The Analyst COMMUNICATION

Studies on neurosteroids

Part XIII. Characterization of catechol estrogens in rat brains using liquid chromatography-mass spectrometry-mass spectrometry†

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The existence of catechol estrogens in rat brains was clarified using liquid chromatography-atmospheric pressure chemical ionization-ion trap-mass spectrometry-mass spectrometry (LC-APCI-MS²). The catechol estrogens were extracted in the presence of ascorbic acid and then derivatized to acetates with acetic anhydride and pyridine. After a successive purification with silica gel mini-column chromatography, reversed-phase solid-phase extraction and preparative HPLC, the obtained fractions containing the catechol estrogen acetates were subjected to LC-APCI-MS². 2-Hydroxyestrone, 2-hydroxyestradiol and their 4-hydroxy isomers were identified as acetates by comparison with authentic samples based on their chromatographic behavior and mass spectral data. The derivatization to acetate was useful for the treatment of labile catechol estrogens.

Introduction

Recently, significant interest has been focused on the biological properties of neurosteroids, such as pregnenolone, dehydroepiandrosterone and their conjugates,[1] and methods of quantitative determination for these steroids, including conjugates, have been developed by us.[2] Also, the usefulness of estrogen-replacement therapy for dementia of the Alzheimer type[3] and the existence of estrogen receptors in the brain have recently been reported,[4] and much interest is focused on the action of estrogens on the central nervous system and on the existence of estrogens in the brain. In the previous paper of this series, we reported the existence of the classical estrogens [estrone (E_1), estradiol (E_2) and estriol] and the guaiacol estrogens (2-hydroxyestrone 3-methyl ether and 4-hydroxyestrone 3-methyl ether) in rat brains using gas chromatography-ion trap-mass spectrometry-mass spectrometry (GC-MS²).[5] This information suggests that the classical estrogens are hydroxylated to the catechol estrogens, the 2- and 4-hydroxylated metabolites, and then *O*-methylated to the guaiacol estrogens in the brain, as well as in other peripheral organs such as the liver.[6] However, ambiguity still remains regarding the existence of catechol estrogens in the brain.[7]

In this report, we characterize the catechol estrogens [2-hydroxyestrone ($2OHE_1$), 4-hydroxyestrone ($4OHE_1$), 2-hydroxyestradiol ($2OHE_2$) and 4-hydroxyestradiol ($4OHE_2$)] in rat brains as acetates (Fig. 1), which was performed using liquid chromatography-atmospheric pressure chemical ionization-ion trap-mass spectrometry and -mass spectrometry-mass spectrometry (LC-APCI-MS and -MS²).

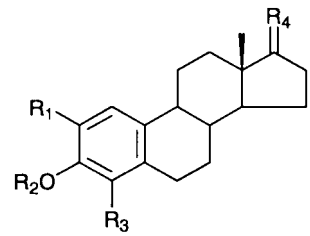
Experimental

Apparatus, materials and reagents

LC-MS^n was performed on a Finnigan MAT LCQ liquid chromatograph-ion trap-mass spectrometer (ThermoQuest, Tokyo, Japan), connected to a JASCO (Tokyo, Japan) PU-980 chromatograph and APCI accessory. The ionization conditions were as follows: ion source current, 5 μA; vaporizer temperature, 400 °C; capillary temperature, 250 °C; capillary voltage, 3 V; sheath gas flow rate, 90 units; auxiliary gas flow rate, 20 units; tube lens offset, 10 V. The relative collision energy for MS² was 20%. Preparative (prep.) HPLC was performed on a Shimadzu LC-6A chromatograph (Kyoto, Japan) equipped with a Shimadzu SPD-10A UV (280 nm) detector. A J'sphere ODS-H80 (4 μm, 150 × 4.6 mm id) (YMC, Kyoto, Japan) was used as the column for LC-MS^n and prep. HPLC at a flow rate of 1.0 ml min^{-1} at 40 °C.

The catechol estrogens ($2OHE_1$, $4OHE_1$, $2OHE_2$ and $4OHE_2$) were prepared from E_1 and E_2 in our laboratories according to the reported methods.[6] The catechol estrogen acetates ($2OHE_1$diAc, $4OHE_1$diAc, $2OHE_2$triAc and $4OHE_2$triAc) were prepared by the usual acetylation using Ac_2O–Py 1 + 1.

ISOLUTE C_{18} (EC) cartridges (500 mg; International Sorbent Tech. Ltd., Hengood, Glamorgan, UK) were purchased from Uniflex (Tokyo, Japan). Silica gel mini-column chromatography (30 × 6 mm id) was performed with silica gel 60 (70–230 mesh; E. Merck, Darmstadt, Germany). All other reagents used were commercially available and of analytical grade.



$2OHE_1$: R_1=OH, R_2=R_3=H, R_4=O
$4OHE_1$: R_1=R_2=H, R_3=OH, R_4=O
$2OHE_2$: R_1=OH, R_2=R_3=H, R_4=β-OH, α-H
$4OHE_2$: R_1=R_2=H, R_3=OH, R_4=β-OH, α-H
$2OHE_1$diAc: R_1=OAc, R_2=Ac, R_3=H, R_4=O
$4OHE_1$diAc: R_1=H, R_2=Ac, R_3=OAc, R_4=O
$2OHE_2$triAc: R_1=OAc, R_2=Ac, R_3=H, R_4=β-OAc, α-H
$4OHE_2$triAc: R_1=H, R_2=Ac, R_3=OAc, R_4=β-OAc, α-H

Fig. 1 Structures of catechol estrogens and their derivatives.

† For Part XII see ref. 9.

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Characterization of $2OHE_1$ and $4OHE_1$ in rat brains

Three Wistar strain rats (10 weeks old, male, 160–180 g, Japan SLC, Hamamatsu, Japan) were decapitated and the whole brains (about 3 g) were homogenized in 10% v/v EtOH containing 0.02% m/v ascorbic acid (20 ml). AcOEt (20 ml) was added to the homogenate, sonicated for 20 min and then shaken at room temperature for 30 min, followed by centrifugation at 1500*g* for 15 min. The precipitate was re-extracted with AcOEt. The suspension was re-centrifuged and all the organic layers were combined. After the solvent had been evaporated under an N_2 gas stream, the residue was treated with Ac_2O–Py 1 + 1 (0.4 ml) at 70 °C for 30 min. The entire solution was extracted with AcOEt, and the organic layer was successively washed with 5% v/v HCl, 5% m/v $NaHCO_3$ and H_2O. After the evaporation of the solvent, the residue was dissolved in hexane–AcOEt 40 + 1 (about 5 ml), subjected to silica gel mini-column chromatography and washed with hexane (10 ml) and hexane–AcOEt 60 + 1 (5 ml). The fraction eluted with hexane–AcOEt 1 + 1 (8 ml) was evaporated under an N_2 gas stream, and the obtained residue was dissolved in 20% v/v EtOH (3 ml) and applied onto an ISOLUTE C_{18} (EC) cartridge. After washing with H_2O (3 ml), hexane (3 ml) and 20% v/v EtOH (3 ml), the eluent with EtOH (3 ml) was evaporated under an N_2 gas stream. The residue was subjected to prep. HPLC (mobile phase, MeOH–H_2O 3 + 2), and fractions containing acetylated $4OHE_1$ ($4OHE_1$diAc; t_R 10.15–12.15 min; fraction A) and $2OHE_1$ ($2OHE_1$diAc; t_R 13.0–15.0 min; fraction B) were separately collected. After the evaporation of the solvent under a N_2 gas stream, the residues were subjected to LC-APCI-MS [mobile phase, MeOH–H_2O 3 + 2 containing 0.1% m/v HCO_2NH_4 or MeCN–H_2O 3 + 2 containing 0.1% m/v HCO_2NH_4; total ion monitoring (TIM), *m/z* 250–400] and LC-APCI-MS^2 (mobile phase, MeOH–H_2O 3 + 2 containing 0.1% m/v HCO_2NH_4; relative collision energy, 20%; precursor ion, *m/z* 388 $[M+NH_4]^+$; product ion monitoring, *m/z* 250–400).

Characterization of $2OHE_2$ and $4OHE_2$ in rat brains

Three Wistar strain rats (10 weeks old, male) were decapitated and the whole brains were treated as already described. After acetylation, the residue obtained was subjected to silica gel mini-column chromatography and washed with hexane (10 ml) and hexane–AcOEt 60 + 1 (5 ml). The fraction eluted with hexane–AcOEt 3 + 2 (8 ml) was evaporated under a N_2 gas stream, and the residue obtained was dissolved in 20% v/v EtOH and applied onto an ISOLUTE C_{18} (EC) cartridge. After washing with H_2O (3 ml), hexane (3 ml) and 30% v/v EtOH (3 ml), the eluent with EtOH (3 ml) was evaporated under an N_2 gas stream. The residue was subjected to prep. HPLC (mobile phase, MeOH–H_2O 3 + 1), and the fractions containing acetylated $4OHE_2$ ($4OHE_2$triAc; t_R 7.10–9.10 min; fraction C) and $2OHE_2$ ($2OHE_2$triAc; t_R 9.10–11.10 min; fraction D) were separately collected. After evaporation of the solvent under an N_2 gas stream, the residues were subjected to LC-APCI-MS (mobile phase, MeOH–H_2O 3 + 1 containing 0.1% m/v HCO_2NH_4 or MeCN–H_2O 7 + 3 containing 0.1% m/v HCO_2NH_4; TIM, *m/z* 250–450) and LC-APCI-MS^2 (mobile phase, MeOH–H_2O 3 + 1 containing 0.1% m/v HCO_2NH_4; relative collision energy, 20%; precursor ion, *m/z* 432 $[M+NH_4]^+$; product ion monitoring, *m/z* 250–450).

Results and discussion

The existence of estrogen 2- and 4-hydroxylase activity[8] and the catechol estrogens in brains has previously been clarified using radioenzymatic assay.[6] However, ambiguity still remains regarding the existence of these metabolites in brains and also their biological significance. For example, Martucci reported that the catechol estrogens have not been detected in rat brains.[7] We also attempted to detect the catechol estrogens in rat brains using GC-MS^2 or LC-MS^2, but poor results were obtained. That is, even the authentic catechol estrogens spiked into the brain homogenate could not be detected. It is well known that the catechol estrogens are unstable; they are easily oxidatively degraded during their isolation and purification and prone to be adsorbed into tissues. These features of the catechol estrogens have hampered the development of an assay for trace amounts of these compounds in a complex matrix such as a brain. These data prompted us to add ascorbic acid to the solvent as an antioxidant during the homogenization and extraction, the catechol estrogens then being derivatized to acetates to prevent oxidation and adsorption.

LC-APCI-MS^n analyses of catechol estrogen acetates

First of all, the authentic catechol estrogen acetates (2/4-OHE_1-diAc and 2/4-OHE_2triAc) were prepared by the usual method and their LC-APCI-MS^n behavior was examined. The mass spectra of authentic catechol estrogen acetates were recorded in LC-APCI-MS using the mobile phase containing HCO_2NH_4. $2OHE_1$diAc and $4OHE_1$diAc produced $[M + NH_4]^+$ (*m/z* 388) as the base peak and some characteristic molecular related ions, such as $[M + H]^+$ (*m/z* 371), $[M + H - 42]^+$ (*m/z* 329) and $[M–AcO]^+$ (*m/z* 311). LC-APCI-MS^2 (precursor ion, *m/z* 388) also gave the $[M + H - 42]^+$. $2OHE_2$triAc and $4OHE_2$triAc produced $[M+NH_4]^+$ (*m/z* 432, base peak) during the LC-APCI-MS, and LC-APCI-MS^2 using this ion as a precursor ion gave the characteristic fragment ions including $[M + H]^+$ (*m/z* 415) and $[M + H\text{-}42]^+$ (*m/z* 373, base peak). It may be postulated that the 42 reduced ions were formed by rearrangement of hydrogen and loss of ketene from phenol acetates. A clear difference has not been observed in the mass spectra of derivatives of 2-hydroxyestrogens and 4-isomers.

Characterization of catechol estrogens in rat brains

Three brains of adult male Wistar strain rats were homogenized in 10% v/v EtOH containing ascorbic acid and extracted with AcOEt, followed by derivatization with acetic anhydride–pyridine. After the purification with silica gel mini-column chromatography, ISOLUTE C_{18} (EC), prep. HPLC, the fractions corresponding to $4OHE_1$diAc (fraction A) and $2OHE_1$-diAc (fraction B) were analyzed using LC-APCI-MS^n. These were identified in comparison with the authentic samples based on their chromatographic behavior during LC-APCI-MS using two mobile phase systems [mobile phase, MeOH–H_2O 3 + 2 containing 0.1% m/v HCO_2NH_4; t_R 12.4 min ($4OHE_1$diAc) and 14.9 min ($2OHE_1$diAc); mobile phase, MeCN–H_2O 3 + 2 containing 0.1% m/v HCO_2NH_4; t_R 10.7 min ($4OHE_1$diAc) and 11.7 min ($2OHE_1$diAc)] and the mass spectra [Fig. 2 (a), (b)]. Furthermore, the product ion mass spectra of these fractions obtained in the LC-APCI-MS^2 were identical with those of authentic samples [Fig. 2 (c)].

The fractions containing $2OHE_2$triAc (fraction C) and $4OHE_2$triAc (fraction D) were also collected and identified by comparison with authentic samples based on chromatographic behavior during LC-APCI-MS using two solvent systems [mobile phase, MeOH–H_2O 3 + 1 containing 0.1% m/v HCO_2NH_4; t_R 8.9 min ($4OHE_2$triAc) and 10.4 min ($2OHE_2$-triAc); mobile phase, MeCN–H_2O 7 + 3 containing 0.1% m/v HCO_2NH_4; t_R 6.5 min ($4OHE_2$triAc) and 7.1 min ($4OHE_2$-triAc)] [Fig. 3 (a)]. The mass spectra and product ion mass spectra of both fractions appeared the same as those of authentic samples [Fig. 3 (b), (c)]. All of the above experiments were carried out twice, and the existence of these estrogens was confirmed in both cases.

In consequence, the form of the catechol estrogens in rat brains was confirmed as acetate using LC-APCI-MS^n. The derivatization to acetate was effective in identifying the labile catechol estrogens in biological substances. The character-

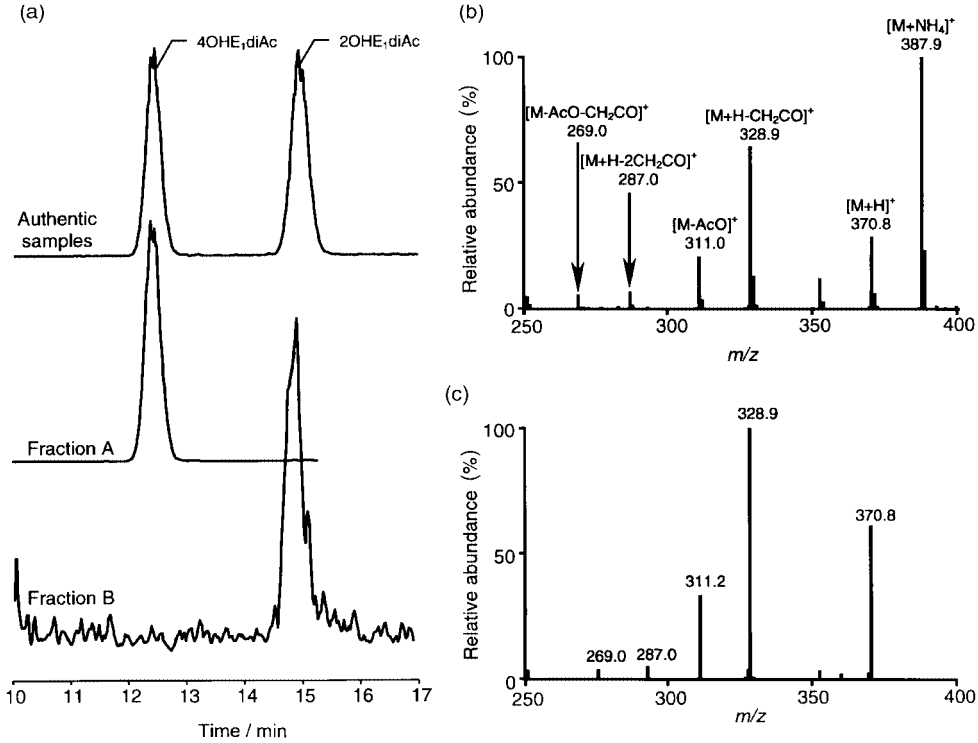

Fig. 2 LC-APCI-MS^n data for $4OHE_1$ and $2OHE_1$ in rat brains as diacetates. (a) Mass chromatograms of $4OHE_1$diAc (fraction A) and $2OHE_1$diAc (fraction B) monitored at *m/z* 388; (b) mass spectrum of $4OHE_1$diAc; and (c) product ion mass spectrum of (b). Conditions: mobile phase, MeOH–H_2O 3 + 2 containing 0.1% m/v HCO_2NH_4; (a, b) TIM; (c) precursor ion , *m/z* 388; relative collision energy, 20%.

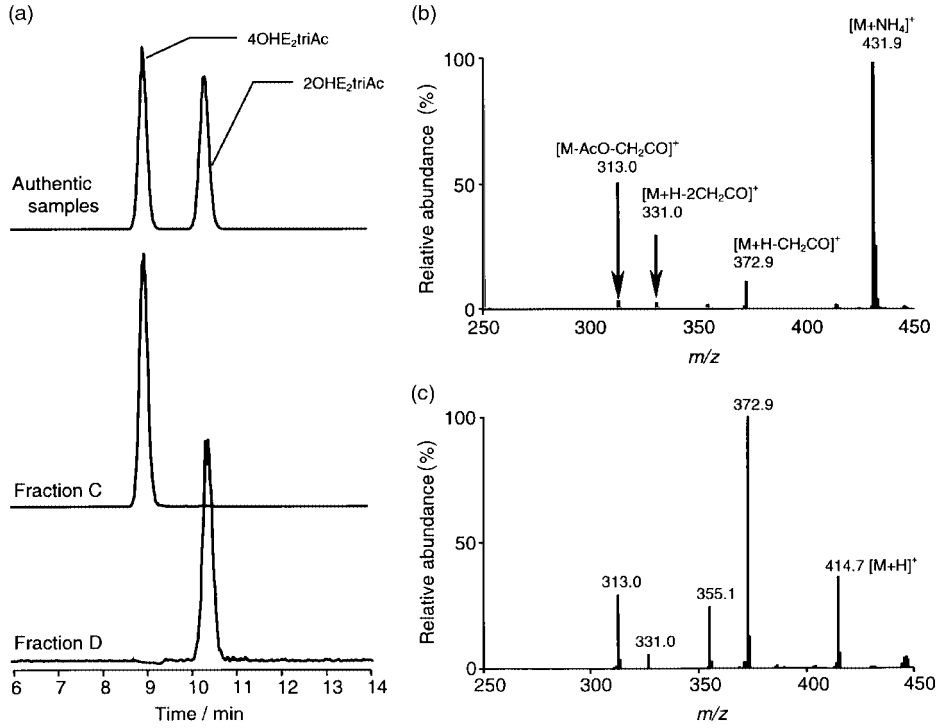

Fig. 3 LC-APCI-MS^n data for the $4OHE_2$ and $2OHE_2$ in rat brains as triacetates. (a) Mass chromatograms of $4OHE_2$triAc (fraction C) and $2OHE_2$triAc (fraction D) monitored at *m/z* 432; (b) mass spectrum of $4OHE_2$triAc; and (c) product ion mass spectrum of (b). Conditions: mobile phase, MeOH–H_2O 3 + 1 containing 0.1% m/v HCO_2NH_4; (a, b) TIM; (c) precursor ion , *m/z* 432; relative collision energy, 20%.

ization of other kinds of catechol estrogens, such as 2-hydroxyestriol, and its 4-isomer, and the development of a quantitative determination method for these estrogens in rat brains is now in progress in our laboratories.

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