Studies on Neurosteroids V: Separation and Characterization of Pregnenolone 3-Stearate in Rat Brains Using High-Performance Liquid Chromatography

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Abstract

The separation and characterization of pregnenolone 3-stearate in rat brains are carried out using high-performance liquid chromatography (HPLC). The pregnenolone 3-stearate is obtained from a whole rat brain by extraction with ethyl acetate followed by silica gel column chromatography. The obtained fraction is derivatized with 1-dimethylaminonaphthalene-5-sulfonylhydrazine (dansylhydrazine) or 4-(*N,N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole, and the derivative is separated by successive preparative HPLC with fluorescence detection. The chromatographic behaviors of both derivatives are identical to those of authentic samples. The former derivative obtained from the rat brain shows satisfactory mass spectral data, and after hydrolysis, dansylpregnenolone is confirmed by HPLC.

Introduction

In a previous paper (1), we clarified the existence of dehydroepiandrosterone and pregnenolone, called neurosteroids, in rat brains by using high-performance liquid chromatography (HPLC) with fluorescence detection. The neurosteroids that exist in rat brains are reported as unconjugated compounds, sulfates, lipoidal esters, and sulpholipids (2). Although the existence of a steroid fatty acid ester has been suggested, its structure still remains poorly understood (3). The precise identification of these steroids is a prerequisite to the establishment of a determination method. This paper deals with the separation and characterization of pregnenolone 3-stearate in rat brains using fluorometric derivatization followed by HPLC and mass spectrometry (MS) (Figure 1).

Experimental

Materials

Oxosteroids were supplied by Teikoku Hormone Mfg. (Tokyo, Japan). Stearic acid, 1-dimethylaminonaphthalene-5-sulfonylhydrazine (dansylhydrazine), and 4-(*N,N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBDH) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Column chromatography was carried out with silica gel 60 (70–230 mesh) (Merck, Damstadt, Germany). Thin-layer chromatography (TLC) and preparative TLC were conducted with 0.25-mm precoated silica gel 60 F_{254} (Merck). All chemicals used were of analytical-reagent grade. Solvents were purified by distillation prior to use.

Apparatus

The proton nuclear magnetic resonance spectrum (^{1}H-NMR) in $CDCl_3$ was recorded using a JEOL JNM-EX 270 (270 MHz) spectrometer (JEOL, Tokyo, Japan) with tetramethylsilane as the internal standard. The following abbreviations were used: s for singlet, d for doublet, t for triplet, m for multiplet, and br for broad. Fast atom bombardment (FAB) and electron impact (EI) MS spectra were recorded with a JEOL JMS-SX102A spectrometer. Electrospray ionization (ESI) MS was recorded using a Jasco Auto Spec EQ (Tokyo, Japan) spectrometer. HPLC was carried out using a Jasco TRI ROTAR chromatograph equipped with a Hitachi F-1050

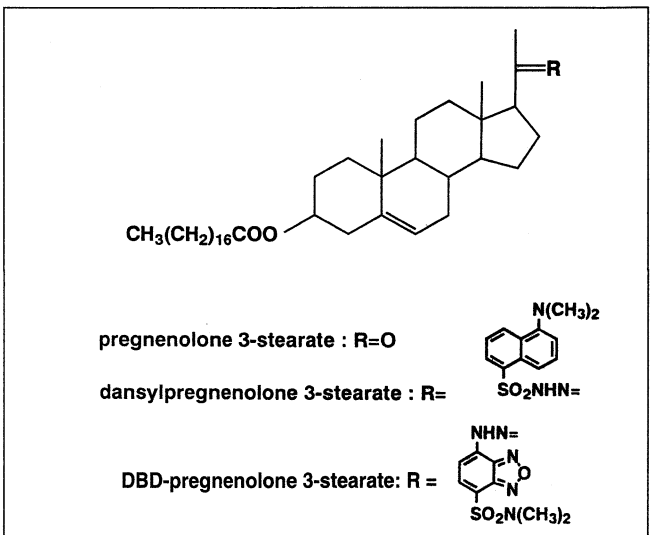

Figure 1. Structures of pregnenolone 3-stearate and its derivatives.

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fluorescence detector (FL) (dansylhydrazine derivative: excitation wavelength, 340 nm; emission wavelength, 525 nm; DBDH derivative: excitation wavelength, 450 nm; emission wavelength, 550 nm)(Hitachi, Tokyo, Japan). Reversed-phase (RP) (YMC-GEL C_8, 15 cm × 0.46-cm i.d., 5-µm film thickness) (YMC, Kyoto, Japan) and normal-phase (NP) (WAKOSIL 5SIL, 15 cm × 0.4-cm i.d., 5-µm film thickness) (Wako Osaka, Japan) columns were used for the identification of derivatized pregnenolone 3-stearate under ambient conditions at a flow rate of 1 mL/min. An RP column, TSKgel ODS-80 (15 cm × 0.46-cm i.d., 5-µm film thickness) (TOSOH, Tokyo, Japan), was used for the identification of dansylpregnenolone under the previously described conditions.

Preparation of authentic pregnenolone 3-stearate

Pregnenolone (170 mg) dissolved in pyridine (2 mL) was mixed with stearoyl chloride (310 mg), and the solution was stirred at room temperature for 30 min. After the addition of ice water to decompose the excess reagent, the reaction mixture was extracted with ether–CH_2Cl_2 (100:1) that was washed with 5% NaOH, 5% HCl, and water and dried with Na_2SO_4. After evaporation of the solvent, the residue was recrystallized from ether to give the desired substances as colorless leaflets (100 mg). The melting point was 92–93°C. The ^{1}H-NMR spectrum was as follows. δ: 0.63 (3H, s, H-18), 0.88 [3H, t, J = 13.2, Hz, $CH_3(CH_2)_{16}$–], 1.02 (3H, s, H-19), 1.25 [30H, br s, $CH_3(CH_2)_{15}$–], 2.12 (3H, s, H-21), 4.62 (1H, m, H-3 α), 5.37 (1H, br d, J = 4.6, Hz, H-6).

Preparation of authentic dansylpregnenolone 3-stearate

Pregnenolone 3-stearate (< 1 mg) dissolved in CH_2Cl_2 (0.05 mL) was added to the solution of dansylhydrazine (< 1 mg) and HCl (one drop) in EtOH (0.5 mL). The whole solution was then kept at room temperature overnight. The reaction mixture was extracted with ether and successively washed with 5% $NaHCO_3$, water, and brine. The organic layer was dried with Na_2SO_4. After evaporation of the solvent, the residue was applied to preparative TLC using hexane–ethyl acetate (40:1, developed three times) as the developing solvent. The zone corresponding to a retardation factor (R_f) of 0.49 was extracted with ether, and the evaporation of the solvent then gave the desired substances as pale yellow amorphous substances. FAB–MS and ESI–MS of the compound showed satisfactory data (*m/z*: 830 $[M + H]^+$).

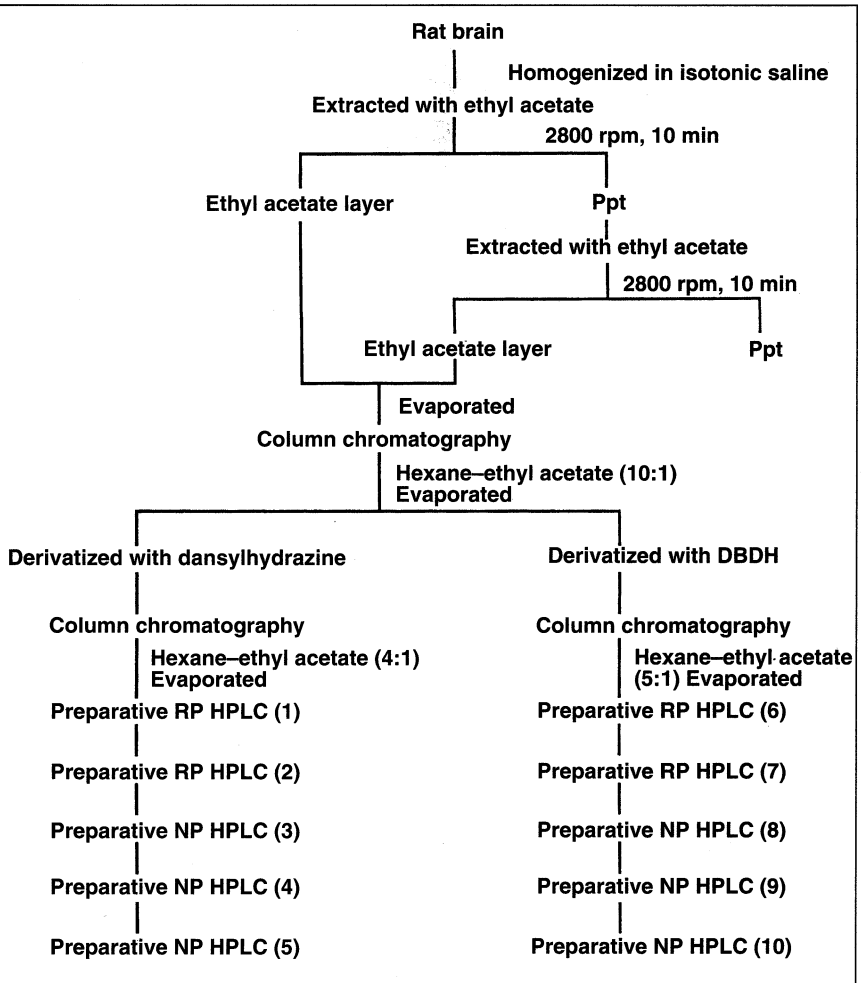

Figure 2. Separation of pregnenolone 3-stearate in the rat brain. Mobile phase and retention time (t_R): (1) MeOH–H_2O (98:2; t_R, 9.1 min), (2) MeCN (t_R, 11 9 min), (3) hexane–ethyl acetate (6:1; t_R, 7.4 min), (4) hexane–acetone (10:1, t_R, 8.0 min), (5) hexane–isopropanol (99:1; t_R, 13.3 min), (6) MeOH–H_2O (97:3; t_R, 12.0 min), (7) MeCN (t_R, 11.9 min), (8) hexane–ethyl acetate (7:1, t_R 10.9 min), (9) hexane–isopropanol (99:1; t_R, 7.3 min), (10) hexane–acetone (10:1; t_R, 7.5 min).

Preparation of authentic DBD-pregnenolone 3-stearate

Pregnenolone 3-stearate (< 1 mg) dissolved in ether (0.05 mL) and EtOH (0.1 mL) was added to the solution of DBDH (< 1 mg) and CF_3COOH (one drop) in EtOH (0.2 mL). The whole solution was then kept at room temperature for 6 h. The reaction mixture was extracted with ethyl acetate and successively washed with brine, 5% $NaHCO_3$, 5% HCl, water, and brine. The organic layer was dried with Na_2SO_4. After evaporation of the solvent, the residue was applied to preparative TLC using hexane–ethyl acetate (5:1, developed twice) as the developing solvent. The zone corresponding to a retardation factor of 0.27 was extracted with ether, and evaporation of the solvent then gave the desired substances as pale yellow amorphous substances. FAB–MS of the compound showed satisfactory data (*m/z*: 822 $[M + H]^+$).

Preparation of authentic dansylpregnenolone

Pregnenolone (< 1 mg) was treated with dansylhydrazine as previously described. The residue obtained was applied to preparative TLC by using hexane–ethyl acetate (2:1) as the developing solvent. The zone corresponding to a retardation factor of 0.40 was extracted with ethyl acetate. Evaporation of the solvent then gave the desired substances as pale yellow amorphous substances. EI–MS of the compound showed satisfactory data (*m/z*: 563 $[M]^+$).

Procedure for separation and characterization of pregnenolone 3-stearate in rat brains

An adult male rat of the Wistar strain (7–8 weeks old, 150–200 g) was decapitated, and the entire brain (2.05 g) was homogenized in 0.9% NaCl (9 mL) and extracted with ethyl acetate (7 mL) for 10 min while being cooled with ice. The mixture was centrifuged (2800 rpm for 10 min), the precipitate was again extracted with ethyl acetate, and the mixture was again centrifuged as described. Both supernatants were combined and evaporated in vacuo to give the colorless amorphous substances. The substances dissolved in ethyl acetate (one drop) and hexane (0.2 mL) were analyzed through silica gel column chromatography (3.5 × 0.5-cm i.d.). After elution with hexane (4 mL), the desired compound was eluted with hexane–ethyl acetate (10:1, 5 mL), which was evaporated in vacuo. Half of the obtained residue dissolved in EtOH (0.2 mL) was added to the solution of dansylhydrazine (0.2 mg) in EtOH (0.2 mL) and 0.1% CF_3COOH in EtOH (0.2 mL). The reaction mixture was vortex-mixed and kept at room temperature overnight. After evaporation of the solvent under an N_2 gas stream, the residue that was dissolved in ethyl acetate (one drop) and hexane (0.2 mL) was applied to silica gel column chromatography (3.5 × 0.5-cm-i.d.). After elution with hexane (4 mL), the desired substances were eluted with hexane–ethyl acetate (4:1, 5 mL), which was evaporated in vacuo. The residue obtained was applied to successive preparative HPLC as shown in Figure 2. The identity of the compound was confirmed through simultaneous chromatography with an authentic sample. ESI–MS of the purified compound showed satisfactory data (*m/z* 830 $[M + H]^+$).

Half of the above residue from the first silica gel column chromatography was dissolved in EtOH (0.2 mL), to which the solution of DBDH (0.2 mg) in EtOH (0.2 mL) and 0.1% CF_3COOH in EtOH (0.2 mL) were added. The whole solution was vortex-mixed and kept at room temperature overnight. After evaporation of the solvent under an N_2 gas stream, the residue that was dissolved in ethyl acetate (one drop) and hexane (0.2 mL) was analyzed by silica gel column chromatography (3.5 × 0.5-cm i.d.). After elution with hexane (4 mL), the desired substances were eluted with hexane–ethyl acetate (6:1, 5:1, 5 mL each), which was evaporated in vacuo. The residue obtained was applied to successive preparative HPLC as shown in Figure 2. The identity of the compound was confirmed through simultaneous chromatography with an authentic sample.

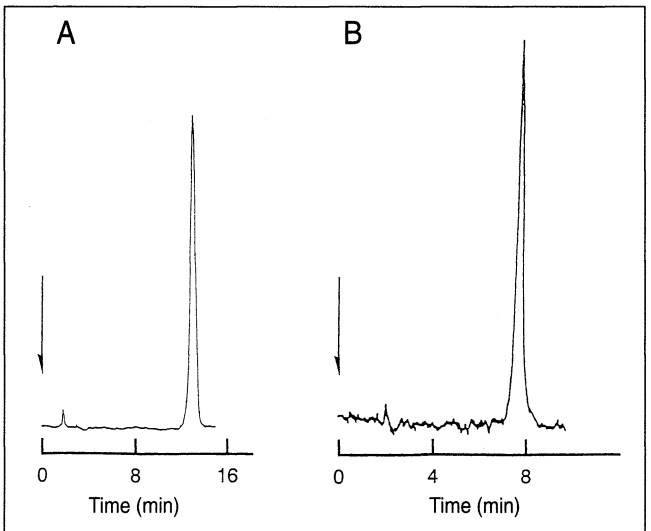

Figure 3. Chromatograms of derivatized pregnenolone 3-stearate from the rat brain: (A) Dansyl derivative, (B) DBD derivative. Column: WAKOSIL 5SIL. Mobile phase: (A) hexane–isopropanol (99:1), (B) hexane–acetone (10:1).

Hydrolysis of dansylpregnenolone 3-stearate

The dansyl derivative obtained from the rat brain was dissolved in 5% KOH–MeOH (0.5 mL) and kept at room temperature for 1 h. The reaction mixture was extracted with ethyl acetate, which was washed with water and evaporated in vacuo. The obtained residue was applied to HPLC (TSKgel ODS 80 column, MeOH–H_2O mobile phase [7:2], 17.5-min retention time), and the fraction corresponding to dansylpregnenolone was collected and evaporated in vacuo. The residue was further applied to HPLC (TSKgel ODS 80 column, MeCN–H_2O mobile phase [3:1], 10.9-min retention time), which showed the corresponding peak in its chromatogram, and the identity of the compound was confirmed by simultaneous chromatography with an authentic sample.

Results and Discussion

Neurosteroids are not very responsive to commonly used HPLC detectors. However, fluorometric derivatization shows promise as a reliable detection method. Dansylhydrazine and DBDH have been chosen as the fluorogenic labeling reagents for the detection of pregnenolone 3-stearate having a 20-oxo group in rat brains.

The entire brain of an adult male Wistar strain rat was treated according to the reported procedures (3). The ethyl acetate extract was purified by silica gel column chromatography followed by derivatization using dansylhydrazine or DBDH. Both derivatization mixtures were purified by silica gel column chromatography, and the desired fraction was applied to preparative RP HPLC (step 1 or 6 in Figure 2) using MeOH as an organic modifier; the corresponding fractions were collected and evaporated in vacuo. The chromatogram of the fraction containing dansyl- or DBD-pregnenolone 3-stearate still showed several peaks, so the fraction was further applied to preparative RP HPLC (step 2 or 7 in Figure 2) using MeCN as an organic modifier. The corresponding peak was further applied to three successive preparative NP HPLC runs as shown in Figure 2 (steps 3–5, and 8–10) to give the derivatized pregnenolone 3-stearate as a single peak in the HPLC chromatogram, which was confirmed through simultaneous chromatography with an authentic sample. Typical chromatograms of the dansyl and DBD derivatives are shown in Figures 3A and 3B, respectively. The dansyl derivative obtained from the rat brain showed satisfactory ESI–MS data and elicited dansylpregnenolone after hydrolysis, which was confirmed by RP HPLC using two other solvent systems. The method using dansylhydrazine was applied to nine other rat brains and gave the same results. These data clarified the existence of pregnenolone 3-stearate in the rat brains. To our knowledge, this is the first reported instance in

which the compound was detected by HPLC with fluorescence detection.

The development of the quantitative determination of pregnenolone 3-stearate in rat brains is now under investigation in our laboratory, the details of which will be reported in the future.

Acknowledgments

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