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Determination of Estrogens in Rat Brains Using Gas Chromatography/Mass Spectrometry/Mass Spectrometry

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Received for Review November 30, 2001. Accepted February 4, 2002.

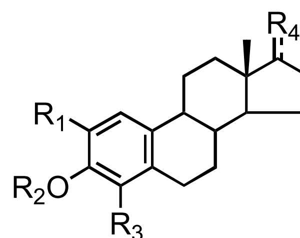
Abstract

A standard addition determination method of estrone, estradiol and guaiacol estrones in rat brains using gas chromatography/mass spectrometry/mass spectrometry (GC/MS/MS) was developed. Estrone and estradiol were extracted with liquid−liquid extraction, purified successively with silica−gel mini−column chromatography, solid−phase extraction and preparative HPLC. The obtained estrogens were derivatized to dimethylethylsilyl ethers followed by GC/MS/MS analysis. The concentrations of estrone and estradiol were 0−44 and 0−159 pg/g tissue (n= 4), respectively. On the other hand, an additional pretreatment procedure was necessary for determination of guaiacol estrones in rat brains. After the liquid−liquid extraction, guaiacol estrones were acetylated with acetic anhydride−pyridine and purified successively with silica−gel mini−column chromatography and solid−phase extraction. The obtained guaiacol estrone acetates were hydrolyzed, derivatized to dimethylethylsilyl ethers and then applied to GC/MS/MS. Although the absolute recovery rates of guaiacol estrones spiked in brain homogenate were higher than 65%, these estrogens in rat brain were not determined.

Keywords: estrone, estradiol, guaiacol estrone, rat brain, standard addition method, gas chromatography/mass spectrometry/mass spectrometry

1. Introduction

The term “neurosteroids” applies to the steroids that are both synthesized in the nervous system, either *de novo* from cholesterol or from steroid hormone precursors, and that accumulate in the nervous system to levels that are at least in part independent of the steroidgenic gland secretion rates. It has been reported that these steroids are found in larger amounts in brain than in plasma [1]; recently, significant interest has been focused on the biological properties of neurosteroids, such as pregnenolone, dehydroepiandrosterone and their conjugates [2]. Also, the existence of estrogen receptor in brain has been clarified [3], and the usefulness of estrogen−replacement therapy for dementia of Alzheimer type has been reported [4]. Much interest is focused on the action of estrogens on the central nervous system and on the existence of estrogens in the brain. In previous papers, we reported the existence of the classical estrogens {estrone (E_1), estradiol (E_2) and estriol}, the guaiacol estrones {2−hydroxyestrone 3−methyl ether ($2OHE_13Me$) and 4−hydroxyestrone 3−methyl ether ($4OHE_13Me$)} [5] (Figure 1) and the catechol estrogens (2− or 4−hydroxy metabolites) [6,7] in rat brains using gas chromatography/mass spectrometry/mass spectrometry (GC/MS/MS) and/or liquid chromatography/mass spectrometry (LC/MS). However, two or three whole brains were used



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
$2OHE_12Me$: 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

Figure 1. Structures of E_1, E_2 and guaiacol estrones.

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for identification at a time; therefore, the quantitative analytical method using part of an entire brain is necessary to clarify the physiological role of these estrogens in the central nervous system.

In this paper, we describe the development of the standard addition determination method of E_1, E_2 and guaiacol estrones in part of a rat brain using GC/MS/MS.

2. Experimental

2.1. Apparatus, materials and animals

GC/MS/MS was performed on a GCQ gas chromatograph/ion trap mass spectrometer (ThermoQuest, San Jose, CA, USA) equipped with a 5% diphenyl–95% dimethylpolysiloxane capillary column (Rtx–5MS, 30m x 0.25mm i.d., 0.25μm df; Restek, Bellefonte, PA, USA), which was used with the following conditions: electron ionization was set at 70 eV and detected with positive ion mode. Carrier gas was helium at a flow rate of 40cm/s. The column oven temperature was set at 60°C for 4 min, ramped to 280°C at 40°C/min and held at 280°C for 10min. The split–less injector temperature was set at 60°C for 1min, ramped to 280°C at 180°C/min and held at 280°C for 18 min. The transfer line temperature and the ion source temperature were set at 275°C and 200°C, respectively.

Preparative HPLC was performed on a LC–6A (Shimadzu, Kyoto, Japan) equipped with a J'sphere ODS–H80 (4μm, 150 x 4.6mm i.d.) (YMC, Kyoto) column and an SPD–10A UV detector (280nm) (Shimadzu). The flow rate and the column temperature were set at 1ml/min and 40°C, respectively. E_1 and E_2 were not separated when MeOH–H_2O (13:7) was used as the mobile phase, so that both compounds were obtained in the same fraction.

E_1 and E_2 were supplied by Teikoku Hormone Mfg. (Tokyo, Japan). Guaiacol estrones (2OHE$_1$2Me, 2OHE$_1$3Me, 4OHE$_1$3Me, 4OHE$_1$4Me) and 16–oxoestradiol (16oxoE$_2$) {internal standard (IS) for guaiacol estrones} were purchased from Steraloids (Newport, RI, USA). [2,4,6,6,9–$^2H_5$]–E_1 and –E_2 (ISs for E_1 and E_2) [8] were prepared in our laboratories from [6,6,9–$^2H_3$]–E_1 and –E_2, respectively, which were kindly supplied by Nippon Kayaku (Tokyo). Silica–gel mini–column chromatography (0.5g, 3 x 0.6cm i.d.) was performed with Silica Gel 60 (70–230mesh; E. Merck, Darmstadt, Germany). ISOLUTE C18 (EC) cartridges (500mg; International Sorbent Technology, Hengoed, Mid Glamorgan, UK) were purchased from Uniflex (Tokyo). Dimethylethylsilylimidazole (DMESI) was obtained from Tokyo Kasei Kogyo (Tokyo), and all the other reagents were analytical grade and commercially available.

Male Wistar strain rats (7weeks old) were obtained from Japan S.L.C. (Hamamatsu, Japan).

2.2. Determination of E_1 and E_2

Wistar strain rat was decapitated and the entire brain (*ca*. 1.8 g) was homogenized in 10% EtOH (10 volumes, w/v) using a Teflon homogenizer, and then divided into three parts so that the content of brain in each tube is approximately 0.6 g of tissue. Standard solutions of E_1 and E_2 (each 0, 50, 100 pg in EtOH) and ISs ([$^2H_5$]–E_1 and –E_2; each 1ng in EtOH) were added to the homogenate. After standing for 10min at room temperature, AcOEt (6ml) was added to the samples and the mixture was then sonicated for 20min in an ultrasonic bath, stirred at room temperature for 30min and centrifuged at 1500*g* for 20min. The organic layer was collected and the residue was re–extracted with AcOEt (6ml), and the entire organic layer was evaporated under a N_2 gas stream. The residue was dissolved in hexane–AcOEt (20:1, 0.5ml) and subjected to silica–gel mini–column chromatography. After washing with hexane (5ml) and hexane–AcOEt (10:1, 5ml), the fraction eluted with hexane–AcOEt (2:1, 8ml) was evaporated under a N_2 gas stream. The residue was dissolved in 10% MeOH (1ml) and applied to an ISOLUTE C18 (EC) cartridge. After washing with H_2O (5ml) and 20% MeOH (2 ml), the eluate with MeOH (3ml) was evaporated under a N_2 gas stream. The obtained residue was purified by preparative HPLC and the fraction containing E_1, E_2 and ISs (5.5–7.5 min) was evaporated *in vacuo* and the residue was derivatized with DMESI (50μl) for 30min at 60°C. The reaction mixture was diluted with $CHCl_3$ (0.5ml), applied to a silica–gel mini–column chromatography, and the eluate with $CHCl_3$ (5ml) was evaporated

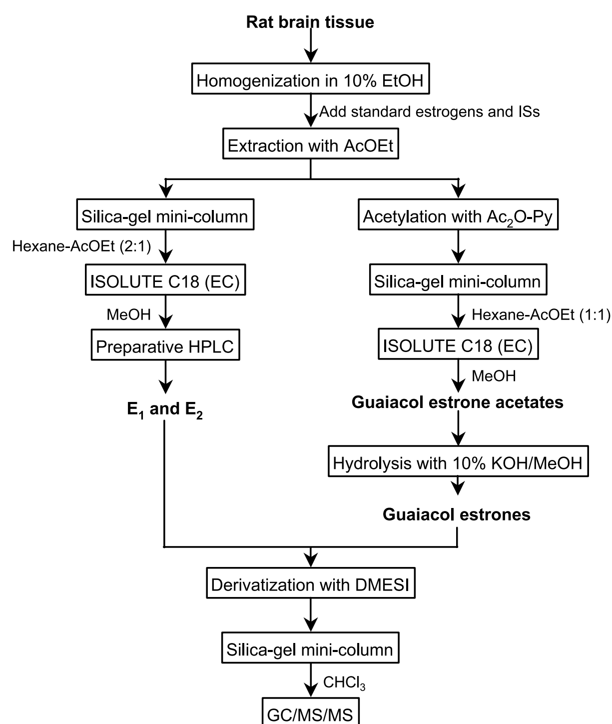

Figure 2. Procedure for determination of E_1, E_2 and guaiacol estrones.

under a N_2 gas stream. The residue was dissolved with hexane (50 μl) and an aliquot of which was subjected to GC/MS/MS {precursor ions, *m/z* 356 and 361 (10–14min, for E_1 and [$^2H_5$]–E_1), 444 and 449 (14–18min, for E_2 and [$^2H_5$]–E_2); excitation voltage, 1.0V for E_1 and [$^2H_5$]–E_1 and 1.2V for E_2 and [$^2H_5$]–E_2; monitoring ions, *m/z* 271 (E_1), 276 ([$^2H_5$]–E_1), 340 (E_2) and 344 ([$^2H_5$]–E_2)} (Figure 2).

2.3. Determination of guaiacol estrones

Standard solutions of guaiacol estrones (each 25–400 pg in EtOH) and IS (16oxoE_2;10 ng in EtOH) were added to the Wistar rat brain homogenate corresponding to 0.5 g of tissue, and the estrogens were extracted with AcOEt as described above. After the solvent was evaporated under a N_2 gas stream, the extract was treated with acetic anhydride–pyridine (1:2, 0.3ml) at room temperature for 30min. After addition of H_2O (1ml), the entire solution was extracted with AcOEt, and the organic layer was successively washed with 5% HCl, 5% $NaHCO_3$ and H_2O. After evaporation of the solvent, the residue was dissolved in hexane–AcOEt (10:1, 1ml), subjected to silica–gel mini–column chromatography and washed with hexane–AcOEt (10:1, 5ml). The fraction eluted with hexane–AcOEt (1:1, 6ml) was evaporated under a N_2 gas stream, the residue was dissolved in 10% MeOH (2ml) and applied to an ISOLUTE C18 (EC) cartridge. After washing with H_2O (5 ml) and 30% MeOH (2ml), the eluate with MeOH (3ml) was evaporated under a N_2 gas stream. The residue was hydrolyzed with 1% KOH/MeOH (0.5ml) for 30min at room temperature. After the neutralization with 5% HCl, the entire solution was extracted with AcOEt and the organic layer was washed with H_2O. The solvent was evaporated under a N_2 gas stream, the obtained residue was derivatized with DMESI, treated as above, and then subjected to GC/MS/MS analysis {precursor ions, *m/z* 342 (14–16 min, for guaiacol estrones), 429 (16–18min, for 16oxoE_2); excitation voltage, 1.0 V; monitoring ions, *m/z* 257 (guaiacol estrones), 337 (16oxoE_2)} (Figure 2).

3. Results

3.1. GC/MS analysis of estrogens

In the previous paper, we chose the methyloxime (MO) and/or trimethylsilyl ether (TMS) as the derivatization form for identification of classical estrogens and guaiacol estrones in rat brain using GC/MS/MS [5]. Although derivatization to MO–TMS was simple and convenient, the complete separation of the four guaiacol estrone isomers was not achieved chromatographically. To find suitable derivatization for E_1, E_2 and guaiacol estrones, we examined *O*–methylhydroxylamine, *O*–pentafluobenzylhydroxylamine (for derivatization of the carbonyl group), *N, O*–bis (trimethylsilyl) trifluoroacetamide, DMESI, dimethyl–*iso*–propylsilylimidazole, heptafluorobutyric anhydride and pentafluorobenzyl bromide (for derivatization of the hydroxy group). Among these derivatization reagents, DMESI appeared to be most promising with respect to resolution, sensitivity and feasibility, and the product ion mass chromatogram was shown in Figure 3.

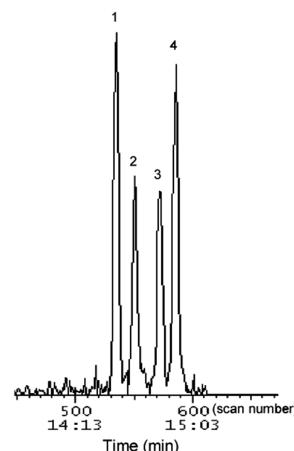

Figure 3. Product ion mass chromatogram of DMES derivatives of guaiacol estrones (100pg/tube).
Peaks: 1, 4OH$E_1$4Me; 2, 2OH$E_1$3 Me; 3, 2OH$E_1$2Me; 4, 4OH$E_1$3Me. GC/MS/MS conditions were described in the experimental section.

The mass spectra of DMES derivatives of E_1, E_2, 2OH$E_1$2Me and 16oxoE_2 showed an ion at *m/z* 356 ([M]$^+$), *m/z* 444 ([M]$^+$), *m/z* 342 ([M–CH_3–C_2H_5]$^+$) and *m/z* 429 ([M–C_2H_5]$^+$), respectively, as the base peak ion, so that these ions were chosen as the precursor ions in the GC/MS/MS (Figure 4a). The product ion mass spectra of E_1– and 2OH$E_1$2Me–DMES showed the prominent ion ([precursor ion–85]$^+$) at *m/z* 271 and *m/z* 257, respectively, which were assigned as the ions formed by dissociation of the steroid C– and D–ring and rearrangement of hydrogen (Figure 4b) [9]. The mass spectra and product ion mass spectra of the other guaiacol estrone isomers did not show such a clear difference from those of 2OH$E_1$2Me. The product ion mass spectrum of E_2–DMES showed ions at *m/z* 299 and *m/z* 340. The former product ion was formed by dissociation of the D–ring and the latter ion corresponded to [M–DMESOH]$^+$. Although the intensity of the latter ion was lower than that of the former, the ion at *m/z* 340 was chosen as the monitoring ion because of responsible peak purity. 16oxoE_2–DMES produced an ion at *m/z* 337 in GC/MS/MS and it was used as a monitoring ion, but the origin was not assigned. Using these product ions as the monitoring ions, the signal to noise ratio (S/N) of every authentic estrogen at 5 pg/injection was higher than 5.

3.2. Determination of E_1 and E_2 in rat brains

A pilot study was performed to optimize the extraction and purification methods. The recovery rates of authentic E_1, E_2 (50–

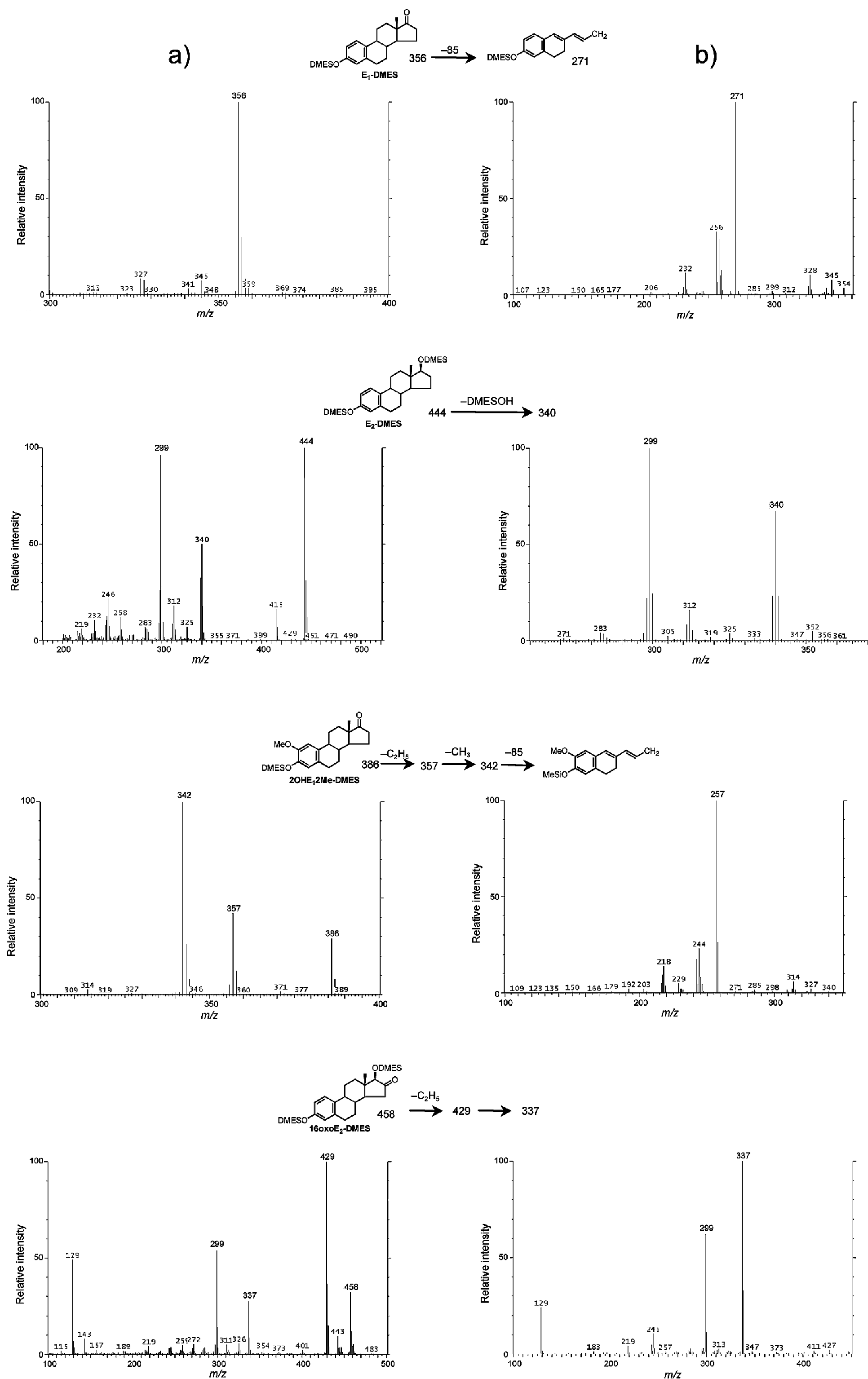

Figure 4. Mass spectra (a) and product ion mass spectra (b) of DMES derivatives of E_1, E_2, $2OHE_12Me$ and $16oxoE_2$.

400pg) and their ISs ([$^2H_5$]–E_1 and –E_2; each 1 ng) from silica–gel mini–column chromatography and solid–phase extraction were around 60–80%. To examine the effect of endogenous substances, ISs were added to the brain homogenate, and then extracted and purified successively by silica–gel mini–column chromatography, solid–phase extraction, and then subjected to GC/MS/MS. The peaks corresponding to the DMES ethers of ISs were detected clearly without interference by other peaks. However, the areas of these peaks tended to decrease as the analyses were repeated owing to endogenous interfering substances; therefore, further purification using preparative HPLC is necessary prior to derivatization to a DMES ether. E_1 and E_2 were separated using the ODS column and MeCN–H_2O system as a mobile phase. On the other hand, these were not separated using a MeOH–H_2O system, so that both estrogens and ISs were obtained in the same fraction.

Then the reproducibility and accuracy were examined in order to validate the method. The overall absolute recovery rates were examined using ISs (1ng) which were added to the brain homogenate. Although the relative standard deviation (RSD) in the same assay was lower than 13%, the recovery rate varied in the individual assay (48–68%, n=3). For the assessment of linearity, increasing amounts of E_1 and E_2 (0, 50, 100pg) and ISs (1ng) were added to the brain homogenate (corresponding to 0.6g of tissue) and assayed, and the typical product ion mass chromatograms were shown in Figure 5. The peak area ratios of the DMES derivatives of E_1 and E_2 toward those of ISs (y) were plotted versus the added amount of E_1 and E_2 (x) to obtain the regression lines (Figures 6a, b). These showed almost satisfactory linearity with correlation coefficient values of more than 0.981 and 0.977, respectively. However, the slope of the lines varied in the individual assay {E_1: 7.15 x 10^{-4}, 20.5%; E_2: 1.05 x 10^{-3}, 49.7% (mean, RSD, n=4)}, and difference from that of standard sample (E_1: 9.00 x 10^{-4}, 4.9%; E_2: 8.82 x 10^{-4}, 6.5%, n=3) was observed.

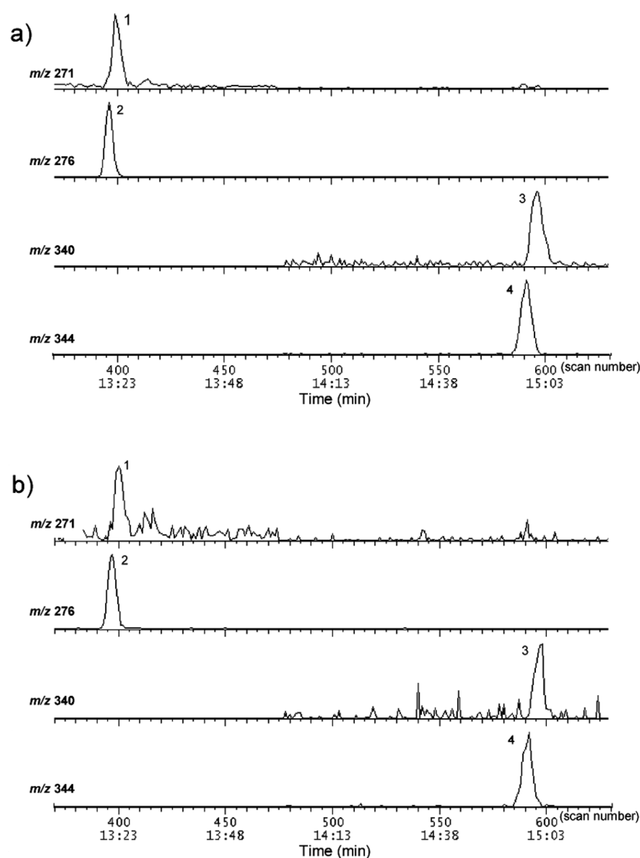

Figure 5. Product ion mass chromatograms of DMES derivatives of E_1, E_2 (50pg/tube) and ISs (1ng/tube).
a) Authentic samples, b) extracted E_1, E_2 and ISs added to rat brain homogenate. Peaks: 1, E_1; 2, [$^2H_5$]–E_1; 3, E_2; 4, [$^2H_5$]–E_2.

Therefore, the standard addition method was applied for the determination of E_1 and E_2 in rat brains (n=4), and the procedure was shown in Figure 2. The concentration of E_1 and E_2 in rat brains was determined from the distance between the intersection of the line with the x axis and the origin of the coordinates. When the peak corresponding to E_1 or E_2 was not detectable on the chromatogram, without addition of estrogens, the concentration of E_1 or E_2 was expressed as 0 pg/g tissue. The measured brain concentration of E_1 and E_2 were 0, 0, 0, 44.2pg/g tissue and 0, 15.4, 159.0, 37.2 pg/g tissue, respectively.

3.3. Determination of guaiacol estrones in rat brains

First of all, we attempted to apply the above pretreatment method for determination of guaiacol estrones in order to analyze E_1 and E_2 simultaneously. Authentic samples (0.1–100ng) were subjected to silica–gel mini column chromatography, solid phase extraction, and the elution conditions were examined. Although the silica–gel mini–column chromatography was effective for purification of biological samples, the recovery rates of low amounts of guaiacol estrones (<10ng) were not satisfactory (<10%). These low recovery rates might be caused by irreversible adsorption of the guaiacol estrones on silica–gel, which may depend on the guaiacol structure of the target compound. In the previous paper, we reported that the derivatization to acetate was useful for the treatment of catechol estrogens in the complex matrix and prevention of adsorption [6, 7]. These data prompted us to apply acetylation for determination of guaiacol estrones. Authentic samples (100pg) were acetylated with acetic anhydride–pyridine, subjected to silica–gel mini–column chromatography, solid–phase extraction, and then hydrolyzed with 1% KOH in MeOH. After derivatization with DMESI, the sample was analyzed with GC/MS/MS as shown in Figure 2. The recovery rate was improved and raised higher than 90%.

It was obvious that a stable isotope labeled compound, such as

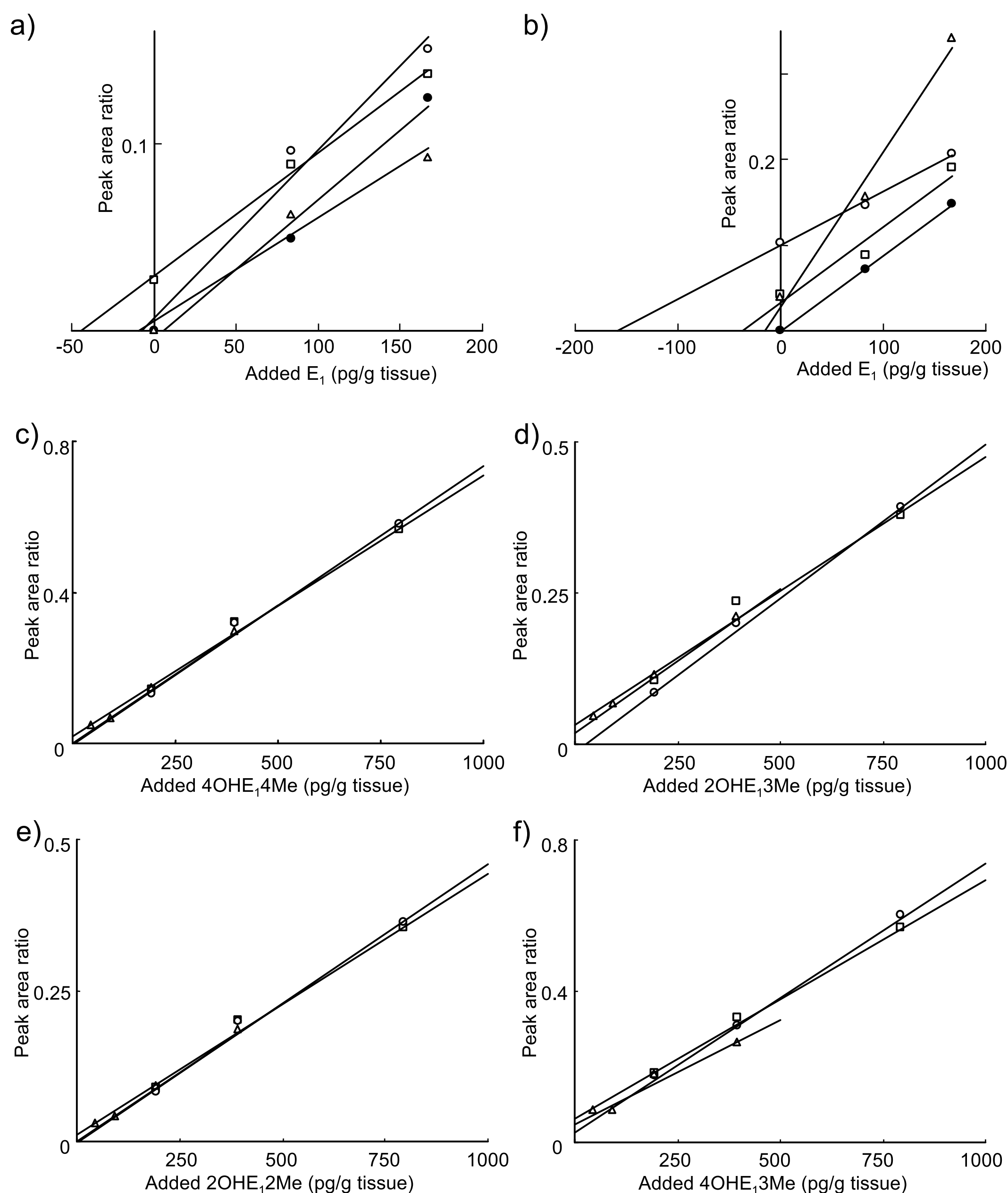

Figure 6. Determination of E_1 (a), E_2 (b) and guaiacol estrones (c–f) in rat brains using standard addition method.

Table 1. Absolute recovery of guaiacol estrones and IS

Add (pg/tube)	Recovery (%)[a]				
	$2OHE_12Me$	$2OHE_13Me$	$4OHE_13Me$	$4OHE_14Me$	$16oxoE_2$
0	N.D.[b]	N.D.	N.D.	N.D.	N.D.
100	65.9	75.5	74.2	65.8	
400	74.3	71.0	70.7	75.7	
(10ng)					63.0

a) Mean (n=3).

b) N.D. = below the detection limit (5pg/ injection; S/N>5).

the deuteride, was superior for IS in GC/MS analysis; however, the hydrolysis step could cause deuterium–hydrogen exchange. We chose 16oxoE_2 as an IS for analysis of guaiacol estrones, which is not an endogenous compound and is clearly separated from guaiacol estrones in the GC/MS.

To estimate the overall recovery rates, guaiacol estrones (0, 100, and 400pg) and IS (10ng) were added to rat brain homogenate (corresponding to 0.5g of tissue), extracted and treated as above, followed by derivatization and GC/MS/MS analysis. The recovery rates were almost satisfactory taking into consideration the above pretreatment method (Table 1); however, the peaks corresponding to guaiacol estrones were not detectable on the chromatogram of the samples where guaiacol estrones were not added. So the intra–assay reproducibility was examined using brain homogenate to which was added guaiacol estrones (100pg) and IS (10ng). RSD (n=4) of the peak area ratios of 2OH$E_1$2Me, 2OH$E_1$3Me, 4OH$E_1$3Me and 4OH$E_1$4Me toward that of IS were 9.4%, 12%, 5.8% and 8.4%, respectively.

In order to assess the linearity, increasing amount of guaiacol estrones (25–400pg) and IS (10ng) were added to the brain homogenate (corresponding to 0.5g of tissue) and assayed. The linearity was satisfactory for all guaiacol estrones; that is, the correlation coefficient values were more than 0.990. Therefore, we attempted to determine guaiacol estrones using the standard addition method, and the obtained regression lines were shown in Figures 6c–f. Although some lines showed the intersection with the *y* axis, the obtained values were quite smaller than the amount of added standard guaiacol estrones, so that the determination was not reliable. Thus, guaiacol estrones in rat brain were not determined by using the method.

4. Discussion

We developed a method to determine E_1, E_2 and guaiacol estrones in rat brains using GC/MS/MS and the standard addition method. The standard addition method needs large amount of sample and is somewhat tedious; however, the method is required to remove the effect of endogenous substances especially for analyzing the low amount of compounds in a complex matrix such as the brain.

Rahimy *et al*. reported the E_2 value in rat brain is *ca*. 150–290pg/g tissue [10], which is measured with radioimmunoassay and compatible with our results. But E_1 and E_2 levels in rat brains were not much higher than those in rat serum (*ca*. 140–240pg/ml, *ca*. 100–150pg/ml, respectively) [11], so it is ambiguous that estrogens act as neurosteroids [1]. The individual differences were also observed in male rat brains regarding E_1 and E_2, although estrogen levels in serum of male rats do not fluctuate unlike those of female rats which have an estrous cycle. Additional experiments are required in order to clarify this ambiguity, and the developed method would be helpful to elucidate the relationship between estrogen levels and the neural state.

Although we reported the existence of guaiacol estrones using two or three whole brains [5], the developed method using a small sample of the brain could not give the reliable determination data of guaiacol estrones. However, we have already demonstrated the existence of hydroxylase and catechol–*O*–methyltransferase in the brain to give catechol estrogens and guaiacol estrogens, respectively [12]. The development of a more sensitive determination method, such as immunoassay, is necessary to overcome this discrepancy.

Acknowledgement

We thank Teikoku Hormone Mfg. and Nippon Kayaku for supplying estrogens. This work was supported in part by a grant from the Ministry of Education, Science, Sports and Culture of Japan.

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