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Studies on neurosteroids XIX Development and validation of liquid chromatography–tandem mass spectrometric method for determination of 5α-reduced pregnane-type neurosteroids in rat brain and serum

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Abstract

A sensitive liquid chromatography–electrospray ionization-tandem mass spectrometric (LC–ESI-MS–MS) method for the simultaneous determination of 5α-reduced pregnan-type neurosteroids, allopregnanolone (AP), epiallopregnanolone and 5α-dihydroprogesterone, in rat brain and serum has been developed and validated. The brain and serum steroids were extracted with methanol–acetic acid, purified using a Strata-X cartridge, derivatized with the permanently charged reagent, 2-hydrazino-1-methylpyridine (HMP), and subjected to LC-positive ESI-MS–MS. The limits of quantitation (LOQ) for brain (0.25 ng/g tissue) and serum (0.25 ng/ml) assays using the derivatization–ESI-MS–MS method are 60–150-fold lower than the LOQs for their atmospheric pressure chemical ionization-MS method without derivatization. [17α,21,21,21-$^{2}H_4$]-AP was used as an internal standard. This method allowed the reproducible and accurate quantification of the brain or serum neurosteroids using a 20 mg or 20 μl sample, respectively. That is, the intra- and inter-assay coefficients of variation were below 8.2 and 6.0%, respectively, and the % accuracy values were 98.5–103.0% for all the steroids in both the brain and serum. The application of the developed method to the analysis of changes in the brain and serum neurosteroid levels by immobilization stress and ethanol administration is also presented.

Keywords: Neurosteroids; LC–ESI-MS–MS; Derivatization; Rat brain; Immobilization stress; Ethanol administration

1. Introduction

Allopregnanolone (AP; 3α-hydroxy-5α-pregnan-20-one), one of the most important endogenous neurosteroids, binds with high affinity to γ-aminobutyric acid type A ($GABA_A$) receptors and positively modulates the action of GABA at these receptors [1]. It has been demonstrated that the brain AP is rapidly elevated to a nanomolar concentration by several acute stress paradigms, such as forced swimming [2,3] and electrical shock [4], in animal models, which is proposed to reflect a homeostatic mechanism for raising the threshold of brain excitability during the response to stressful stimuli [1]. For example, Purdy et al. found that the stress-induced increase in brain AP started within 5 min after exposure to the stressor and the concentration returned to the baseline level by 2 h [2]. Therefore, the down-regulation of the AP synthesizing activity may be involved in the stress-elicited disorders, such as depression [5]. In addition, there is evidence that the sedative and anxiolytic effects of ethanol [6,7] and the anti-depressant activity of selective serotonin reuptake inhibitors (SSRIs), such as fluoxetin [8], occur through the promotion of AP synthesis. On the other hand, epiallopregnanolone (EAP; 3β-hydroxy-5α-pregnan-20-one), the 3β-isomer of AP, can antagonize the GABAergic function of AP in rats [9], but its change in the brain and serum levels by acute stress or drug administration has been poorly examined. With this background information, a method for simultaneous determination of AP, EAP and their precursor, 5α-dihydroprogesterone (DHP; 5α-pregnane-3,20-dione), which also affects the brain functions via an intracellular progesterone receptor [10], in the brain and serum can contribute to the elucidation of their physiological roles and the development of new antipsychotic agents targeting neurosteroidogenesis.

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The brain and serum/plasma AP levels following exposure of animals to several acute stress paradigms or drug administration have been conventionally measured by radioimmunoassay (RIA) [2,4,6]. However, RIA requires handling of radioactive materials and extensive sample purification schemes, including preparative high-performance liquid chromatography (HPLC) due to interference from other endogenous steroids and lipids. Among the alternative methods, gas chromatography (GC)–electron capture negative chemical ionization-mass spectrometry (MS) has been proposed as the analytical procedure of choice for the determination of AP [3,7,8]. The studies using this technique demonstrated that the brain AP in the unstressed or saline administered (control) rat is in the trace level (practically none) and the forced swimming and ethanol administration elevated it to the ng/g tissue level [3,7]. Thus, GC–MS is a good methodology for neurosteroid analysis, but the method of Uzunov et al. also required extensive sample purification schemes [8]. The method developed by Vallée et al. [3] employed a simple procedure, i.e., a one-step solid phase extraction, for sample purification, but required a two-step derivatization.

Liquid chromatography (LC) coupled with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI)-MS has been recently proposed as the analytical procedure of choice for the determination of neurosteroids due to its specificity and versatility [11–13]. However, neurosteroids have rather low responses using either ESI or APCI and often cannot be quantified at low levels. Although the introduction of a newly developed API technique, atmospheric pressure photoionization (APPI), has also been examined for steroids [14,15], Leinonen et al. reported that APPI provided lower sensitivity than ESI for anabolic steroids, and the matrix effect appeared more sensitively in APPI than APCI [14]. Although APPI may be a promising technique for steroids, its performance in neurosteroid analysis has still not been proven. Based on this idea, derivatization suitable for each ionization method has been examined [11,12,16]. The best detectability with ESI-MS has been achieved in the analysis of compounds that are either ionic or can be readily ionized in solution. Based on this, Griffiths et al. used Girard reagent P (GP) as a reagent having a permanently charged moiety for the analysis of oxosteroids [17,18] and we developed a new permanently charged reagent, 2-hydrazino-1-methylpyridine (HMP), for oxosteroids [19]. The GP derivatives can be detected at the sub-picograms level and give structurally informative ions by ESI-MS–MS [17]. Thus, the GP derivatives has a significant advantage for the analysis of oxosteroids, but we have reported that the HMP derivatives are superior to the GP derivatives not only in their sensitivity, but also in their chromatographic behavior [19]. In the present paper, we describe an LC–ESI-tandem MS (MS–MS) method employing derivatization with HMP for the simultaneous determination of AP, EAP and DHP in the rat brain and serum using 20 mg of tissue or 20 μl of serum (Fig. 1). The application of the method to the analysis of changes in the steroid levels by immobilization stress and ethanol administration is also reported.

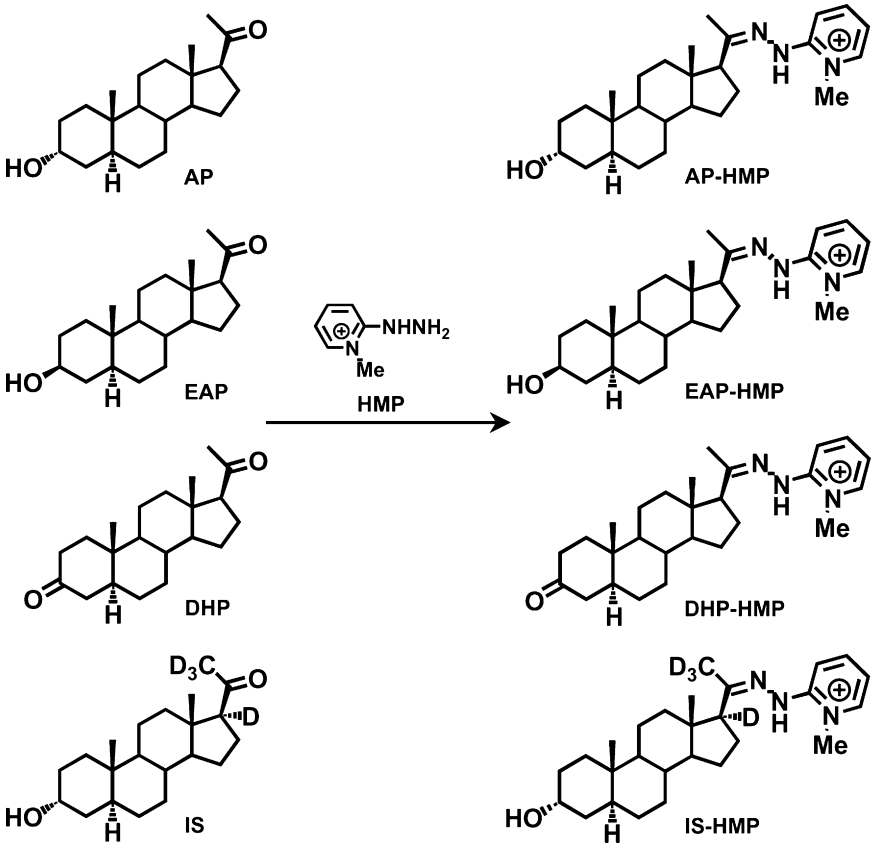

Fig. 1. Derivatization of 5α-reduced pregnane-type neurosteroids with HMP. D = deuterium (^{2}H).

2. Experimental

2.1. Materials and chemicals

AP, EAP, DHP, pregnanolone (P; 3α-hydroxy-5β-pregnan-20-one), epipregnanolone (EP; 3β-hydroxy-5β-pregnan-20-one) and 5β-dihydroprogesterone (5β-DHP; 5β-pregnane-3,20-dione) were purchased from Steraloids (Newport, RI, USA). Pregnenolone (PREG; 3β-hydroxypregn-4-en-20-one) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Stock solutions of AP, EAP and DHP were prepared as 100 μg/ml solutions in ethanol. Subsequent dilutions were carried out with ethanol to prepare 0.5, 1, 2, 5, 10 and 20 ng/ml solutions. [17α,21,21,21-$^2H_4$]-AP (internal standard: IS) was synthesized by a known method [20], dissolved and diluted with ethanol to prepare a 20 ng/ml solution. HMP was synthesized in our laboratories as previously reported [19]. Strata-X cartridges (60 mg adsorbent; Phenomenex, Torrance, CA, USA) were successively washed with ethyl acetate (2 ml), methanol (2 ml) and water (2 ml) prior to use. All other reagents and solvents were of analytical grade.

2.2. LC–MS–MS

LC–MS–MS was performed using an Applied Biosystems API 2000 triple stage quadrupole-mass spectrometer (Foster City, CA, USA) connected to a Shimadzu LC-10AT chromatograph (Kyoto, Japan). The HMP derivatives of steroids were analyzed by ESI-MS in the positive-ion mode and the conditions were as follows: declustering potential, 80 V; focusing potential, 200 V; entrance potential, 10 V; ion spray voltage, 5 kV; curtain gas (nitrogen), 45 psi; ion source gas 1 (nitrogen), 80 psi; ion source gas 2 (nitrogen), 80 psi; turbo gas temperature, 500 °C and interface heater, on. Nitrogen was used as the collision gas in the selected reaction monitoring (SRM) mode with a collision energy of 60 eV. The precursor and monitoring ions of the HMP derivatives were as follows: AP-HMP and EAP-HMP, *m/z* 424.3 and 108.2, DHP-HMP, *m/z* 422.3 and 108.2, IS-HMP, *m/z* 428.3 and 108.2. A YMC-Pack C8 column (5 μm, 150 × 2.0 mm i.d.; YMC, Kyoto) was used at 40 °C. Methanol–10 mM ammonium formate (8:3, v/v) was used at a flow rate of 0.2 ml/min as the mobile phase unless otherwise indicated. Intact steroids were analyzed with the selected ion monitoring (SIM) mode of positive APCI-MS and the conditions were as follows: declustering potential, 30 V; focusing potential, 380 V; entrance potential, 10 V; nebulizer current, 2 μA; curtain gas (nitrogen), 45 psi; ion source gas 1 (nitrogen), 80 psi; ion source gas 2 (nitrogen), 15 psi; vaporizer temperature, 450 °C and interface heater, off. The monitoring ions of the steroids were as follows: AP and EAP, *m/z* 319.0 and DHP, *m/z* 317.0. A J'sphere ODS H-80 column (4 μm, 150 mm × 2.0 mm i.d.; YMC) was used at 40 °C. Methanol–water (4:1, v/v) was used at a flow rate of 0.2 ml/min as the mobile phase. The data were collected and quantified using Applied Biosystems Analyst software (version 1.3.1). The smoothing options were as follows: default number of smooths, 5; previous point weight, 0.5; current point weight, 1 and next point weight, 0.5.

2.3. Treatment of rats

Wistar strain rats (7-week-old, male, 190–200 g) obtained from Japan S.L.C. (Hamamatsu, Japan) were assigned either to an untreated group ($n = 10$), a group subjected to immobilization stress ($n = 10$), a group receiving an intrapertioneal (i.p.) saline (5 ml/kg, Otsuka normal saline, Otsuka Pharmaceutical, Tokyo) injection ($n = 5$) or a group receiving an i.p. ethanol (99.5%, Wako Pure Chemicals, Osaka, Japan) injection ($n = 5$). Ethanol was diluted in saline and administered as a 20% (v/v) solution at a dose of 0.8 g/kg. All the animals were unrestrained in a quiet place for 2 h before the experiments. The untreated rats were then immediately euthanized by decapitation. The second group rats were immobilized on their backs on a board for 20 min. After immobilization, the rats were unrestrained for 30 min and then euthanized [11]. In the saline and ethanol-administration groups, 30 min after treatment the rats were euthanized. Blood was collected from the cut end immediately after the decapitation, left at 4 °C for 3 h, then centrifuged at 1500 × *g* (4 °C, 15 min). The serum was separated and stored at −20 °C. All animal studies were performed between 13:30 and 15:00 h. All animal care and use were approved by the Institutional Animal Care and Use Committee of Kanazawa University.

2.4. Pretreatment procedures

The whole brain (1.5–1.7 g) was homogenized in methanol–acetic acid (99:1, v/v) (ca. 10 ml) [21] using an ultrasonic homogenizer. The homogenate was centrifuged at 1500 × *g* for 15 min and the supernatant was saved. The precipitate was further extracted with methanol–acetic acid (99:1, v/v) (ca. 3 ml) and centrifuged. The supernatants were combined, diluted to their concentrations of 100 mg brain tissue/ml with methanol–acetic acid (99:1, v/v) and stored at −20 °C prior to use (this solution is called the brain extract).

The brain extract (0.2 ml: corresponding to 20 mg of brain tissue) was pipetted into a tube. After the addition of an ethanolic solution of IS (200 pg in 10 μl) and water (0.5 ml), the sample was passed through a Strata-X cartridge. After washing with water (2 ml), methanol–water (7:3, v/v) (2 ml) and hexane (1 ml), the steroids were eluted with ethyl acetate (1 ml). After evaporation, the residue was subjected to derivatization with HMP as described below.

Serum (20 μl) was added to methanol–acetic acid (99:1, v/v) (0.1 ml) containing IS (200 pg), vortex-mixed for 30 s and centrifuged at 1500 × *g* (4 °C, 5 min). The supernatant was diluted with water (0.5 ml) and purified in the same way as the brain sample. After evaporation, the residue was subjected to derivatization with HMP as described below.

2.5. Derivatization reaction

To the pretreated brain and serum samples in ethanol (30 μl), a freshly prepared solution of HMP (10 μg) in ethanol (50 μl) containing 25 μg of trifluoroacetic acid was added, and the mixture was subjected to an ultrasonic treatment in a CS-20

water bath (Shibata Scientific Technology, Tokyo; oscillation frequency, 46 kHz) at ambient temperature (ca. 20 °C) for 15 min. After removal of the solvents, the products were dissolved in methanol–water (1:1, v/v, 40 μl), 10 μl of which was subjected to LC–MS–MS.

2.6. Calibration curves for AP, EAP and DHP

Because AP, EAP and DHP were not detected in the brain extract and serum obtained from an untreated rat, these samples were used as the blank brain extract and blank serum, respectively, for the construction of calibration curves and validation studies (Sections 2.7 and 2.8). The blank brain extract (0.2 ml) or blank serum (20 μl) was spiked with AP, EAP and DHP [5,10, 20, 50 or 100 pg each: corresponding to 0.25, 0.5, 1.0, 2.5 or 5.0 ng/g tissue (brain) or ng/ml (serum)] and IS (200 pg), which was then pretreated, derivatized and subjected to LC–MS–MS. The calibration curves were constructed by plotting the peak area ratios [AP/IS, EAP/IS or DHP/IS] against the concentration of AP, EAP or DHP [ng/g tissue (brain) or ng/ml (serum)].

2.7. Recoveries of AP, EAP, DHP and IS during pretreatment

An ethanolic solution of AP, EAP and DHP (50 pg each in 10 μl) or ethanol (10 μl, control sample) was added to the blank brain extract (0.2 ml) or blank serum (20 μl) and the resulting samples were pretreated. AP, EAP and DHP (50 pg each) was then added to only the control sample and IS (200 pg) was added to both samples. After derivatization, the samples were subjected to LC–MS–MS. The recoveries of AP, EAP and DHP during pretreatment were calculated from the peak area ratios (AP/IS, EAP/IS or DHP/IS) of the spiked and control samples.

The ethanolic solution of IS (200 pg in 10 μl) or ethanol (10 μl, control sample) was added to the blank brain extract (0.2 ml) or blank serum (20 μl), and the resulting samples were pretreated. IS (200 pg) was then added to only the control sample and AP (50 pg) was added to both samples. After derivatization, the samples were subjected to LC–MS–MS. The recovery of IS during pretreatment was calculated from the peak area ratios (IS/AP) of the spiked and control samples.

2.8. Assay precision and accuracy

Quality control (QC) samples were prepared by adding an ethanolic solution of AP, EAP and DHP (200 pg or 2.0 ng each in 10 μl) to the blank brain extract (5.0 ml; corresponding to 0.5 g of brain tissue) or blank serum (0.5 ml). Final concentrations of QC samples were 0.4 or 4.0 ng/g tissue (brain) or ng/ml (serum). The intra-assay precision and accuracy were evaluated by analyzing multiple replicates ($n = 5$) of QC samples on the same day. The inter-assay precision and accuracy were evaluated by analyzing the QC samples on 5 days.

The inter-assay ($n = 5$) and intra-assay ($n = 5$) reproducibility of the assay was also evaluated by analyzing the brain and serum samples of rats that had been stressed. These samples contained endogenous neurosteroids in significant amounts.

3. Results and discussion

3.1. LC–ESI-MS–MS of HMP derivatives

The derivatization rates of standard AP and EAP with HMP were quantitative, because after derivatization of AP and EAP (10 ng each), intact steroids were not detected in LC–APCI-MS [the minimum detectable amounts were 70 pg (0.7% of the initial amounts) of AP and EAP without derivatization]. Also, when 10 ng of DHP was reacted, the remaining DHP [the minimum detectable amount was 50 pg (0.5% of the initial amount) of DHP without derivatization] was also not detected. Although DHP has two oxo-groups at the C-3 and 20 positions, the HMP-derivatization produced the mono-HMP derivative as the main product, and the yield of the 3,20-bis-HMP derivative was small; its peak area (monitoring ion: *m/z* 527.3 $[M–1]^+$) was below 7% of that of the mono-HMP derivative. Due to the formation of the *E*- and *Z*-isomers in the derivatization of oxosteroids with HMP, the derivatives often have twin peaks in their chromatograms, but 20-oxosteroids, such as AP and EAP, showed single peaks under the LC conditions of the present study. On the contrary, the HMP derivative of 3-oxosteroids, such as 5α-dihydrotestosterone, showed twin peaks with almost equal peak areas under these LC conditions. The mono-HMP derivative of DHP showed a single peak just like AP and EAP. Based on these data, the chemical structure of the HMP derivative of DHP was inferred to be that shown in Fig. 1.

For the ESI-MS operating in the positive-ion mode, the HMP derivatives of AP, EAP, DHP and IS provided only their molecular cations, $[M]^+$ (Fig. 2). The use of the SRM mode may allow for the discrimination and quantification of the neurosteroids from a biological matrix without the need for a long chromatographic separation, due to its high specificity. The product ion mass spectrum of the HMP derivatives employing the respective $[M]^+$ as the precursor ions and a 60 eV collision energy are shown in the insets of Fig. 2, in which a base product ion was observed at *m/z* 108.2. The product ion was assigned to be [*N*-methylpyridine + NH]$^+$ formed by the cleavage of the N–N bond of hydrazone. Based on these results, the SRM mode using the $[M]^+$ (AP and EAP, *m/z* 424.3; DHP, *m/z* 422.3 and IS, *m/z* 428.3) and [*N*-methylpyridine + NH]$^+$ (AP, EAP, DHP and IS, *m/z* 108.2) as the precursor and monitoring ions, respectively, was employed in the following studies.

Because the *m/z* values of precursor and monitoring ions of AP-HMP and EAP-HMP are exactly the same, complete chromatographic separation of these derivatives is necessary for their accurate quantification. When a YMC-Pack C8 column with a mobile phase of methanol–10 mM ammonium formate (8:3, v/v) was used, a satisfactory chromatographic separation of the derivatives was achieved with a short chromatographic run time (10 min per assay); AP-HMP, retention time (t_R) 8.2 min; EAP-HMP, t_R 7.0 min; DHP-HMP, t_R 7.3 min and IS-HMP, t_R 8.0 min (Fig. 3).

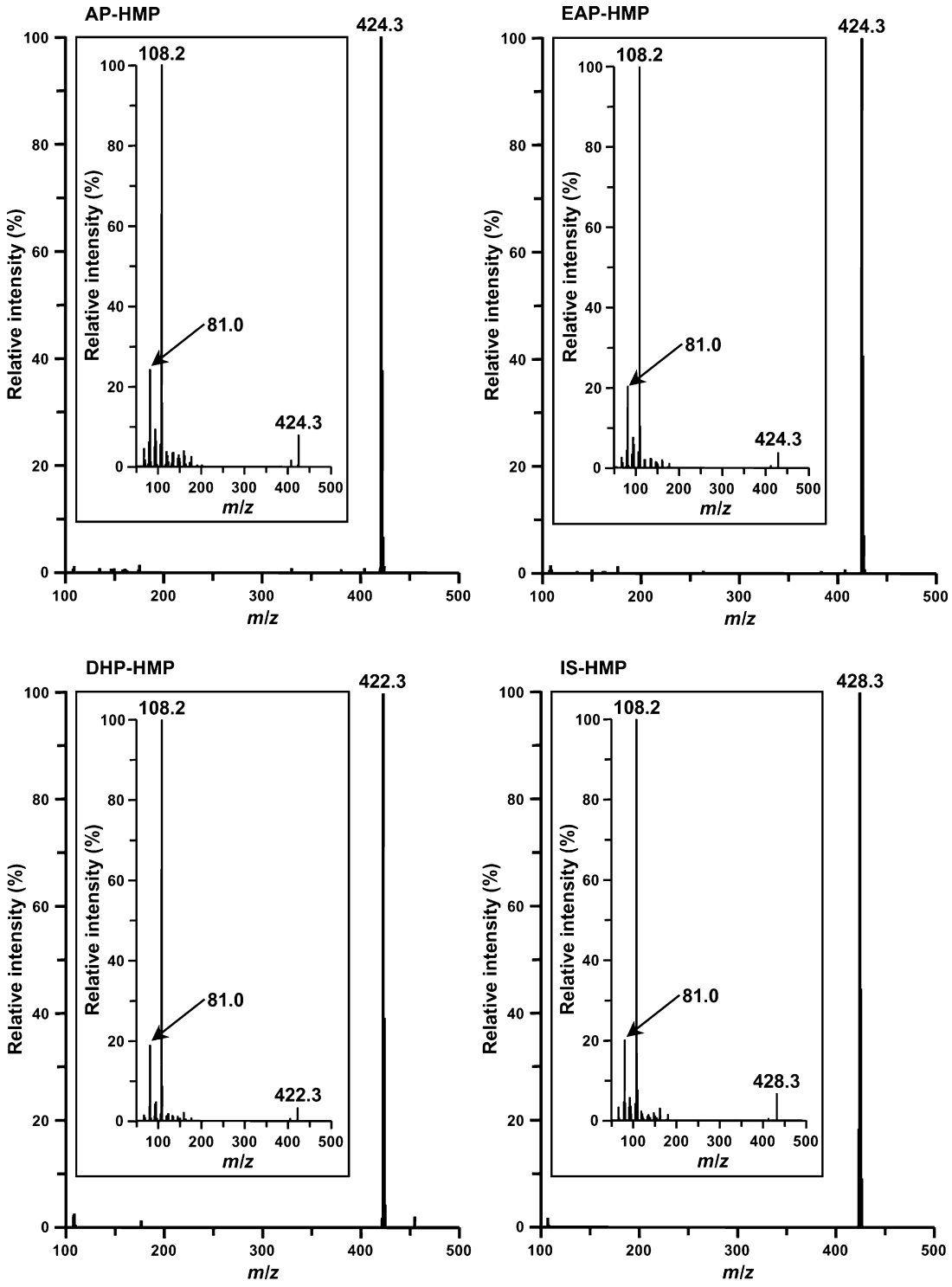

Fig. 2. ESI-MS and ESI-MS–MS spectra of the HMP derivatives of AP, EAP, DHP and IS. MS–MS spectra (insets) were recorded by the collisional activation of the respective $[M]^+$ with a 60 eV of collision energy. Other conditions were described in Section 2.2.

3.2. Pretreatment procedures

The brain sample was homogenized with methanol containing acetic acid according to the method of Liere et al., by which steroids are quantitatively extracted from the brain tissue [21], and the brain extract was then purified using a Strata-X cartridge that has a reversed-phase polymeric sorbent. The serum sample was deproteinized in methanol containing acetic acid and purified using the same cartridge. The neurosteroid fractions obtained from the brain and serum were then treated with excess HMP. Thus, our method employed only one step of SPE for the purification of the brain and serum samples, which is much simpler than the previously reported RIA [2,4,6] and GC–MS [8] methods. The recovery rates [mean ± standard

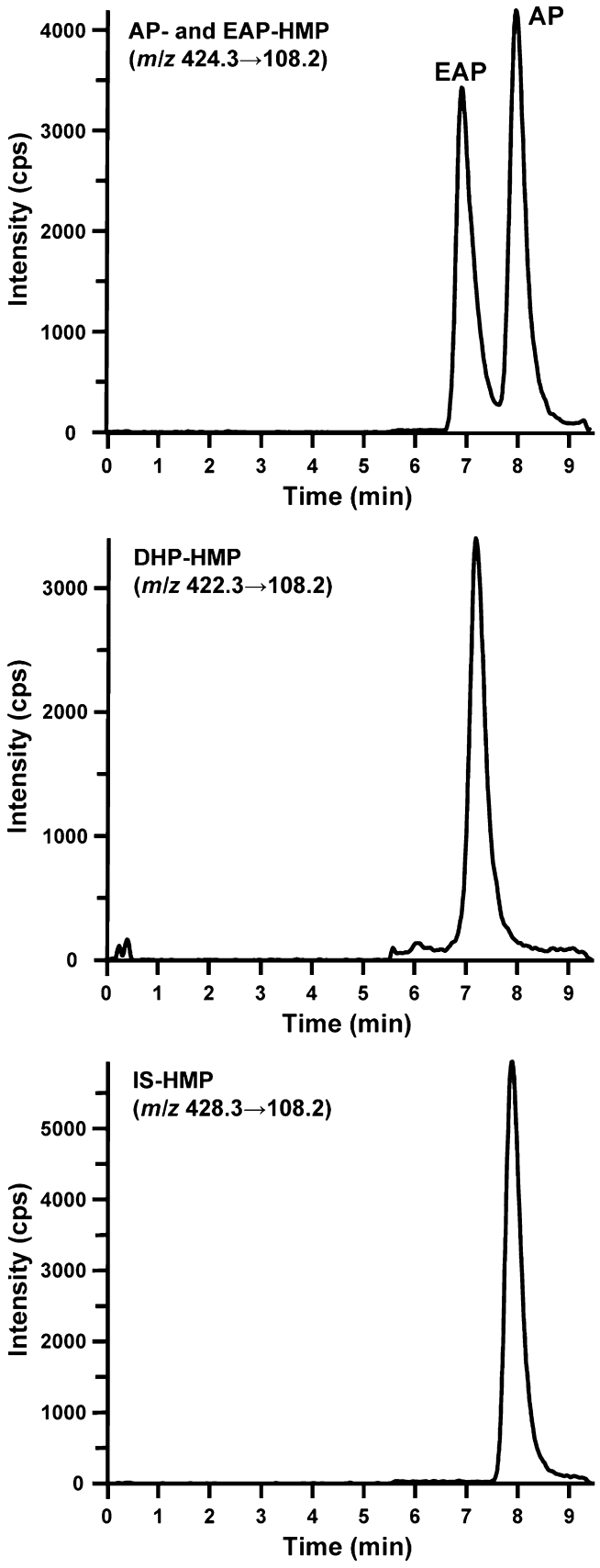

Fig. 3. Chromatograms of standard AP-, EAP-, DHP- and IS-HMP. The mixture of AP, EAP, DHP (100 pg each) and IS (200 pg) was derivatized and dissolved in methanol–water (1:1, v/v, 40 μl), 10 μl of which was subjected to LC–ESI-MS–MS. The LC–ESI-MS–MS conditions were described in Section 2.2.

deviation (S.D.), three different rats] of AP, EAP, DHP and IS during the pretreatment were 95.6 ± 1.5, 95.8 ± 2.1, 92.8 ± 1.8 and 93.7 ± 1.2%, respectively, in the brain assay and 85.5 ± 2.6, 85.1 ± 1.4, 85.8 ± 2.2 and 83.7 ± 2.7%, respectively, in the serum assay. The reproducibility of the recovery rates was satisfactory, and there was no significant difference between the compounds.

3.3. Specificity

The chromatograms shown in Figs. 4a and 5a were obtained from an untreated rat brain and serum, respectively, in which AP, EAP and DHP were not detected. These chromatograms revealed that there was no interfering peak derived from the endogenous components and the derivatization reagent at the elution positions of the derivatized AP, EAP and DHP.

Typical chromatograms of brain and serum samples obtained from a stressed rat are shown in Figs. 4b and 5b, respectively. The peaks corresponding to the derivatized AP, EAP and DHP were clearly observed at 8.2, 7.0 and 7.3 min, respectively, in the brain sample. In the serum sample, AP and DHP were detected, but the concentration of EAP was below the limit of quantitation (LOQ, see Section 3.5). Incidentally, the huge peak eluted at 6.2 min in the chromatograms for the brain and serum DHP is derived from PREG, whose brain level was over 40 ng/g tissue in the stressed rat in the previous study [11]. The HMP derivatives of P and EP, the 5β-isomers of AP and EAP, respectively, showed their molecular cations and product ions at *m/z* 424.3 and 108.2, respectively, which were quite the same as those of AP- and EAP-HMP. Moreover, P-HMP (t_R 7.9 min) and EP-HMP (t_R 7.1 min) were closely eluted with AP- and EAP-HMP, respectively. Therefore, the peak purities of the brain and serum samples were confirmed by a different mobile phase system; the t_R values of the derivatized AP (14.7 min) and EAP (11.9 min) in the brain and that of the derivatized AP in the serum completely agreed with those of the standard samples under the LC conditions using acetonitrile–10 mM ammonium formate (9:11, v/v) as the mobile phase, where the derivatized P (t_R 13.6 min) and EP (t_R 12.5 min) showed different t_R values. We further examined the occurrence of P and EP in the brain and serum samples obtained from five different rats, and they were not detected at all. Although the separation of the HMP derivatives of DHP and 5β-DHP (t_R 7.4 min) could not be achieved even if different columns and mobile phases were used, the latter was reported not to be present in the rat brain [22]. These data demonstrate that the other endogenous steroids do not interfere with the present assays.

3.4. Comparison of the sensitivity of ESI-MS analysis of HMP derivatives and APCI-MS analysis of intact steroids

To the brain extract (0.2 ml; corresponding to 20 mg brain tissue) obtained from an untreated rat, AP, EAP and DHP (5 pg each) were spiked (concentration, 0.25 ng/g tissue). This sample was pretreated and then derivatized with HMP. As shown in Fig. 6a, when the steroids were analyzed as their HMP derivatives by ESI-MS, the peaks were clearly observed with a signal

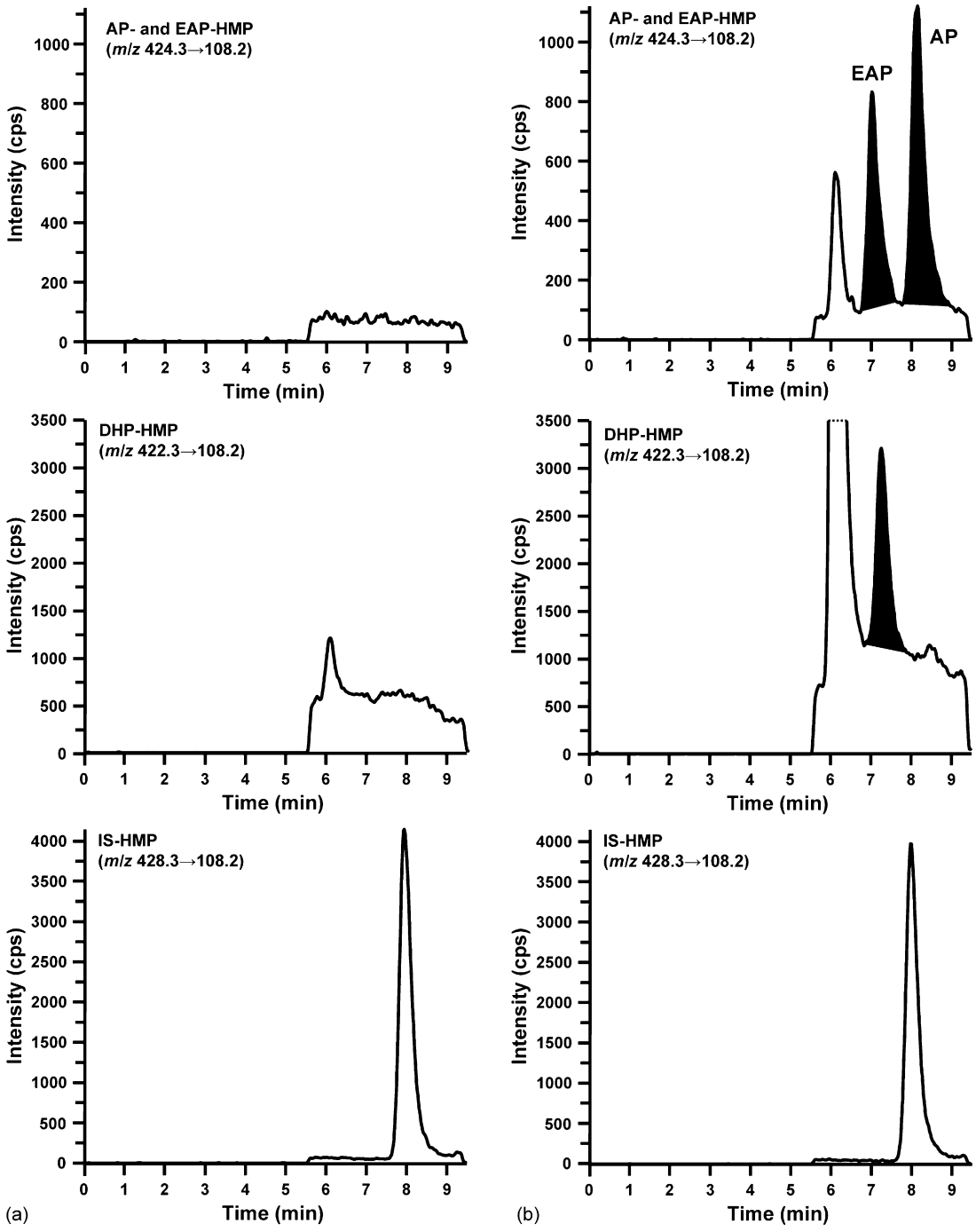

Fig. 4. Chromatograms of derivatized AP, EAP, DHP and IS in the brain of (a) the untreated and (b) stressed rats. The LC–ESI-MS–MS conditions were described in Section 2.2. The measured concentrations of AP, EAP and DHP in the stressed rat were 2.24, 1.31 and 3.73 ng/g tissue, respectively.

to noise ratio (S/N) of more than 6 at the concentration of 0.25 ng/g tissue. The S/N values were manually calculated by division of the peak height of a target analyte (painted in black in Fig. 6) by the noise level around the peak. On the contrary, when the same brain sample was analyzed without derivatization by APCI-MS, equal S/N values were at least obtained at the concentration of 25 ng/g tissue of AP, 37.5 ng/g tissue of EAP and 15 ng/g tissue of DHP [this sample was prepared by spiking AP (500 pg), EAP (750 pg) and DHP (300 pg) to the same brain extract (0.2 ml)] (Fig. 6b). These results prove that the HMP-derivatization increases the assay sensitivity by 100-, 150- and 60-fold for intact AP, EAP and DHP analyzed by APCI-MS, respectively.

3.5. Calibration curves for AP, EAP and DHP assays, LOQ and limits of detection (LOD)

The regression lines obtained from the combination of five different curves for AP, EAP and DHP are summarized in Table 1. All the CV values of the slope of five curves were

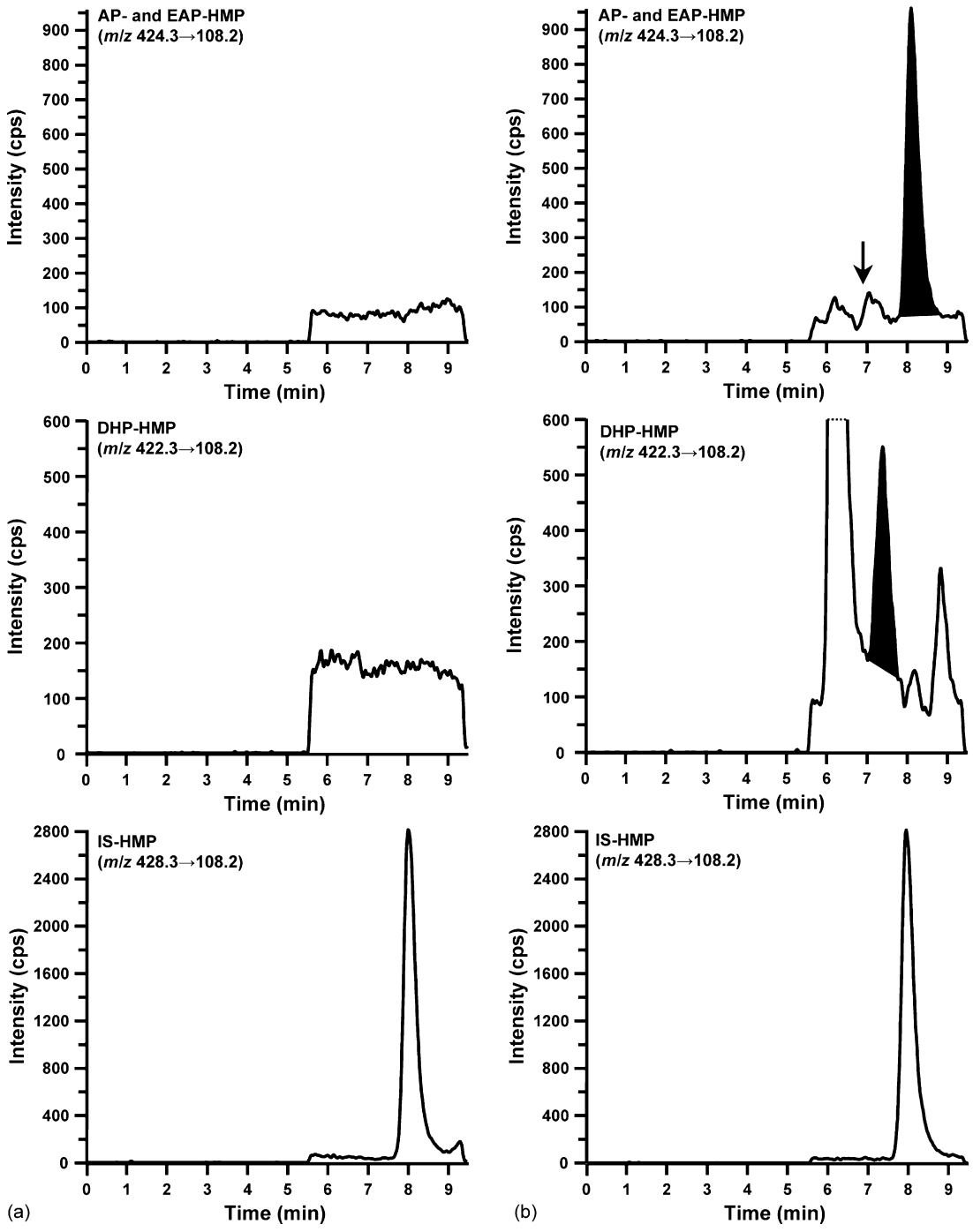

Fig. 5. Chromatograms of derivatized AP, EAP, DHP and IS in the serum of (a) the untreated and (b) stressed rats. The LC–ESI-MS–MS conditions were described in Section 2.2. The arrow indicates the elution position of the EAP derivative. The measured concentrations of AP and DHP in the stressed rat were 2.13 and 1.48 ng/ml, respectively.

below 5.3%. The CV values and relative errors (RE) of the back-calculated concentrations at the minimum point (0.25 ng/g tissue or 0.25 ng/ml) were less than 9.4% and ranging from −8.0 to −1.2%, respectively. The LOQ was defined as the lowest concentration on the calibration curve of the analyte measured with an acceptable precision and accuracy (i.e., CV and RE < 15%) and with at least S/N of 5. On the basis of this criterion and the above results, the LOQs of AP, EAP and DHP were determined to be 0.25 ng/g tissue in the brain assay when a 20 mg tissue sample was used or 0.25 ng/ml in the serum assay when a 20 μl serum sample was used.

When the LOD is defined as the signal equivalent to 5 times the noise, those of AP, EAP and DHP were 0.15, 0.16 and 0.20 ng/g tissue, respectively, in the brain assay and 0.17, 0.18 and 0.21 ng/ml, respectively, in the serum assay.

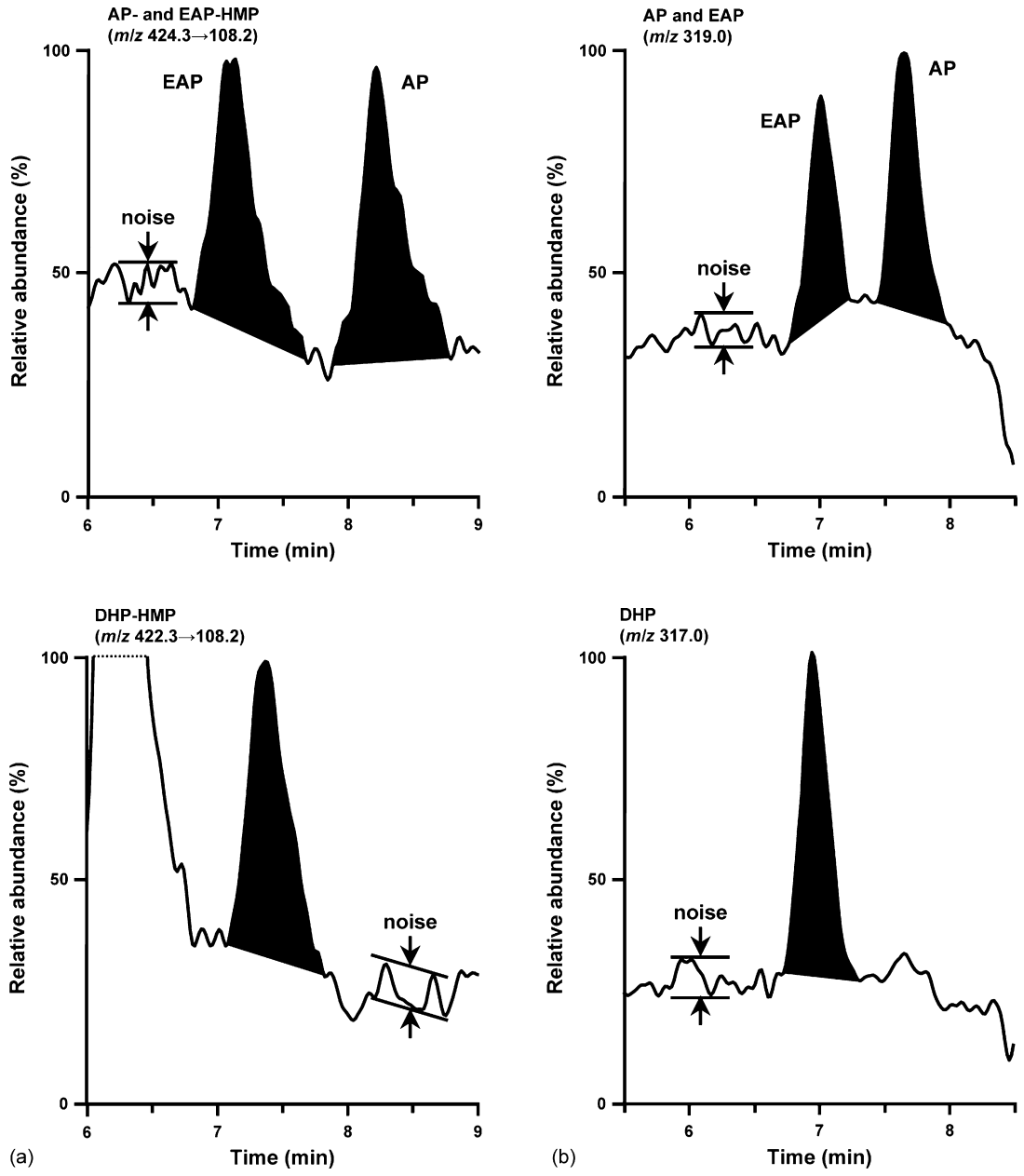

Fig. 6. Comparison of the sensitivity of ESI-MS analysis of HMP derivatives and APCI-MS analysis of intact steroids. (a) Steroids (0.25 ng/g tissue each) were analyzed by ESI-MS after HMP-derivatization and (b) steroids (AP, 25 ng/g tissue; EAP, 37.5 ng/g tissue and DHP, 15 ng/g tissue) were analyzed by APCI-MS without derivatization.

Table 1
Summary of calibration curves for neurosteroids

	Slope[a] (CV)	Intercept[a]	Correlation coefficient (r^2)	Back-calculated concentration[b]
Brain AP	0.1186 ± 0.0063 (5.3%)	0.0045 ± 0.0037	0.99996[c]	0.244 ± 0.018 (CV 7.4%, RE −2.4%)
Brain EAP	0.1253 ± 0.0039 (3.1%)	0.0042 ± 0.0025	0.99979[c]	0.242 ± 0.014 (CV 5.8%, RE −3.2%)
Brain DHP	0.0905 ± 0.0029 (3.2%)	0.0005 ± 0.0022	0.99988[c]	0.230 ± 0.017 (CV 7.4%, RE −8.0%)
Serum AP	0.1184 ± 0.0023 (1.9%)	0.0065 ± 0.0020	0.99999[d]	0.244 ± 0.010 (CV 4.1%, RE −2.4%)
Serum EAP	0.1272 ± 0.0037 (2.9%)	0.0055 ± 0.0015	0.99997[d]	0.247 ± 0.019 (CV 7.7%, RE −1.2%)
Serum DHP	0.0938 ± 0.0030 (3.2%)	0.0025 ± 0.0028	0.99999[d]	0.245 ± 0.023 (CV 9.4%, RE −2.0%)

[a] Mean ± S.D., $n = 5$.
[b] Back-calculated concentration (mean ± S.D., $n = 5$) at 0.25 ng/g tissue (brain) or 0.25 ng/ml (serum). R.E., relative error.
[c] Measurable range, 0.25–5.0 ng/g tissue.
[d] Measurable range, 0.25–5.0 ng/ml.

Table 2
Intra- and inter-assay precision and accuracy for determination of neurosteroids in rat brain and serum

		Brain (0.4 ng/g tissue)[a]			Brain (4.0 ng/g tissue)[a]		
		AP	EAP	DHP	AP	EAP	DHP
Intra-assay	Measured concentration[b]	0.404 ± 0.012	0.402 ± 0.018	0.412 ± 0.019	4.08 ± 0.065	4.05 ± 0.085	3.99 ± 0.088
	CV (%)	3.0	4.5	4.6	1.6	2.1	2.2
	Accuracy (%)	101.0	100.5	103.0	102.0	101.3	99.8
Inter-assay	Measured concentration[b]	0.400 ± 0.012	0.400 ± 0.010	0.408 ± 0.013	4.07 ± 0.089	3.97 ± 0.138	4.09 ± 0.093
	CV (%)	3.0	2.5	3.2	2.2	3.5	2.3
	Accuracy (%)	100.0	100.0	102.0	101.8	99.3	102.3
		Serum (0.4 ng/ml)[a]			Serum (4.0 ng/ml)[a]		
		AP	EAP	DHP	AP	EAP	DHP
Intra-assay	Measured concentration[b]	0.412 ± 0.024	0.395 ± 0.018	0.395 ± 0.018	3.94 ± 0.082	4.04 ± 0.120	4.07 ± 0.085
	CV (%)	5.8	4.3	4.6	2.1	3.0	2.1
	Accuracy (%)	103.0	98.8	98.8	98.5	101.0	101.8
Inter-assay	Measured concentration[b]	0.408 ± 0.011	0.398 ± 0.015	0.396 ± 0.011	4.04 ± 0.065	4.05 ± 0.094	4.05 ± 0.082
	CV (%)	2.7	3.8	2.8	1.6	2.3	2.0
	Accuracy (%)	102.0	99.5	99.0	101.0	101.3	101.3

[a] Nominal concentration.
[b] Mean ± S.D. [ng/g tissue (brain) or ng/ml (serum), $n = 5$].

3.6. Assay precision and accuracy

The assay precision and accuracy were examined using QC samples with two different concentrations. The intra-assay ($n = 5$) CV values for the brain AP, EAP and DHP were less than 4.6%, and good inter-assay ($n = 5$) CV values (less than 3.5%) were also obtained, as shown in Table 2. The present method is superior to the previously reported GC–MS method [3] in assay precision; the intra- and inter-assay CV values of the GC–MS method were over 10% in samples with less than 2 ng/g tissue. The intra- ($n = 5$) and inter-assay ($n = 5$) CV values for the serum AP, EAP and DHP were also below 5.8% (Table 2). Moreover, we examined the assay reproducibility by analyzing the brain and serum samples of two different stressed rats, which contained endogenous neurosteroids in significant amounts. Table 3 demonstrates that the reproducibility is satisfactory in both the brain and serum assays.

Satisfactory % accuracy values ranging from 99.3 to 103.0% in the brain assay and from 98.5 to 103.0% in the serum assay were also obtained (Table 2). These data indicate that the present method is highly reproducible and accurate.

3.7. Applicability of the developed method: changes in brain and serum AP, EAP and DHP levels by immobilization stress and ethanol administration

In order to examine the applicability of the proposed method for a pharmacological study, changes in the AP, EAP and DHP levels in the brain and serum due to immobilization stress, a representative physical stress, and ethanol administration were

Table 3
Reproducibility in determination of neurosteroids in stressed rat brain and serum

		Brain 1			Brain 2		
		AP	EAP	DHP	AP	EAP	DHP
Intra-assay	Mean ± S.D. (ng/g tissue, $n = 5$)	1.15 ± 0.092	0.40 ± 0.022	1.65 ± 0.084	2.33 ± 0.029	1.20 ± 0.098	3.42 ± 0.225
	CV (%)	8.0	5.5	5.1	1.2	8.2	6.6
Inter-assay	Mean ± S.D. (ng/g tissue, $n = 5$)	1.08 ± 0.065	0.44 ± 0.027	1.74 ± 0.068	2.35 ± 0.141	1.27 ± 0.052	3.60 ± 0.126
	CV (%)	6.0	6.1	3.9	6.0	4.1	3.5
		Serum 1			Serum 2		
		AP	EAP	DHP	AP	EAP	DHP
Intra-assay	Mean ± S.D. (ng/ml, $n = 5$)	1.10 ± 0.053	ND[a]	0.59 ± 0.033	2.94 ± 0.086	ND	1.44 ± 0.072
	CV (%)	4.8	–	5.6	2.9	–	5.0
Inter-assay	Mean ± S.D. (ng/ml, $n = 5$)	1.09 ± 0.019	ND	0.59 ± 0.025	3.01 ± 0.057	ND	1.44 ± 0.032
	CV (%)	1.7	–	4.2	1.9	–	2.2

[a] Not detected.

Table 4
Brain and serum levels of AP, EAP and DHP

	Brain (ng/g tissue, mean ± S.D.)			Serum (ng/ml, mean ± S.D.)		
	AP	EAP	DHP	AP	EAP	DHP
Nontreated ($n = 10$)	ND[a]	ND	<LOQ[b]	ND	ND	ND
Stressed ($n = 10$)	1.74 ± 0.71	0.58 ± 0.30	2.74 ± 1.12	1.31 ± 0.72	ND	0.94 ± 0.36
Saline-administered ($n = 5$)	ND	ND	<LOQ	ND	ND	ND
Ethanol-administered ($n = 5$)	0.57 ± 0.19	<LOQ	0.85 ± 0.34	0.44 ± 0.15	ND	0.33 ± 0.04

[a] Not detected.
[b] Less than limit of quantitation (0.25 g/g tissue or 0.25 ng/ml).

analyzed using the developed method (Table 4). In the brain of stressed rats, AP, EAP and DHP were determined to be 0.83–3.20, 0.27–1.27 and 1.57–4.78 ng/g tissue, respectively, but the levels in the untreated group were less than LOD for AP and EAP and below LOQ for DHP. The increase in the brain AP level observed here is certainly a defensive response to acute stress. The serum AP and DHP levels were also dramatically elevated by the stress (0.52–3.01 and 0.45–1.44 ng/ml, respectively). These brain and serum AP levels agreed well with those in the swim-stressed male Wistar rats measured by GC–MS [mean level ($n = 6$); brain, ca. 1.3 ng/g tissue and plasma, ca. 1.5 ng/ml] [3], but in another report that used RIA [2], the brain AP level was much higher (ca. 5 and 12 ng/g tissue in the untreated and swim-stressed male Sprague–Dawley rats, respectively) than our results. One potential explanation for this discrepancy is that our LC–MS method is more specific for the brain samples than the RIA previously employed [2,4,6].

Acute administration of ethanol (0.8 g/kg, i.p.) also elevated the brain AP and DHP levels (0.35–0.80 and 0.40–1.30 ng/g, respectively) and serum AP and DHP levels (0.25–0.62 and 0.25–0.39 ng/ml, respectively) (Table 4). A similar result was found for AP levels in the brain (ca. 1.3 ng/g tissue) and plasma (ca. 0.6 ng/ml) of male Wistar rats 30 min after acute ethanol (2 g/kg) administration [7]. The brain EAP level was also increased by ethanol administration but was below the LOQ. Saline administration did not affect the neurosteroid levels.

In contrast to AP and DHP, EAP was not detected in the serum even in the stressed and ethanol-administered rats. This result indicates that EAP was independently synthesized in the brain. Although no previous report describes the changes in the brain and circulating EAP levels due to the ethanol administration, the present study demonstrate that this treatment does not significantly influence the EAP level.

4. Conclusion

In this study, we developed the LC–MS–MS method for the simultaneous determination of the brain and serum AP, EAP and DHP after converting them to highly detectable derivatives in positive ESI-MS. The method was sensitive, specific and reproducible, and enabled the analysis of changes in the brain and serum neurosteroid levels by immobilization stress and ethanol administration using a small amount of sample (20 mg of brain tissue or 20 μl of serum).

The animal studies demonstrated that the brain AP, EAP and DHP levels and serum AP and DHP levels were rapidly elevated by immobilization stress or ethanol administration. The studies also found that EAP is the brain-specific product. Because EAP can antagonize the GABAergic function of AP [9], the simultaneous determination of AP and EAP is indispensable for examining the relationship between endogenous neurosteroid levels and stress-induced disorders. To our knowledge, our LC–MS–MS method is the first one that can simultaneously determine AP and EAP in rat brain.

As mentioned in Section 1, the anti-depressant activity of SSRIs occurs through the promotion of AP synthesis [8]. Therefore, it is expected that the developed method can be applied for the screening of antipsychotic agents targeting neurosteroidogenesis.

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