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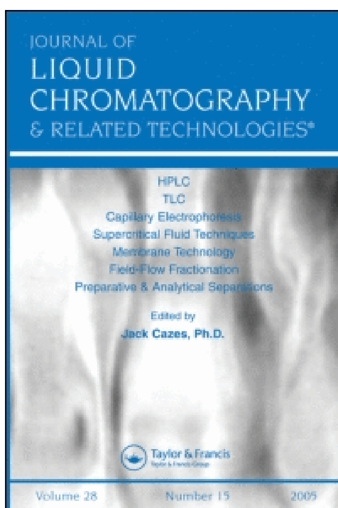

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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Studies on Neurosteroids. IV. Quantitative Determination of Pregnenolone in Rat Brains Using High-Performance Liquid Chromatography

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To cite this Article Shimada, Kazutake and Nakagi, Toshiro(1996) 'Studies on Neurosteroids. IV. Quantitative Determination of Pregnenolone in Rat Brains Using High-Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 19: 16, 2593 — 2602
To link to this Article: DOI: 10.1080/10826079608014040
URL: http://dx.doi.org/10.1080/10826079608014040

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J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2593–2602 (1996)

STUDIES ON NEUROSTEROIDS. IV. QUANTITATIVE DETERMINATION OF PREGNENOLONE IN RAT BRAINS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The quantitative determination of pregnenolone in rat brain was done using reverse phase high performance liquid chromatography with fluorescence detection and the internal standard method. The desired fraction was obtained from a rat brain with the combined use of a Bond Elut C8 cartridge for the solid-phase extraction and a lipophilic gel (piperidino-hydroxypropyl Sephadex LH-20) for the ion-exchange chromatography. The fraction was derivatized with 1-anthroyl cyanide followed by purification with 2 successive silica gel columns to remove the excess or decomposed reagent. Separation of the compounds was performed on a J'sphere ODS-L80 column and the calibration graph was linear from 10 to 60 ng/tube (*ca.* 1 g tissue of brain). The method was applied to the determination of pregnenolone in rat brains of Wistar and Sprague-Dawley strains, most of which showed much lower amounts than that previously reported.

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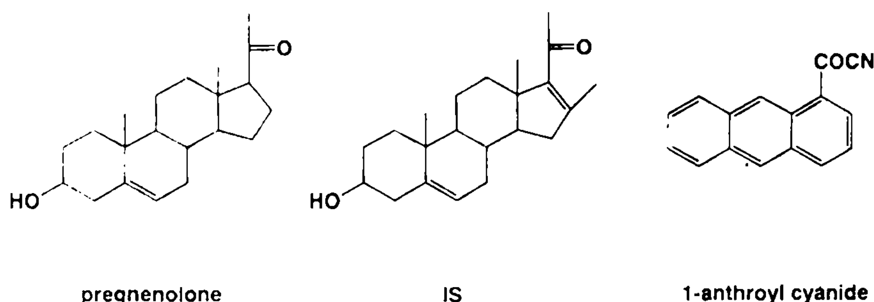

Figure 1. Structures of pregnenolone, IS, and 1-anthroyl cyanide

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INTRODUCTION

Since the discovery of dehydroepiandrosterone in rat brains, several 17- and 20-oxosteroids, called "neurosteroids", have been elucidated in mammalian brains,[1] in which pregnenolone is one of the main steroids. Significant interest has thus been focused on their biological properties in this organ.[1] The determination of neurosteroids [65±15 ng/g tissue of rat brain; mean ± standard deviation (S.D.)] has usually been done by gas chromatography-mass spectrometry or radioimmunoassay;[1,2] however, these methods have some problems regarding their simplicity and versatility. High performance liquid chromatography (HPLC) shows promise as a convenient determination method, but highly sensitive derivatization is necessary because neurosteroids are not very responsive to the commonly used detectors.

In a previous paper of this series, we clarified the existence of dehydroepiandrosterone and pregnenolone in rat brains by HPLC using fluorescence (FL) detection.[3] This paper deals with the quantitative determination of pregnenolone in rat brains using HPLC with FL detection and the internal standard (IS) method (Fig. 1).

EXPERIMENTAL

Materials

Oxosteroids were kindly supplied by Teikoku Hormone Mfg. (Tokyo, Japan). 3β-Hydroxy-16-methylpregna-5,16-dien-20-one was prepared in this laboratory and used as an IS.[4] 1-Anthroyl cyanide was purchased from Wako Pure Chemical Ind. (Osaka, Japan).

Bond Elut-C18 and -C8 cartridges, 500 mg (Varian Sample Preparation Products, Harbor City, CA, U.S.A.) were successively washed with hexane, $CHCl_3$, MeOH, and H_2O prior to use. Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) was prepared using the method reported by Goto et al.[5]

Apparatus

HPLC was carried out using a JASCO TRI ROTAR chromatograph equipped with a Hitachi F-1000 FL detector (λ_{ex} 370 nm, λ_{em} 470 nm)(Hitachi, Tokyo). Reverse phase column (J'sphere ODS-L80, 4 μm, 15 cm x 0.46 cm i.d.) (YMC, Kyoto, Japan) was used under ambient conditions at a flow rate of 1 mL/min.

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Procedure for Determination of Pregnenolone in a Rat Brain

Adult male Wistar and Sprague-Dawley rats (7-8 weeks old: 190-215 g) were kept for 2 days at 25 °C under a 12hr/12hr lighting schedule (8:00-20:00). The rat was decapitated and half (*ca.* 1 g tissue) of the entire brain was homogenized in isotonic saline (2 mL) under ice-cooling. MeOH (2 mL) and IS (50 ng) in EtOH (50 μL) were added to the homogenate and centrifuged at 2,800 rpm for 15 min. The precipitate was again suspended in MeOH (2 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 (45 mL) and the whole solution was applied to a Bond Elut C8 cartridge, washed with H_2O (6 mL) and eluted with 90% EtOH (5 mL), which was applied to a PHP-LH-20 column (0.6 cm i.d.). The eluate with 90% EtOH was concentrated *in vacuo* to a volume of 1 mL, which was diluted with H_2O (4 mL) and applied to a Bond Elut C18 cartridge. After washing with H_2O (6 mL), the desired compounds were eluted with 80% MeOH (5 mL) which was then evaporated and dried *in vacuo*. The residue was next used for the derivatization reaction. 1-Anthroyl cyanide (500 μg) in MeCN (0.1 mL) containing 0.24% quinuclidine in MeCN (0.05 mL) was added to the residue and kept at 60 °C for 30 min, 2 drops of MeOH was added to decompose the excess reagents and then evaporated under an N_2 gas stream. The residue from the derivatization mixture was applied to 2 successive silica gel columns (each 3 x 0.6 cm i.d.) as described below. After washing with hexane (3 mL) and hexane-AcOEt (20:1, 6 mL), the eluate with hexane-AcOEt (20:1, 20 mL) was evaporated in vacuo and the residue was applied to the next silica gel column.

After washing with hexane (3 mL) and hexane-acetone (60:1, 9 mL), the desired compounds were eluted with hexane-acetone (60:1, 13 mL). After evaporation of the solvent, the residue was dissolved in EtOH and an aliquot of which was applied to HPLC using MeCN-H_2O (15:2) as the mobile phase.

Absolute Recovery Rate Before Derivatization Reaction

A solution of pregnenolone (30 ng) in EtOH (300 μL) was added to the rat brain (*ca.* 1 g tissue) homogenate, deproteinized, treated with Bond Elut C8 cartridge, PHP-LH-20 column and Bond Elut C18 cartridge as described above. A solution of IS (50 ng) in EtOH (50 μL) was addded to the residue and evaporated *in vacuo*. The residue was derivatized with 1-anthroyl cyanide as described above and the recovery rate was calculated using the peak height ratio method.

The above described solutions of pregnenolone (30 ng) and IS (50 ng) were mixed, evaporated, derivatized with 1-anthroyl cyanide and treated with 2 successive silica gel columns followed by HPLC analysis as previously described and the peak height ratio of which was taken as 100%.

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Calibration Graph

In the standard addition method, solutions of pregnenolone (10, 20, and 30 ng) in EtOH (100, 200, and 300 μL) and IS (50 ng) in EtOH (50 μL) were added to the rat brain (*ca.* 1 g tissue) homogenate, and each of the resulting solutions was assayed using the proposed method. In the IS method, solutions of pregnenolone (10, 20, 40, and 60 ng) in EtOH (100, 200, 400, and 600 μL) and IS (50 ng) in EtOH (50 μL) were mixed and each of the resulting solutions was applied to a Bond Elut C8 cartridge using the proposed method.

Method Validation

The solutions of pregnenolone (10 and 30 ng) in EtOH (100 and 300 μL) and IS (50 ng) in EtOH (50 μL) were added to the rat brain (*ca.* 1 g tissue) homogenate, and each of the resulting solutions was assayed using the proposed method.

RESULTS AND DISCUSSION

Cleanup of Pregnenolone in Rat Brain

Neurosteroids are not very responsive to the commonly used HPLC detectors; however, fluorometric derivatization shows promise as a reliable determination method. In a previous paper, we used 4-(*N,N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBDH) and

1-anthroyl cyanide as fluorogenic labeling reagents for the separation and characterization of pregnenolone and dehydroepiandrosterone in rat brains.[3] In the preliminary experiment, 1-anthroyl cyanide is superior to DBDH in the derivatization procedure, because the latter reagent produced many decomposed compounds. These data prompted us to use 1-anthroyl cyanide as the derivatization reagent.

The entire brains of adult male Wistar rats were homogenized in isotonic saline and deproteinized with methanol. The supernatant was applied to solid-phase extraction, ion-exchange chromatography, solid-phase extraction and then derivatization with 1-anthroyl cyanide (Fig. 2). The reaction mixture was purified with two successive silica gel columns, applied to HPLC and a typical chromatogram is shown in Fig. 3.

No interference from the endogenous substances in the rat brain was detected at the retention time of IS. To confirm the purity of the peak, the eluate from the corresponding peak was collected, reapplied to HPLC using MeOH-H_2O (9:1) as the mobile phase and showed a single peak, in which any potential interference with the other oxosteroids was clearly minimized as shown in Fig. 4.

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Absolute Recovery Rate and Calibration Graph

The absolute recovery rate before the derivatization reaction at 30 ng/tube was examined according to the cleanup procedure. Reasonable net recovery rates (65.0 ± 6.1%, n=5) were obtained throughout the pretreatment (four steps). The calibration graph constructed using the standard addition (added 10-30 ng) and peak height ratio methods showed good linearity (y=0.040x+0.66, r=0.998). Next the usual IS method was examined as follows. The solutions of pregnenolone at four levels (10 - 60 ng) and IS were mixed and each of the resulting solutions was applied to a Bond Elut C8 cartridge followed by the proposed method. The constructed calibration graph also showed good linearity (y=0.039x+0.043, r=0.996, 10-60 ng/tube), which had almost the same slope as that obtained using the standard addition method. It was used as the calibration graph in the following experiments.

Method Validation

After spiking of the standard sample (10 and 30 ng) to the rat brain homogenate, the concentrations of pregnenolone were determined using the proposed method. The obtained data are summarized in Table 1. Satisfactory recovery rates were obtained with a relative S.D. (R.S.D.) of less than 13%.

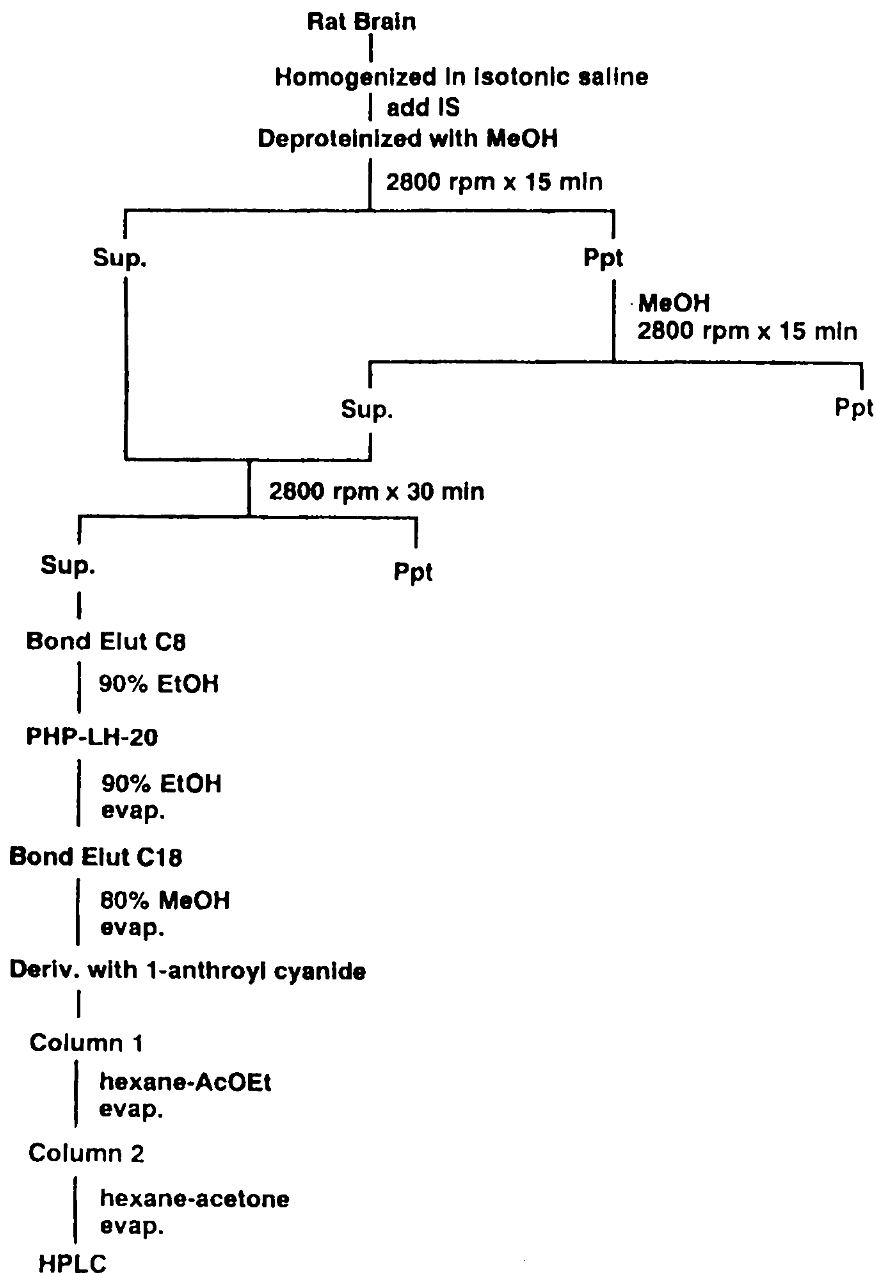

Figure 2. Procedure for determination of pregnenolone in rat brain

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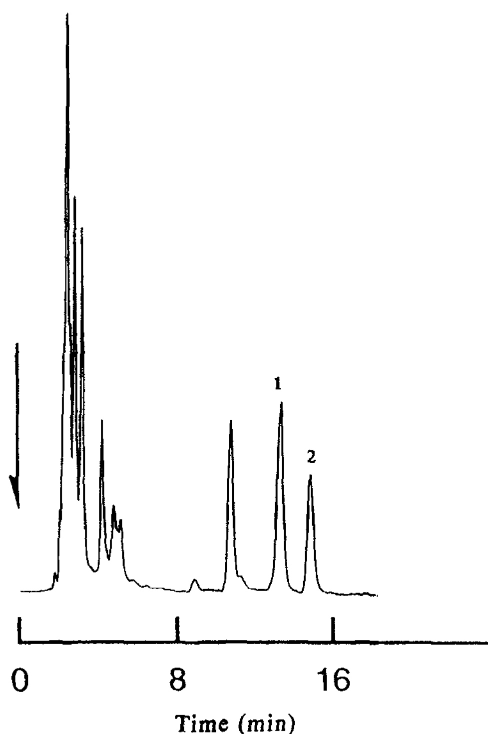

Figure 3. Chromatogram of pregnenolone derivatized with 1-anthroyl cyanide in rat brain. 1. pregnenolone; 2. IS. Conditions: mobile phase, MeCN-H_2O (15:2).

Application of the Method

The quantity of pregnenolone in rat brains of Sprague-Dawley strain is reported as 65 ± 15 ng/g tissue.[2] Brain neurosteroids undergo large circadian variations, with the largest values around the time of lights off under a 12hr/12hr light regimen.[1]

The measurement of pregnenolone was made for the whole brain of young adult male rats and the obtained data are shown in Table 2. Only one rat in five of Wistar (Entry 1) or Sprague-Dawley (Entry 3) strain contained more than 10 ng of pregnenolone/g tissue and circadian variations have not been found as shown in Entries 1 and 2. Also, the other brains contained less than 1 ng of pregnenolone (ng/g tissue) or the steroid could not be detected under these conditions.

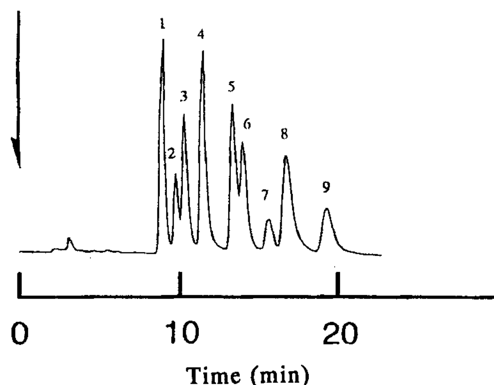

Figure 4. Separation of oxosteroids derivatized with 1-anthroyl cyanide.
1. 3α-hydroxy-5β-androstan-17-one, 3β-hydroxy-5β-androstan-17-one; 2. 3α-hydroxy-5α-androstan-17-one; 3. dehydroepiandrosterone; 4. 3β-hydroxy-5α-androstan-17-one; 5. 3α-hydroxy-5β-pregnan-20-one; 6. 3α-hydroxy-5α-pregnan-20-one; 7. 3β-hydroxy-5β-pregnan-20-one; 8. pregnenolone; 9. 3β-hydroxy-5α-pregnan-20-one; Conditions: mobile phase, MeOH-H_2O (9:1).

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Table 1

Accuracy and Precision of Pregnenolone in Rat Brain

Pregnenolone (ng/Tube)			Difference[c]
Added	**Found**	**Expected**	**(%)**
0	16.0[a]		
10	26.0 ± 1.6 (6.2)[b]	26.0	0.0
0	21.0[a]		
30	53.1 ± 6.7 (12.6)[b]	51.0	7.0

[a] mean (n=3), *ca.* 1g tissue.
[b] n=5, mean ± S.D. (R.S.D., %).
[c] % mean difference = 100x (mean of net found - added)/added.

Table 2

Pregnenolone in Rat Brain

Entry	Concentration (ng/g Tissue)					
1[a]	10.5	<1	<1	nd[c]	nd	(Wistar strain)
2[b]	13.1	5.5	<1	<1	<1	(Wistar strain)
3[a]	22.2	<1	nd	nd	nd	(Sprague-Dawley strain)

[a] after 1 hr of light exposure
[b] after 10 hr of light exposure
[c] nd = not detectable

CONCLUSIONS

The quantitative determination of pregnenolone in rat brain was done by reverse phase HPLC with FL detection using 1-anthroyl cyanide as the derivatization reagent and 3 β-hydroxy-16-methylpregna-5,16-dien-20-one was used as the IS. The method was applied to the determination of this steroid in the rat brains of Wistar and Sprague-Dawley strains, most of which showed the much lower amounts than that previously reported. No circadian variations have been found. As the reasons for this difference are not clear at this time, we are now investigating this discrepancy and the significance of this steroid in the brain will be reported in the future.

ACKNOWLEDGEMENTS

Part of this work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan. The authors thank Teikoku Hormone Mfg. for providing the oxosteroids.

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Received March 8,1996
Accepted March 20, 1996
Manuscript 4101

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