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Studies on neurosteroids XII. Determination of enzymatically formed catechol estrogens and guaiacol estrogens by rat brains using liquid chromatography–mass spectrometry–mass spectrometry

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Abstract

Enzymic formations of catechol- and guaiacol-estrogens by rat brains have been investigated using classical- and catechol-estrogens as substrates, respectively. The incubation mixtures were pretreated with liquid–liquid and/or solid-phase extraction, and the products were identified by comparison with authentic samples using liquid chromatography–mass spectrometry (–mass spectrometry) {LC–MS (–MS)}. The enzymic activities were also determined by measuring the formed products with LC–MS. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Neurosteroids; Catechol estrogens; Guaiacol estrogens

1. Introduction

Recently, significant interest has been focused on the biological properties of neurosteroids in the brain, and quantitative determination methods of these steroids including conjugates have been developed by us [1]. The hitherto known neurosteroids consist of several 17- or 20-oxosteroids such as dehydroepiandrosterone, pregnenolone and their conjugates [2]. On the other hand, 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 estrogen 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 not only the classical estrogens {estrone (E_1), estradiol (E_2), estriol} but also the guaiacol estrogens {2-hydroxyestrone 3-methyl ether ($2OHE_13Me$), 4-hydroxyestrone 3-methyl ether ($4OHE_13Me$)} in rat brains using gas chromatography–mass spectrometry–mass spectrometry (MS–MS) (Fig. 1) [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 guaiacol estrogens in the brain, as well as in other

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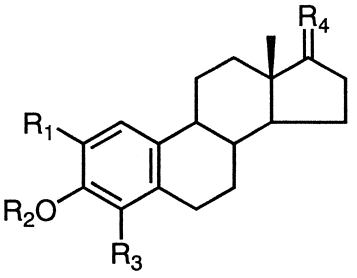

E_1: R_1=R_2=R_3=H, R_4=O
E_2: R_1=R_2=R_3=H, R_4=β-OH, α-H
$2OHE_1$: R_1=OH, R_2=R_3=H, R_4=O
$2OHE_2$: R_1=OH, R_2=R_3=H, R_4=β-OH, α-H
$4OHE_1$: R_1=R_2=H, R_3=OH, R_4=O
$4OHE_2$: R_1=R_2=H, R_3=OH, R_4=β-OH, α-H
$2OHE_12Me$: R_1=OMe, R_2=R_3=H, R_4=O
$2OHE_13Me$: R_1=OH, R_2=Me, R_3=H, R_4=O
$4OHE_13Me$: R_1=H, R_2=Me, R_3=OH, R_4=O
$4OHE_14Me$: R_1=R_2=H, R_3=OMe, R_4=O

Fig. 1. Structures of estrogens.

peripheral organs such as the liver [6]. It has been recognized that the enzymes catalyzing these reactions, estrogen-2/4-hydroxylase and catechol-*O*-methyltransferase (COMT), existed in the brain [7–9]. However these approaches were based on using a radioactive substrate or co-enzyme, so the ambiguity has remained regarding the structures of the formed products, such as 2- or 4-isomers.

In this report, we clarified the structures of enzymatically formed catechol- and guaiacol-estrogens by rat brains using liquid chromatography (LC)–MS (–MS). The enzymic activities were determined by measuring the formed products with LC–MS.

2. Experimental

2.1. Materials and reagents

E_1 and E_2 were donated by Teikoku Hormone Mfg. (Tokyo, Japan), catechol- ($2OHE_1$, $4OHE_1$, $2OHE_2$ and $4OHE_2$) and guaiacol-estrogens ($2OHE_12Me$, $2OHE_13Me$, $4OHE_13Me$ and $4OHE_14Me$) were prepared in our laboratories according to the reported methods [6]. *S*-Adenosyl-L-methionine (SAM) was purchased from Daiichi Kagaku (Tokyo), $NADP^+$, glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49, from *Leuconostoc mesenteroides*) were obtained from Oriental Yeast (Osaka, Japan). Empore Octadecyl (C_{18}) HD disk cartridges (7 mm; 3M Filtration Products, St. Paul, MN, USA) and Isolute C_{18} (EC) cartridges (500 mg; International Sorbent Tech., Ltd., Glamorgan, UK) were purchased from GL Sciences (Tokyo) and Uniflex (Tokyo), respectively. All other reagents used were commercially available and of analytical grade.

2.2. Apparatus

LC–MS (–MS) was performed on a Finnigan MAT LCQ liquid chromatograph–ion trap mass spectrometer (ThermoQuest, Tokyo) connected to a JASCO PU-980 (Tokyo) chromatograph. The ionization conditions were as follows. Electrospray ionization (ESI) (negative ion mode): ion source voltage, 5 kV; capillary temperature, 200°C; capillary voltage, −10 V; sheath gas flow-rate, 90 units, auxiliary gas flow-rate, 20 units; tube lens offset, −30 V. Atmospheric pressure chemical ionization (APCI) (positive ion mode): ion source current, 5 μA; vaporizer temperature, 450°C; capillary temperature, 200°C; capillary voltage, 1 V; sheath gas flow-rate, 80 units; tube lens offset, 5 V. A YMC-Pack ProC18 (5 μm, 150×2.0 mm I.D.) (YMC, Kyoto, Japan) or a J'sphere ODS-H80 (4 μm, 150×4.6 mm I.D.) (YMC) was used as a column at a flow-rate of 0.1 or 1.0 ml/min, respectively, at 40°C.

2.3. Animals and tissue preparations

Wistar strain rats (10 weeks old, male, 160–180 g, Japan S.L.C., Hamamatsu, Japan) were decapitated, and the whole brains were homogenized in 0.15 *M* KCl to provide a final concentration of 20% (w/v). Microsomal fractions were prepared from the homogenates by centrifugation at 9 000 *g* at 4°C for 10 min and recentrifugation of the resultant supernatant at 100 000 *g* at 4°C for 60 min (CP 56G, Hitachi, Tokyo). The obtained microsomal pellets were rinsed and suspended in 2 ml of 10 m*M*

potassium phosphate buffer (pH 7.5) (PB). The 100 000 *g* supernatants were used as the soluble fractions. All assays were performed on freshly prepared enzyme sources. Protein concentrations were estimated by the method of Lowry et al. [10] using bovine serum albumin as a standard.

2.4. Formation of the catechol estrogens

2.4.1. Standard incubation conditions

The incubation medium contained substrate {E_2, 800 nmol (218 μg) in 40 μl of EtOH; final concentration was 1.0 mM}, $NADP^+$ (800 nmol in 10 μl of PB), G6P (4 μmol in 10 μl of PB), G6PDH (800 mU in 10 μl of PB), $MgCl_2$ (1.2 μmol in 80 μl of PB), Tween 80 (800 nl in 10 μl of PB), ascorbic acid (500 nmol in 10 μl of PB), microsomal fraction (2 mg protein) and PB in a total volume of 800 μl. The mixture was incubated at 37°C for 3 h, and the reaction was stopped with the denaturation by the addition of 1 ml of CH_3CN.

2.4.2. Identification of the enzymatically formed catechol estrogens

After the addition of CH_3CN, the incubation mixture was centrifuged at 1500 *g* for 10 min, and the supernatant was diluted with H_2O, so that the concentration of CH_3CN was adjusted to 10%. The resulting solution was applied on an Empore disk cartridge, washed with 5 ml of 10% EtOH containing 0.02% ascorbic acid, and the eluate with 3 ml of EtOH containing 1% CH_3CO_2H was dried under a N_2 gas stream. The residue was redissolved in 50 μl of MeOH, and an aliquot was subjected to LC–ESI-MS {column, YMC-Pack ProC18; mobile phase, MeOH–10 m*M* HCO_2NH_4 (pH 4.0; adjusted with HCO_2H) (1:1); full scan monitoring (*m*/*z* 200–400); $2OHE_2$ t_R, 15.1 min; $4OHE_2$ t_R, 12.6 min} or LC–ESI-MS–MS {precursor ion, *m*/*z* 333 $[M+HCO_2]^-$; relative collision energy, 15%; product ion monitoring (*m*/*z* 200–350)}.

2.4.3. Assay of the estrogen-2/4-hydroxylase activity

After an incubation mixture was subjected to the pretreatment as previously described, the desired fraction was subjected to LC–ESI-MS (full scan monitoring: *m*/*z* 200–400), and the concentrations of the catechol estrogens were obtained using the absolute calibration curve method. The calibration curves were constructed by the peak areas of the authentic samples ($2OHE_2$ and $4OHE_2$; 2–100 ng each/tube) in the mass chromatograms (monitoring ion: *m*/*z* 333: $2OHE_2$, $Y=1.77\times10^5X+3.41\times10^5$, $r^2=0.999$; $4OHE_2$, $Y=3.83\times10^5X+2.19\times10^6$, $r^2=0.998$; Y=peak area, X=steroid value).

2.4.4. Recoveries of the catechol estrogens

The authentic catechol estrogens (10, 20, 50, 100 ng each/tube; $n=2$) were added to the standard incubation medium (without substrate), and the resulting solution was treated and subjected to LC–ESI-MS in a full scan mode (*m*/*z* 200–400) as previously described. The regression lines were constructed using the peak areas of the mass chromatograms (monitoring ion: *m*/*z* 333: $2OHE_2$, $Y=1.81\times10^5X-3.31\times10^5$, $r^2=0.999$; $4OHE_2$, $Y=3.41\times10^5X+9.656\times10^5$, $r^2=0.996$; Y=peak area, X=steroid value), and the recoveries of these estrogens were calculated using the ratios of the slopes to those of the calibration curves.

2.5. Formation of the guaiacol estrogens

2.5.1. Standard incubation conditions

The incubation medium contained the substrate {$2OHE_1$ or $4OHE_1$, 160 nmol (48 μg) in 40 μl of EtOH; final concentration was 200 μ*M*}, SAM (400 nmol in 50 μl of PB), $MgCl_2$ (1.2 μmol in 80 μl of PB), the microsomal fraction or soluble fraction (2 mg protein) and PB in a total volume of 800 μl. After the incubation at 37°C for 3 h, the reaction was stopped by the addition of 1 ml of 0.8 *M* borate buffer (pH 10.7).

2.5.2. Identification of the enzymatically formed guaiacol estrogens

The products were extracted with 2 ml of AcOEt, followed by separation of the organic phase by centrifugation at 1500 *g* for 1 min. The obtained organic phase was evaporated under a N_2 gas stream, then the residue redissolved in 10% EtOH was applied to an Isolute C_{18} cartridge. After washing with 3 ml of H_2O and 2 ml of 20% EtOH, the eluate with 3 ml of MeOH was dried under a N_2 gas stream, redissolved in 50 μl of MeOH, and an

aliquot was subjected to LC–APCI-MS {column, J'sphere ODS-H80; mobile phase, MeOH–H_2O (3:2); full scan monitoring (m/z 280–310) or selected ion monitoring mode (SIM) (m/z 301 $[M+H]^+$); $2OHE_1$2Me, t_R 10.2 min; $2OHE_1$3Me, t_R 11.4 min; $4OHE_1$3Me, t_R 16.0 min; $4OHE_1$4Me, t_R 9.7 min} or LC–APCI-MS–MS {precursor ion, m/z 301, relative collision energy, 30%; product ion monitoring (m/z 250–300)}.

2.5.3. Assay of COMT activity

After the incubation, an internal standard (IS; E_1 1 μg/tube) was added to the incubation mixture and then subjected to the pretreatment as previously described. The desired fraction was subjected to LC–APCI-MS in a SIM mode (m/z 301 and 271 $[M+H]^+$ for the guaiacol estrogens and I.S., respectively), and the concentrations of the guaiacol estrogens were obtained using the calibration curve method. The calibration curves were constructed by the peak area ratio of the authentic samples ($2OHE_1$2Me, $2OHE_1$3Me, $4OHE_1$3Me, $4OHE_1$4Me; 10–1000 ng each/tube) to I.S. ($2OHE_1$2Me, $Y=8.41\times10^{-4}X-2.15\times10^{-3}$, $r^2=0.999$; $2OHE_1$3Me, $Y=1.43\times10^{-3}X-5.98\times10^{-3}$, $r^2=0.999$; $4OHE_1$3Me $Y=1.86\times10^{-3}X-7.23\times10^{-4}$, $r^2=0.999$; $4OHE_1$4Me, $Y=9.79\times10^{-4}X-5.15\times10^{-4}$, $r^2=0.999$; Y=peak area ratio, X=steroid value).

2.5.4. Recoveries of guaiacol estrogens

The authentic guaiacol estrogens (50 or 500 ng each/tube; $n=2$) were added to the standard incubation medium (without substrate), and the resulting solution was treated as previously described.

3. Results

3.1. Identification of enzymatically formed catechol- and guaiacol-estrogens

The mass spectra of authentic catechol- and guaiacol-estrogens were recorded by APCI- or ESI-MS (–MS) (both polarities) with flow injection analysis. The catechol estrogens ($2OHE_2$ and $4OHE_2$) produced $[M+CH_3CO_2]^-$ (m/z 347) or $[M+HCO_2]^-$ (m/z 333) as the base ion in the negative ion mode of both ionization methods using the mobile phase containing $CH_3CO_2NH_4$ or HCO_2NH_4, respectively. After the careful optimization of the ionization conditions, the signal-to-noise ratio (S/N) using ESI and HCO_2NH_4 was found to be higher than that using APCI and/or $CH_3CO_2NH_4$. ESI-MS–MS (precursor ion, m/z 333; relative collision energy, 15%) also gave the product ion at m/z 287 corresponding to $[M-H]^-$. A clear difference has not been observed in the mass spectra of $2OHE_2$ and its 4-isomer. The molecular related ions were not observed in the positive ion mode. The guaiacol estrogens produced $[M+H]^+$ (m/z 301) ions in the positive ion mode of APCI using MeOH–H_2O as the mobile phase, although the predominant ions were not obtained in the negative ion mode or ESI. APCI-MS–MS (precursor ion, m/z 301; relative collision energy, 30%) also gave the ion at m/z 283 corresponding to $[M+H-H_2O]^+$. As with the catechol estrogens, a clear difference has not been observed in the mass spectra of these positional isomers, except for the ion intensity ($2OHE_1$2Me< $2OHE_1$3Me, $4OHE_1$4Me<$4OHE_1$3Me).

These data prompted us to use LC–ESI-MS (-MS) {semi-micro ODS column; MeOH-10 mM HCO_2NH_4 (pH 4.0); negative ion mode} and LC–APCI-MS (–MS) (conventional ODS column; MeOH–H_2O; positive ion mode) for the determination of catechol- and guaiacol-estrogens, respectively, whose positional isomers were clearly separated each other under the proposed conditions as previously described.

The catechol estrogens were extracted from incubation mixture containing substrate (E_2), rat brain microsomal fraction and NADPH generating system by solid-phase extraction. During the incubation and pretreatment, ascorbic acid or acetic acid was added to the mixture in order to prevent the oxidative degradation of catechol estrogens. The peaks corresponding to $2OHE_2$ and $4OHE_2$ were detected in the mass chromatogram of LC–ESI-MS selected at m/z 333 as shown in Fig. 2a. LC–MS and LC–MS–MS gave the predominant ion at m/z 333 and m/z 287 corresponding to $[M+HCO_2]^-$ and $[M-H]^-$, respectively (Fig. 2b,c), and these were the same as those of the authentic samples.

The guaiacol estrogens were extracted from incubation mixture containing substrate ($2OHE_1$ or

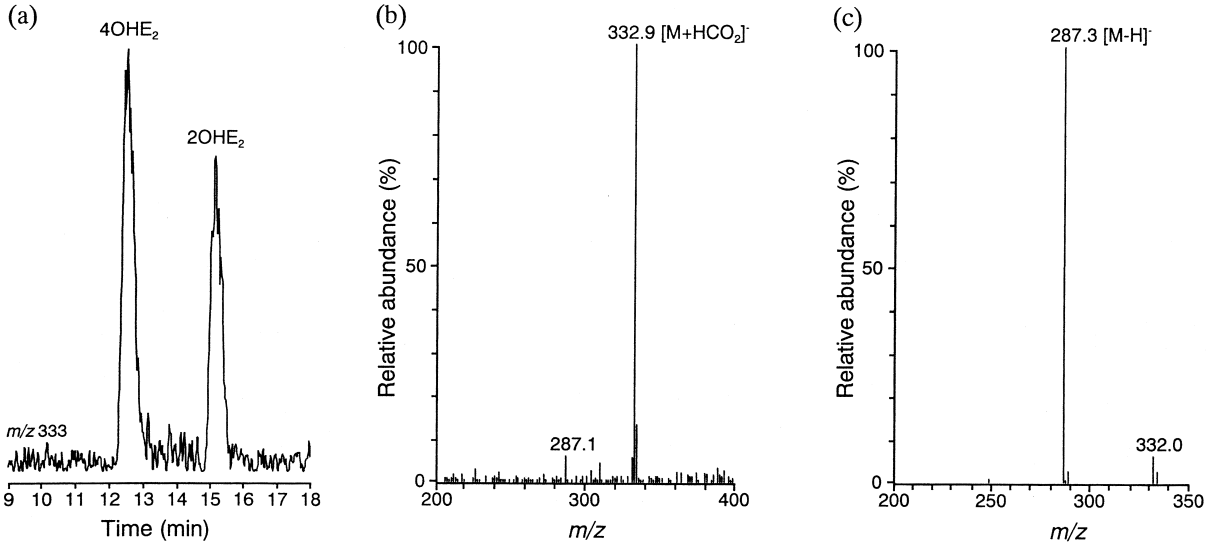

Fig. 2. Typical LC–ESI-MS (–MS) data for the enzymatically formed catechol estrogens. (a) Mass chromatogram of the extracts from an incubation mixture, (b) mass spectrum of $2OHE_2$ from an incubation mixture, (c) product ion mass spectrum of (b) (precursor ion, m/z 333).

$4OHE_1$), rat brains (microsomal fraction or soluble fraction) and SAM by liquid–liquid and solid-phase extraction. In the incubation mixture, which contained $2OHE_1$ as the substrate, the peaks corresponding to $2OHE_1$2Me and $2OHE_1$3Me were detected in the mass chromatogram and selected ion chromatogram of LC–APCI-MS monitored at m/z 301 $[M+H]^+$ (Fig. 3a). The peaks corresponding to $4OHE_1$3Me and $4OHE_1$4Me were also formed from $4OHE_1$ (Fig. 3b). LC–MS and LC–MS–MS gave the predominant ions at m/z 301 $[M+H]^+$ and m/z 283 $[M+H-H_2O]^+$, respectively, and these were the same as those of the authentic samples (Fig. 3c,d).

All these data confirmed the structures of enzymatically formed products and peak purity.

3.2. Activity of estrogen-2/4-hydroxylase

The formed catechol estrogens ($2OHE_2$ and $4OHE_2$) were extracted from incubation mixture and quantified using LC–ESI-MS (Fig. 2a). Although an appropriate IS was not available for the determination, the absolute calibration graphs constructed with peak areas of the authentic samples (2–100 ng/tube) exhibited satisfactory linearity ($r^2>0.99$). The regression lines, which were constructed using the peak area of the authentic samples (10–100 ng/tube) added to the incubation medium (without substrate) and then pretreated, also exhibited satisfactory linearity ($r^2>0.99$), and the recoveries of $2OHE_2$ and $4OHE_2$ calculated by the slope values of the lines were 102.1% and 89.2%, respectively. The concentrations of these estrogens obtained using the absolute calibration curve method were corrected by the recovery rates. The detection and quantitative limits were 0.4 ng/injection (2.8 ng/ml of incubation mixture) ($S/N=5$).

The effects of time and substrate concentration on the formation of catechol estrogens were studied by performing the incubation at different times (1, 2, 3 h) and substrate concentrations (0.1–1.0 m*M*). The product formations were increased up to 3 h. Although the substrate solubility was beginning to become saturated at about 0.3 m*M*, the formed products were not detected at E_2 concentration lower than 0.5 m*M*. Therefore, incubation times of 3 h and 1.0 m*M* of substrate concentration were used for assaying estrogen-2/4-hydroxylase activity in rat brain microsomal fractions. The results are summarized in Table 1, but K_m and V_{max} values have not been obtained owing to the above reason. The ratio of formed $2OHE_2$ to $4OHE_2$ (2OH/4OH) was ca. 1.4, which was much lower than that (ca. 50) obtained from rat liver homogenate [11].

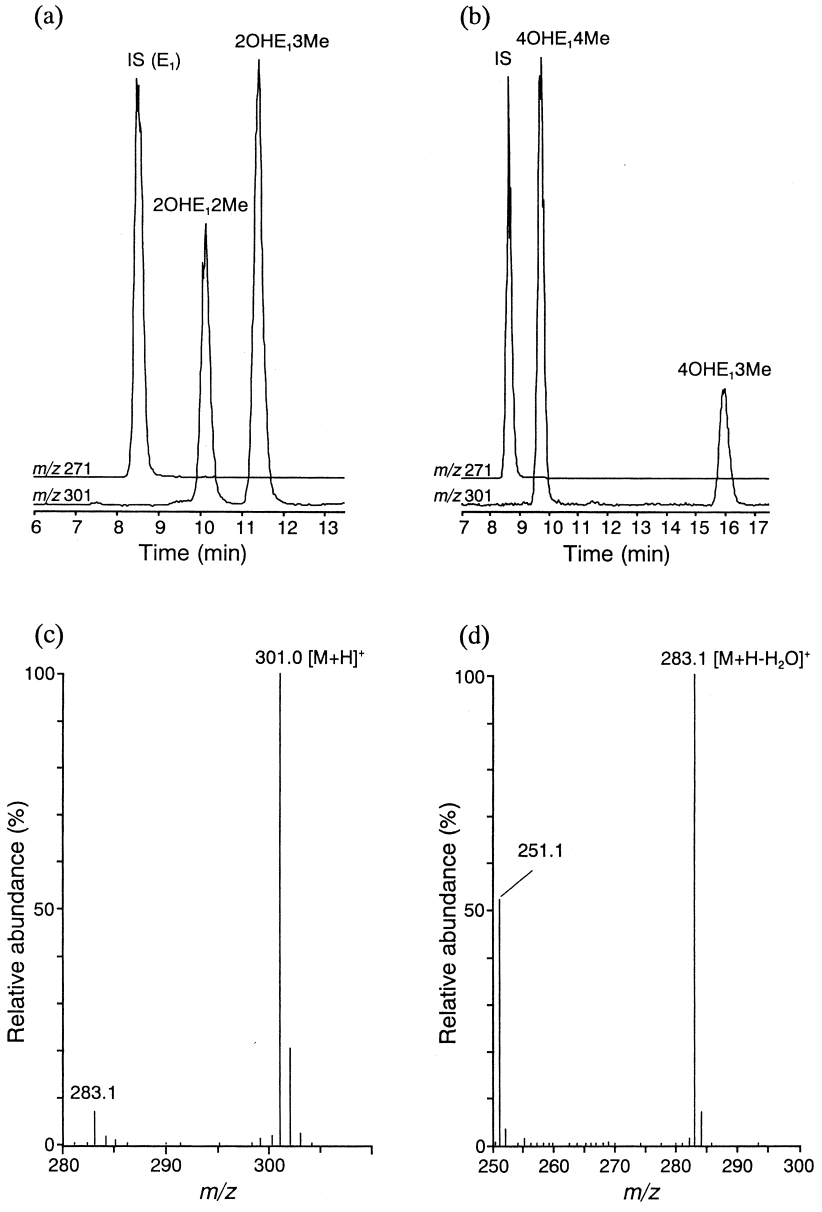

Fig. 3. Typical LC–APCI-MS (–MS) data for the enzymatically formed guaiacol estrogens by soluble fraction. (a) Selected ion chromatogram of the extracts from an incubation mixture with $2OHE_1$ as the substrate, (b) selected ion chromatogram of the extracts from an incubation mixture with $4OHE_1$ as the substrate, (c) mass spectrum of $4OHE_1$4Me from an incubation mixture with the soluble fraction, (d) product ion mass spectrum of (c) (precursor ion, m/z 301).

3.3. Activity of COMT

The formed guaiacol estrogens ($2OHE_1$2Me, $2OHE_1$3Me, $4OHE_1$3Me and $4OHE_1$4Me) were extracted from the incubation mixture together with IS and then quantified using LC–APCI-MS in a SIM mode (Fig. 3a,b). The calibration graphs constructed with the peak area ratio of the authentic samples (10–1000 ng/tube) to IS (E_1) exhibited satisfactory linearity ($r^2>0.99$). The recovery rates of guaiacol

Table 1
Activity of estrogen-2/4-hydroxylase in rat brains[a]

Substrate	Product (ng/h/mg protein)[b]		
	$2OHE_2$	$4OHE_2$	2OH/4OH
E_2	1.08±0.91	0.76±0.55	1.38±0.40

[a] Standard incubation conditions as described in the experimental section.
[b] Mean±SD, n=9.

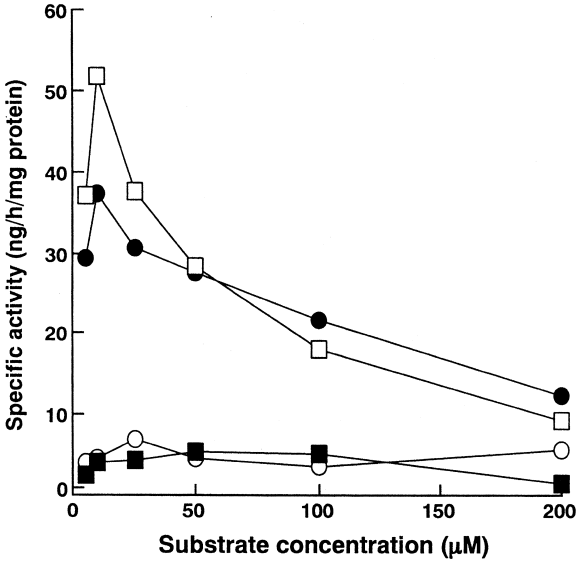

Fig. 4. Effects of substrate concentration on the formation of guaiacol estrogen by the rat brain microsomal fraction. $2OHE_1$2Me(●); $2OHE_1$3Me (○); $4OHE_1$3Me (■); $4OHE_1$4Me (□). Standard incubation conditions were used except for substrate concentration.

estrogens were more than 84.1% (50 ng/tube, n=2, mean) and 81.9% (500 ng/tube), and the S/N values of these four isomers were more than 4 at the 2 ng/injection (14.8 ng/ml of incubation mixture). A difference has not been observed in the slope values of the calibration graph and those obtained from the spiked samples.

The effects of time and substrate concentration on the formation of guaiacol estrogens were studied by performing the incubation at different times (1–6 h) and substrate concentrations (5–200 μM) using microsomal and soluble fractions. The product formations were increased linearly up to 6 h and were inhibited by $2OHE_1$ or $4OHE_1$ at concentrations greater than 10–25 μM (Fig. 4), therefore, K_m and V_{max} values have not been obtained. Although the substrate inhibition may exist, a large excess (200 μM) of substrate was used for assaying COMT activity in microsomal and soluble fractions.

The results are summarized in Table 2. In both cases using $2OHE_1$ and $4OHE_1$ as the substrate, the enzymic activities in soluble fractions were greater than those in microsomal fractions. The ratios of $2OHE_1$2Me to $2OHE_1$3Me (2Me/3Me), which were formed from $2OHE_1$ using both enzyme sources, were ca. 1.3, and the positional specificity of COMT has not been observed. On the other hand, $4OHE_1$ gave $4OHE_1$4Me predominantly, and the 4Me/3Me values in the soluble fractions were around six times greater than that in the microsomal fractions, it was suggested that the nature of COMT in the rat brain microsome is different from that in the soluble fraction. The formation ratios (2Me/3Me 1.3; 4Me/3Me 12.9) of guaiacol estrogens obtained from brain

Table 2
Activity of COMT in rat brains[a]

Enzyme source	Substrate	Product (ng/h/mg protein)[b]				2Me/3Me or 4Me/3Me
		$2OHE_1$2Me	$2OHE_1$3Me	$4OHE_1$3Me	$4OHE_1$4Me	
Microsomal fraction[c]	$2OHE_1$	9.60±3.84	7.86±3.90			1.32±0.45
	$4OHE_1$			3.36±0.60	7.26±2.28	2.15±0.35
Soluble fraction[d]	$2OHE_1$	65.9±27.8	52.3±23.7			1.27±0.10
	$4OHE_1$			12.8±20.7	92.9±36.7	12.9±5.87

[a] Standard incubation conditions as described in the Experimental section.
[b] Mean±SD.
[c] n=5.
[d] n=8.

soluble fractions were similar to those (2Me/3Me ca. 1.1; 4Me/3Me ca. 11.7) obtained from rat liver homogenate [12].

4. Discussion

Recently we have clarified the existence of catechol estrogens in rat brains [13] and it has been demonstrated that the catechol estrogens in the brain inhibit tyrosine hydroxylase and COMT, the enzymes involved in the synthesis and degradation of catecholamines, respectively [14]. In this report, we determined the enzymatically formed catechol- and guaiacol-estrogens by rat brains using LC–MS (–MS). The ratio of 2OH/4OH formed by rat brain microsomal fractions was different from that by rat liver homogenate; on the other hand, the ratios of guaiacol estrogens (2Me/3Me and 4Me/3Me) formed by rat brain soluble fractions were similar to those by rat liver homogenate. On the other hand, 4Me/3Me values were significantly increased by rat brain microsomal fractions, which means that the nature of COMT in the rat brain microsome is different from that in the soluble fraction.

All these data have suggested that the catechol estrogens exist as neuroactive neurosteroids in rat brains. The further investigation of biological significance of these estrogens is now in progress in our laboratories.

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