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Quantification of N-acetylaspartylglutamate, an N-terminal blocked dipeptide neurotransmitter candidate, in brain slice superfusates by gas chromatography–mass spectrometry

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ABSTRACT

N-Acetylaspartylglutamate is a highly promising neurotransmitter candidate. A method for its quantification has been developed that allows to investigate its stimulation-induced release from brain slices. The method is based on ion-exchange prepurification, derivatization with HCl in methanol, separation by capillary gas chromatography and quantification by ammonia chemical ionization mass spectrometry with selected-ion monitoring. Deuterium-labelled N-acetylaspartylglutamate is used as internal standard The method has been validated, also with respect to possible interfering compounds A limit of quantification in the low pmol to high fmol range has been achieved, which is clearly sufficient for the intended purpose. A detailed analytical procedure is given, and alternatives for some of the different steps are discussed. Derivatization with diazomethane instead of methanolic HCl turned out to be impracticable. The method may well be applicable to certain other N-terminal blocked di- and tripeptides and to acylated amino acids.

INTRODUCTION

Knowledge of the compounds that are responsible for interneuronal communication is an important goal of neurochemistry and of great relevance to medicine and pharmacology. New candidates for neurotransmitters or neuromodulators are frequently proposed, yet in many cases the evidence for such a function is incomplete. Within the class of the N-terminal blocked small peptides, N-acetylaspartylglutamate (NAAG) is the most extensively investigated neurotransmitter candidate, but others have been suggested [1,2]. NAAG has been shown to fulfill many of the criteria required for acceptance as a neurotransmitter in some parts of the mammalian brain [1]. However, an important piece of evidence, an indication for its stimulation-induced release from neurons, has been lacking for a long time. Using the technique of brain slice superfusion, we have been able to demonstrate a Ca^{2+}-dependent increase of NAAG concentration in superfusates

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upon stimulation of the neurons by increasing the K^+ concentration in the superfusion medium from 5 to 50 m*M*. This suggests a stimulation-induced release of NAAG from a neuronal compartment, such as nerve terminals, in the parts of the rat brain that were investigated. The neurochemical aspects of this work, together with the details of the *in vitro* technique, have been published elsewhere [3]. Here we present the details of the analytical method.

Methods for quantification of NAAG using either esterification with diazomethane and packed-column GC [4] or anion-exchange high-performance liquid chromatography (HPLC) with gradient elution [5] or isocratic elution [6] and UV detection at wavelengths of *ca.* 210 nm have been published. The achieved limits of detection between 0.1 and 1 nmol are adequate for studies of the NAAG content of tissue homogenates and brain synaptosomal extracts but are insufficient for our purpose. We therefore developed our own method based on gas chromatography–mass spectrometry (GC–MS). Because of the volatility constraints of GC, NAAG has to be derivatized. HPLC is actually more adapted to this type of compound, but detection at low wavelengths is neither selective nor very sensitive, and well established methods for coupling with chromophoric or fluorescent groups, allowing detection with better sensitivity, require a free amino group that is not present in NAAG. MS detection not only provides high selectivity but also very good reliability of the quantification because a stable isotope-labelled analogous compound can be used as internal standard.

EXPERIMENTAL

Materials

NAAG was purchased from Bachem (Bubendorf, Switzerland) and N-acetylaspartyl[3,4-^{3}H]glutamate (44.1 Ci/mmol) from New England Nuclear (Boston, MA, U.S.A.). N-([2,2,2-$^2H_3$]Acetyl)aspartylglutamic acid (NAAG-d_3; 97% d_3) was synthesized from Asp-Glu (Bachem) as described elsewhere [3]. An analogous procedure was used for the acetylation of Asp-Gln (Bachem) to yield N-acetylaspartylglutamine (N-Ac-Asp-Gln) and for acetylation of Asp-Asp, Glu-Glu and Glu-Asp (Bachem). A solution of 0.5 *M* HCl in methanol was obtained in ampoules (Supelco, Bellefonte, PA, U.S.A.) and stored at −20°C. Solutions of HCl in butanol at different concentrations were prepared by adding acetyl chloride to ice-cooled butanol (both from Fluka, Buchs, Switzerland; puriss. p.a. grade). Diazomethane was produced by adding 40% aqueous KOH (w/v) to N-methyl-N′-nitro-N-nitrosoguanidine (Fluka). The gaseous diazomethane[a] was transported by a slow stream of nitrogen to a separate flask where it was bubbled through diethyl ether (Merck, Darmstadt, F.R.G.; spectroscopic grade, freshly

[a] Because of the well known dangerous properties of gaseous diazomethane, the procedure was carried out in a closed hood The base was added cautiously from outside the hood by pushing it from a syringe through thin PTFE tubing into the reaction vessel

distilled before use). According to GC analysis this method provided a much cleaner solution of diazomethane than the usual procedure using an aqueous–ethereal two-phase system. AG1-X8 200–400 mesh anion exchanger was purchased from Bio-Rad (Richmond, CA, U S.A.) in the chloride form, converted into the formate form as described by the manufacturer and washed extensively with 3 *M* formic acid in water–methanol 9:1 (v/v). Solvents were of analytical grade (Merck). Water was obtained from a Milli-Q system (Millipore, Bedford, MA, U.S.A.). For superfusion of the rat brain slices under resting conditions, an Earl's solution of the following composition was used: 154 m*M* Na^+, 5 m*M* K^+, 2 m*M* Ca^{2+}, 1 m*M* Mg^{2+}, 141.75 m*M* Cl^-, 20 m*M* HCO_3^-, 1.25 m*M* $H_2PO_4^-$, 1 m*M* SO_4^{2-}, 10 m*M* D-glucose, saturated with 5% CO_2–95% O_2. The compositions of the other superfusion media, the preparation of brain material and details of the release experiments are described elsewhere [3].

Instrumentation

For GC a Varian (Palo Alto, CA, U.S.A.) Model 3700 gas chromatograph was used, equipped with a Varian split/splitless injector, a Carlo Erba (Milan, Italy) on-column injector, a flame ionization detector and a nitrogen–phosphorus specific thermoionic detector, both from Varian. The GC–MS system consisted of a Carlo Erba Fractovap 4200 gas chromatograph coupled to a Vacuum Generators (Manchester, U.K) 7070F mass spectrometer by an interface of the kind described by Wetzel and Kuster [7], and a Vacuum Generators data system Model 2225, release 6. The instrument was operated in the multiple-ion monitoring mode using the digital MID unit and the SIR software of VG. The slit widths were adjusted such that flat-top peaks were obtained at a resolution of *ca.* 500 (10% valley definition). For GC as well as GC–MS open tubular glass capillary columns (20 m × 0.3 mm I.D.) with film thicknesses typically *ca.* 0.25 μm were used. The columns were made in the laboratory according to the procedures given by Grob [8]. Silicon PS255 or PS264 (Fluka) were coated as stationary phases onto glass surfaces pretreated by persilylation. Samples were injected in the on-column mode. Helium was used as carrier gas at optimized pressure [8].

Prepurification

Superfusate fractions of 5 ml, collected in ice-cooled tubes containing 250–500 pmol of NAAG-d_3 as internal standard, were put onto small columns (5 mm I.D.) containing 1.5 ml of strong anion exchanger (Bio-Rad AG1-X8, 200–400 mesh, formate form). The columns were rinsed with 20 ml of water–methanol (9:1, v/v) followed by 5 ml of 3 *M* formic acid in water–methanol (9:1, v/v) under gravity flow conditions. The acidic eluents were evaporated under vacuum at 30–40°C, redissolved in small volumes of water and transferred to Reacti-Vials (Pierce, Rockford, IL, U.S.A.). After evaporation to dryness, the samples were stored in a desiccator until further treatment.

Derivatization

Esterification of each sample was carried out with 100 μl of 0.5 *M* HCl in methanol at 60°C for 10 min. The reagent was evaporated in a gentle stream of dry nitrogen, and the product was redissolved in 75 μl of water, which was extracted twice with 150 μl of CH_2Cl_2 by agitation on a vortex mixer for 2 min and centrifugation for phase separation. The organic phases were combined in a Reacti-Vial and evaporated in a stream of nitrogen The residue was redissolved in 5 μl of toluene–ethyl acetate (9:1, v/v), of which 0.5–1 μl was injected on-column for GC–MS analysis.

Separation and quantification by GC-MS

The column temperature was 90°C at injection, then increased at 10°C/min to 280°C, where it was held for 5 min. $[M + H]^+$ ions from NAAG and NAAG-d_3 trimethyl esters (*m/z* 347 and *m/z* 350, respectively) were produced by ammonia chemical ionization at a source temperature of 150°C and a pressure in the ion source pumping line of 5 10^{-3} Pa, and continuously recorded by selected-ion monitoring. From the ratios of the areas under the peaks on these two mass traces and the amount of NAAG-d_3 added, absolute amounts of NAAG in each superfusate fraction were calculated using a calibration curve (see Results and discussion). 1,2-Diiodobenzene (Aldrich, Milwaukee, WI, U.S.A., 98%) was introduced via the reference inlet, and its steady signal at *m/z* 330 ($M^{+\bullet}$) was used as a lock mass.

RESULTS AND DISCUSSION

Prepurification

Anion-exchange chromatography was chosen for the prepurification of NAAG in brain superfusate fractions because NAAG is multiply negatively charged at neutral pH and electrically neutral at *ca.* pH 2. This distinguishes it from all constituents of the Earl's solution used for superfusion except phosphate, which was shown, however, to elute substantially later than NAAG from the ion exchanger under the conditions chosen. It is possible therefore to separate NAAG (and similar compounds) completely from all the salts and the glucose of the Earl's solution by the procedure given in Experimental. By means of tritiated NAAG and HPLC with radioactivity monitoring it was verified that amounts of NAAG as low as 2 pmol are recovered in the acidic fraction quantitatively and without degradation. 3 *M* Formic acid is sufficiently acidic for elution of NAAG, whereas 10 ml of 0.5 *M* formic acid in water–methanol (9:1, v/v) did not displace NAAG from the column. The ion exchanger was discarded after a single use. Freezing of superfusates before ion-exchange prepurification is not recommended because the Earl's solution is unstable and forms a precipitate when frozen. This solid does not dissolve on thawing and is trapped on the ion-exchange column at neutral pH, only to dissolve and elute with NAAG under acidic conditions.

Therefore the ion-exchange procedure was performed immediately after collection of the superfusate fractions.

Derivatization

In trace analysis by GC or GC–MS the sensitivity is often limited by a derivatization step. We therefore tried to keep the derivatization of NAAG as simple as possible. Its trimethyl ester turns out to be volatile enough to produce a narrow GC peak with almost no tailing if a suitable column is chosen. This derivative can be formed easily by treating a methanolic solution of synthetic NAAG with diazomethane in diethyl ether for 30 min (or shorter) at room temperature. However, the reaction failed for NAAG that was added to Earl's solution and recovered again, using the ion-exchange procedure described. Even for amounts as large as 100 nmol of NAAG, yields[a] of the trimethyl ester were very low (less than 5%) when the ion-exchange eluates were evaporated, redissolved in methanol and treated with an excess of diazomethane (that maintained its yellow colour till termination of the reaction). A number of experiments, using typically 30 nmol of NAAG, were carried out to find the reason for this failure. With tritium-labelled NAAG it was discovered that the compound had become almost insoluble in methanol after the ion-exchange procedure whereas before it dissolved well. It is conceivable that NAAG is eluted from the ion-exchange column in a not completley protonated form and therefore might be neither soluble in methanol nor esterifiable with diazomethane. Such an explanation, however, is not confirmed by the observation that good yields (*ca.* 80%) of NAAG trimethyl ester were obtained when an aqueous solution of NAAG, containing three equivalents of sodium acetate, was evaporated, taken up in methanol and then treated with diazomethane. Neither can deprotonation of NAAG be responsible for the variable and mostly low yields of its trimethyl ester obtained on dissolving NAAG in 3 *M* formic acid in water–methanol (9:1, v/v), evaporating the solution, redissolving in methanol and then treating with diazomethane. The reason for the failure of the diazomethane esterification has not become evident.

Because no way of improving the yields was found, esterification with diazomethane was abandoned and HCl in methanol was used instead, even though diazomethane would have been a milder reagent. Problems with esterification using diazomethane after acid treatment of peptides have been mentioned in the literature [4,9] without comment.

With 0.5 *M* HCl in methanol (Fischer esterification) an optimal yield of NAAG trimethyl ester was obtained when the reaction was carried out at 60°C for 10 min. Harsher conditions (longer reaction time or higher temperature) or milder conditions gave lower yields because of decomposition and incomplete reaction, respectively Ammonia chemical ionization MS (see below) was used to assess the stability of the deuterium label in NAAG-d_3. No loss of deuterium was

[a] All yields were estimated by comparing with external standards

detected during the esterification (using the above conditions) or during the ion-exchange prepurification or the following evaporation of the acidic fraction. When the esterification was carried out at 100°C for 40 min, the degree of deuteration dropped from 97 to 92% NAAG-d_3 but 50% of NAAG decomposed under these conditions. NAAG-d_3 is therefore a suitable internal standard for this analytical method.

To exclude the possibility that larger peptides containing an N-terminal N-Ac-Asp-Glu-sequence could be cleaved to yield NAAG, the conversion of N-Ac-Asp-Gln into NAAG was investigated. It can be expected that NAAG would be formed from a larger peptide at a lower rate than from N-Ac-Asp-Gln, making the latter a worst-case situation. During the ion-exchange procedure, including evaporation of the acidic fraction, no detectable amounts of NAAG were formed from N-Ac-Asp-Gln. However, during esterification of N-Ac-Asp-Gln with 0.5 *M* HCl in methanol, NAAG trimethyl ester was obtained with the following yields: 3–4% during 15 min esterification at 35°C, *ca.* 10% during 10 min at 60°C and *ca.* 30% during 40 min at 60°C. It is possible therefore that peptide bond cleavages occur during esterification. For this reason, in one series of experiments, samples from all brain regions investigated were esterified for 15 min at 35°C, then, after GC–MS measurement of an aliquot, were esterified again at 60°C for 40 min and measured once more. No significant differences were found between samples esterified using the two different conditions. We therefore conclude that the superfusates we investigated did not contain compounds that are converted into NAAG by our procedure.

Extraction of the methylation products with CH_2Cl_2 was added to the procedure after the first few experiments. Without this extraction low sensitivities and very broad GC peaks were encountered occasionally, without apparent reason. Sporadically present polar impurities with strong adsorption properties might have been responsible for these outliers. It should be pointed out, however, that also in such cases the results can still be considered reliable, as long as quantifiable signals are obtained, because the internal standard can be expected to behave in the same way as the component to be quantified.

Use of the tributyl instead of the trimethyl ester of NAAG was considered The former gave a more symmetrical GC peak than the latter. Optimal yields of the tributyl ester were obtained with 3 *M* HCl in butanol when the reaction proceeded for 15 min at 50°C. However, this converted N-Ac-Asp-Gln into NAAG tributyl ester with 35% yield. No milder conditions (lower concentration of HCl, lower temperature, shorter reaction time) could be found representing a good compromise between esterification yield and minimal amide hydrolysis. Therefore methylation has to be considered more suitable than butylation in this case.

Separation and quantification by GC–MS

GC separation was performed on glass capillary columns as described in Experimental. Silicon PS255 and PS264 were equally suitable as stationary phases

when coated onto glass surfaces pretreated by persilylation, as opposed to barium carbonate pretreatment, which led to strong adsorption effects with the same stationary phases. Samples were injected in the on-column mode. Splitless injection was unsuccessful with our equipment because of adsorption or thermal degradation in the injector. With a temperature programme of 10°C/min, starting from 90°C, the trimethyl ester of NAAG eluted from a PS255 column at *ca.* 250°C. At an elution temperature 5°C lower an additional signal was sometimes observed, which disappeared on cutting the first 30 cm from the column. Hence this signal was due to decomposition of NAAG trimethyl ester caused by deposits on the head of the column.

Optimized conditions for ionization in the mass spectrometer were as follows: chemical ionization with ammonia as reactant gas ($5 \cdot 10^{-3}$ Pa in the ion source pumping line); ion-source temperature at or below 150°C. Under these conditions the spectrum of NAAG trimethyl ester shown in Fig. 1A was obtained. The $[M + H]^+$ ion at m/z 347 represents the base peak and provides good sensitivity for quantification by selected-ion monitoring at a mass high enough to show no background signals from contaminants in the sample or the ion source. At higher

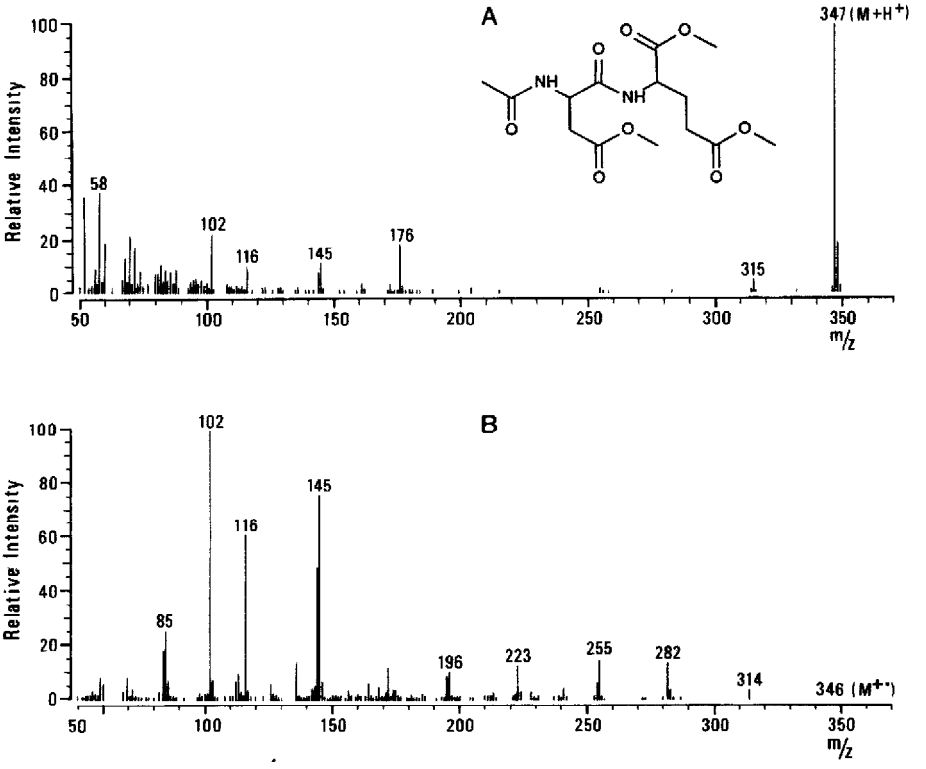

Fig 1 Mass spectra of NAAG trimethyl ester (A) Ammonia chemical ionization at 150°C ion-source temperature; (B) electron-impact ionization at 250°C ion-source temperature and 70 eV ionization energy

ion-source temperatures the intensity of fragment ions is increased at the expense of the quasi-molecular ion. With electron-impact ionization, NAAG trimethyl ester gave the spectrum shown in Fig. 1B. Almost no molecular ions were observed and intense fragment ions were found only in the low mass region. With methane or isobutane chemical ionization, $[M + H]^+$ ions of NAAG trimethyl ester were observed but did not represent the base peaks in the spectra (*ca* 120°C ion-source temperature). In addition, background signals at every mass in the molecular ion region, probably caused by clustering reactions in the ion source, were observed, especially with isobutane but also with methane. A substantially higher limit of detection for NAAG trimethyl ester can therefore be expected when using methane or isobutane instead of ammonia.

With ammonia chemical ionization the total ion chromatogram showed a severely tailing peak on injection of 1 nmol of NAAG trimethyl ester (Fig. 2A). This tailing did not disappear when the ion-source temperature was increased to 250°C. A mass spectrum from the tailing portion of the peak was found to contain intense signals only in the low mass region. Accordingly this tailing is not visible on the *m/z* 347 mass trace (Fig. 2B). There was no such tailing visible in the total ion chromatograms obtained with electron impact ionization (Fig. 2C) and methane or isobutane chemical ionization (120 and 250°C ion-source temperature, not shown). Reactions on the source walls in the presence of an ammonia plasma may be responsible for this tailing. Similar effects have been observed in negative-ion mass spectrometry [10,11]

The linearity of the relationship between the ratio of the amounts of NAAG and NAAG-d_3 trimethyl ester injected and the ratio of the signal areas at *m/z* 347 and 350 was checked and found to be unsatisfactory, especially for certain choices of acquisition parameters. A calibration curve was therefore established which was shown to be reproducible when keeping acquisition parameters constant. The cause of the non-linearity was tracked down to a misconception in the con-

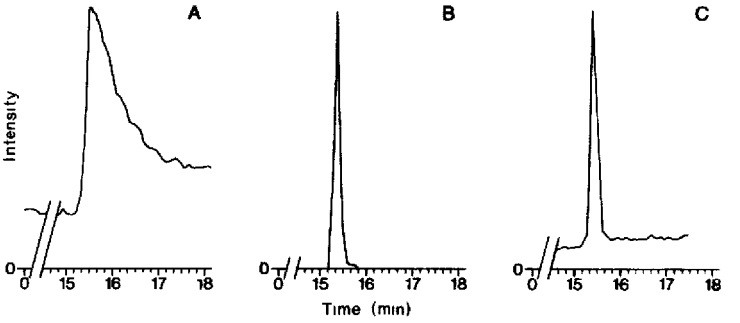

Fig. 2. Reconstructed ion chromatograms from injection of 1 nmol of NAAG trimethyl ester onto the GC column. (A) Total ion chromatogram (*m/z* 50–400) following ammonia chemical ionization, ion source at 150°C, (B) selected-ion chromatogram of *m/z* 347, conditions as in A, (C) total ion chromatogram (*m/z* 50–400) following electron-impact ionization, ion soure at 250°C, 70 eV ionization energy

trol by the data system of the automatic gain-adjusting electronics of the signal amplifier in our instrument.

Characteristics of the procedure

For the method described in Experimental the following characteristics were determined. A signal-to-noise ratio greater than 10 was obtained on the mass trace of *m/z* 347 on injection of 500 fmol of NAAG trimethyl ester on-column, together with 50 pmol of internal standard. Ratios of signal areas at *m/z* 347 and 350 showed relative standard deviations below ± 5% for amounts above 2 pmol injected. A non-linearity, not inherent to the technique itself but due to the instrumentation used, was corrected for by using a calibration curve. The two mass traces did not show any peaks in addition to those of NAAG and NAAG-d_3 trimethyl ester. N-Acetylglutamylaspartic acid trimethyl ester was found to elute 14 s later than NAAG trimethyl ester under our standard conditions. Therefore our method clearly distinguishes between the two isomers, of which only NAAG has been found in brain. The deuterium labelling in NAAG-d_3 was stable under all conditions of sample treatment used. No decomposition of NAAG occurred during ion exchange, evaporation of the acidic fraction or esterification. The probability that NAAG is formed from a different compound during sample preparation is low, but highest during the esterification step. This possibility could be excluded for the brain superfusates investigated by comparing results obtained on esterification under milder and harsher conditions than optimal.

It might be possible to use GC instead of GC–MS for quantification of NAAG in *in vitro* brain superfusates. Preliminary experiments with a nitrogen–phosphorus specific thermoionic detector revealed a sensitivity similar to the one obtained with our GC–MS instrumentation. The low number of GC signals in the range where the NAAG trimethyl ester is eluting suggests that interfering compounds will probably not become a problem. N-Ac-Asp-Asp could be used as internal standard. N-Ac-Glu-Glu may not be as suitable because its trimethyl ester was found to decompose on the GC column with variable yield to a compound eluting very close to NAAG trimethyl ester. An instrumentally less demanding GC procedure might therefore provide sufficient specificity and accuracy.

In conclusion, we have developed a method for quantification of NAAG in brain superfusates. The technique is clearly adequate for this purpose with respect to specificity, accuracy and sensitivity Amounts of NAAG found in superfusates of rat brain slices were in the range 0.2–3 pmol/mg of protein per min [3]. The method has been applied to confirm a Ca^{2+}-dependent, stimulation-induced release of NAAG from neuronal tissue [3], which represents an important piece of evidence in favour of a neurotransmitter role of NAAG. The method may be easily adaptable to chemically similar compounds.

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