Research Paper

LC/ESI-MS/MS analysis of progesterone-derived steroids produced in SH-SY5Y cells

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Abstract Many animal studies have demonstrated that progesterone (PROG) is reduced to various metabolites in the brain, and some of them have the regulatory effect of neuronal excitation. Although the SH-SY5Y cells have been used in various studies of neuroactive steroids as the human neuronal model cells, a complete understanding of the metabolism of PROG in these cells remains to be elucidated. The objective of this study was to identify the PROG-derived steroids produced in the SH-SY5Y cells by high-performance liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS).

The SH-SY5Y cells were cultured in serum-free medium supplemented with PROG for 24 h, then the steroids released into the culture supernatant were extracted. The steroids were derivatized with Girard reagent P (GP) to enhance their detectability and facilitate fragmentation in the positive ESI-MS/MS. By using two different mobile phases, allopregnanolone (AP), epiallopregnanolone (EAP), pregnenolone, 3*β*-dihydroprogesterone (3*β*-DHP), 20*α*-DHP and 20*β*-DHP released into the supernatant were separated and detected as the GP derivatives. The peak corresponding to either 5*α*-DHP or 5*β*-DHP was also found in the chromatograms. Although the derivatized 5*α*-DHP and 5*β*-DHP were not chromatographically separated, the C5-reduced form of PROG was identified as 5*α*-DHP based on the presence of only AP and EAP, which are the downstream metabolites of 5*α*-DHP, in the culture supernatant. Thus, this study revealed that the 5*α*-reduction followed by 3*α*-/3*β*-reduction, and independent 20*α*-/20*β*-reduction of PROG occurred in the SH-SY5Y cells to produce the various metabolites.

Key words: progesterone, SH-SY5Y cells, metabolite, Girard reagent P, LC/ESI-MS/MS

Introduction

Many animal studies have demonstrated that progesterone (PROG), which is commonly known as a luteal hormone, is reduced to various metabolites in the brain, some of which have neuroexcitatory modulatory effects[1−3]. Allopregnanolone (AP; 3*α*,5*α*-tetrahydroprogesterone), one of the metabolites of PROG, acts as a positive allosteric modulator of the *γ*-aminobutyric acid type A ($GABA_A$) receptor and strongly enhances the Cl^- influx effect of GABA[3]; thereby, AP exhibits anxiolytic, anticonvulsant, sedative, and analgesic effects[4]. On the other hand, epiallopregnanolone (EAP; 3*β*,5*α*-tetrahydroprogesterone), the stereoisomer of AP, has been reported to inhibit the action of AP on the $GABA_A$ receptor[5]. Steroids that act on the central nervous system (CNS), whether they are of endogenous or exogenous origin, are called neuroactive steroids and their association with psychiatric disorders, such as depression and anxiety disorders, has attracted attention[3,6].

The biosynthesis of PROG is initiated by the incorporation of cholesterol into mitochondria by the 18 kDa translocator protein (TSPO)[7]. Cholesterol is first converted into

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pregnenolone (PREG) by the action of the cholesterol side-chain cleavage enzyme (P450scc) in mitochondria, then PREG is converted into PROG by 3*β*-hydroxysteroid dehydrogenase (3*β*-HSD). PROG is reduced to 5*α*-dihydroprogesterone (5*α*-DHP) by 5*α*-reductase (5*α*-Red), then further reduced to AP by 3*α*-HSD or to EAP by 3*β*-HSD as necessary[2]. In addition, PROG is known to be metabolized to its 3*α*-/3*β*- and 20*α*-/20*β*-reduced forms, and accordingly, various metabolites are present in the human body[8–10] (Fig. 1).

The SH-SY5Y cells are a cell line that has been often used as an *in vitro* model of the human CNS[11]. This cell line was obtained by isolating and subcloning the original cell line called SK-N-SH from a medullary biopsy of a 4-year-old female patient with neuroblastoma[12]. There is a report suggesting the presence of 5*α*-Red and 3*α*-HSD in the SH-SY5Y cells[13], therefore, PROG would likely be converted to 5*α*-DHP and AP in the cells. Another study demonstrated that 3*β*-HSD is expressed in the SH-SY5Y cells[14], which suggests the production of EAP in the cells. However, a complete understanding of metabolism of PROG in the SH-SY5Y cells remains to be elucidated. Clarification of the overall picture of this metabolism could help to characterize this cell line, and eventually, to evaluate its utility as an *in vitro* model of the human CNS in the neuroactive steroid studies. Although other cell lines derived from rodents, such as the rat cerebellar granule cells[15] and rat brain glioma cells (C6 cells)[16,17], have been also used in the neuroactive steroid studies, these cells may fail to reflect the PROG metabolism in the human CNS due to the species difference.

The steroids examined in this study were AP, EAP, pregnanolone (P; 3*α*,5*β*-tetrahydroprogesterone), epipregnanolone (EP; 3*β*,5*β*-tetrahydroprogesterone), 3*α*-/3*β*-/5*α*-/5*β*-/20*α*-/20*β*-DHP and PREG (precursor of PROG) (Fig. 1). Due to the structural similarity of these steroids, a highly specific method is required for the analysis; high-performance liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) is considered suitable

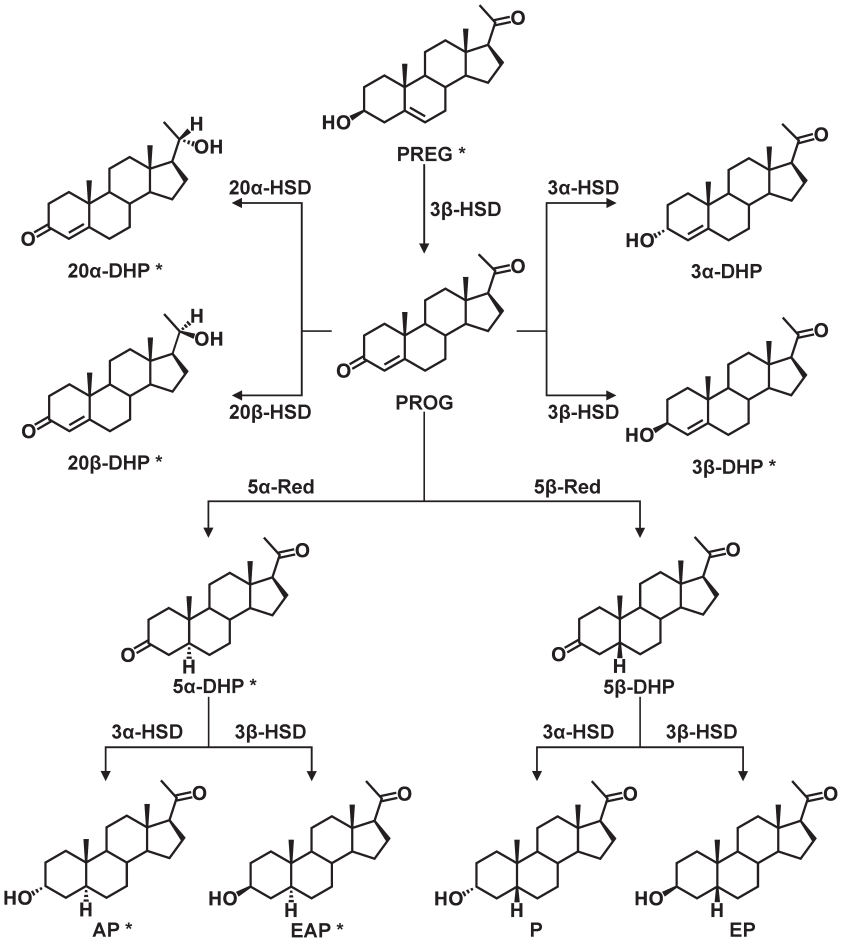

Fig. 1. Metabolic pathways of PROG in humans.
* represents the steroids detected in the culture supernatant of SH-SY5Y cells in this study.

due to the high separation power of LC and high discrimination capability of MS/MS. However, LC/ESI-MS/MS does not demonstrate the required sensitivity and identification capability for some steroids with a low ESI efficiency and poor fragmentation behavior in MS/MS. It well known that derivatization is an effective procedure to improve the detectability and fragmentation behavior of steroids during LC/ESI-MS/MS[18]. Among the various derivatization reagents, Girard reagent P (GP) has been widely used for the LC/ESI-MS/MS of ketosteroids[19].

Based on this background information, the objective of this study was to clarify the major metabolic pathway of PROG in the SH-SY5Y cells. To achieve this objective, we first developed a method for the separation and detection of eleven PROG-related steroids by LC/ESI-MS/MS combined with the GP derivatization. The steroids released in the culture supernatant of the SH-SY5Y cells were then identified using the developed method.

Experimental

Materials and chemicals

AP was purchased from Abcam (Cambridge, MA, USA). EAP, P, EP and 3*α*-/3*β*-/5*α*-/5*β*-/20*α*-/20*β*-DHP were from Steraloids (Newport, RI, USA). PREG, PROG and GP were obtained from Tokyo Chemical Industry (Tokyo, Japan). Stock solutions of 100 μg/mL for each of the steroids were prepared in ethanol, then diluted with ethanol to prepare the working solutions. Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F12) and penicillin-streptomycin solution (×100) were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). Fetal bovine serum (FBS) was from Capricorn Scientific (Ebsdorfergrund, Germany). DMEM/F12 supplemented with 50 U/mL of penicillin, 50 μg/mL of streptomycin and 10% heat-inactivated FBS was described as "complete DMEM/F12" in this study. "Serum-free DMEM/F12" was the complete DMEM/F12 without the heat-inactivated FBS. All other reagents and solvents were of the highest grade commercially available or LC/MS grade.

Cell culture

The SH-SY5Y cells obtained from the American Type Culture Collection (Manassas, VA, USA) were cultured in complete DMEM/F12 at 37°C under 5% CO_2 and saturated humidity. The cells were cultured until 80−90% confluence, then harvested with 0.025% trypsin-0.1 mM EDTA solution and plated in a culture dish at a density of 2×10^4 cells/cm^2.

Cell treatment

The cells were plated at a density of 5×10^4 cells/cm^2 in the complete DMEM/F12 in a petri dish and incubated for 24 h. After the medium was removed, the cells were gently washed with serum-free DMEM/F12, then incubated again in serum-free DMEM/F12 containing 100 nM PROG for 24 h. The culture supernatant was then collected and used for the subsequent experiments.

Extraction of steroids from cell culture supernatant

Ethyl acetate (1.5 mL) was mixed with 1.5 mL of the cell culture supernatant to extract the steroids, then 1.0 mL of the ethyl acetate layer was collected after centrifugation (1500×*g*, 20°C, 5 min). The solvent was evaporated under an N_2 gas stream.

Derivatization

GP (1 mg/mL in methanol; 20 μL) was added to the steroid standards or the culture supernatant extracts dissolved in methanol-acetic acid (99 : 1, v/v; 50 μL). The mixture was heated at 80°C for 15 min, then the solvent was evaporated under an N_2 stream. The residue was dissolved in the mobile phase (100 μL), a part of which was injected into the LC/ESI-MS/MS.

LC/ESI-MS/MS

LC/ESI-MS/MS was performed using a Waters Quattro Premier XE connected to a Waters LC-e2695 chromatograph (Milford, MA, USA). A YMC-Triart C18 column (5 μm, 100×2.0 mm i.d.; YMC, Kyoto, Japan) was used at the temperature of 40°C and the flow rate of 0.2 mL/min. The mobile phase consisted of methanol-10 mM ammonium formate (1 : 1, v/v, isocratic elution) or acetnitrile-10 mM ammonium formate (1 : 2, v/v, isocratic elution). The ESI-MS/MS conditions for the GP-derivatized steroids were as follows; capillary voltage: 3.00 kV, cone voltage: 50 V, CE: 30 eV (AP, EAP, P, EP, PREG, 3*α*-/3*β*-/5*α*-/5*β*-DHP) or 32 eV (20*α*-/20*β*-DHP), source temperature: 120°C, desolvation temperature: 400°C, desolvation gas (N_2) flow rate: 700 L/h and cone gas (N_2) flow rate: 50 L.

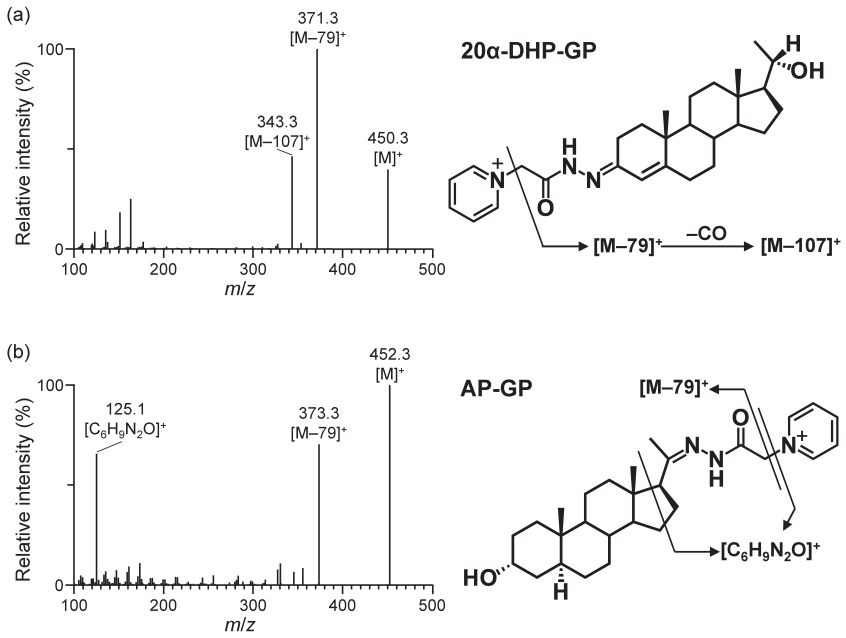

Fig. 2. ESI-MS/MS spectra and fragmentation processes of the GP-derivatized steroids.
(a) 20*α*-DHP and (b) AP.

Results

ESI-MS/MS behaviors of GP-derivatized steroids

GP has been widely used as the derivatization reagent for the LC/ESI-MS/MS of ketosteroids due to the presence of the permanently charged moiety that enhances the ESI-MS sensitivity of the resulting derivative as well as the high reactivity to a keto group[18,19]. Furthermore, the resulting ketosteroid derivatives with GP are known to provide characteristic product ions during MS/MS and their fragmentation behavior has been thoroughly analyzed[19]. Due to these advantageous features, GP was employed in this study. All the steroids examined in this study satisfactorily reacted with GP and the ensuing derivatives provided the intense molecular cations ($[M]^+$) in the ESI-MS operating in the positive-ion mode.

All the GP-derivatized steroids produced the intense product ions expressed as $[M-79]^+$ ($[M-C_5H_5N]^+$), which were formed by the neutral loss of pyridine from their $[M]^+$, during MS/MS (Fig. 2). Although the loss of CO from the $[M-79]^+$ also occurred in all the steroids, this fragmentation was more predominant in the steroids having a keto group only at the C3-position (3-ketosteroids), *i.e.*, 20*α*-/20*β*-DHP; accordingly, the product ions expressed as $[M-107]^+$ ($[M-C_5H_5N-CO]^+$) could be used for detecting these steroids (Fig. 2a). On the other hand, the derivatized 20-ketosteroids including AP, EAP, P, EP, 3*α*-/3*β*-DHP and PREG provided a specific product ion at *m*/*z* 125, which was assigned as $[C_6H_9N_2O]^+$; this ion was formed by the cleavage of the C13−17 and C15−16 bonds and elimination of the pyridine (Fig. 2b). These fragmentations were identical to those previously reported[19]. Although 5*α*-/5*β*-DHP are 3,20-diketosteroids, GP reacted only with their C20-keto groups under the stated derivatization conditions to yield the C20-monohydrazones. Therefore, the ESI-MS/MS behaviors of the derivatized 5*α*-/5*β*-DHP were similar to those of the derivatized 20-ketosteroids. Thus, when GP was used, two specific product ions were obtained from all the steroids, which was advantageous for the detection and identification of trace steroids in the cell samples.

Several studies have reported that the GP-derivatization provided a several-fold greater detectability for the ketosteroids[18,19]. In this study, the detectability before and after the derivatization was compared using AP as a model steroid; the GP-derivatized AP showed a 10-fold higher sensitivity compared to the intact AP. Thus, the GP-derivatization was beneficial not only for facilitating the specific fragmentation but also for enhancing the sensitivity for the PROG-derived steroids.

LC behaviors of GP-derivatized steroids

The derivatives of the ketosteroids with GP inevitably consisted of the *E*- and *Z*-isomers[18,19]. The derivatized 3-ketosteroids (20*α*-/20*β*-DHP) gave two peaks on the chromatogram due to their *E*- and *Z*-isomers, whereas the other

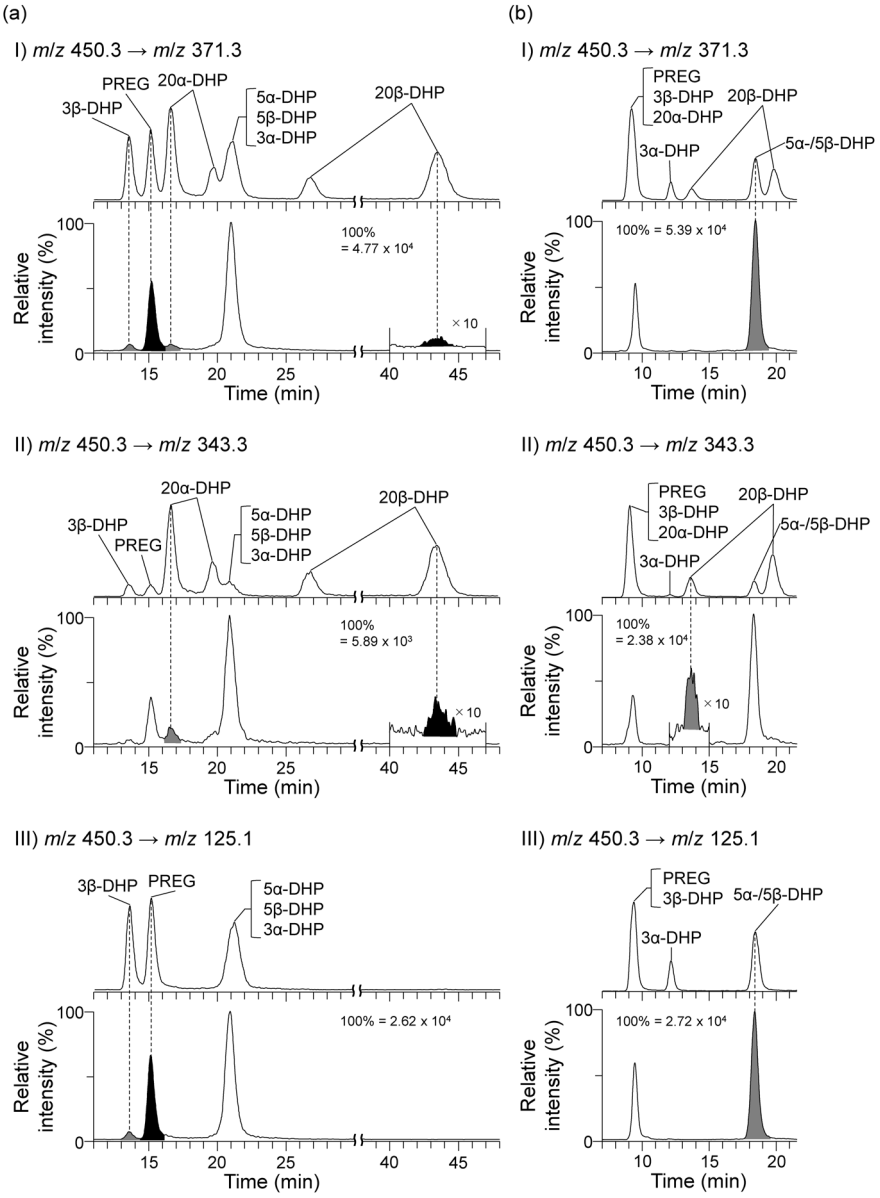

Fig. 3. SRM chromatograms of GP-derivatized dihydro-metabolites and PREG using (a) methanol- and (b) acetonitrile-based mobile phases.

Three different SRM transitions (I−III) suitable for the respective steroids were monitored. The upper and lower chromatograms were obtained from the authentic standards and cell culture supernatant samples, respectively.

derivatized steroids (AP, EAP, P, EP, PREG, 3*α*-/3*β*-/5*α*-/5*β*-DHP) gave single peaks because their *E*- and *Z*-isomers co-eluted. Thus, the use of GP somewhat complicated the chromatogram but its effects of enhancing the sensitivity and specificity outweighed this negative.

When the YMC-Triart C18 column was used with the mobile phase consisting of methanol-10 mM ammonium formate (1 : 1, v/v), the derivatized 3*β*-DHP, PREG, 20*α*-DHP (major isomer), 20*β*-DHP (major isomer), EAP and EP were separated from the other steroids (Figs. 3a and 4a, upper chromatograms). However, the derivatized 3*α*-DHP, 5*α*-DHP, 5*β*-DHP, AP and P co-eluted with the other steroids. The replacement of the mobile phase by acetnitrile-10 mM ammonium formate (1 : 2, v/v) gave a better result in separation of the derivatized 3*α*-DHP, AP and P from the other steroids (Figs. 3b and 4b, upper chromatograms). Based on these results, two LC conditions with different mobile phases were complementarily used to separate and detect the PROG-derived steroids in the cell culture supernatant samples. However, the separation of 5*α*-DHP and 5*β*-DHP from each other could not be achieved in spite of all our efforts. For comparison, even by the

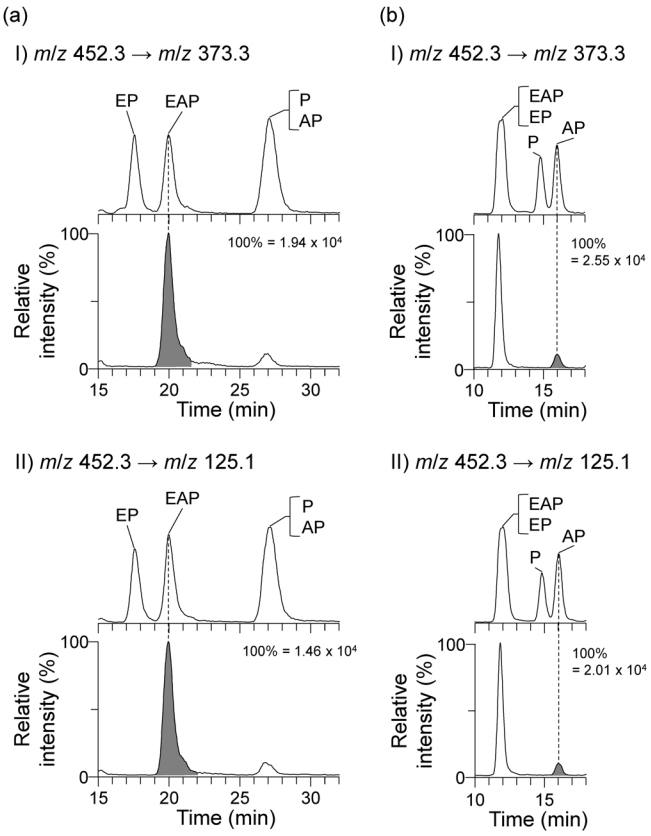

Fig. 4. SRM chromatograms of GP-derivatized tetrahydro-metabolites using (a) methanol- and (b) acetonitrile-based mobile phases.

Two different SRM transitions (I and II) were monitored. The upper and lower chromatograms were obtained from the authentic standards and cell culture supernatant samples, respectively.

mobile phase consisting of three solvents, *i.e.*, methanol, acetonitrile and 10 mM ammonium formate, a poor result was generated for the separation of 20α-DHP and PREG, and separation of 3α-DHP and 5α/β-DHP.

Performance of analytical system

To evaluate the robustness of the analytical system, triplicate measurements were performed for each steroid. The coefficients of variation (CVs) of the retention times (t_Rs) and peak areas did not exceed 0.6% and 5.6%, respectively, for all the derivatized steroids, indicating that the system enabled the reproducible analysis of the steroids (Table 1). Furthermore, the limits of detection (LODs, S/N = 3) of the derivatized steroids were 0.6−2.8 fmol, which demonstrated that our method had sufficient sensitivity to detect trace amounts of the steroids (Table 2).

Table 1. Robustness of analytical system

Steroids	CV (%) for t_R	CV (%) for peak area		
		$[M]^+ \rightarrow [M-79]^+$	$[M]^+ \rightarrow [C_6H_9N_2O]^+$	$[M]^+ \rightarrow [M-107]^+$
AP[a)]	0.6	3.5	3.4	—
EAP[b)]	0.0	2.0	2.7	—
P[a)]	0.3	0.7	0.3	—
EP[b)]	0.4	5.1	1.9	—
PREG[b)]	0.2	0.7	1.7	—
3α-DHP[a)]	0.5	0.4	2.4	—
3β-DHP[b)]	0.4	1.0	1.2	—
5α-DHP[a)]	0.4	2.2	3.0	—
5β-DHP[a)]	0.4	0.8	0.8	—
20α-DHP[b)]	0.4	3.6	—	3.3
20β-DHP[b)]	0.3	0.8	—	1.2

The steroids (1.0 ng each) were derivatized with GP, then one-tenth of which was injected into LC/ESI-MS/MS (triplicate measurements). *a*) Acetonitrile-10 mM ammonium formate (1 : 2, v/v) or *b*) methanol-10 mM ammonium formate (1 : 1, v/v) was used as the mobile phase.

Detection and identification of PROG-derived steroids in SH-SY5Y cell culture supernatant

Figs. 3a and 4a (lower chromatograms) show the SRM

chromatograms of the SH-SY5Y cell culture supernatants using the methanol-based mobile phase. Several peaks were observed when the $[M]^+ \rightarrow [M-79]^+$ transition was monitored (Figs. 3a-I and 4a-I, lower chromatograms). Some peaks were assigned as the derivatized 3*β*-DHP (t_R 13.5 min), PREG (t_R 15.1 min), 20*α*-DHP (t_R 16.6 min), 20*β*-DHP (t_R 43.5 min) and EAP (t_R 19.9 min) by co-chromatography with the authentic standards. When the $[M]^+ \rightarrow [M-107]^+$ transition was used for detection of 20*α*-DHP and 20*β*-DHP, the trace peaks corresponding to these steroids were also observed (Fig. 3a-II, lower chromatogram). By the transition of $[M]^+ \rightarrow [C_6H_9N_2O]^+$, the derivatized 3*β*-DHP, PREG and EAP were specifically detected (Figs. 3a-III and 4a-II, lower chromatogram). Although the methanol-based mobile phase could separate the derivatized EP (t_R 17.5 min, Figs. 4a-I and -II, upper chromatograms) from the other steroids, the peak corresponding to this steroid did not appear in the cell culture supernatant, which indicated the absence of EP in the supernatant (Figs. 4a-I and -II, lower chromatograms). We subsequently analyzed the cell samples using the acetonitrile-based mobile phase and observed the separated peak corresponding to AP (t_R 16.0 min) in the two SRM chromatograms ($[M]^+ \rightarrow [M-79]^+$ and $[M]^+ \rightarrow [C_6H_9N_2O]^+$) (Figs. 4b-I and -II, lower chromatograms). The analysis using the acetonitrile-based mobile phase revealed that P (t_R 14.8 min) and 3*α*-DHP (t_R 12.1 min) were not present in the cell culture supernatant (Figs. 3b-I, -III, 4b-I and -II).

Table 2. LODs of GP-derivatized steroids

Steroids	Methanol-based mobile phase	Acetonitrile-based mobile phase
AP	2.2 (0.7)	0.9 (0.3)
EAP	1.9 (0.6)	0.9 (0.3)
P	2.5 (0.8)	0.9 (0.3)
EP	2.2 (0.7)	0.9 (0.3)
PREG	1.3 (0.4)	0.6 (0.2)
3*α*-DHP	2.5 (0.8)	1.6 (0.5)
3*β*-DHP	2.2 (0.7)	0.9 (0.3)
5*α*-DHP	2.2 (0.7)	0.6 (0.2)
5*β*-DHP	2.8 (0.9)	1.3 (0.4)
20*α*-DHP	1.0 (0.3)	0.6 (0.2)
20*β*-DHP	1.6 (0.5)	0.9 (0.3)

Unit; fmol/injection. The values in parentheses are amounts (pg) converted into intact steroids.

As previously mentioned, the derivatized 5*α*-DHP and 5*β*-DHP could not be separated by our method. The peak was observed at 18.4 min in the cell culture supernatant sample when using the acetonitrile-based mobile phase (Figs. 3b-I and -III, lower chromatograms); these results suggested the occurrence of either 5*α*-DHP or 5*β*-DHP, or both of the steroids in the cell culture supernatant. Given the fact that AP and EAP (5*α*-reduced metabolites) were detected in the cell culture supernatant, whereas P and EP (5*β*-reduced metabolites) were not, it was natural that only 5*α*-DHP was produced in the SH-SY5Y cells as the C5-reduced form of PROG.

To more reliably identify the PROG-derived steroids found in the cell culture supernatant, the peak area ratios in the two SRM chromatograms were compared between the steroids in the cell culture supernatant samples and the authentic standards. The results are shown in Table 3; the peak area ratios of the seven steroids detected in the cell culture supernatant were almost identical to those of the standards. Thus, the analysis using the two specific SRM transitions revealed that AP, EAP, PREG, 3*β*-DHP, 5*α*-DHP, 20*α*-DHP and 20*β*-DHP were released from the SH-SY5Y cells. For comparison, none of the steroids including PROG were detected in the supernatant collected from the SH-SY5Y cells cultured without PROG.

Table 3. Peak area ratios in two SRM chromatograms

Steroid	Transition	Peak area ratio (mean ± SD, n = 3)	
		Culture supernatant	Standard
AP	$([M]^+ \rightarrow [C_6H_9N_2O]^+) / ([M]^+ \rightarrow [M-79]^+)$	0.749 ± 0.015	0.739 ± 0.003
EAP	$([M]^+ \rightarrow [C_6H_9N_2O]^+) / ([M]^+ \rightarrow [M-79]^+)$	0.763 ± 0.003	0.769 ± 0.006
PREG	$([M]^+ \rightarrow [C_6H_9N_2O]^+) / ([M]^+ \rightarrow [M-79]^+)$	0.664 ± 0.003	0.665 ± 0.008
3*β*-DHP	$([M]^+ \rightarrow [C_6H_9N_2O]^+) / ([M]^+ \rightarrow [M-79]^+)$	0.612 ± 0.015	0.623 ± 0.003
5*α*-DHP	$([M]^+ \rightarrow [C_6H_9N_2O]^+) / ([M]^+ \rightarrow [M-79]^+)$	0.589 ± 0.008	0.583 ± 0.009
20*α*-DHP	$([M]^+ \rightarrow [M-107]^+) / ([M]^+ \rightarrow [M-79]^+)$	0.395 ± 0.014	0.400 ± 0.010
20*β*-DHP	$([M]^+ \rightarrow [M-107]^+) / ([M]^+ \rightarrow [M-79]^+)$	0.453 ± 0.015	0.443 ± 0.006

Discussion

PROG and its metabolites exert various effects for neuronal and glial functions[1–3]. Abundant evidence also suggests that AP, one of the PROG-derived neuroactive steroids, is relevant to the antipsychotic drug action and pathophysiology of depression and anxiety disorder[3,20–22]. Furthermore, the AP preparation has been recently approved as a therapeutic agent for postpartum depression[23]. Thus, it is accepted that the PROG metabolites play important roles in neuronal regulation, but it is not fully understood what specific metabolites are produced in the SH-SY5Y cells, which have been used as an *in vitro* model of human CNS[11]. Based on this background, we attempted the identification of the PROG-derived steroids produced in the SH-SY5Y cells.

First, to achieve this objective, we developed an LC/ESI-MS/MS method for the separation and detection of the metabolites of PROG. To enhance the detectability and fragmentation of the steroids in ESI-MS/MS, the GP derivatization was employed in this study; the GP-derivatized steroids produced several characteristic product ions including $[M-79]^+$, $[M-107]^+$ and $[C_6H_9N_2O]^+$ with sufficient intensities. These remarkable advantages of the GP derivatization significantly helped to detect and identify the steroids in the culture supernatant of the SH-SY5Y cells. We unsuccessfully attempted to separate the eleven steroids of interest in a single LC run due to their very similar structures and physicochemical properties. However, the combined use of two mobile phases consisting of different organic modifiers provided satisfactory results for the steroid separation.

In this study, the SH-SY5Y cells were cultured in the PROG-added medium because no steroids were detected in the culture supernatant without the addition of PROG. We successfully identified AP, EAP, 3*β*-DHP, 20*α*-DHP and 20*β*-DHP as the PROG metabolites formed in the cells by the satisfactory separation from other steroids and discriminable detection using the double SRM transitions. Because 5*α*-DHP and 5*β*-DHP could not be chromatographically separated, the SRM chromatograms shown in Figs. 3b-I and -III indicated only that the peak eluted at 18.4 min corresponded to either 5*α*-DHP or 5*β*-DHP. However, we concluded that 5*α*-DHP was present in the cell culture supernatant based on the presence of its downstream metabolites (AP and EAP) and absence of their 5*β*-isomers (P and EP) in the supernatant. These results suggested that 5*α*-Red is expressed at sufficient levels in the SH-SY5Y cells and 5*β*-Red is significantly less or not active. It is a well-known fact that 5*α*-Red is expressed in the human CNS[2,24], whereas to the best of our knowledge, the 5*β*-Red activity is not apparent in the human CNS. Therefore, in the stereoselective reduction of *Δ*4-bond of PROG, the SH-SY5Y cells can be used as the *in vitro* model of the human CNS.

When discussing 3*α*-HSD and 3*β*-HSD, the latter activity was found to be higher in the SH-SY5Y cells because EAP (3*β*,5*α*-reduced form) was more abundantly produced than AP (3*α*,5*α*-reduced form), and 3*β*-DHP was detected but 3*α*-DHP was not. There is a report suggesting the presence of 3*α*-HSD in the SH-SY5Y cells[13]. Moreover, 3*β*-HSD has been reported to be expressed in the human glioblastoma cells (U87 cells)[25] as well as in the SH-SY5Y cells[14]. To the best of our knowledge, there is no study that clearly demonstrates whether 3*α*-HSD or 3*β*-HSD is dominantly expressed in the human CNS. Cruz *et al*. revealed that the AP concentrations were similar to the EAP concentrations in the orbital frontal cortex of subjects with post-traumatic stress disorder and controls[26].

This study revealed that the SH-SY5Y cells also have the 20*α*- and 20*β*-HSD activities because 20*α*- and 20*β*-DHP were detected in the cell culture supernatant. In humans, aldo-keto reductase 1C1 (AKR1C1) works as the 20*α*-HSD for PROG and AP, and it is thought that this enzyme decreases the AP concentrations in the brain by inactivating AP and eliminating its precursor, PROG, from the synthetic pathways[27]. However, based on the amounts of 20*α*- and 20*β*-DHP found in this study, the 20*α*- and 20*β*-HSD activities were not considered to be significant in the SH-SY5Y cells. To summarize these results, the major metabolic pathway of PROG in the SH-SY5Y cells was the 5*α*-reduction of the *Δ*4-bond followed by the 3*β*-reduction of the C3-keto group; 5*α*-DHP and EAP were the most and second abundant metabolites produced in the SH-SY5Y cells, respectively.

Although a validation for the quantification of the PROG-derived steroids in the cell culture supernatant was not performed, their approximate concentrations (mean, $n = 3$) were determined by comparison with known amounts of the authentic standards; 80 pg/mL for AP, 630 pg/mL for EAP, 490 pg/mL for PREG, 50 pg/mL for 3*β*-DHP, 1660 pg/mL for 5*α*-DHP, 20 pg/mL for 20*α*-DHP and 10 pg/mL for 20*β*-DHP. As the steroids produced in the SH-SY5Y cells were diluted with the cell culture medium,

it was difficult to speculate the steroid concentrations in the localized areas of the target tissues based on the measured concentrations in the cell culture supernatant. Further research is needed to determine whether the amounts of steroids produced in the SH-SY5Y cells are sufficient to have activity in the target tissues.

It is noted that a considerable amount PREG was detected in the culture supernatant of the SH-SY5Y cells although it is not metabolically formed from PROG. We interpreted this phenomenon as follows. The SH-SY5Y cells constantly produce PREG from cholesterol and also PROG from PREG, but the amounts of PREG and PROG produced are usually very small. Therefore, none of the steroids were detected in the culture supernatant of the SH-SY5Y cells without the addition of PROG by our method. When a significant amount of PROG is added to the cells, the cells suspend the use of PREG to produce PROG, probably due to the negative feedback mechanism, and the surplus PREG may be extracellularly released.

Most previous studies on the PROG metabolism in the cultured neuronal cells exclusively focused on AP[15–17]. Moreover, these studies sometimes undiscriminatingly analyze the stereoisomers, such as AP and EAP, which could lead to an erroneous result. Additionally, many previous studies monitored only the mRNA and protein expressions of the PROG metabolizing enzymes in the cultured cells and did not show what steroids were actually produced in the cells[2,14,25]. As contrasted with these studies, the present study comprehensively analyzed the PROG metabolites produced in the SH-SY5Y cells with the discrimination between the regioisomers and stereoisomers, especially for dihydro-metabolites (3*α*-/3*β*-/5*α*-/5*β*-/20*α*-/20*β*-DHP) and tetrahydro-metabolites (AP, EAP, P and EP), which resulted in the reliable identification of the six PROG metabolites (AP, EAP and 3*β*-/5*α*-/20*α*-/20*β*-DHP) and PREG. Surprisingly, to the best of our knowledge, the present study is the first reported instance showing that AP, a potent neuroactive steroid, is really formed in the SH-SY5Y cells.

The SH-SY5Y cell-based LC/ESI-MS/MS assay system presented here will be applicable to the analysis of the changes in the PROG-derived neuroactive steroid levels caused by various stimuli, such as the drug treatment. This will be useful for elucidating the mechanism of the neuroactive steroid-related drug actions. For example, finasteride is a 5*α*-Red inhibitor used to treat and relieve benign prostatic hyperplasia and prostate cancer, while depression-like symptoms have been reported as adverse reactions, which are possibly due to the decreased AP concentration in the brain[28,29]. Our assay system might be helpful to demonstrate the effect of finasteride on the AP production in the CNS. Furthermore, some drugs increase the brain AP concentration (*de novo* AP biosynthesis in the brain) and exert subsequent activation of $GABA_A$ receptors to have an anxiolytic effect[3,20–22]. Our assay system will enable the screening of compounds with the anxiolytic effect *via* the AP biosynthesis.

Conclusion

We developed a method for the separation and identification of the PROG-derived steroids produced in the SH-SY5Y cells by LC/ESI-MS/MS using two mobile phases. Using this method, AP, EAP, 3*β*-DHP, 5*α*-DHP, 20*α*-DHP, 20*β*-DHP and PREG were detected in the cell culture supernatant. The findings of this study can be a help not only for evaluating the SH-SY5Y cells as the *in vitro* model of the human CNS, but also for understanding the metabolism of PROG, *i.e*., the biosynthesis of neuroactive steroids, in the human CNS. Furthermore, the developed method is also expected to be a new tool for assessing the mechanisms of drug action *via* promotion/inhibition of the biosynthesis/metabolism of neuroactive steroids in the CNS.

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Conflicts of Interest

The authors declare no conflicts of interest.

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