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# Determination of *N*-methyl-D-aspartate in tissues of bivalves by high-performance liquid chromatography

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**Abstract**

The natural occurrence of *N*-methyl-D-aspartate (NMDA) is limited to the foot muscle of *Scapharca broughtonii*; it is a well known compound for its neuroexitatory activity. This paper describes a high-performance liquid chromatographic (HPLC) method for the determination of NMDA in biological extracts. The method involves removal of neutral and basic substances by anion-exchange chromatography and removal of acidic primary amino acids by treatment with *o*-phthalaldehyde before derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate, followed by HPLC with isocratic elution with a selected mobile phase that separates the two diastereomers formed. The identity of the detected NMDA has been confirmed by a procedure using (−)-1-(9-fluorenyl)ethyl chloroformate as a derivatizing agent. The identification has been further supported by the disappearance of the peak of the NMDA derivative by pretreatment of the sample with D-aspartate oxidase. Application of the method has shown the presence of NMDA in several tissues of *S. broughtonii* and *Scapharca subcrenata*.


*Keywords:* *N*-Methyl-D-aspartate

## 1. Introduction

*N*-Methyl-D-aspartate (NMDA) is a well known neuroexitatory compound [1] that is specific to one of the subtypes of glutamate receptors in higher animals [2], which are consequently known as NMDA receptors. NMDA was regarded as an artificial compound until its isolation from the bivalve *Scapharca broughtonii* by Sato et al. [3]. The discovery of the natural occurrence of NMDA has suggested the possibility that this compound is distributed in many other living organisms with some significant functions. However, there has been little information on the occurrence of this compound in organisms other than *S. broughtonii*, except a report of the presence of *N*-methyl-D,L-aspartate in certain marine algae [4]. This is probably because of the lack of suitable methods for determination of minute concentrations of NMDA in tissues of organisms. The first identification of this compound in the bivalve was achieved through crystallization after purification from a huge amount of foot muscle of the organism [3].

Several methods have been developed for the resolution of D- and L-enantiomers of primary amino

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acids by high-performance liquid chromatography (HPLC). These methods depend on the preparation of diastereomeric derivatives of amino acids with chiral reagents, such as *o*-phthalaldehyde (OPA) and *N*-acetyl-L-cysteine [5], 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide [6], and (+)-1-(9-fluorenyl)ethyl chloroformate [(+)-FLEC)] [7]. Of these reagents, only (+)-FLEC has been shown to react with secondary amino acids such as NMDA, although the separation of this compound from its enantiomer, *N*-methyl-L-aspartate (NMLA) has not been accomplished yet [8].

In the present paper, we report a method for the determination of NMDA by HPLC of their (+)-FLEC and (−)-1-(9-fluorenyl)ethyl chloroformate ((−)-FLEC) derivatives, after removal of primary amino acids by treatment with OPA. Application of the method to the determination of NMDA in the tissues of bivalves is also described.

## 2. Experimental

### 2.1. Chemicals

NMDA and NMLA were purchased from Sigma (St. Louis, MO, USA), and (+)- and (−)-FLEC from Aldrich (Milwaukee, WI, USA). Solvents for HPLC, acetonitrile and tetrahydrofuran of HPLC grade were obtained from Nacalai Tesque (Kyoto, Japan). D-Aspartate was a gift from Tanabe (Osaka, Japan) and all the other chemicals were analytical-reagent grade and purchased from Nacalai Tesque. The anion-exchange resin, Dowex 1×2 (100–200 mesh, chloride form) obtained from Muromachi Chemicals (Tokyo, Japan), was converted to acetate form before use by washing with saturated sodium acetate solution, followed by washing with water.

### 2.2. Enzymes

Catalase from bovine kidney (47 000 units/mg protein, 115 mg/ml) was obtained from Sigma. D-Aspartate oxidase was purified as previously reported [11] and had a specific activity of 463 nmol/min per mg protein.

### 2.3. Animals and preparation of tissue extracts

Live bivalves, *S. broughtonii* and *Scapharca subcrenata* being cultured in Miyagi and Saga Prefecture, Japan, respectively were obtained from a fish farm and kept in sea water which was bubbled with air. They were opened and the foot, gills, adductors and mantles were removed. Some tissues of the larger *S. broughtonii* were further divided: the foot was cut into two parts, the more intensely colored outside part (about 46% of the total mass) and the other less intensely colored inside part. The mantles were also divided into two parts, the circular fringe part and the other inner part. The tissues were washed in ice-cold saline and stored at −40°C until use. The frozen tissues were thawed and homogenized with a high-speed microhomogenzier (Physcotron, Niti-on, Funabashi, Japan) or a Potter-Elvehjem homogenizer equipped with a PTFE pestle in 10 volumes of 8% perchloric acid under cooling in ice water. The homogenate was centrifuged for 20 min at 12 000 *g* at 4°C and the supernatant was neutralized with KOH. After centrifugation to remove the precipitated $KClO_4$, the supernatant (2–5 ml) was applied onto a column (2 ml) of Dowex 1×2 (100–200 mesh, acetate form). After washing the column with 20 ml of water to remove neutral and basic substances, *N*-methylaspartates and acidic amino acids were eluted with 20 ml of 1 *M* acetic acid. The eluate was concentrated to dryness under reduced pressure with a centrifugal evaporator (VC-960, Taitec, Saitama, Japan) at 40°C. The residue was dissolved in 3 ml of 0.1 *M* sodium borate buffer (pH 9.0) and subjected to the precolumn derivatization process.

### 2.4. Derivatization of N-methylaspartates and amino acids with (+)- and (−)-FLEC

The derivatization based on the reactions shown in Fig. 1 was performed essentially according to Hayashi and Sasagawa [8] with some minor modifications. In a 1.5-ml microcentrifuge tube (Treff, Degelsheim, Switzerland), a 20-μl volume of the sample solution in 0.1 *M* sodium borate buffer (pH 9.0) was mixed with 15 μl of acetonitrile and 5 μl of 7.2 m*M* (+)- or (−)-FLEC in acetone. After reaction for 15 min at 50°C, a 10-μl volume of 100 m*M*

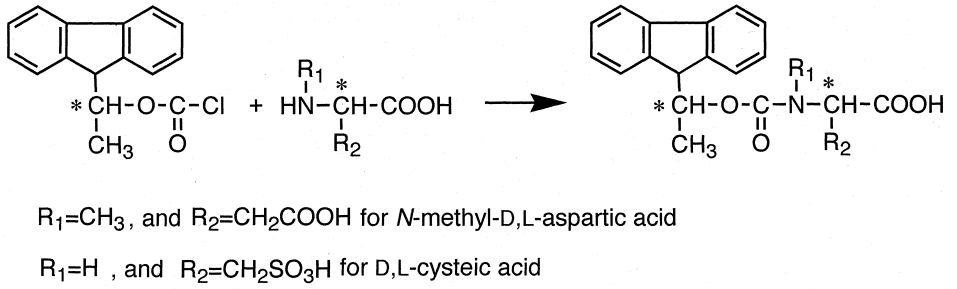


Fig. 1. Structure of (+)- and (−)-1-(9-fluorenyl)ethyl chloroformate and their reactions with enantiomers of *N*-methylaspartate and cysteic acid. Asterisks denote chiral carbon atoms.

aqueous L-cysteic acid in 0.1 *M* sodium borate buffer (pH 9.0) was added to remove the remaining FLEC, and the mixture was allowed to react for another 7 min at the same temperature. After addition of 150 μl of 0.1 *M* sodium acetate buffer (pH 4.0), the mixture was filtered through a 0.45-μm filter (Nacalai Tesque) and injected into the HPLC system. The injection volume was 10 μl for mixtures of authentic compounds and 10–30 μl for tissue extracts.

### *2.5. Derivatization of N-methylaspartates with (+)- and (−)-FLEC after OPA treatment of amino acids*

In a 1.5-ml microcentrifuge tube, a 20-μl volume of sample solution in 0.1 *M* sodium borate buffer (pH 9.0) was mixed with 10 μl of OPA solution (5 mg/ml) in acetonitrile and was kept at 50°C for 15 min. To the mixture, 5 μl of 18 m*M* (+)- or (−)-FLEC in acetone and 5 μl of acetonitrile were added, and the mixture underwent the same FLEC derivatization reaction and subsequent procedure as described in Section 2.4.

### *2.6. HPLC of (+)- and (−)-FLEC derivatives of N-methylaspartates and amino acids*

The chromatography was performed with a Shimadzu (Kyoto, Japan) HPLC system consisting of two LC-10AD pumps, a SIL-10A autoinjector equipped with a sample cooler S (3°C), a CTO-10A column oven, a SCL-10A system controller, a DUG-3A degasser, an RF-10A fluorescence detector and a Chromatopak C-R5A data processor. The analytical column was a reversed-phase J'sphere ODS-M80 (250×4.6 mm I.D.) (YMC, Kyoto, Japan).

Unless otherwise stated, the mobile phase consisted of 0.1 *M* sodium acetate (pH 5.6)–acetonitrile–tetrahydrofuran (86:7:7, v/v/v), and isocratic elution was carried out at a flow-rate of 1.2 ml/min and at a column temperature of 40°C. For fluorometric detection of eluted FLEC derivatives the excitation and emission wavelengths were set at 260 and 315 nm, respectively.

### *2.7. Thin-layer chromatography of reaction products of OPA with L-aspartate and L-glutamate*

A 10-μl volume of OPA in acetonitrile (4 mg/ml) was mixed with 20 μl of L-aspartate or L-glutamate in 0.1 *M* sodium borate buffer (pH 9.0) and the mixtures were subjected, after reaction for 15 min at 50°C, to thin-layer chromatography on a precoated Wakogel FM plate (glass, 20×20 cm) (Wako, Osaka, Japan). The solvent systems used were solvent A: *n*-butyl alcohol–acetic acid–water (12:3:5, v/v) and solvent B: *n*-butyl alcohol–acetic acid–water (3:1:1, v/v). The chromatogram was examined under UV light (256 nm) or by the ninhydrin method described by Spies [12].

### *2.8. Preparation of D-aspartate oxidase treated tissue extracts*

To 0.4 ml of the tissue extract dissolved in 0.1 *M* sodium borate buffer (pH 9.0) described above (Section 2.3), 20 μl of 0.5 m*M* FAD, 10 μl of catalase (2.5 mg/ml), 40 μl of 300 m*M* sodium pyrophosphate buffer (pH 8.0) and 30 μl of D-

aspartate oxidase (4.14 μmol/min per ml) from pig kidney were added, and the mixture was incubated at 37°C for 30 min before addition of 4.5 ml of 16% perchloric acid. The supernatant was taken and neutralized with KOH, then the supernatant was concentrated to dryness and the residue was again dissolved in 0.1 *M* sodium borate buffer (pH 9.0).

## 3. Results

Fig. 2A shows that NMDA and NMLA derivatized with (+)-FLEC were completely resolved under the HPLC conditions employed. The retention times of their derivatives were switched as shown in Fig. 2B when (−)-FLEC was used for derivatization. A comparable resolution was obtained with the solvent system consisting of 84–90% (v/v) 0.1 *M* sodium acetate buffer (pH 4.4–5.8), 5–8% (v/v) acetonitrile and the same volume of tetrahydrofuran as acetonitrile. These conditions for the resolution that had not been achieved before, were discovered by testing carefully the effect of different solvent systems, using a rather longer run time. Fig. 2C shows that NMDA was detected under the same conditions for Fig. 2A even in the presence of D- and L-enantiomers of aspartate and glutamate, although NMLA was not separated from one of these acidic primary amino acids. By raising the pH of the solvent buffer from 5.6 to 5.8, NMLA became detectable, while NMDA, in turn, became unseparable from the acidic amino acids.

Using the same HPLC conditions as for Fig. 2A, attempts were made to identify NMDA in tissues of *S. broughtonii*. Extracts were prepared as described including anion-exchange chromatography to remove neutral and basic amino acids, and then underwent the (+)-FLEC treatment and HPLC. As shown in Fig. 3A, the peak of NMDA could not be detected because of the interference by overwhelmingly large peaks of the acidic amino acids.

We tested various ways to remove primary amino acids from samples before derivatization with (+)-FLEC, and the treatment with OPA gave satisfactory results: When the same mixture of authentic *N*-methylaspartates and acidic amino acids as in Fig. 2C was treated with OPA before (+)-FLEC treatment, the chromatogram showed only the presence

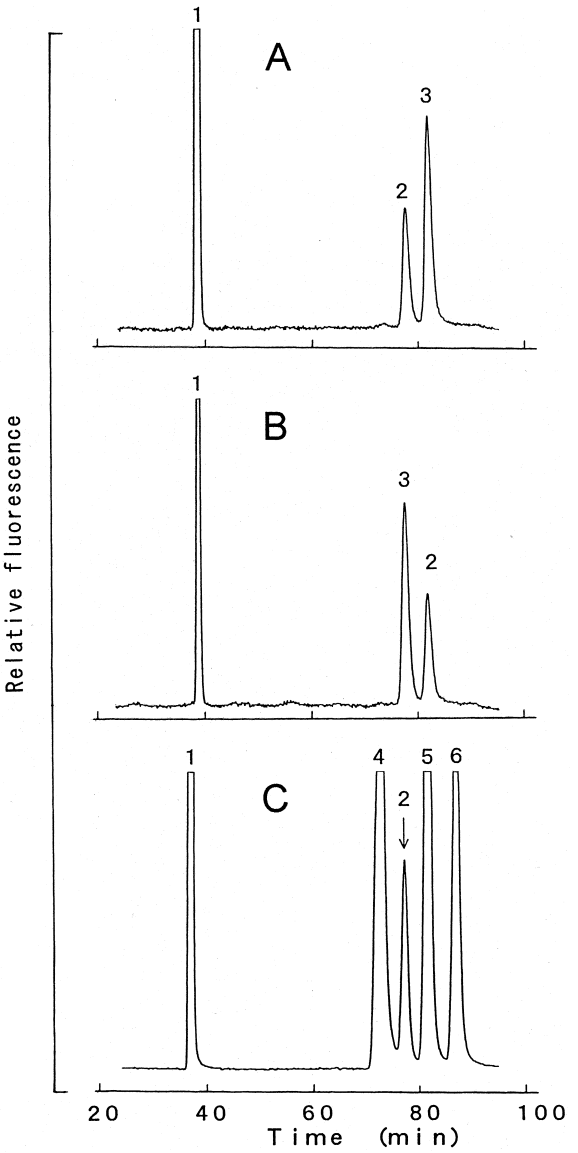


Fig. 2. HPLC chromatogram of (A) (+)-FLEC derivatives of authentic *N*-methylaspartate enantiomers, (B) (−)-FLEC derivatives of authentic *N*-methylaspartate enantiomers, and (C) (+)-FLEC derivatives of authentic *N*-methylaspartate, aspartate and glutamate enantiomers. A mixture of 5 pmol of NMDA and 7.5 pmol of NMLA was treated with (+)- and (−)-FLEC for (A) and (B), respectively, and a mixture of 10 pmol each of L-enantiomers and 5 pmol each of D-enantiomers was treated with (+) FLEC for (C). HPLC was performed with isocratic elution with 0.1 *M* sodium acetate buffer (pH 5.6)–acetonitrile–tetrahydrofuran (86:7:7, v/v/v) at a flow-rate of 1.2 ml/min and at 40°C, as described in the Section 2. Peaks: 1=L-Cysteic acid; 2=NMDA; 3=NMLA; 4=D- and L-aspartate; 5=D-glutamate and NMLA; 6=L-glutamate .

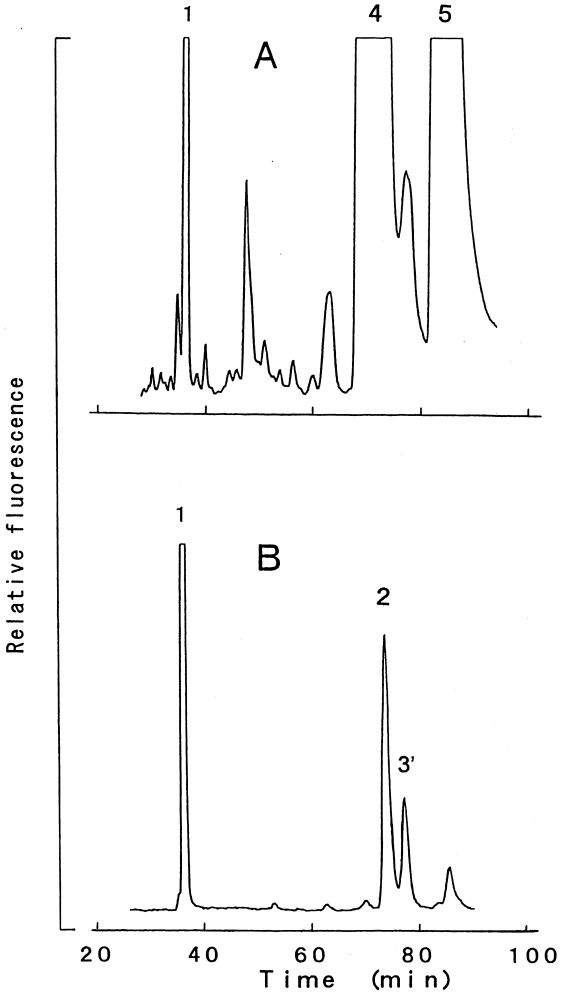


Fig. 3. Effect of OPA treatment before (+)-FLEC derivatization of a tissue extract of *S. broughtonii* on HPLC chromatogram. The extract was prepared from the outside part of mantle of the bivalve and underwent the (+)-FLEC derivatization (A) without and (B) with the prior treatment with OPA, before injection into the HPLC system as described in the Section 2. Peaks: 1=L-cysteic acid; 2=probably NMDA; 3′=unknown; 4=mainly D- and L-aspartate; 5=mainly D- and L-glutamate.

of *N*-methylaspartates with all the acidic amino acids disappeared (data not shown). When the OPA treatment was applied to the tissue extract, the peak of NMDA was clearly observable on the chromatogram as shown in Fig. 3B. The chromatogram also showed a peak of unknown substance that behaved as derivatized NMLA, which so far has been discovered only in a species of algae [9]. To check the identity of the substances corresponding to these peaks, we used (−)-FLEC in place of (+)-FLEC for derivatization employing otherwise same procedures. Comparison of Fig. 4A and B with Fig. 2A and B obviously supports the identity of NMDA and not of NMLA.

Usually, OPA is used together with a thiol to derivatize amino acids as exemplified by the report by Aswad [5]. However, in the present study we used OPA alone to remove primary amino acids, since we found that the presence of the thiol severely

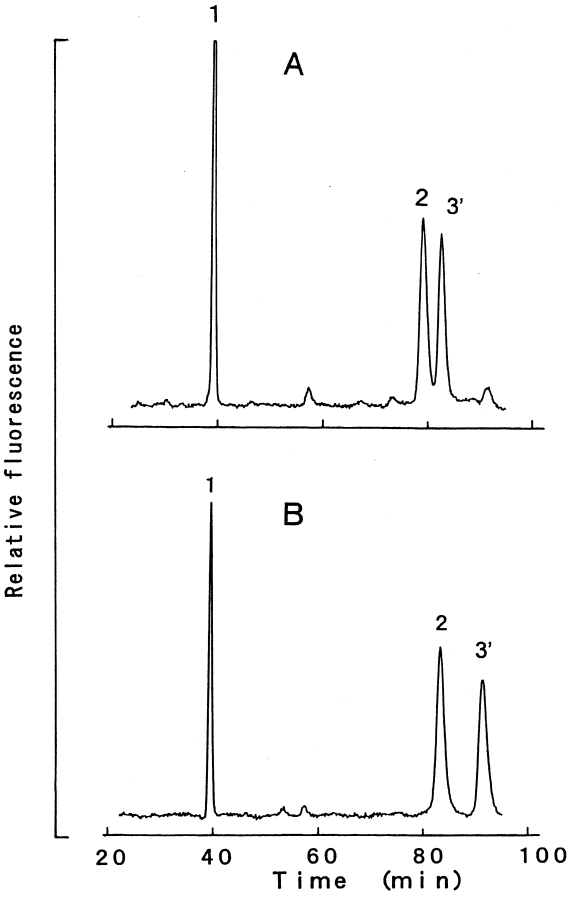


Fig. 4. HPLC chromatograms of a tissue extract of *S. broughtonii* derivatized with (A) (+)-FLEC and (B) (−)-FLEC after OPA treatment. The extract was prepared from the inside part of foot of the bivalve, treated with OPA and derivatized with (+)-FLEC or (−)-FLEC before injection into the HPLC system as described in Section 2. Peaks: 1=L-cysteic acid; 2=NMDA; 3′=unknown.

affected the formation of FLEC derivatives of *N*-methylaspartates, probably because the thiol also reacted with (+)-FLEC. To our knowledge, there has been no report on the reaction of primary amino acids with OPA alone except the condensation of histidine with OPA in alkaline solution [10]. We therefore examined reaction mixtures of an excess of OPA with L-aspartate and L-glutamate, respectively, by thin-layer chromatography. The chromatogram developed with Solvent A indicated that the reaction mixtures did not contain L-aspartate and L-glutamate, which should have given ninhydrin-positive spots at $R_F$ 0.35 and 0.45, respectively, but contained, instead, some new substances which gave UV-absorbing spots at $R_F$ 0.74 and 0.83, respectively, in addition to the spot of OPA at $R_F$ 0.96. Similar results were obtained with Solvent B: in place of L-aspartate ($R_F$ 0.42) and L-glutamate ($R_F$ 0.49), UV-absorbing substances at $R_F$ 0.74 and 0.83, respectively were detected in addition to OPA. It is therefore evident that OPA alone converted acidic primary amino acids to some derivatives of OPA. Further work is required for elucidation of the structure of the derivatives.

To further confirm the identity of the peak of NMDA that became detectable, we treated the bivalve tissue extracts with pig kidney D-aspartate oxidase, which is known to oxidize NMDA as one of its specific substrates [11]. As shown in Fig. 5, the peak of NMDA derivative was abolished by the enzyme treatment, confirming the identification. The detection limit for NMDA was $<1$ pmol at a signal-to-noise ratio of 3 to 1, and the peak areas of their (+)- or (−)-FLEC derivative were linear up to 150 pmol with $r^2 \geq 0.995$. It should be added that the OPA treatment did not significantly affect the detection sensitivity, as indicated by the experiments with authentic NMDA.

The method thus established was applied to the determination of NMDA in the tissues of the blood shell, *S. broughtonii* and its closely related species, *Scapharca subcrenata*. Table 1 shows that several tissues of *S. broughtonii*, including the foot where NMDA was first discovered [3], contain substantial concentrations of NMDA. Among the tissues examined, the outside part of mantle had the highest concentration of NMDA with respect to the mean values. In addition, the concentration in this tissue

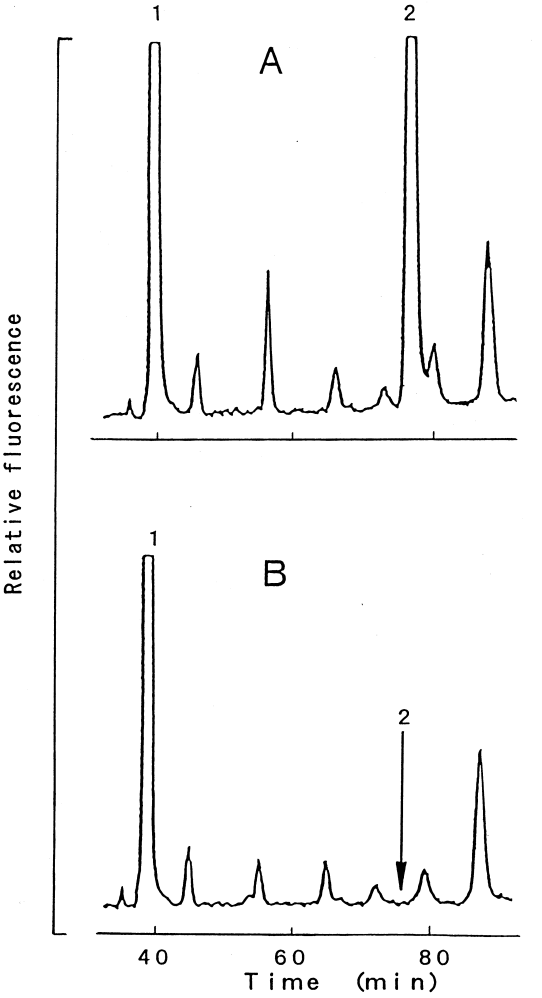


Fig. 5. Effect of D-aspartate oxidase treatment of a tissue extract of *S. broughtonii* on HPLC chromatogram. (A) A portion of the extract from the outside part of mantle of the bivalve was processed just as for Fig. 3B, and (B) another portion was first treated with D-aspartate oxidase as described in Section 2, then underwent the same procedure as the untreated extract. Peaks: 1=L-cysteic acid; 2=NMDA.

was significantly higher than those in the other tissues except the gill. The tissues of *S. subcrenata* also contained substantial concentrations of NMDA, comparable to those in *S. broughtonii*. The foot had a significantly higher NMDA concentration than found in the other tissues of this species and in the foot of *S. broughtonii*.

Table 1
Content of NMDA in tissues of *S. broughtonii* and *S. subcrenata*: extracts were prepared from the tissues of bivalves, treated with OPA and subjected to HPLC analysis after derivatization with (+)-FLEC[a]

| Species | Tissue | NMDA (nmol/g wet tissue) |
|---|---|---|
| *S. broughtonii* | Adductor | 17.7±9.6 |
| | Foot (inside) | 34.7±29.7 |
| | Foot (outside) | 25.1±12.6 |
| | Gill | 82.2±37.9 |
| | Mantle (inside) | 36.8±18.0 |
| | Mantle (outside) | 110.5±70.0[b] |
| *S. subcrenata* | Adductor | 21.2±21.1 |
| | Foot | 203.1±70.6[c] |
| | Gill | 41.5±13.0 |
| | Mantle | 107.0±42.6 |

[a] Values are means±S.D. for 4–7 individual animals (ND: not detectable). The statistical significance was determined by Student's *t*-test for unpaired comparison.
[b] Statistical significance ($P<0.05$) as compared with corresponding values for all tissues except the gill of the same species.
[c] Statistical significance ($P<0.05$) as compared with corresponding values for all the tissues of the same species and for foot (inside) and foot (outside) of the other species.

## 4. Discussion

The present study has established an HPLC method for the determination of NMDA in living organisms by finding conditions for the resolution of NMDA and NMLA derivatized with (+)- and (−)-FLEC as well as a method to remove acidic primary amino acids which are amply present in samples from organisms and interfere with the detection of *N*-methylaspartate enantiomers. The removal of the amino acids is carried out by treatment with OPA before derivatization with FLEC. The treatment evidently converts L-aspartate and L-glutamate to some new type of OPA derivative as indicated by their behavior on thin layer chromatography. Although the nature of the products has not been reported as far as we know, and therefore remains to be clarified, the OPA treatment is obviously useful for a practical purpose.

The identity of NMDA detected in biological extracts by the HPLC of (+)-FLEC treated samples was confirmed by the procedure employing (−)-FLEC as a derivatizing agent. The identification was further supported by the pretreatment of samples with pig kidney D-aspartate oxidase to degrade NMDA specifically. It should be pointed out that the use of (−)-FLEC also excluded the presence of NMLA that appeared possible from the chromatogram of (+)-FLEC treated samples. This indicates that the use of both enantiomers of FLEC is very effective for reliable identifications.

Application of the method to the determination of NMDA has revealed its presence in several tissues of *S. broughtonii* and *S. subcrenata* in addition to the foot muscle of the former previously reported [3]. The discovery of NMDA in *S. subcrenata* means that the compound is not limited to one species and may be distributed and metabolized in many living organisms, and perform some physiological functions. The present HPLC method will be useful in studies to explore this possibility.

In recent years, a large number of papers dealing with NMDA have been published, and in almost all of them this compound has been employed as the agonist of NMDA receptors and given to animals, neural tissues and cells to induce various effects. As far as we are aware, little is known on the subsequent fate of NMDA. The present method would help solve this kind of problem.

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