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-HPLC determination of acidic α -amino acids and their *N*-methyl derivatives in biological tissues

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Abstract

α -aspartate (α -Asp) and *N*-methyl- α -aspartate (NMDA) occur in the neuroendocrine systems of vertebrates and invertebrates where they play a role in hormone release and synthesis, neurotransmission, and memory and learning. *N*-methyl- α -glutamate (NMDG) has also been detected in marine bivalves. Several methods have been used to detect these amino acids, but they require pretreatment of tissue samples with *o*-phthalaldehyde (OPA) to remove primary amino acids which interfere with the detection of NMDA and NMDG. We report here a one step derivatization procedure with the chiral reagent *N*- α -(5-fluoro-2,4-dinitrophenyl)-(D or L)-valine amide, FDNP-Val-NH₂, a close analog of Marfey's reagent but with better resolution and higher molar absorptivity. The diastereomers formed are separated by HPLC on an ODS-Hypersil column eluted with TFA/water – TFA/MeCN. UV absorption at 340 nm permits detection levels as low as 5–10 picomoles. D -Asp, NMDA and NMDG peaks are not obscured by other primary or secondary amino acids; hence pretreatment of tissues with OPA is not required. This method is highly reliable and fast (less than 40 minutes HPLC run). Using this method, we have detected D -Asp, NMDA and NMDG in several biological tissues (octopus brain, optical lobe, and buccal mass; foot and mantle of the mollusk *Scapharca broughtonii*), confirming the results of other researchers.

Keywords

HPLC; D -Asp; *N*-methyl- D,L -Asp; NMDA; *N*-methyl- D,L -Glu

INTRODUCTION

α -Aspartic acid (α -Asp) is an endogenous amino acid found for the first time in the nervous system of the mollusk *Octopus vulgaris* (D'Aniello and Giuditta, 1977, 1978). Since then, this amino acid has been discovered in the nervous and endocrine systems of many other animal phyla from mollusks to mammals (for reviews see D'Aniello, 2007; H. Homma, 2002), including humans (Fisher *et al.*, 1991, 1994, 1998). α -Asp has been found localized particularly in the synaptic vesicles of nerve endings, where there is evidence that α -Asp acts as a neurotransmitter (Spinelli *et al.*, 2006).

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N-methyl-D-aspartate (NMDA) is a potent neuroexcitatory amino acid (Curtis and Watkins, 1962; Watkins and Evans, 1981) that has specific action as an agonist for one of the glutamate sub-type receptors in the central nervous system of invertebrates and vertebrates (Monaghan and Cotman, 1986; Monaghan, Bridges, and Cotman, 1989; Hashimoto and Oka, 1997). NMDA receptors play an important role in learning and memory (Mondadori *et al.*, 1989; Rison and Stanton, 1995). NMDA has also been shown to improve social recognition potency in rats when administered systemically to adult rats (Hlinak and Krejci, 2002).

Endogenous NMDA was first extracted from the muscle of the mollusk *Scapharca broughtonii* (Sato *et al.*, 1987) and has subsequently been found in nervous tissues and endocrine glands of various animal phyla (D'Aniello, *et al.*, 2000a, 2000b, 2003, 2007). *N*-Methyl-L-aspartate (NMLA) also occurs naturally in certain marine algae (Sciuto, Piattelli and Chillemi, 1979; Sato *et al.*, 1987). NMDA and NMLA, as well as *N*-methyl-(D & L)-glutamate (NMDG and NMLG), have been found in other mollusk bivalves (Shibata, *et al.*, 2001; Tarui, *et al.*, 2003).

Several methods have been developed for resolution of D- and L-enantiomers of primary amino acids by high performance liquid chromatography (HPLC) following precolumn preparation of diastereomeric derivatives of the amino acids with chiral reagents. However, very few chiral reagents are known to react with secondary amino acids such as *N*-methyl-D,L-aspartate and *N*-methyl-D,L-glutamate. D'Aniello *et al.* (2002) developed an indirect method for determination of NMDA based on the HPLC detection of the methylamine (CH₃NH₂) produced by the oxidation of NMDA by D-aspartate oxidase (D-AspO, EC 1.4.3.1), an enzyme that catalyzes oxidization of acidic D-amino acids. This method, however, would not distinguish between NMDA and NMDG since both of these amino acids could be oxidized by D-AspO, producing methyl amine.

Todoroki *et al.* (1999) developed an HPLC method for determination of NMDA in biological tissues using precolumn derivatization with the chiral reagent (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) followed by isocratic resolution of the two diastereomers formed. This method, however, requires the removal of neutral and basic substances by anion-exchange chromatography and removal of primary amino acids by treatment with *o*-phthalaldehyde (OPA) before derivatization of the NMDA. Subsequently, Shibata *et al.* (2001) and Tarui *et al.* (2003) used (+)- and (-)-FLEC chiral derivatizing reagents and HPLC to determine NMDA, NMLA, NMDG, and NMLG in bivalve mollusks. Again, these methods required prior removal of neutral and basic substances and primary amino acids, and the HPLC runs were extremely long (80 mins).

The Homma group in Japan (Sekine *et al.*, 2002) also developed an automated two step column-switching HPLC system for separation of the *N*-methyl aspartates from primary amino acids followed by resolution on a chiral column of the NMDA and NMLA derivatives formed by reaction with the fluorescent reagent 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). The disadvantages of this method are the high cost and short life-time of the chiral column and long procedure times for the two step column-switching HPLC system.

In this report, we present a sensitive, one-step derivatization and HPLC resolution method for determination of NMDA, NMLA, NMDG, NMLG as well as D- and L-Asp. This method is based on a derivatization procedure with the chiral reagent *N*-α-(5-fluoro-2,4-dinitro-phenyl)-(D or L)-valine amide (FDNP-Val-NH₂), a close analog of Marfey's reagent 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (see reviews of Marfey's reagent by Bhushan and Brückner, 2004; B'Hymer et al., 2003; Harada et al., 1995). The use of Val amide instead of Ala amide has previously been shown to improve amino acid resolution and detection (Brückner and Keller-Hoehl, 1990). The diastereomers formed (see Scheme 1) are separated on an inexpensive reversed phase ODS-Hypersil column with elution by 0.11% TFA/water – 0.11% TFA/MeCN (90:10) isocratically for 2 min, followed by a linear gradient up to 70% TFA/MeCN. UV absorption at 340 nm permits detection levels as small as 5–10 picomoles.

EXPERIMENTAL

Materials

NMDA, NMLA, NMDG, NMLG, (D & L)-Asp and other amino acids were purchased from Sigma-Aldrich (St. Louis, MO, USA). FDNP-(D or L)Val-NH₂ derivatizing reagents were purchased from NovaBiochem (La Jolla, CA, USA), and were >99.5% pure as ascertained by HPLC and had >99.9% optical purity as ascertained by optical rotation (manufacturer's specifications). AG50W-X8 cation exchange resin was from Bio-Rad (Hercules, CA, USA). All solvents were HPLC grade. The reversed-phase ODS-Hypersil 5μm column (25 × 0.46 cm) was purchased from Column Engineering (Ontario, CA, USA). The HPLC system consisted of a SpectraSYSTEM P4000 quaternary pump connected to a SpectraSYSTEM UV2000 variable wavelength detector (Thermo Electron Corp., San Jose, CA, USA), an Alltech in-line degasser (Alltech Associates, Inc., Deerfield, IL, USA), and a Hitachi D-7500 computing integrator (Hitachi Instrument Co., Japan). Trifluoroacetic acid (TFA), acetonitrile (MeCN), and methanol (MeOH) were reagent grade. Water was purified through a Modulab™ PureOne water purification system, Continental Water Systems, Corp.

Tissue sample preparation

Biological samples consisting of 100–500 mg of tissue were homogenized with 0.2 M perchloric acid (PCA) or 10% w/v trichloroacetic acid (TCA) in a ratio of 1:10, and centrifuged at 5000 g. The supernatant was passed through an AG50W-X8 (100–200 mesh, H⁺ form) cation exchange column (~4 × 1 cm) to purify the sample from peptides and organic pigments. After applying the sample, the column was first washed with 0.01 M HCl, followed by 4 M NH₄OH to elute the amino acids. The ammonia was evaporated under a hood at 40°C and the remaining solution was freeze dried. After freeze drying, the residual solid was dissolved in 0.5 M NaHCO₃ followed by the derivatization procedure described below.

Derivatization

D,L-amino acids or tissue samples were derivatized with D- or L-FDNP-Val-NH₂, essentially according to the procedure for Marfey's reagent as reported by Szókán *et al.* (1988) (Scheme 1). 2.5 mmol of amino acid or an aliquot of tissue homogenate from above was

dissolved in 100 mL of 0.5 M NaHCO₃ to which 400 mL of a 1% solution of FDNP-Val-NH₂ in acetone was added. The mixture was incubated for 90 min at 40°C in a Thermomixer, and after cooling acidified with 2 M HCl to pH 4. After 100–160 fold dilution with MeOH, 10 mL were injected into the HPLC. The derivatized amino acids are very stable when stored at 4°C and can be re-analyzed for a year.

HPLC analyses

The derivatized diastereomers formed were separated by HPLC on an 5 µm ODS-Hypersil reversed phase column (25.0 × 0.46 cm) using a gradient pump, with detection at 340 nm, connected to a computing integrator. Two solvent mixtures were used: A) 0.11% TFA in water (v/v); B) 0.11% TFA in MeCN (v/v). The following HPLC program was used: isocratic for 2 min with 80% A:20% B, then a linear gradient to 62% A:38% B over the next 46 minutes, followed by a 4 min wash with 5% A:95% B, then back to 80% A:20% B. All flows rates are 1.1 mL/min. The diastereomers were detected with a UV-Vis detector at 340 nm, and peak areas were quantified using a computing integrator data handling system.

RESULTS

Figure 1 shows HPLC chromatograms for standards of (A) NMLA and NMDA, (B) D- and L-Asp, and (C) NMDG and NMLG when derivatized with FDNP-D-Val-NH₂. The enantiomers of each set are well resolved. The order of elution of the enantiomers is reversed when derivatized with FDNP-L-Val-NH₂ (data not shown). A large peak seen around 38 min is unreacted FDNP-Val-NH₂ derivatizing reagent. The NMDA, NMDG, and D-Asp peaks disappeared or diminished in size when the sample was pre-incubated with D-aspartate oxidase which oxidizes these D-amino acids (data not shown).

Figure 2 shows an HPLC chromatogram of a standard mixture of all the amino acids that elute in the region from 15 to 45 min. It can be seen that the enantiomers of each amino acid are fairly well resolved and each pair of amino acids is fairly well separated from the others. Primary amino acids eluting in the same region (e.g. Ser, Asp and Glu) show little interference with the N-methyl amino acids. However, D-Thr coelutes with NMLA. This should not be a problem since most biological samples probably do not contain D-Thr. Also, when the sample is derivatized with FDNP-L-Val-NH₂ then L-Thr and NMDA coelute, and NMLA is resolved. Thus, NMLA can be identified. Other primary amino acids elute either before or after this region and do not interfere with NMDA, NM(D&L)G or (D&L)-Asp.

Figure 3 shows the HPLC chromatogram analysis of tissues from the brain and optical lobe of *Octopus vulgaris*. The presence of NMDA is clearly seen in octopus brain (Fig. 3A) and in optical lobe (Fig. 3B). Proof that the peak is actually NMDA is shown in Fig. 3C after the octopus optical lobe tissue sample was spiked with racemic D,L-N-methyl aspartate, derivatized and re-run on the HPLC. As seen in Fig. 3C, now there is an increase in the NMDA peak and the appearance of a new peak corresponding to NMLA. A peak corresponding to D-Asp is also found in both of these octopus tissues.

Fig. 4 shows the HPLC chromatogram of the mantle of the mollusk *Scapharca broughtonii*. The presence of both NMDA and NMDG is shown in this mollusk. Our results confirm the

results of previous researchers who also found both NMDA and NMDG in *Scapharca broughtonii*.

DISCUSSION

We have developed an HPLC method for determination of D-Asp, N-Me-D-Asp, and N-Me-D-Glu using FDNP-(D or L)Val-NH₂ chiral derivatizing agent, a close analog of Marfey's reagent. FDNP-Val-NH₂ derivatizing reagents give better resolution [greater separation between retention times, t_R (Brückner and Keller-Hoehl, 1990)] and higher molar absorptivity of the derivatives of N-methyl amino acid enantiomers than with Marfey's reagent. This is presumably due to the increased hydrophobicity of the derivative by replacing alanine amide by valine amide, thereby interacting more strongly with the reversed phase HPLC column. In addition, the derivatives of the amino acids are stable for more than one year.

Our method is highly reliable and fast (less than 40 min HPLC run time). After extensive studies of all 20 natural amino acids plus gamma amino butyric acid (GABA) and taurine, we found that only D-threonine partially overlaps with N-Me-L-Asp while N-Me-D-Asp is well separated from other amino acids. However, if one is interested in detecting specifically the N-Me-L-Asp, this problem can be easily overcome by using the other enantiomer of the derivatizing agent (FDNP-L-Valine-NH₂). Then the order of elution will be reversed, and now N-Me-L-Asp will stay separated and N-Me-D-Asp will be partially obscured by L-threonine.

Another definitive advantage of our method is that pretreatment of tissue samples with OPA to remove primary amino acids is not necessary since none of the diastereomers of the primary amino acids overlap with D-Asp or N-Me-D-Asp. Elimination of the OPA treatment step also ensures higher recovery of the N-methyl amino acids from tissue samples.

We have presented this method primarily as a qualitative method for detecting acidic amino acids and their N-methyl analogs in biological tissues. In actual application, peak areas as a function of concentrations can be used to quantify and calculate of the concentrations of these amino acids in tissue samples once calibration curves have been determined using amino acid standards. Quantification and calibrations, however, are dependent on individual lab's HPLC instruments, columns, and data handling systems. Using this method, we have detected D-Asp, NMDA and NMDG in several biological tissues (for example: octopus brain, optical lobe, and buccal mass; foot and mantle of the mollusk *Scapharca broughtonii*), confirming the results of other researchers. Future work will be carried out to accurately quantify these amino acids in biological tissues.

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Figure 1. HPLC chromatograms for standards of (A) NMLA and NMDA, (B) D-Asp and L-Asp, and (C) NMDG and NMLG, each derivatized with FDNP-D-Val-NH₂. (Reprinted with permission from *D-Amino Acids: A New Frontier in Amino Acid and Protein Research: Practical Methods and Protocols*, Konno, Brückner, D'Aniello, Fisher, Fujii, and Homma, eds., Nova Science Publishers, New York, 2007, M. Tsesarskaia et al., Chapter 2.4, pp. 41–47.)

Figure 2.

HPLC chromatogram of a standard mixture of amino acids derivatized with FDNP-D-Val-NH₂. The order of elution of the individual amino acids is reversed when derivatized with FDNP-L-Val-NH₂ (data not shown). (Reprinted with permission from *D-Amino Acids: A New Frontier in Amino Acid and Protein Research: Practical Methods and Protocols*, Konno, Brückner, D'Aniello, Fisher, Fujii, and Homma, eds., Nova Science Publishers, New York, 2007, M. Tsesarskaia et al., Chapter 2.4, pp. 41–47.)

Figure 3.

HPLC chromatogram of tissues from the brain and optical lobe of *Octopus vulgaris*.

Octopus brain (3A) and optical lobe (3B) clearly show presence of NMDA in both of these tissues. Proof that the peak is actually NMDA is shown in Fig. 3C after the octopus optical lobe tissue sample was spiked with racemic D,L -*N*-methyl aspartate, derivatized and re-run on the HPLC; now there is an increase in the NMDA peak and the appearance of a new peak corresponding to NMLA.

Figure 4.
HPLC chromatogram of the mantle of the mollusk *Scapharca broughtonii*, showing the presence of both NMDA and NMDG.

Scheme 1.