ELSEVIER

Journal of Chromatography A, 847 (1999) 171–178

JOURNAL OF CHROMATOGRAPHY A

Short communication

Studies on neurosteroids IX. Characterization of estrogens in rat brains using gas chromatography–tandem mass spectrometry

Kazutake Shimada*, Kuniko Mitamura, Miho Shiroyama, Kenichiro Yago

Faculty of Pharmaceutical Sciences, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-0934, Japan

Abstract

The characterization of the classical estrogens (estrone, estradiol, estriol) and guaiacol estrogens (2-hydroxyestrone 3-methyl ether, 4-hydroxyestrone 3-methyl ether) in rat brains was performed using gas chromatography–tandem mass spectrometry (GC–MS–MS). Estrogens were purified from Wistar strain rat brains by some chromatographic pre-treatments, such as solid-phase extraction, preparative thin-layer chromatography or preparative high-performance liquid chromatography. After the derivatization with *O*-methylhydroxylamine and/or *N,O*-bis(trimethylsilyl)trifluoroacetamide, estrogens were identified by comparison of their chromatographic behavior during GC–MS–MS operating in the product ion scan mode and comparison with the product ion MS spectra of an authentic sample. These evidences suggested that estrogens exist in rat brains as neurosteroids or neuroactive steroids.

Keywords: Steroids; Neurosteroids; Estrogens

1. Introduction

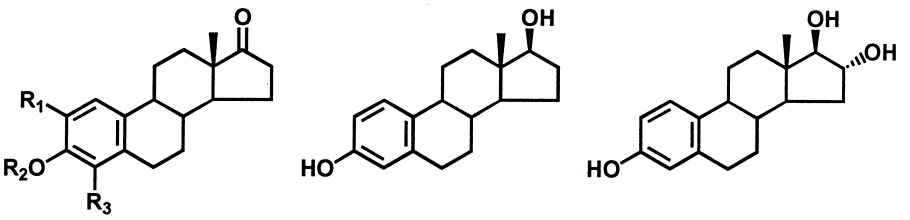
Since the discovery of dehydroepiandrosterone in rat brains, several 17- and 20-oxosteroids, called ‘neurosteroids’, have been elucidated in mammalian brains [1]. The neurosteroids that exist in rat brains are reported as the free form, sulfates, lipoidal esters and sulfolipids [2], and significant interest has thus been focused on their biological properties in this organ [3]. We previously clarified the existence of pregnenolone, dehydroepiandrosterone and their conjugates (sulfate, fatty acid ester) in rat brains using liquid chromatography–atmospheric pressure chemical ionization-mass spectrometry (MS) [4,5]. We also clarified that pregnenolone and its 3-stearate in rat brains increased and decreased after acute stress, respectively [6]. These data suggested that the fatty acid ester is the storage form of the free steroid in rat brains, which is excreted after acute stress.

Although the usefulness of estrogen-replacement therapy for dementia of the Alzheimer type [7], and the existence of estrogen receptors in brains [8] have recently been reported, the ambiguity still remains regarding the existence of estrogens in brains.

In this report, we characterized the classical estrogens (estrone (E_1), estradiol (E_2), estriol (E_3)) and guaiacol estrogens (2-hydroxyestrone 3-methyl ether ($2OHE_13Me$), 4-hydroxyestrone 3-methyl ether ($4OHE_13Me$)) in rat brains, which was performed using gas chromatography–tandem MS (GC–MS–MS) (Fig. 1).

*Corresponding author. Fax: +81-76-2344459.

0021-9673/99/$ – see front matter
PII: S0021-9673(98)01083-8



E_1: R_1=R_2=R_3=H E_2 E_3

2OH$E_1$2Me: R_1=OCH_3, R_2=R_3=H

2OH$E_1$3Me: R_1=OH, R_2=CH_3, R_3=H

4OH$E_1$3Me: R_1=H, R_2=CH_3, R_3=OH

4OH$E_1$4Me: R_1=R_2=H, R_3=OCH_3

Fig. 1. Structures of estrogens.

2. Experimental

2.1. Materials, reagents and animals

Classical estrogens (E_1, E_2, E_3) were donated by Teikoku Hormone Mfg. (Tokyo, Japan) and guaiacol estrogens (2OH$E_1$2Me, 2OH$E_1$3Me, 4OH$E_1$3Me, 4OH$E_1$4Me) were prepared in our laboratories [9]. Silica gel mini-column chromatography (6×0.6 cm I.D.) was performed with Silica gel 60 (70–230 mesh; E. Merck, Darmstadt, Germany). Preparative (prep.) thin-layer chromatography (TLC) (20×20 cm) was conducted with 0.5 mm pre-coated Silica gel $60F_{254}$ (E. Merck). ISOLUTEC18 (EC) cartridges (500 mg; International Sorbent Tech., Mid-Glamorgan, UK) were obtained from Uniflex (Tokyo, Japan). *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and NH_2OCH_3·HCl were obtained from Wako (Osaka, Japan) and Tokyo Kasei Kogyo (Tokyo), respectively, and all other reagents were of analytical grade and commercially available. Wistar strain rats (7 weeks old, 120–190 g) were obtained from Japan S.L.C. (Hamamatsu, Japan).

2.2. Apparatus

A Finnigan MAT GCQ gas chromatograph–ion trap mass spectrometer (Austin, TX, USA) equipped with a 5% diphenyl–95% dimethylpolysiloxane capillary column Rtx-5MS (30 m×0.25 mm I.D., 0.25 μm d_f; Restex, Bellefonte, PA, USA) was used with the following conditions: electron impact ionization (70 eV), positive ion mode, carrier gas (helium; linear flow-rate, 40 cm/s), transfer line temperature (275°C), ion source temperature (200°C), and injector temperature (300°C). The column oven temperature was set at 60°C for 1 min, ramped to 280°C at 25°C/min and held at 280°C for 5 min. The applied collision energy used for the MS–MS analysis was 1.5 V (for derivatives of E_1 and guaiacol estrogens) or 1.0 V (for derivatives of E_2 and E_3).

Preparative high-performance liquid chromatography (prep. HPLC) was performed on a Shimadzu LC-6A chromatograph (Kyoto, Japan) equipped with a Shimadzu SPD-10A UV (280 nm) detector. A Develosil ODS-5 (15×0.46 cm I.D., 5 μm; Nomura Chem., Seto, Japan) column was used at the flow-rate of 1 ml/min under ambient conditions.

2.3. Derivatization of estrogens

2.3.1. Methyloxime (MO)

Two percent NH_2OCH_3·HCl in pyridine (0.3 ml) was added to the sample and the mixture was heated at 70°C for 1 h. The entire solution was extracted with EtOAc and the organic layer was successively washed with chilled 5% HCl and water, and then the solvent was evaporated under a N_2 gas stream.

2.3.2. Trimethylsilyl ether (TMS)

BSTFA was added to the sample and the mixture was heated at 60°C for 30 min. The solvent was evaporated under a N_2 gas stream.

2.4. Procedure for characterization of estrone and guaiacol estrogens in rat brains

Wistar strain rats (2 males, 2 females) were decapitated and the whole brains (ca 1 g) were homogenized in isotonic saline (2 ml) under ice-cooling. The homogenate was deproteinized with EtOH (3 ml) and centrifuged at 1500 *g* for 30 min. The precipitate was further extracted with EtOH (3 ml) and all the supernatants were recentrifuged at 1500 *g* for 30 min. The obtained supernatant was diluted with water (50 ml) and applied on an ISOLUTEC18 (EC) cartridge. After washing with water (5 ml), 20% EtOH (2 ml), and hexane (2 ml), the desired steroids were eluted with 80% EtOH (2.5 ml) and the solvent was evaporated under a N_2 gas stream. After the derivatization with $NH_2OCH_3 \cdot HCl$, the obtained residue was applied on a silica gel mini-column chromatograph and washed with hexane (10 ml) and hexane–EtOAc (10:1) (5 ml). The fraction eluted with hexane–EtOAc (7:1) (10 ml) was evaporated under a N_2 gas stream and the obtained residue was applied on a GC–MS–MS for comparison with authentic samples (E_1, retention time (t_R) 12.85 min; $4OHE_1$4Me, t_R 13.58 min; $2OHE_1$2Me, t_R 13.80 min; $4OHE_1$3Me, t_R 14.02 min; $2OHE_1$3Me, t_R 14.17 min: precursor ion; m/z 268 $[M-OCH_3]^+$ (for E_1), m/z 329 $[M]^+$ (for guaiacol estrogens)).

Part of the above residue was subjected to trimethylsilylation and then applied on a GC–MS–MS (E_1, t_R 12.80 min; $4OHE_1$4Me, t_R 13.63 min; $2OHE_1$3Me, t_R 13.90 min; $2OHE_1$2Me, t_R 13.97 min; $4OHE_1$3Me, t_R 14.08 min: precursor ion; m/z 340 $[M-OCH_3]^+$ (for E_1), m/z 401 $[M]^+$ (for guaiacol estrogens)).

2.5. Procedure for characterization of estradiol in rat brains

Two Wistar strain rats (2 males, 2 females) were decapitated and the whole brains (total 3 g) were homogenized in isotonic saline and the homogenate was extracted with EtOAc–$CHCl_3$ (6:1) (10 ml) and centrifuged at 1500 *g* for 10 min. The precipitate was further extracted twice and all the supernatants were combined and evaporated in vacuo. The obtained residue was applied on a silica gel mini-column chromatograph and washed with hexane (10 ml), hexane–AcOEt (10:1) (10 ml) and hexane–EtOAc (4:1) (15 ml). The fraction containing E_2 was eluted with hexane–EtOAc (2:1) (10 ml) and the solvent was evaporated under a N_2 gas stream. The obtained residue was applied on a prep. TLC plate and developed twice with the same developing solvent ($CHCl_3$–AcOEt (20:1)). The zone corresponding to R_f 0.32–0.59 was extracted with EtOAc and the solvent was evaporated in vacuo. The residue was applied on a prep. HPLC (mobile phase, MeOH–water (3:1); t_R 7.5–9.0 min). After evaporation of the solvent in vacuo, the obtained residue was subjected to trimethylsilylation and then applied on GC–MS–MS (E_2, t_R 12.95 min: precursor ion, m/z 416 $[M]^+$ or 285 $[M-C_6H_{15}OSi]^+$).

2.6. Procedure for characterization of estriol in rat brains

Two Wistar strain rats (2 males, 2 females) were decapitated and the whole brains (total 3 g) were homogenized in isotonic saline and treated as already described. The obtained residue was applied on a silica gel mini-column chromatograph and washed with hexane (10 ml), hexane–EtOAc (10:1) (10 ml), hexane–EtOAc (4:1) (15 ml), hexane–EtOAc (2:1) (10 ml) and hexane–EtOAc (1:2) (10 ml). The fraction containing E_3 was eluted with hexane–EtOAc (1:4) (10 ml) and the solvent was evaporated under a N_2 gas stream. The obtained residue was applied on a prep. TLC using $CHCl_3$–MeOH (10:1) as the developing solvent. The zone corresponding to R_f 0.30–0.53 was extracted with EtOAc and the solvent was evaporated in vacuo. The obtained residue was applied on a prep. HPLC (mobile phase, MeOH–water (2:3); t_R 10.3–12.0 min). After evaporation of the solvent in vacuo, the obtained residue was subjected to trimethylsilylation and then applied on GC–MS–MS (E_3, t_R 14.98 min: precursor ion, m/z 504 $[M]^+$ or 414 $[M-C_3H_{10}OSi]^+$).

3. Results

3.1. GC–MS–MS analysis of estrogens derivatives

The mass spectra of authentic estrogens deriva-

tives were recorded in GC–MS, and the predominant ions were selected as the precursor ions in GC–MS–MS. The major ions obtained in product ion mass spectra were listed in Table 1. The sum of the predominant product ions of each estrogen's derivative was selected as a monitoring ion in GC–MS–MS chromatograms.

3.2. Characterization of estrone and guaiacol estrogens

The brains of adult Wistar strain rats were homogenized, deproteinized and subsequently applied on a solid-phase extraction. The fraction containing E_1 and guaiacol estrogens was derivatized with *O*-methylhydroxylamine followed by purification using silica gel mini-column chromatography, and then the obtained residue was applied on GC–MS–MS using the product ion scan mode. The fragment ion corresponding to $[M-OCH_3]^+$ and the molecular ion were selected as the precursor ions for the derivatives of E_1 and guaiacol estrogens, respectively. Part of the above residue was further derivatized with BSTFA and subsequently applied on a GC–MS–MS. The peaks corresponding to both derivatives of E_1, $2OHE_13Me$ and $4OHE_13Me$, were detected in the mass chromatograms as shown in Fig. 2. The existence of these estrogens was also confirmed by comparison of their product ion mass spectra with authentic samples (Fig. 3), but the positional isomers ($2OHE_12Me$ and $4OHE_14Me$) of these guaiacol estrogens have not been clearly detected. All the examined specimens showed the existence of these estrogens independent of the sex of the animals used.

3.3. Characterization of estradiol and estriol

Compared with E_1 or guaiacol estrogens, much smaller amounts of E_2 and E_3 existed in the rat brains, so the following pre-treatment was employed to detect these classical estrogens. The brains of adult Wistar strain rats were homogenized, extracted with organic solvent and subsequently applied on a silica gel mini-column chromatograph, and then further purification using prep. TLC and prep. HPLC was necessary. The fraction containing E_2 or E_3 was treated with BSTFA and then applied on GC–MS–MS. The product ion mass spectra of both fractions showed the same ones as those of the authentic samples. The typical mass spectra of E_2 and E_3 obtained from rat brains using the corresponding molecular ion as the precursor ions are shown in Fig. 4. All the examined specimens showed the existence of these estrogens independent of the sex of the animals used.

Table 1
GC–MS–MS data on estrogens derivatives

Compound	Derivative	M_w	Precursor ion (m/z)	Product ion (m/z)
E_1	MO	299	268 $[M-OCH_3]^+$	251 (91)[a], 211 (100), 157 (74)
$2OHE_12Me$	MO	329	329 $[M]^+$	298 (100)
$2OHE_13Me$	MO	329	329 $[M]^+$	298 (100)
$4OHE_13Me$	MO	329	329 $[M]^+$	298 (100)
$4OHE_14Me$	MO	329	329 $[M]^+$	298 (100)
E_1	TMS-MO	371	340 $[M-OCH_3]^+$	323 (75), 283 (66), 231 (100)
$2OHE_12Me$	TMS-MO	401	401 $[M]^+$	371 (100), 370 (50)
$2OHE_13Me$	TMS-MO	401	401 $[M]^+$	371 (100), 370 (42)
$4OHE_13Me$	TMS-MO	401	401 $[M]^+$	371 (100), 370 (45)
$4OHE_14Me$	TMS-MO	401	401 $[M]^+$	371 (57), 370 (100)
E_2	TMS_2	416	416 $[M]^+$	326 (89), 285 (100)
			285 $[M-C_6H_{15}OSi]^+$	269 (83), 256 (88), 229 (100), 205 (81)
E_3	TMS_3	504	504 $[M]^+$	414 (100), 324 (41)
			414 $[M-C_3H_{10}OSi]^+$	324 (100)

[a]Relative intensity (%).

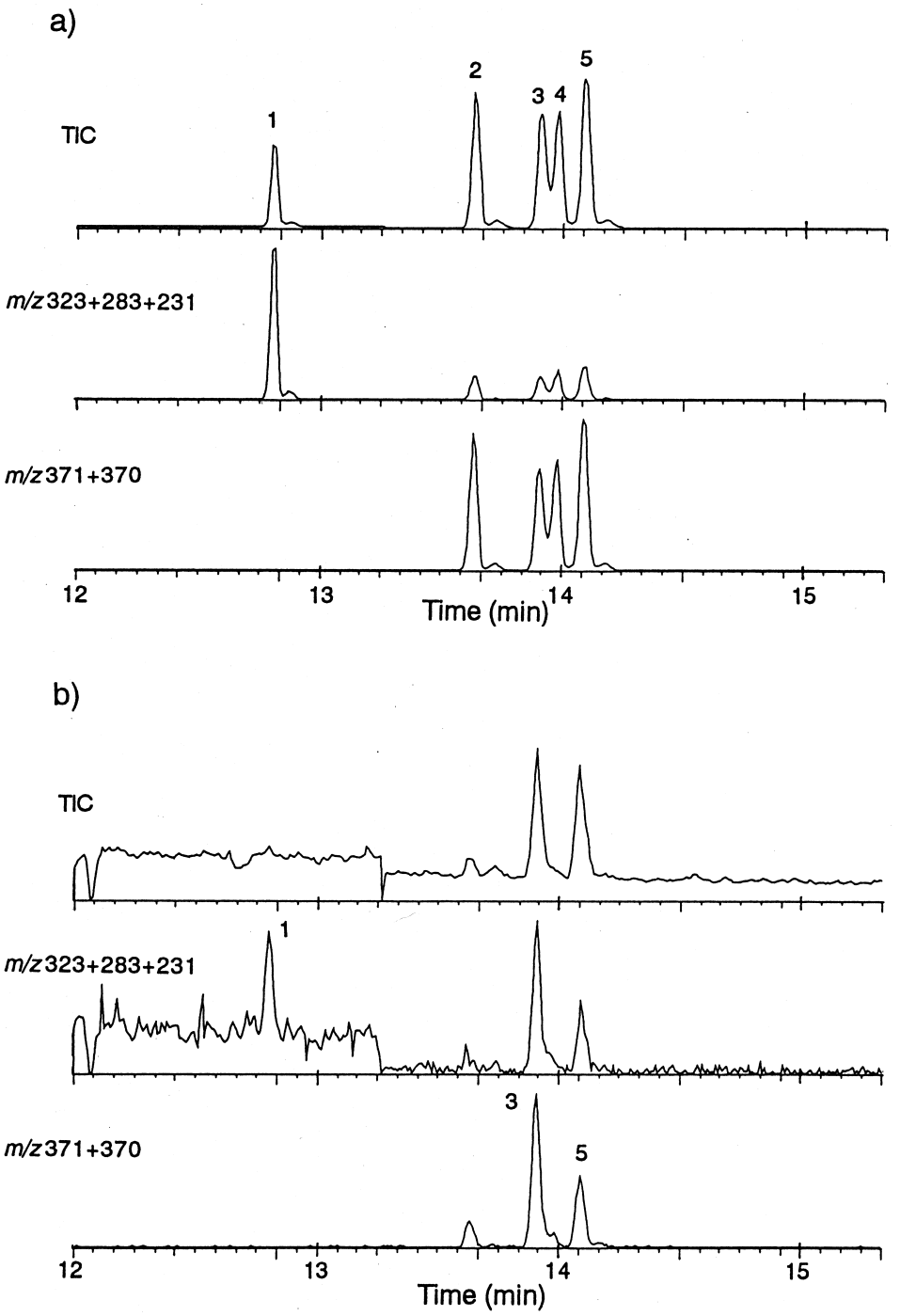


Fig. 2. GC–MS–MS chromatograms of E_1 and guaiacol estrogens as TMS-MO derivatives. (a) Authentic samples, (b) extracts obtained from rat brains. (1) E_1; (2) $4OHE_1$4Me; (3) $2OHE_1$3Me; (4) $2OHE_1$2Me; (5) $4OHE_1$3Me. Precursor ion, m/z 340 for E_1 (12.00–13.25 min) and m/z 401 for guaiacol estrogens (13.25–16.00 min); scan range, m/z 200–450.

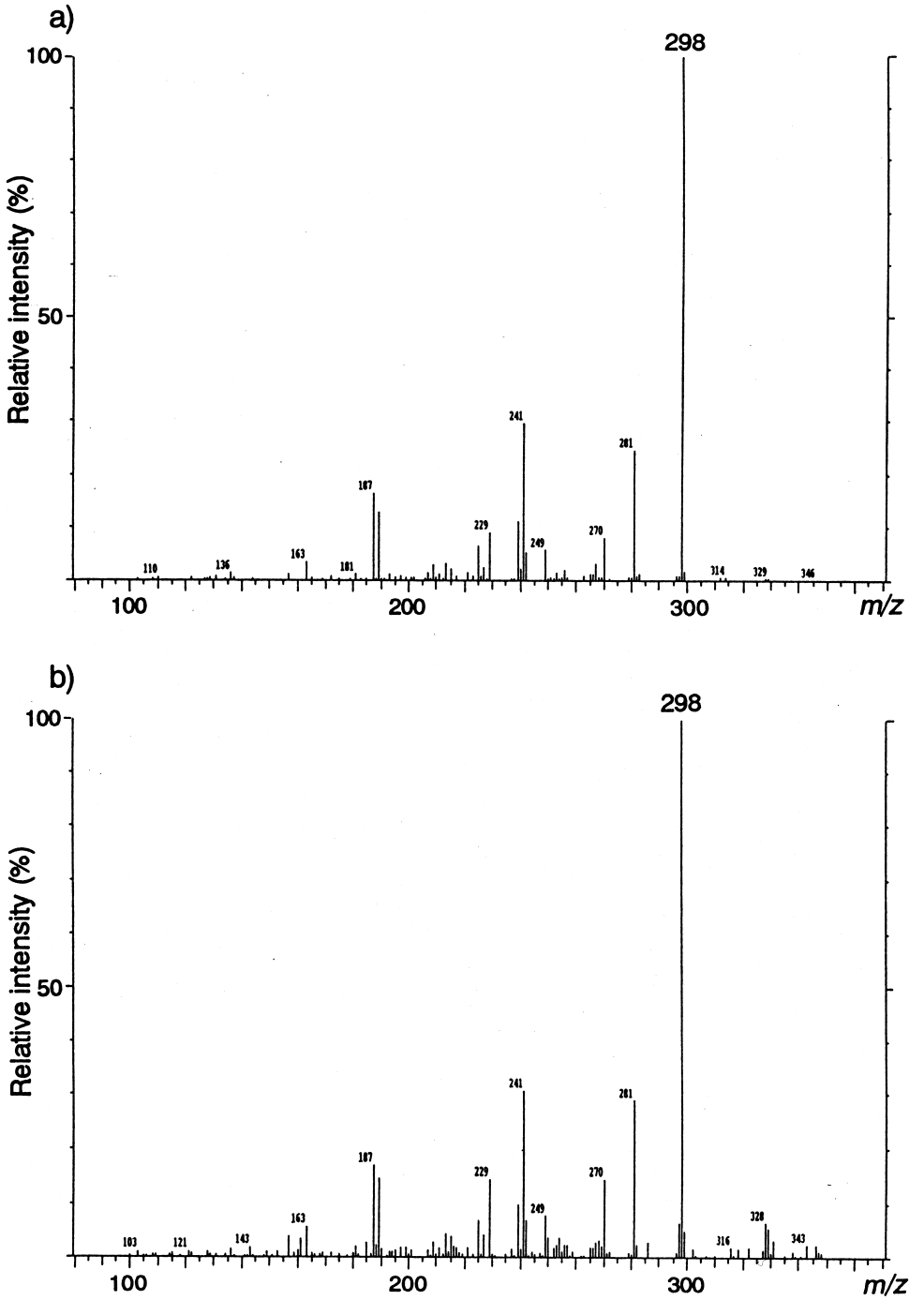


Fig. 3. Product ion mass spectra of 2OHE$_1$3Me as MO derivative. (a) Authentic sample, (b) extracts obtained from rat brains. Precursor ion, m/z 329.

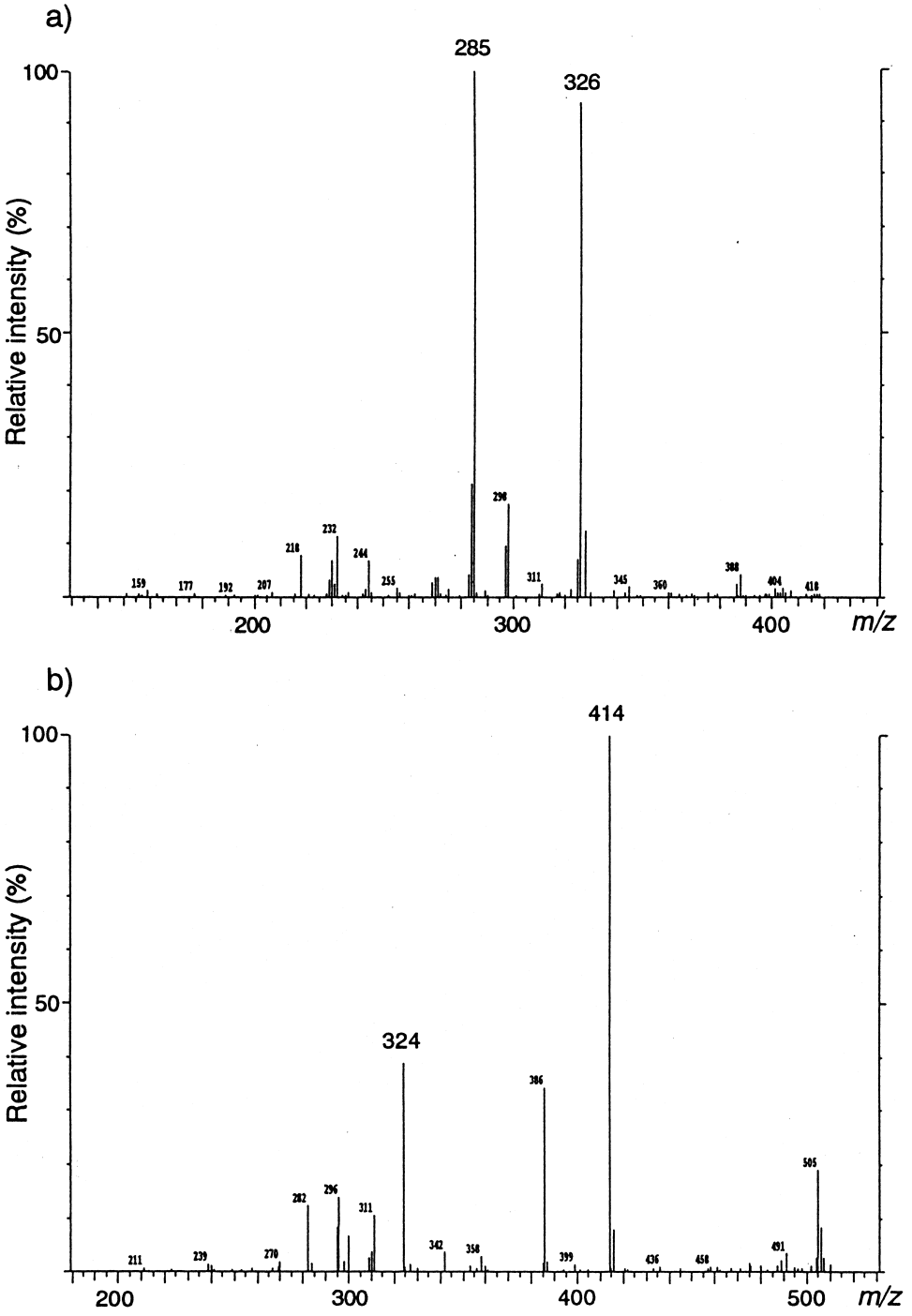


Fig. 4. Product ion mass spectra of E_2 and E_3 obtained from rat brains as TMS derivatives. (a) E_2, (b) E_3. Precursor ion, (a) m/z 416, (b) m/z 504.

4. Discussion

The usefulness of estrogen-replacement therapy for dementia of the Alzheimer type and the existence of estrogen receptors in brains have recently been reported, and much interest is focused on estrogens in brains. On the basis of radioimmunoassay, Bixo et al. reported the existence of E_2 in brains, but that of other classical estrogens (E_1, E_3) has not been clarified [10]. Although estrogens 2- and 4-hydroxylase activities in rat brains have been reported [11], ambiguity still remains for the existence of catechol estrogens in brains, together with their biological significance. For instance, Martucci reported that catechol estrogens ($2OHE_1$) and guaiacol estrogens ($2OHE_12Me$) have not been detected in rat brains [12]. In this report, we characterized the classical estrogens (E_1, E_2, E_3) and guaiacol estrogens ($2OHE_13Me$, $4OHE_13Me$) in rat brains, using GC–MS–MS.

Estrogens were purified from Wistar strain rat brains by some chromatographic pre-treatments, such as solid-phase extraction, prep. TLC or prep. HPLC. After the derivatization with *O*-methylhydroxylamine and/or BSTFA, estrogens were identified by comparison of its chromatographic behavior during GC–MS–MS and the obtained mass spectrum with an authentic sample. These results showed that not only E_2 but also other classical estrogens (E_1 and E_3) together with $2OHE_13Me$ and $4OHE_13Me$ were detected in the rat brains. We could not detect 2-methoxyestrogens, which is compatible with a previous report [12]. It is well known that 2- or 4-methoxyestrogens were detected in human serum [9], but 3-methoxyestrogens were obtained from rat brains instead of the 2- or 4-methoxyisomers as shown above. A study using the enzyme (catechol *O*-methyltransferase) which catalyzes *O*-methylation will clarify these discrepancies. All the above data suggest that estrogens exist in rat brains as neurosteroids or neuroactive steroids.

The development of a quantitative determination method for these estrogens in rat brains is now in progress in our laboratories, which will be used to clarify the physiological significance of these steroids.

Acknowledgements

This work was supported in part by a grant from the Ministry of Education, Science, Sport and Culture of Japan. We are grateful to Dr S. Honma (Teikoku Hormone Mfg.) for his helpful discussion and kind supply of classical estrogens.

References

[1] P. Robel, Y. Akwa, C. Corpéchot, Z.-Yi Hu, I. Jung-Testas, K. Kabbadj, C. Le Goascogne, R. Morfin, C. Vourch, J. Young, E.-E. Baulieu, in: M. Motta (Ed.), Brain Endocrinology, Raven Press, New York, 1991, pp. 105–132.
[2] C. Mathur, V.V.K. Prasad, V.S. Raju, M. Welch, S. Lieberman, Proc. Natl. Acad. Sci. USA 90 (1993) 85.
[3] P. Robel, E.-E. Baulieu, Trends Endocrinol. Metab. 5 (1994) 1.
[4] K. Shimada, Y. Mukai, A. Nakajima, Y. Naka, Anal. Commun. 34 (1997) 145.
[5] K. Shimada, Y. Mukai, K. Yago, J. Liq. Chromatogr. Relat. Technol. 21 (1998) 765.
[6] K. Shimada, Y. Mukai, J. Chromatogr. B 714 (1998) 153.
[7] H. Honjo, K. Tanaka, T. Kashiwagi, M. Urabe, H. Okada, M. Hayashi, K. Hayashi, Horm. Metab. Res. 27 (1995) 204.
[8] S. Mosselman, J. Polman, R. Dijkema, FEBS Lett. 392 (1996) 49.
[9] P. Ball, R. Knuppen, Acta Endocrinol. Suppl. 232 (1980) 1.
[10] M. Bixo, T. Backstrom, B. Winblad, A. Andersson, J. Steroid Biochem. Mol. Biol. 55 (1995) 297.
[11] C.N. Theron, V.A. Russell, J.J.F. Taljaard, J. Steroid Biochem. 28 (1987) 533.
[12] C.P. Martucci, J. Steroid Biochem. 19 (1983) 635.