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# N-Acetyl-aspartylglutamate (NAAG) in human cerebrospinal fluid: Determination by high performance liquid chromatography, and influence of biological variables

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**Summary.** NAAG is one of the neuropeptides found in highest concentrations in the CNS. The presence of micromolar concentrations of NAAG in human CSF was demonstrated by using two different and complementary analytical approaches: 1) isocratic separation of endogenous NAAG by reverse-phase high performance liquid chromatography (HPLC) with dual wavelength detection and 2) derivatization of endogenous NAAG with acidic methanol and subsequent HPLC analysis of the derivative NAAG-trimethyl ester. The NAAG concentration was between 0.44 $\mu$mol/l and 7.16 $\mu$mol/l (mean of 2.19 $\pm$ 1.53 $\mu$mol/l) in CSF samples from forty neuropsychiatric patients. Endogenous NAAG or [$^3$H]NAAG added to CSF samples were not significantly degraded when the CSF was incubated at 37 °C during one hour, suggesting that the peptide is a highly stable metabolite in the subarachnoid space. In addition, evidence is provided that NAAG does not present a concentration gradient along the lower subarachnoid space.

**Keywords:** Amino acids – NAAG – N-Acetyl-aspartyl-glutamate – N-Acetyl aspartyl-glutamate trimethyl ester – N-Acetyl-glutamyl-glutamate – Cerebrospinal fluid-HPLC

## Introduction

Several lines of evidence suggest that the dipeptide N-acetyl-aspartyl-glutamate (NAAG) plays a modulatory or neurotransmitter role in the mammalian central nervous system (CNS). NAAG is one of the neuropeptides found in highest concentration in the CNS, being localized in an heterogeneous population of neurons (for review see Blakely and Coyle, 1988). Although NAAG contains equimolar amounts of the excitatory transmitters, glutamate and aspartate, it exhibits a very low intrinsic excitatory action at post-synaptic sites (Whitemore and Koerner, 1989; Luini et al., 1984; Riveros

and Orrego, 1984). However, it is known that NAAG may be hydrolized by a N-acetyl-aspartate-α-linked-acidic-dipeptidase (NAALADase) present in synaptic plasma membranes, into N-acetyl-aspartate and glutamate, and is thus a potential source of these excitatory amino acids (Blakely et al., 1988; Serval et al., 1990). Accordingly, extracellular NAAG may influence neuronal excitability through the indirect activation of excitatory amino acid receptors and in this manner, be involved in the genesis of some neurodegenerative disorders (Plaitakis and Constantakakis, 1993, Tsai et al., 1991).

Cerebrospinal fluid (CSF) is considered to reflect the composition of the extracellular brain space and thus, sampling of CSF is a valuable tool for the assessment of neurochemical parameters of CNS function and pathology in man. Several methods are available to determine monoaminergic, peptidergic and amino acid neurotransmitters in human CSF (Nyberg and Terenius, 1982; Wallasch et al., 1988; Pitkänen et al., 1989; Kornhuber et al., 1988; Ferraro and Hare, 1985). Recently, the levels of N-acetyl-aspartate and NAAG in CSF have been assessed by gas chromatography-mass spectrometry and HPLC-UV detection, respectively (Swahn, 1990; Rothstein et al., 1990). However, protocols for NAAG analysis based on UV detection at 210 nm, such as that used in Rothstein's report, are considered quite unspecific, particularly for the assessment of relatively low concentrations of the peptide in complex samples like CSF. Moreover, little is known about eventual factors influencing NAAG levels in the CSF, such as gender differences, peptide stability and distribution along the lumbar subarachnoid space. In the present study, by using two complementary and highly specific HPLC approaches we report the existence of micromolar concentrations of NAAG in human CSF and we provide preliminary information on some biological factors influencing CSF NAAG levels.

## Material and methods

### *Material*

NAAG and glutamyl-α-linked-L-glutamate were obtained from Bachem (Switzerland); N-acetyl-aspartyl-L(3,5-$^3$H)glutamate (54.7 Ci/mol) from New England Nuclear, and Dowex resins AG 50W-X8 and AG 1-X2 from Bio-Rad. All other reagents were from Sigma Chem. Co. and Baker. N-acetyl-glutamyl-glutamate (NGG) was synthesized from glutamyl-glutamate and acetic anhydride according to Koller et al. (1984). The derivative NAAG trimethyl ester (NAAG-E) was synthesized from NAAG and acidic methanol according to Zollinger et al. (1990). Both compounds were purified and tested for contaminating NAAG, N-acetyl-glutamate and/or glutamyl-glutamate by HPLC.

### *CSF samples*

CSF samples were obtained from a CSF bank of patients enrolled in a cross-cultural study on depression carried out jointly by the Psychiatric Clinics from the Karolinska Institute (Sweden) and the Psychiatric Hospital (Montevideo), with the corresponding approval of the Ethical Committees from both countries. Other samples were kindly supplied by Dr Mario Medici from the Neurology Clinic of the Hospital de Clínicas (Montevideo), following the same ethical rules. The samples analyzed were obtained from 26 psychiatric patients of either sex (43 ± 10 years old) with diagnosis of major depressive disorder and

schizophrenia, and from 14 neurological patients of either sex (50.7 ± 19 years old) with diagnosis of amyotrophic lateral sclerosis (ALS) and muscular distrophy. None of the patients had undergone pharmacological treatment in the week previous to the study, with the exception of anxiolitic doses of benzodiazepines in the case of psychiatric patients. In all cases, lumbar punctures (L3–L4 or L4–L5 interspace) were performed between 8 a.m. and 2 p.m. in lying position and after receiving the patient's consent. Two successive fractions of 5 ml of CSF were drawn from each patient, immediately chilled on ice and frozen to −80 °C until analysis. Only clear, colourless CSF was used. To investigate the stability of NAAG in the CSF, some untreated CSF aliquots were incubated in the presence of [$^3$H]NAAG (3 nM) at 37 °C during 60 min and then processed for HPLC analysis in order to determine endogenous NAAG levels and comparative recoveries of NGG and [$^3$H]NAAG.

*Sample preparation*

NAAG levels were measured in 500 µl of CSF acidified with 200 µl of 0.1 M perchloric acid and centrifuged at 15.000 g during 5 min at 4 °C. The NAAG analog, NGG (10 µl, $5 \times 10^{-3}$ M) and/or [$^3$H]NAAG (20 µl, $10^{-7}$ M) were added to CSF aliquots and used as internal standards during sample preparation. Acidified CSF aliquots were loaded on microcolumns packed with 500 µl of Dowex AG 50 resin previously washed with 0.02 M sodium phosphate buffer, pH 5.5. This column allows the N-substituted peptides such as NAAG and NGG to pass through, retaining free amino acids and other peptides with unsubstituted amino terminal groups. The eluate was combined with the washings and adjusted to pH 5.5 with concentrated NaOH using a colorimetric pH marker (Prolabo, 10 µl per sample). It was then applied to an anion exchange column packed with 400 µl of Dowex AG-1 (formate form), previously treated with 1 ml of 50 mM sodium phosphate buffer, pH 5.5. Acidic compounds were retained in the column, while other compounds were washed through with 1.5 ml of 50 mM phosphate buffer. Finally, NAAG and NGG were eluted with 4.5 ml of 0.2 M formic acid and lyophilized overnight. Residues were resolubilized in 200 µl of mobile phase, centrifuged at 10.000 g for 10 minutes and injected to the HPLC system.

*Derivatization of NAAG in CSF samples*

Two ml of CSF were purified as described above and the lyophilizate was directly derivatized according to Zollinger et al. (1990), using 500 µl of 2 N methanolic-HCl during 2 h at room temperature. The solvent was then evaporated under a gentle stream of dry nitrogen, and the residue was redissolved in 100 µl of water for HPLC determination. Preliminary studies showed that under these conditions, more than 95% of the endogenous NAAG was derivatized to NAAG trimethyl ester (NAAG-E).

*HPLC analysis*

The HPLC system (LKB-Pharmacia, Sweden) consisted of a LKB 2150 pump provided with a low Pressure Mixer, a LKB 2252 LC Controller and a sample injector with a 50 µl injection loop. Detection of peptides was performed with a 2141 LKB dual variable wavelength monitor at 210 and 254 nm. Data were recorded and quantified in a Chromjet integrator (Spectraphysics). NAAG analysis under isocratic conditions was carried out according to Siciliano et al. (1991) using a reversed-phase column (Ultrosphere ODS, 5 µm, 25 cm × 4.6 mm i.d., Beckman) eluted with 50 mM potassium phosphate (monobasic), adjusted to pH 3.0 with phosphoric acid at 0.8 ml/min. The concentration of NAAG in the CSF was calculated from the ratio of its peak area compared with the peak area in the external standard, and corrected for NGG recovery. In some cases, the recovery of [$^3$H]NAAG was calculated by collecting the column eluate during the NAAG peak and counting the radioactivity by scintillation spectrometry (Beckman LS 100C). Esterified NAAG derivative (NAAG-E) was assessed by HPLC using a reversed-phase

column (Nucleosil C18, 5 $\mu$m, 4.6 × 150 mm, Interchrom) eluted with a linear gradient (0% to 50% acetonitrile in 0.05% trifluoroacetic acid), during 50 min at 0.8 ml/min, and measured with LKB UV detector at 210 nm.

*Statistics*

Statistical analysis was performed by applying the Student's two tailed t-test using the PRESTA-PC version kindly provided by Dr. V. Abraira, Instituto Ramón y Cajal, Spain.

## Results

Figure 1 shows typical chromatograms from isocratic runs detected at dual wavelength of a standard solution containing NAAG and NGG and of a representative CSF sample. NAAG in the CSF was identified as a single peak showing the same retention time as the standard peptide and displaying the same absorbance spectrum. NAAG is an acidic peptide and its retention in reverse phase columns is only achieved at pH < 3.6 (Siciliano et al., 1991). In the present experimental conditions, a mobile phase pH 3 was required to separate NAAG from another peak present in the CSF but also absorbing at 254 nm (see Fig. 1D). Thus, dual wavelength detection was currently used to monitor proper peak separation after column replacement. The internal standard NGG which exhibits a close chemical analogy with NAAG, was also resolved in the same chromatogram. Initial studies showed that NGG and [$^3$H]NAAG behave similarly during the sample preparation procedures with a mean recovery of 92 ± 4%, indicating that the use of NGG as internal standard improves the reproducibility and accuracy of the analytical process.

In order to further confirm the presence of NAAG in the CSF, the samples were treated with acidic methanol previously to the analysis, a reaction allowing the rapid and complete derivatization of NAAG into NAAG-trimethylester (NAAG-E). NAAG-E was then resolved by a HPLC procedure involving an elution gradient with acetonitrile. Figure 2 shows typical chromatograms of the NAAG-E standard, and of CSF samples before and after derivatization with acidic methanol. Endogenous esterified NAAG was identified in derivatized samples as a single peak with identical retention time than the NAAG-E standard.

Figure 3 shows the distribution of NAAG levels determined for each subject in the two successive 5 ml fractions (I and II) of CSF drawn from forty patients of both genders. NAAG levels showed a relatively wide dispersion within the population of subjects studied. However, small and non-systematic variations in NAAG levels between the first and second fractions of CSF drawn from each patient were found. On the other hand, no significant gender differences were observed in each of these fractions (Table 1).

NAAG stability in the CSF was studied in aliquots containing exogenous [$^3$H]NAAG incubated at 0 °C or at 37 °C during 60 min. Endogenous and tritiated NAAG were then simultaneously assessed in each sample. As shown in Table 2, no evidence of peptide degradation was obtained in five different CSF samples.

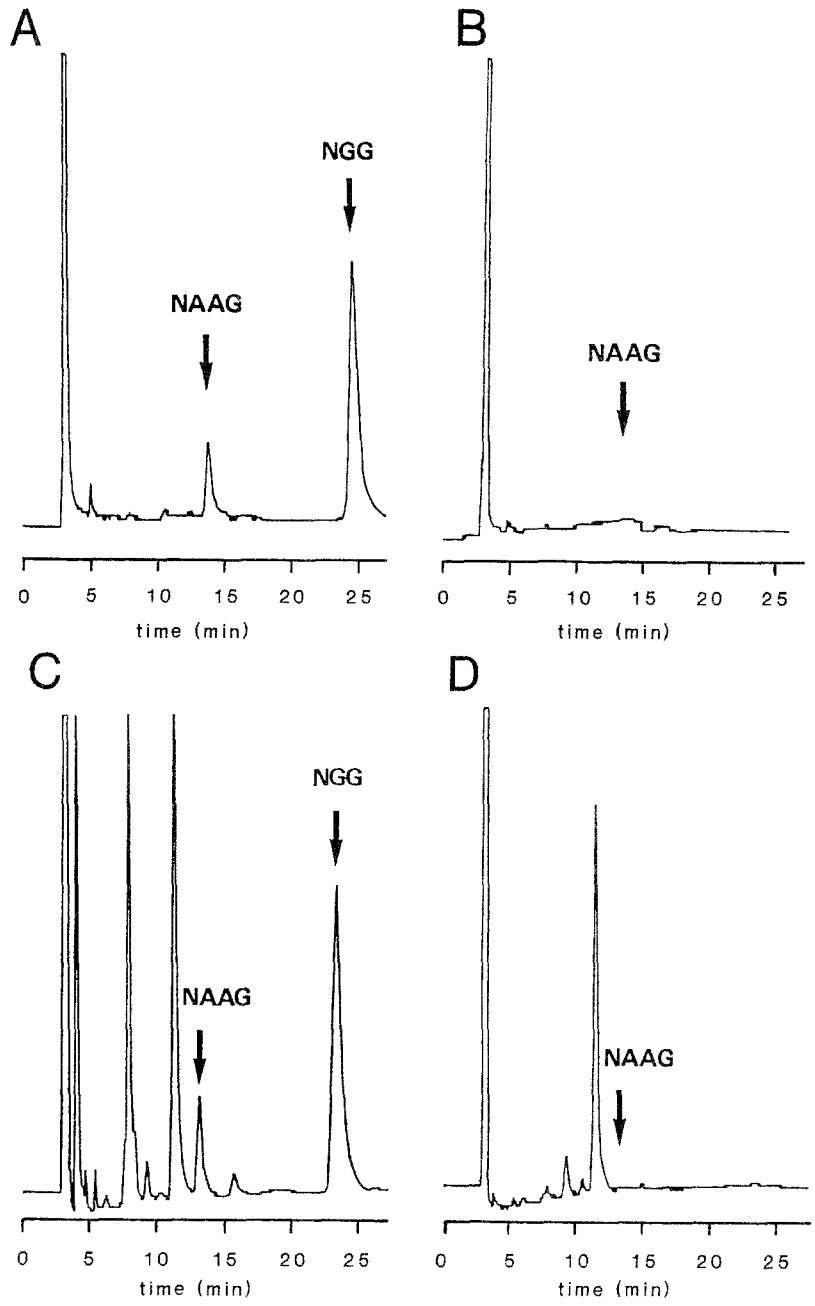


**Fig. 1.** Identification and quantification of NAAG in the CSF by HPLC. **A** External standard mixture containing NAAG (500 pmol) and NGG (1,5 nmol). Detector sensitivity was 2 AUFS and integrator attenuation was 4 mV full scale (Wavelength monitor at 210 mm). **B** Same chromatogram but monitored at 254 nm. Note that no peptide was detected at this wavelength. **C** Typical chromatogram of a CSF sample prepared as described in Material and methods. **D** Same chromatogram but monitored at 254 nm. NAAG was clearly separated from a contaminating peak absorbing at 254 nm

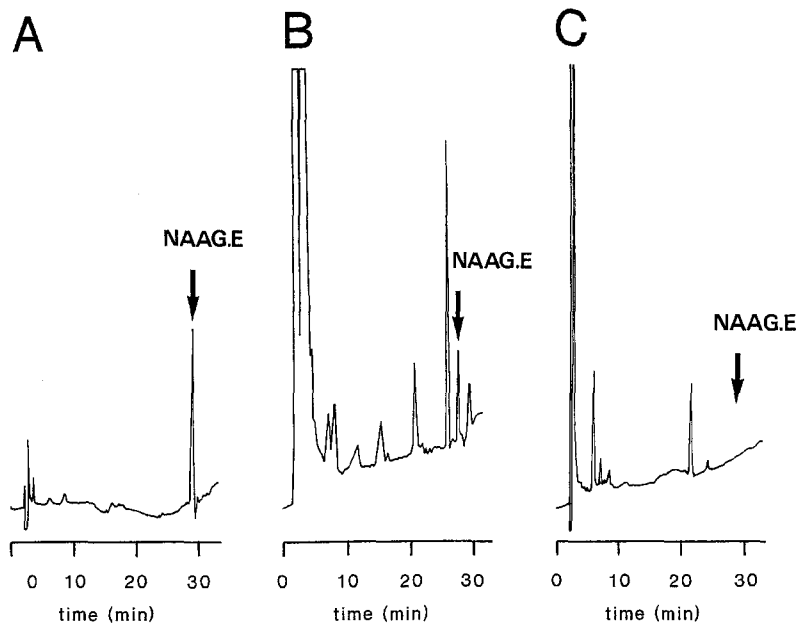


**Fig. 2.** Chromatographic profile of a CSF sample after derivatization with acidic methanol. **A** 3.0 nmol of a standard solution of NAAG trimethyl ester (NAAG.E). Detector sensitivity was 0.2 AUFS and integrator attenuation was 16 mV full scale. **B** CSF sample derivatized as described in Material and Methods. The peak of NAAG.E corresponds to 1.6 nmol of endogenous NAAG. **C** Same sample as **B** but without undergoing derivatization

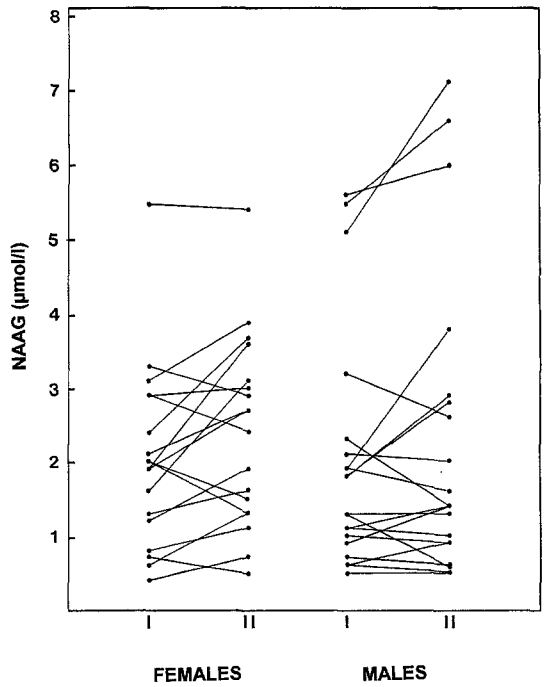


**Fig. 3.** NAAG levels in the first and second 5 ml fractions (**I** and **II**) of CSF collected. NAAG was determined in a group of forty subjects of either sex as described in Material and methods. Each line links the NAAG values (expressed in $\mu$mol/l) obtained for each individual in the respective fractions. Note that no systematic variations between fraction I and II can be observed

**Table 1.** Mean concentration of NAAG in the different CSF fractions[a]

| | | NAAG (μmol/l) | | |
|---|---|---|---|---|
| | n | Fraction I | Fraction II | $\bar{X}$ |
| Female | 18 | 2.03 ± 1.22 | 2.40 ± 1.27 | 2.22 ± 1.22 |
| Male | 22 | 2.04 ± 1.55 | 2.26 ± 1.98 | 2.15 ± 1.74 |
| Total | 40 | 2.04 ± 1.38 | 2.33 ± 1.66 | – |

[a] NAAG levels were determined in the first and second 5 ml fractions (I and II) of CSF collected. Values are expressed as mean ± S.D.; $\bar{X}$ denotes mean ± S.D. taken into account NAAG levels in fractions I and II.

**Table 2.** Metabolism of NAAG and [$^3$H]NAAG in human CSF[a]

| Samples | Endogenous NAAG levels (μmol/l) | | | [$^3$H]NAAG (DPM) | | |
|---|---|---|---|---|---|---|
| | 0 °C | 37 °C | % change | 0 °C | 37 °C | % change |
| I | 0.60 | 0.50 | −17 | 14372 | 14208 | −1 |
| II | 1.44 | 1.59 | +9 | 14234 | 15036 | +5 |
| III | 2.05 | 2.25 | +9 | 22840 | 20086 | −12 |
| IV | 4.39 | 4.53 | +3 | 13808 | 13938 | +1 |
| V | 6.99 | 7.18 | +3 | 13056 | 15246 | +14 |

[a] [$^3$H]NAAG was added to five different CSF samples to a final concentration of 3 pmol/ml. Each sample was then divided into two aliquots of 0.5 ml that were incubated during 60 min at 0 °C and 37 °C, respectively. The amount of endogenous and tritiated NAAG was determined in each sample as described in Methods. The difference between the values obtained at 0 °C and 37 °C was expressed as a percentage of change.

## Discussion

The present data show that the neuropeptide NAAG can be accurately separated and quantified by HPLC with dual wavelength detection in isocratic conditions. Two successive steps of ion exchange chromatography were necessary before analysis in order to avoid the co-elution of NAAG with CSF constituents, a situation that may lead to over-estimate NAAG levels. Moreover, we validate the use of a new internal standard, the NGG, which greatly improves the calculations of NAAG recoveries during sample preparation. The experiments showing equivalent concentrations of NAAG-E in samples derivatized with acidic methanol confirm the presence of NAAG in the CSF and validate the isocratic HPLC approach.

The wide dispersion of NAAG concentrations determined in a group of neuropsychiatric patients (from 0.44 μmol/l to 7.16 μmol/l) was not linked to gender differences. Indeed, it may reflect variations of peptide levels associated to different pathologies or simply non-systematic interindividual fluctuations. However, it is worth mentioning that globally considered, the

NAAG concentrations reported in this study are two or three times lower than those reported by Rothstein et al. (1990) using an anion exchange HPLC approach.

Although mean concentrations of NAAG are not indicated in Rothstein's report (1990), the average levels were between 7 $\mu$mol/l in the control group and 14 $\mu$mol/l in ALS patients. It is unlikely that the higher levels found by these authors may be explained by the differences inherent to the subjects studied, since NAAG concentration in ALS patients included in our group was $1.34 \pm 0.55$ $\mu$mol/l, thus, 10 times lower than that reported by Rothstein et al. The experiments with [$^3$H]NAAG or NGG show that in the present experimental conditions, endogenous NAAG is neither lost during the sample preparation procedures nor underestimated during chromatography. Therefore, the higher NAAG concentrations found by Rothstein et al., must be associated with the analytical procedure utilized by these authors for NAAG measurements.

The CSF concentration of NAAG did not seem to follow a gradient along the lower part of the lumbar subarachnoid space, as it has been described for other neurochemicals such as GABA, homocarnosine, homovanilic acid and 5-hydroxyindoleacetic acid (Sjostrom et al., 1975; Grove et al., 1982). However, due to the small volumes of CSF collected in this study, the existence of a NAAG concentration gradient between more rostral levels and the lumbar region, e.g. a cysternal-lumbar gradient, cannot be excluded. As the spinal cord posses one of the highest levels of NAAG within the CNS (Ory-Lavollee et al., 1987), it may be considered a potential source of NAAG diffusing to the CSF and accordingly, the highest concentrations of NAAG could be expected to occur in the second 5 ml CSF fraction. However, our results can be explained by considering that due to its stability in CSF, NAAG accumulation may become considerable allowing the homogeneous distribution of the peptide in the subarachnoid space.

According to our data, NAAG did not suffer significant degradation after incubation of CSF samples at 37 °C during one hour, suggesting that NAAG degrading enzymes have a low activity in the CSF. The latter could be restricted to neuronal and glial cell membranes (Blakely et al., 1988). In this sense, NAAG differs from other neuropeptides such as enkephalins and substance P, which are substrates of highly potent enzymes present in CSF (Nyberg et al., 1985).

In conclusion, the present study demonstrates the presence of micromolar levels of NAAG in human CSF by using two complementary HPLC approaches and shows that the peptide is a highly stable metabolite in the CSF, being homogeneously distributed in the lower part of the lumbar subarachnoid space. Further studies should be made to determine the normal levels of NAAG in the CSF in control subjects, and the possible diagnostic use of these measurements in neuropychiatric disorders.

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