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Development, validation, and application of a surrogate analyte method for determining *N*-acetyl-L-aspartyl-L-glutamic acid levels in rat brain, plasma, and cerebrospinal fluid

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ABSTRACT

A bioanalytical strategy for the simple and accurate determination of endogenous substances in a variety of biological matrices using liquid chromatography-tandem mass spectrometry is described. The robust method described here uses two stable isotope-labeled compounds as a surrogate analyte and an internal standard to construct calibration curves with authentic matrices that can be applied to determine *N*-acetyl-L-aspartyl-L-glutamic acid (NAAG) levels in rat brain, plasma, and cerebrospinal fluid (CSF) using a simple extraction and with a short analysis time of 4 min. The validated lower limits of quantification were 1.00 nmol/g for brain and 0.0100 nmol/mL for plasma and CSF. Using this method, regional differences in NAAG levels in the brain as well as plasma and CSF levels that were much lower than those in the brain were successfully confirmed in treatment-naïve rats. Moreover, after the rats were treated with the intraventricular administration of a NAAG peptidase inhibitor, the NAAG levels increased rapidly and dramatically in the CSF and slightly in the plasma in a time-dependent manner, while the brain levels were not affected. Thus, the procedure described here was easily applied to the determination of NAAG in different matrices in the same manner as that used for xenobiotics, and this method would also be easily applicable to the accurate measurement of endogenous substances in a variety of biological matrices.

1. Introduction

The identification, qualification, and quantification of endogenous substances, including biomarkers, are becoming key success factors for drug discovery and development, and many bioanalytical techniques for molecular biomarkers have been developed and reported, such as radioimmunoassay, high-performance liquid chromatography, and liquid chromatography-tandem mass spectrometry (LC–MS/MS). As changes in disease status or the conditions of experimental animals or patients as a result of the administration of drug candidates may lead to changes in biomarker disposition, a simple and accurate bioanalytical method for detecting endogenous substances in a variety of biological matrices is very important for enhancing drug discovery and development.

The search for clinically relevant biomarkers for serious disorders such as schizophrenia, which is a chronic and severe psychiatric disorder that affects 1% of the population, has a long history. Although some molecules have been proposed as diagnostic or prognostic biomarkers for schizophrenia, their measurement is associated with varying degrees of error that must be considered when making decisions [1]. The acidic dipeptide *N*-acetyl-L-aspartyl-L-glutamic acid (NAAG) is a neuropeptide which is considered to act not only as an agonist for metabotropic glutamate receptor-3 (mGlu3 receptor) but also as an antagonist for the *N*-methyl D-aspartate (NMDA) receptor in glutamatergic neurotransmission [2–4]. In addition, NAAG is hydrolyzed to *N*-acetyl-L-aspartate (NAA) and L-glutamate by glutamate car-

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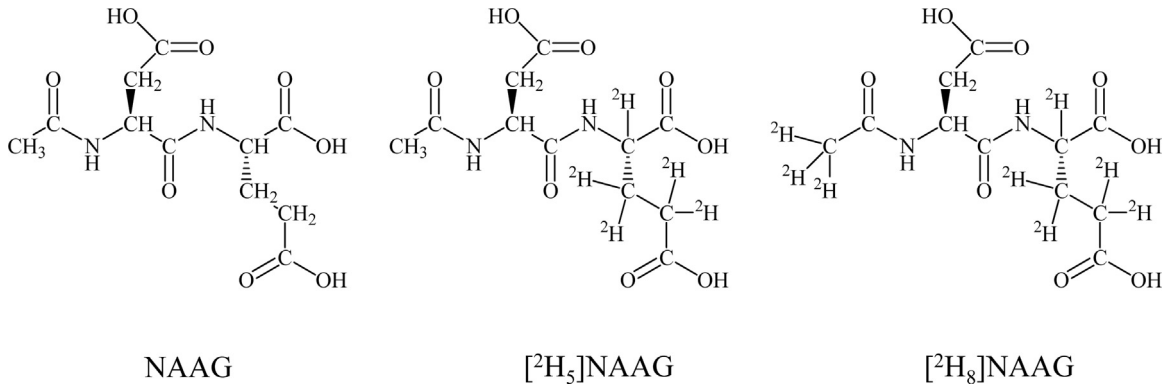

Fig. 1. Chemical structures of NAAG, [$^2H_5$]NAAG, and [$^2H_8$]NAAG.

boxypeptidase II (GCPII, also known as NAAG peptidase), and its function is thought to range from a neurotransmitter to a transmitter pool of glutamate [5]. Based on these physiological roles of NAAG, the extent of changes in NAAG levels not only in the brain, but also in other tissues may reflect the severity of symptoms or the effects of drug candidates. As a matter of fact, regional differences in NAAG distribution in the brain, compared with normal subjects, have been found in schizophrenia patients [6,7]. Thus, NAAG is a potential biomarker for diseases involving glutamatergic abnormalities. To utilize endogenous substances as biomarkers, the biological samples must be easily obtainable. A need to evaluate NAAG levels in plasma and cerebrospinal fluid (CSF) as well as the brain is therefore apparent.

Among quantitative bioanalysis techniques, LC–MS/MS is a powerful and widely used modality, especially from the viewpoint of selectivity and sensitivity. For accurate analyses using LC–MS/MS, the analyte of interest should be spiked into an analyte-free authentic matrix to prepare the calibration standards to ensure that analyte recovery and analytical interferences by the matrix components are leveled and compensated, respectively. In the case of the bioanalysis of endogenous substances including biomarkers, alternative approaches, such as a standard addition method and a surrogate matrix method, are often chosen because of the lack of analyte-free authentic matrices [8–12]. A standard addition is the method of choice for the accurate determination of endogenous substances. However, complicated and time-consuming procedures are required for sample preparation and analysis, especially when numerous samples must be analyzed, and this technique is thus unsuitable for the high-throughput demands of pharmaceutical industries. Moreover, each sample has to be divided into at least three aliquots to construct a regression curve for determination, which is not a practical approach for rare matrices such as CSF. An alternative choice for the bioanalysis of endogenous substances, especially for high-throughput analyses, is a surrogate matrix method, which is the most commonly used method for NAAG determination [13–15]. However, components existing in surrogate matrices, such as buffers, are obviously different from those in authentic matrices, and the degree of difference in the matrix effects on the ionization of the analytes severely affects the accuracy of the data. To compensate for such differences, not only a thorough sample clean-up procedure corresponding to each type of matrix, but also the use of stable isotope-labeled internal standards is absolutely necessary. For these reasons, the use of standard addition and surrogate matrix methods requires great care and should be justified.

From the viewpoint of versatility and capability, the use of a surrogate analyte to prepare calibration standards with authentic matrices, that is to say, the surrogate analyte method, has the potential to overcome the aforementioned disadvantages of the standard addition and the surrogate matrix methods [8,16]. However, very few applications of the surrogate analyte method have been reported for endogenous substances. To the best of our knowledge, NAAG quantification using the surrogate analyte method has not been reported. In addition, the FDA draft guidance on the validation of bioanalytical methods mentions the need for a validation approach in the development of methods for endogenous substances so as to give diagnostic and/or prognostic power to biomarkers [17]. The objective of this study was to demonstrate the utility of a bioanalytical strategy for endogenous substances using LC–MS/MS with a surrogate analyte technique through the development, validation, and application of an assay method for NAAG in rat brain, plasma, and CSF.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals were of analytical grade unless otherwise stated. NAAG was obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol, acetonitrile, ammonium formate, and formic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). *N*-[[[(1*S*)-1-carboxy-3-methylbutyl]amino]carbonyl]-L-glutamic acid (ZJ43) was obtained from Tocris (Bristol, UK). Stable isotope-labelled NAAGs, [$^2H_5$]NAAG and [$^2H_8$]NAAG, were synthesized in reference to the method [18] using commercially available [$^2H_5$]L-glutamate, *N*-protected-L-aspartic acid, and acetyl chloride for [$^2H_5$]NAAG, and [$^2H_5$]L-glutamate, *N*-protected-L-aspartic acid, and [$^2H_3$]acetyl chloride for [$^2H_8$]NAAG, respectively. The chemical structures of all the analytes are shown in Fig. 1.

Table 1
Mass spectrometer parameter settings for NAAG, [$^2H_5$]NAAG and [$^2H_8$]NAAG.

Analyte	SRM transition	Polarity	Declusteringpotential (V)	Collisionenergy (V)	Collision exit potential (V)
NAAG	$m/z303 \rightarrow m/z128$	negative	−50	−26	−13
[$^2H_5$]NAAG	$m/z308 \rightarrow m/z133$	negative	−50	−22	−13
[$^2H_8$]NAAG	$m/z311 \rightarrow m/z133$	negative	−50	−22	−13