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Simultaneous determination of *N*-acetylaspartylglutamate and *N*-acetylaspartate in rat brain homogenate using high-performance liquid chromatography with pre-column fluorescence derivatization

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ABSTRACT

Simultaneous determination method of *N*-acetyl-L-aspartyl-L-glutamate (NAAG), an endogenous agonist at type3 metabotropic glutamate receptor, and its degradation product, *N*-acetyl-L-aspartate (NAA) was developed by using reversed-phase high-performance liquid chromatography (HPLC) with pre-column fluorescence derivatization using 4-*N*,*N*-dimethylaminosulfonyl-7-*N*-(2-aminoethyl)amino-2,1,3-benzoxadiazole. The detection limits of NAAG and NAA were approximately 12 and 34 fmol on the column, respectively (signal to noise ratio 3). The proposed HPLC method was applied to determine NAAG and NAA simultaneously in the rat brain homogenate. Both concentrations of NAAG and NAA in the male rat cerebrum (13 weeks old) were 5.7 ± 0.30 and $2.1 \times 10^2 \pm 9.2$ nmol/mg protein, respectively ($n = 6$), while those in the hippocampus were 6.8 ± 0.48 and $1.9 \times 10^2 \pm 8.5$ nmol/mg protein, respectively ($n = 5$). Hippocampal NAA concentration was significantly increased in the ketamine-treated rats as compared to the control rats ($p < 0.01$).

1. Introduction

N-Acetyl-L-aspartyl-L-glutamate (NAAG) is a dipeptide neurotransmitter that acts an endogenous agonist at type3 metabotropic glutamate receptor, and is distributed abundantly in the mammalian brain [1–3]. NAAG is enzymatically cleaved to *N*-acetyl-L-aspartate (NAA) and glutamate (Glu) by glutamate carboxypeptidase (GCP) II or III [4], and therefore, NAAG also receives attention as a storage form of Glu, which elicits the excitatory neurotransmission to an ionotropic glutamate receptor [5]. Chronic administration of phencyclidine (PCP) induces schizophrenia-like changes in both NAAG and NAA in the rat hippocampus [6]. Brain NAAG and NAA concentrations have been reported to be changed by *in vivo* activity of GCP II or III, and the inhibitors of GCP have thus far been receiving attention to develop a new type of drug to overcome neurological disorders [7–9].

Brain NAA concentration has been reported to be significantly altered in psychiatric and/or neurological diseases [10–16] by using magnetic resonance spectroscopy. Hence, the brain NAA may be associated with the etiology of serious diseases.

In our previous paper [17], we developed a sensitive determination method of NAA by using high-performance liquid chromatography. A fluorescence derivatization of NAA with a fluorescence reagent, 4-*N*,*N*-dimethylaminosulfonyl-7-*N*-(2-aminoethyl)amino-2,1,3-benzoxadiazole (DBD-ED) [18], was employed to increase the detection sensitivity. As a result, successful determination of rat cerebrum NAA was enabled using methylsuccinic acid (MSA) as an internal standard. As described above, both NAAG and NAA are considered to play a crucial role for neuronal functions, and therefore, simultaneous determination of NAAG and NAA can provide useful information on the etiology of neurological diseases.

Thus, in the present study, the fluorescence derivatization technique using DBD-ED is extended to NAAG, which has three carboxyl groups in its chemical structure, and the simultaneous determination of rat brain NAAG and NAA by HPLC equipped with an octadecylsilica (ODS) column is attempted. Finally, using the proposed HPLC method, we investigate NAAG and NAA concentrations in the cerebrum or hippocampus of ketamine-

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treated rats, which are reported to be an animal model for schizophrenia [19].

2. Experimental

2.1. Chemicals

NAAG and NAA were purchased from Sigma Co., Ltd. (St. Louis, MO, USA). (±)-MSA, 4-dimethylaminopyridine (DMAP), dimethylformamide (DMF), ketamine hydrochloride, and trifluoroacetic acid (TFA) were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). DBD-ED and 1-ethyl-*N*,*N*-dimethylaminocarbodiimide hydrochloride (EDC) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Water was purified using an ADVANTEC GSR-200 (Nihon Millipore K.K., Tokyo, Japan). Acetonitrile (CH_3CN) and methanol (MeOH) were special grade and were purchased from Kanto Kagaku Kogyo Co., Ltd. (Tokyo, Japan).

2.2. Fluorescence derivatization

NAAG and NAA were dissolved in H_2O (40 μM and 0.80 mM, respectively). The fluorescence derivatization procedure was performed according to our previous paper with minor modifications [17]. Each 10 μL of NAAG, NAA, and 0.20 mM MSA (I.S., in CH_3CN) was mixed vigorously with 470 μL of MeOH, following which, 50 μL of the mixture was evaporated *in vacuo*. The obtained residue was dissolved in 25 μL of DMF and serially added with 25 μL of DBD-ED (40 mM, in CH_3CN), 25 μL of EDC (100 mM, in CH_3CN), and 25 μL of DMAP (100 mM, in CH_3CN); it was then mixed vigorously using a vortex mixer. Subsequently, the reaction mixture was heated with a water bath at 60 °C for 45 min, and the reaction mixture was diluted fivefold with 0.10% TFA in H_2O/CH_3CN (50/50), and a 50-μL aliquot of the solution was loaded onto a solid-phase extraction (SPE) cartridge bearing a membrane coated with sulfonated poly(styrenedivinylbenzene)polymer (SDB-RPS, Empore™, GL Sciences, Tokyo, Japan), initialized with 50 μL of 0.10% TFA in H_2O/CH_3CN (50/50). After the centrifugation of the cartridge, the eluate through the membrane was collected; subsequently, the membrane was rinsed once with 50 μL of 0.10% TFA in H_2O/CH_3CN (50/50). After filtration using the Millex-LG® (0.20 μm membrane, Nihon Millipore K.K., Tokyo, Japan), 20 μL of the final solution was injected into our HPLC system. For the confirmation of the chemical structure of the derivatives, the HPLC-mass spectrometer (MS) was used , and the condition was the same as our previous paper [17].

2.3. HPLC conditions

The HPLC system comprised the auto sampler AS-2059 *plus* (JASCO Co., Tokyo, Japan), with a 100 μL sample loop, the pump PU-2080 *plus* (JASCO Co., Tokyo, Japan), with a gradient unit LG-2090-04 (JASCO Co., Tokyo, Japan) and a degasser DG-2080-54 (JASCO Co., Tokyo, Japan), the column oven CO-2060 *plus* (JASCO

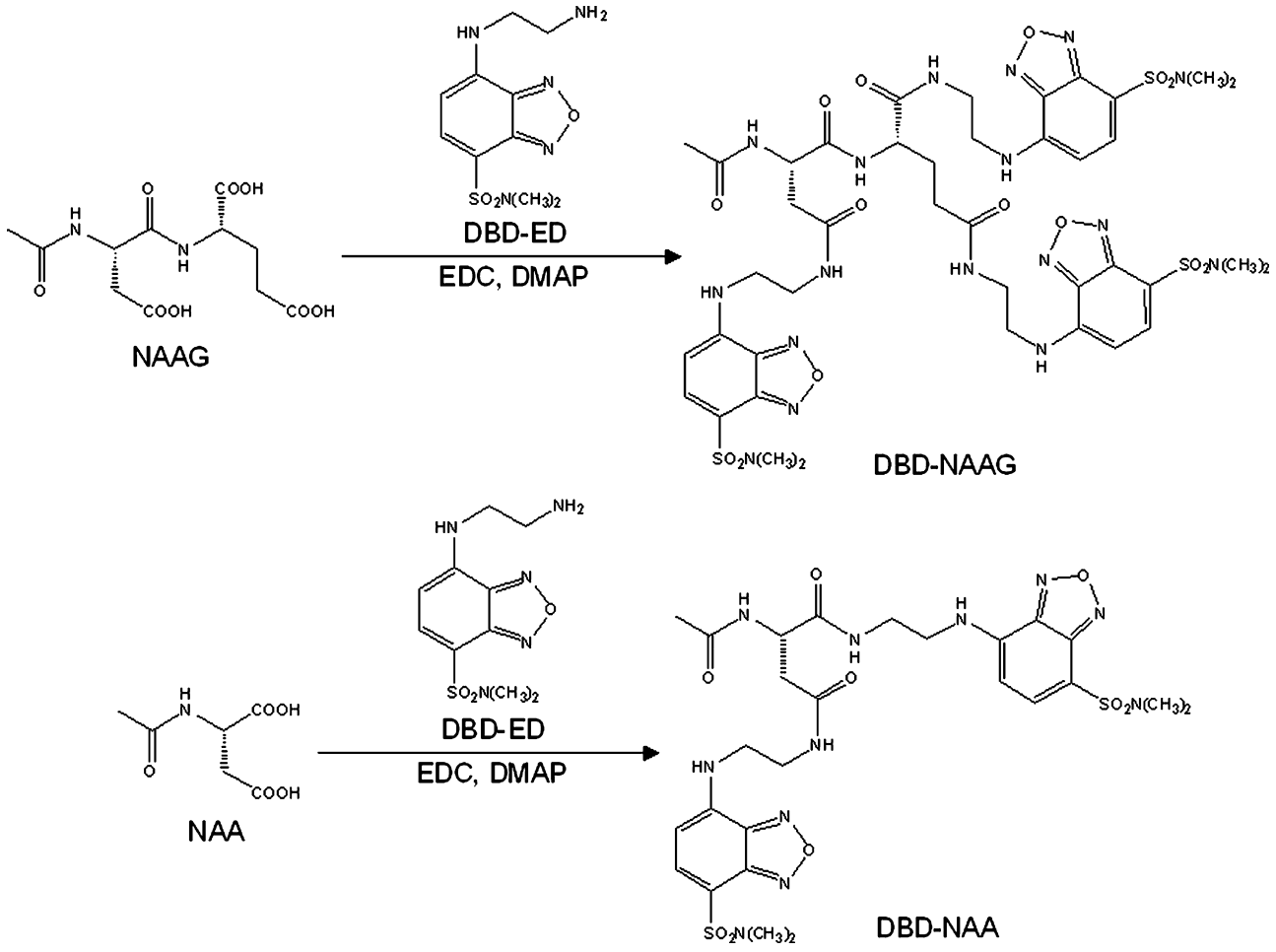

Fig. 1. Fluorescence derivatization scheme of NAAG and NAA with the fluorescence reagent DBD-ED.

Co., Tokyo, Japan), and the fluorescence detector FP-2025 *plus* (JASCO Co., Tokyo, Japan). The ODS column used was Cadenza CD-C18 (250 mm × 4.6 mm i.d., 3 μM, Imtact Co., Kyoto, Japan), with a guard column, Cadenza CD-C18 (5 mm × 2 mm i.d., 3 μM, Imtact Co., Kyoto, Japan). The mobile phases A and B used in this study were 0.10% TFA in H_2O and 0.10% TFA in CH_3CN. The mobile phase was eluted according to the following program: 0–30 min; B% = 33 (isocratic), 30–50 min; B% = 33–75 (linear gradient). The flow rate was constantly maintained at 0.80 mL/min. The whole time-program was operated automatically using a software, namely, Borwin ver. 1.50 (JASCO Co., Tokyo, Japan). The column temperature was set at 40 °C and the detection wavelength was set at 562 nm with 438 nm of excitation.

2.4. Animal experiments

Male Sprague–Dawley rats were purchased from Charles River Japan (Kanagawa, Japan) and housed in an environmentally controlled room for at least one week prior to use. All the animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Shizuoka.

Ketamine (Ket)-treated rats were prepared according to previous paper [19,20]. For the control rats, saline was injected daily (1.0 mL/kg) instead of Ket. Both rats (13 weeks old) were sacrificed by drawing blood from the abdominal aorta under light anesthesia with diethylether, and the whole brain was rapidly removed. Subsequently, the brain was dissected after a slight rinse with chilled saline, and the cerebrum and hippocampus were immediately homogenized using a potter-type homogenizer with 10 volumes of phosphate-buffered saline (Nissui pharmaceutical Co., Ltd., Tokyo, Japan) under ice-cooled conditions. The resultant homogenates were stored at −80 °C until use.

For the derivatization of NAAG and NAA with DBD-ED, an aliquot of the homogenate was thawed at 2 °C and centrifuged at 3000 *g* for 10 min. A 10-μL aliquot of the supernatant was sampled to a transparent tube (1.5 mL); subsequently, 10 μL of 0.20 mM I.S. in CH_3CN was added followed by the addition of 480 μL of MeOH, and the resultant solution was mixed vigorously. After centrifugation at 3000 *g* for 5 min, the 50 μL of supernatant was evaporated into dryness at 40 °C *in vacuo*, and the residue was dissolved in 25 μL of DMF and derivatized with the fluorescence reagent, DBD-ED. The reaction mixture was heated at 60 °C for 45 min. Subsequently, the reaction mixture was treated in a manner similar to the procedure described above and the final solution was injected into our HPLC system.

Table 1
Precision (RSD) and accuracy (RME) for the determination of NAAG and NAA in rat cerebrum spiked with NAAG or NAA by the proposed HPLC system ($n = 3$)

	Intra-day	Inter-day
NAA		
Precision (%)	0.46–2.8	2.0–3.8
Accuracy (%)	−6.5 to −6.4	−7.4 to −3.2
Recovery (%)	88	86
NAAG		
Precision (%)	0.67–4.2	0.63–5.6
Accuracy (%)	−12 to −9.8	−12 to −10
Recovery (%)	78	77

2.5. Determination of rat brain NAAG and NAA

NAAG and NAA concentrations in rat cerebrum and hippocampus homogenate were expressed as moles of NAAG or NAA/protein (mg) concentration. The calibration curves for NAAG and NAA were constructed by plotting the fluorescence peak area ratio of NAAG or NAA to I.S. against the concentration of NAAG (5.0, 10, 20, and 40 μM) or NAA (0.20, 0.40, 0.80, and 1.6 mM). The derivatization condition was set at 60 °C for 45 min.

The protein concentration of the rat brain homogenate was assayed using a commercial kit bicinchoninic acid (BCA™) protein assay (Pierce Biotechnology Inc., Rockford, IL, USA), as previously reported [17]. Statistical analyses were performed by Student's unpaired *t*-test and a p value of <0.05 was regarded as significant difference.

3. Results and discussion

3.1. Simultaneous determination of NAAG and NAA

In our previous study, fluorescence derivatization of NAA, which has two carboxyl groups, with DBD-ED was successfully carried out in the presence of EDC with DMAP as the condensing agents. DBD-ED was effective for the complete fluorescence labeling of NAA,

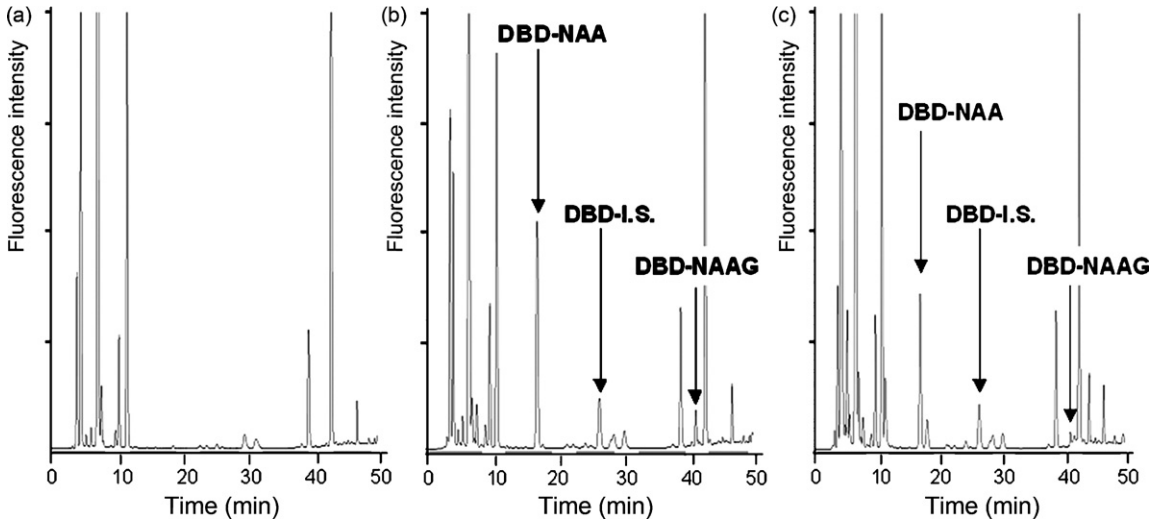

Fig. 2. Chromatograms of (a) blank, (b) authentic sample of NAAG and NAA (0.80 and 16 pmol, respectively) derivatized with DBD-ED, and (c) NAAG and NAA in rat cerebrum homogenate sample derivatized with DBD-ED.

Table 2
Concentrations of NAAG and NAA in the cerebrum (frontal and dorsal) and the hippocampus homogenate of saline- and ketamine-treated rats (mean ± SE, $n = 5–6$)

Brain region	NAAG (nmol/mg protein)		NAA (nmol/mg protein)	
	Saline-treated	Ketamine-treated	Saline-treated	Ketamine-treated
Cerebrum				
Frontal	5.72 ± 0.300	5.78 ± 0.227	214 ± 9.15	215 ± 7.00
Dorsal	5.83 ± 0.286	6.17 ± 0.237	212 ± 5.17	212 ± 5.17
Hippocampus	6.79 ± 0.479	7.29 ± 0.287	188 ± 8.49	216 ± 1.77**

** $p < 0.01$ *vs.* saline-treated.

because it has a flexible aminoethyl moiety as the reaction site. In order to detect NAAG and NAA simultaneously with fluorescence detection, NAAG, which has three carboxyl groups, had to be derivatized with DBD-ED, as shown in Fig. 1.

First, authentic samples of NAAG, NAA, and I.S. were derivatized with DBD-ED. Distinct fluorescence peaks for NAAG (DBD-NAAG) and NAA (DBD-NAA) were observed with few interfering peaks (Fig. 2(b)). As previously reported [17], MSA was employed as an I.S., because of its structural similarity with NAA. The derivatization of NAAG, NAA, and I.S. with DBD-ED in the presence of EDC and DMAP as condensing reagents reached a plateau after 45 min at 60 °C (data not shown).

Next, the fluorescence derivatives were isolated and evaporated *in vacuo*, and these fractions were subjected to HPLC-MS in order to confirm the resultant fluorescence derivative of NAAG, NAA, or I.S. with DBD-ED. The obtained mass spectrum of NAAG derivative (DBD-NAAG) exhibited a pseudo molecular ion [$(M+H)^+$ 1106.4], indicating that three carboxyl groups in NAAG were completely labeled with DBD-ED. NAA and I.S. could also be derivatized with DBD-ED (DBD-NAA and DBD-I.S., respectively), and their chemical structures were assigned by HPLC-MS [$(M+H)^+$ 710.4] and [$(M+H)^+$ 667.3], respectively; the carboxyl groups were completely labeled with DBD-ED. The excitation and emission spectra of DBD-NAAG and -NAA were almost similar, and their optimum wavelengths for excitation were 440 and 438 nm and those for fluorescence were 561 and 562 nm, respectively. Therefore, the wavelengths of 438 and 562 nm were employed for the simultaneous detection of DBD-NAAG and -NAA.

3.2. Validation study

Both calibration curves of NAAG and NAA showed good linearities in the concentration range of 5.0–40 μM and 0.20–1.6 mM with a correlation coefficient of 0.999, respectively ($n = 3$). The detection limits of NAAG and NAA were approximately 12 and 34 fmol on the column, respectively (signal to noise ratio 3).

Table 1 shows the validation data obtained in this study. Ten microliters of NAAG (0, 5.0, and 10 μM) and NAA (0, 0.20, and 0.40 mM) were added to 10 μL of the rat cerebrum homogenate ($n = 3$). As a consequence, intra- and inter-day precisions (RSD) were within 0.46–4.2% and 0.63–5.6%, respectively, and intra- and inter-day accuracies (RME) were within −12 to −6.4% and −12 to −3.2%, respectively. Although derivatization procedure with DBD-ED was included in the proposed method, the sensitivity obtained was superior to that in the reported method using UV detection [6]. Thus, the proposed HPLC method has an advantage to need a lesser amount of biological specimens.

3.3. NAAG and NAA in rat cerebrum and hippocampus homogenate

Subsequently, the proposed HPLC method was applied to determine NAAG and NAA in rat brain homogenate. In addition, each dissected cerebrum was divided into the frontal and dorsal regions. In this study, the cerebrum and hippocampus were chosen because several researches on NAAG and NAA have targeted the cerebrum or hippocampus in preclinical and clinical studies on schizophrenia [10,12]. In terms of the pre-treatment of the brain homogenate, a purification procedure was carried out according to our previous paper [17]. Fluorescence peaks with DBD-ED reached a plateau level for 45 min at 60 °C (data not shown). Fig. 2(c) shows the representative chromatograms of NAAG, NAA, and I.S. with DBD-ED in the rat cerebrum homogenate.

Consequently, distinct fluorescence peaks of NAAG, NAA, and I.S. were clearly observed in the chromatograms of the homogenates. The obtained chromatogram from hippocampus homogenate was almost similar to that from cerebrum.

The concentrations of NAAG and NAA in the male rat cerebrum (13 weeks old) were 5.7 ± 0.30 and $2.1 \times 10^2 \pm 9.2$ nmol/mg protein, respectively ($n = 6$). In the case of the hippocampus, they were 6.8 ± 0.48 and $1.9 \times 10^2 \pm 8.5$ nmol/mg protein, respectively ($n = 5$). Hippocampal NAA concentration was significantly increased in the ket-treated rats as compared to the control rats ($p < 0.01$), while that in the cerebrum showed no significant changes (Table 2). In addition, there were no significant changes of NAAG in both the cerebrum and hippocampus. It has been reported that the repeated administration of ket caused schizophrenia-like behaviors in rats [19,21]. In contrast, it has recently been reported that increased neurogenesis was observed in the hippocampus of the ket-treated rats [22]. Therefore, the increased NAA might be suggested to have some relations with etiology of schizophrenia or neurogenesis in the hippocampus. Further researches should be necessary for elucidating the functions of NAA in the hippocampus.

4. Conclusion

Using the proposed HPLC method, NAAG and NAA were simultaneously determined with high sensitivity. The HPLC method was successfully applied to determine NAAG and NAA in rat brain homogenate sample, and a significant increase of NAA in the hippocampus of Ket-treated rat was found for the first time.

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