were 75.6% and 76.3%, respectively (mean, n=2).

We attempted to determine the concentration of PS using the proposed calibration graph method, however, the peak corresponding to PS-DBD in most of the samples was below the quantitative limit (4 pmol/tube). Therefore, we calculated the concentration of PS in the brains of male Wistar strain rats using standard addition method with the addition of 4 levels of PS (Fig. 4). The obtained concentrations were 0.53±0.28 ng/g tissue (1.33±0.7 pmol/g tissue; mean±SD, n=10), which were much lower than that reported in a previous study [*ca.* 20 ng/g tissue (*ca.* 50 pmol/g tissue)].[2,3]

Discussion

PS has been known as one of the neurosteroids, which act as a positive and negative modulator of the NMDA and $GABA_A$ receptor, respectively.[9] An intensive investigation of its biosynthetic pathway and biological properties has been done. Although the quantitative determination of PS in rat brains has been already done with GC/MS or RIA, these were indirect methods which determined the genin before and after the solvolysis. We tried to develop the determination method using DBD-H as a fluorescent derivatization reagent, but satisfactory results were not obtained as regards selectivity. That is, the endogenous interfering substances co-eluted with the desired compound during

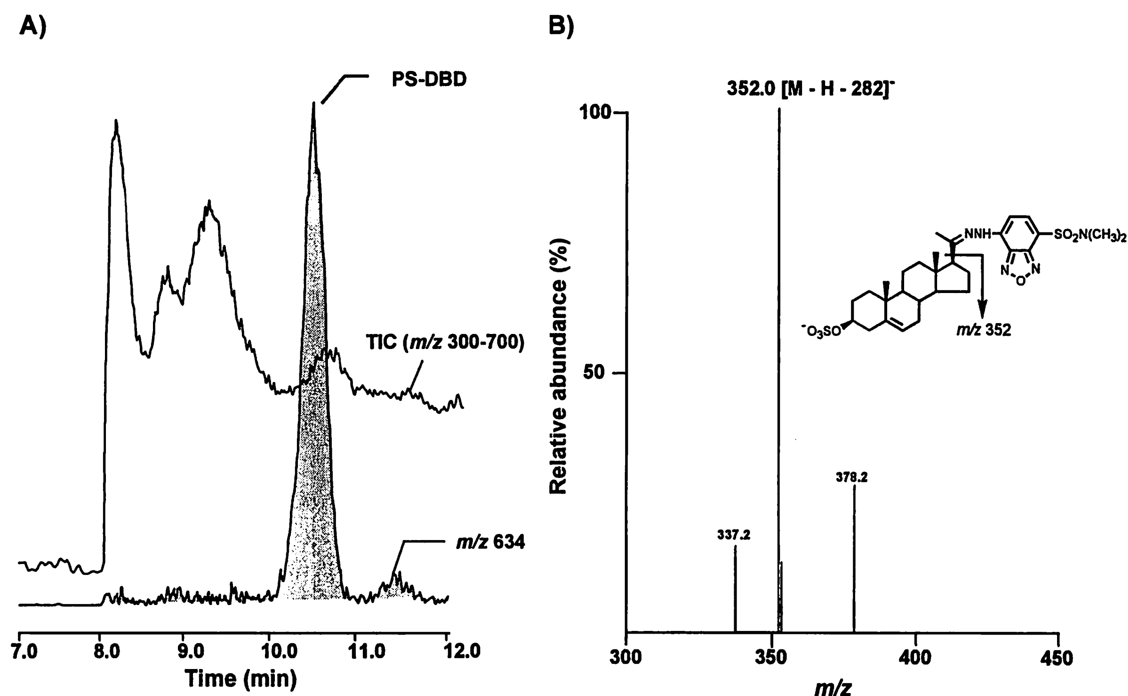

Fig. 3 LC/ESI-MS data of PS from rat brains as DBD derivative. A) Mass chromatogram, B) product ion mass spectrum. Column, YMC-Pack Pro C18; mobile phase, MeOH-4 mM $AcONH_4$ (3:1); flow rate, 0.4 ml/min; B) precursor ion, *m/z* 634; relative collision energy, 30%.

Table 2 Accuracy and precision of quantitative determination of PS

Entry	Concentration/pmol		Recovery, %[b]	RSD, %	
	Added	Found[a]		Intra-assay	Inter-assay
1	4	4.4±0.2		3.7	
2	4	4.8±0.3		5.7	
3	4	4.2±0.2		4.5	
4	4	3.8±0.1		1.7	
5	4	4.6±0.1		2.1	
6	4	4.4±0.1		2.8	
1	8	8.3±0.2	97.4±6.3	6.5	
2	8	9.1±0.3	107.7±7.2	3.2	
3	8	8.9±0.2	117.3±4.3	6.7	
4	8	7.5±0.5	91.0±12.1	13.3	11.2
5	12	13.3±0.3	108.5±3.4	3.1	
6	12	12.9±0.2	106.8±2.2	2.1	

a. Mean±SD, *n*=5, 0.4 g tissue/tube. b. The recovery was calculated from the PS concentration found in the added sample (8 or 12 pmol/tube minus 4 pmol/tube). Recovery (%)=$(F-F')/(A-4)\times100$; *F*=found concentration of PS in added sample (8 or 12 pmol/tube); *F'*=found concentration of PS in added sample (4 pmol/tube); *A*=amount of PS added to the tube; mean±SD, *n*=5.

HPLC with fluorescence detection. The developed method using LC/ESI-MS does not require solvolysis and has proved to be satisfactory in its accuracy and precision. The derivatization with DBD-H not only increased the sensitivity but also stabilized the deuterated IS in LC/MS. That is, the derivatization protected the hydrogen-deuterium exchange reaction at the 17 and 21 positions of the steroids under ionization.[4,5] The quantitative limit of PS was 4 pmol/tube (1.6 ng/tube), which could be sensitive enough to determine the PS in 0.4 g of rat brain tissue, that is, the reported concentration of that steroid was *ca.* 20 ng/g tissue (*ca.* 50 pmol/g tissue). However, the levels of PS in the brains of the observed rats were much lower than that of the previous report, so the standard addition method was used for the quantitative determination to give the fol-

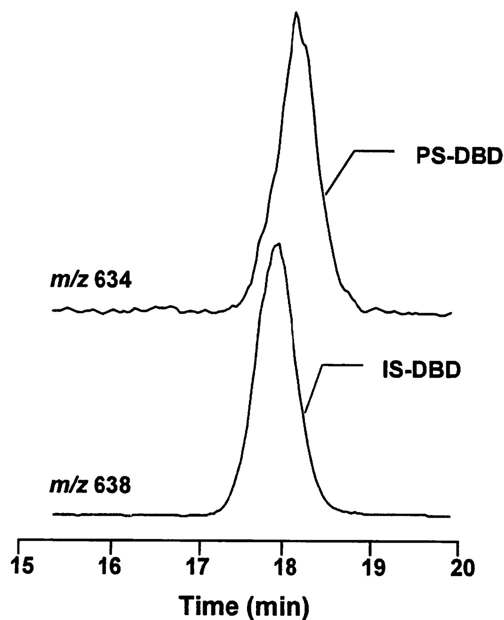

Fig. 4 LC/ESI-MS/MS chromatograms of PS- and IS-DBD PS (8 pmol) and IS (200 pmol) were added to rat brain (0.4 g tissue/tube). Column, Develosil ODS-HG-5; mobile phase, MeOH-4 mM $AcONH_4$ (2:1); flow rate, 0.1 ml/min; precursor ion, *m/z* 634 and 638; relative collision energy, 10%.

lowing data; *ca.* 0.53 ng/g tissue (*ca.* 1.33 pmol/g tissue). Although the reason of this discrepancy is not clear, these results suggest that a more sensitive method is necessary to determine PS in the part of the brain (< 50 mg tissue). The development of RIA for the analysis of PS or dehydroepiandrosterone 3-sulfate without deconjugation is now in progress in our laboratories and the details will be reported in the near future.

This work is supported by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

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(Received May 31, 1999)
(Accepted June 29, 1999)