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Determination of D- and L-aspartate in cell culturing medium, within cells of MPT1 cell line and in rat blood by a column-switching high-performance liquid chromatographic method

Zhiqun Long, Noriyuki Nimura, Minako Adachi, Masae Sekine, Toshihiko Hanai, Hiroaki Kubo, Hiroshi Homma*

School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

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Abstract

HPLC fluorometric methods have been used to analyze trace amounts of D-amino acids in biological samples. In this study, we established an expedient column-switching fluorometric HPLC system that would improve the analysis of D-amino acids, in particular D-aspartate (Asp). Our system consists of the fluorogenic derivatization of amino acids with NBD-F and two chromatographic steps, one that separates individual amino acids in reverse phase mode and another that separates the chiral forms of each amino acid in normal-phase mode. The two separation steps are linked through a trapping column by an automated column-switching system. In addition, sample preparation is simplified and improved, where trichloroacetic acid is used for deproteinization, and borate buffer, pH 9.5 is employed for the fluorescent derivatization. The detection limit for D-Asp in culturing medium is 5 n*M*. The resulting peak heights correlated well with concentrations that ranged from 12.5 to 250 n*M* for both D- and L-Asp. The present method was applied to determine D- and L-Asp levels in cell culturing medium, and within cells of MPT1 cell line. The detected cellular levels of D- and L-Asp agree with those detected by our previous method. In addition, this method was used to measure D- and L-Asp levels in rat blood samples, and the results are consistent with the reported values.

Keywords: Enantiomer separation; Column switching; Aspartate

1. Introduction

D-Amino acids have been reported to be widely distributed in many living organisms ranging from bacteria and archaea to mammals [1–3], and many studies have been made to elucidate their biological function and metabolism. With regard to free D-amino acids, D-serine (Ser) and D-aspartate (Asp) appear to play significant roles in the mammalian central nervous system [4] and in endocrine and/or neuroendocrine organs [5,6], respectively. Specifically, D-Ser is believed to be an endogenous ligand for the glycine site in *N*-methyl-D-aspartic acid (NMDA) receptors and to act as a neuromodulator in the central nervous system [1,7,8]. D-Asp appears to be a novel messenger that is involved in prolactin release in the anterior pituitary gland [9], in melatonin secretion in the pineal gland [10,11], in oxytosin

*Corresponding author. Tel.: +81-3-5791-6229; fax: +81-3-3444-4745.

E-mail address: hommah@pharm.kitasato-u.ac.jp (H. Homma).

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and/or vasopressin production in the posterior pituitary gland [12], and in testosterone synthesis in the testis [13,14]. Recently, it was reported that NMDA stimulates luteinizing hormone-release in the rat hypothalamus, and that D-Asp may be a candidate precursor for NMDA synthesis [15]. In addition to these two abundant D-amino acids, the presence and metabolism of various D-amino acids have also been reported [2]. It is now increasingly being realized that trace amounts of these D-amino acids may have some, yet unknown, biological functions in the mammalian body.

Enzymatic [16], chromatographic [17] and immunohistochemical methods [18,19] have been developed to investigate the presence of D-amino acids in biological samples. Of these methods, HPLC fluorometric methods are the most superior in view of their high sensitivity and reliability. However, a negative point about these latter methods is that the samples often need to be purified by tedious methods (such as ion-exchange resins or liquid–liquid extraction) that will remove endogenous interfering substances or excess reagents (e.g. trichloroacetic acid or fluorescent reagents) [15,19–23]. Our previous report [5] describes a reliable and sensitive method that we developed to detect D-amino acids in tissue samples. In this latter method, amino acids are fluorogenically derivatized with NBD-F and then separated by two different chromatographic steps, namely, one that separates the individual amino acids and another that separates the chiral forms of each amino acid. We used this method to demonstrate for the first time that D-Asp is indeed synthesized within mammalian cells (rat pheochromocytoma 12 cells [24] and rat pituitary tumor cells [9]).

In the study reported here, we have linked the two chromatographic steps in our method by an automated column-switching system. This entailed inserting a trapping step for NBD-amino acids that would link the reverse and normal phase separation steps. The time-consuming and tedious procedures for sample preparation that we used in our previously described method [5] have also been improved and simplified. The method thus developed is expedient, more accurate and highly sensitive. The application of this method would greatly advance the current understanding of regulation and dynamics of D-Asp homeostasis and its significance in the mammalian body. In addition, this method can be adapted for the quantification of other amino acids and thus has a wide applicability.

2. Experimental

2.1. Apparatus

The HPLC apparatus consist of a system controller (model 802-SC), two pumps (model 880-02), an autosampler (model 851-AS), a ternary gradient unit (model 880-02) for solvent selection, a column select unit (model HV-1592-01) for column-switching, a column oven (model 860), two fluorescence detectors (model 920) and two integrators (model 807-IT). All instruments were products of Jasco (Tokyo, Japan).

2.2. Materials

D- and L-Asp, L-glutamate (Glu) and L-cysteine sulfinic acid (LCSA) were purchased from Sigma (St. Louis, MO, USA). 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was from Wako (Osaka, Japan), and other reagents and solvents were of the highest grade commercially available. The analytical columns, Sumichiral OA2500S and OA3100, were kindly supplied by Sumika Analysis Service (Osaka, Japan). The reverse phase separation column, LiChroCART Superspher RP-8 (125×4 mm I.D., 5 μm, C8), and the trap column, LiChrospher 100RP-18 (50×4 mm I.D., 5 μm, C_{18}) were both obtained from Merck (Germany). MPT1 cells were generous gift from Professor Bruce D. Howard (University of California at Los Angeles, School of Medicine). The cells were selected by Denton and Howard [25] from pheochromocytoma 12 (PC12) cell line according to their resistance to the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Dulbecco's modified Eagle's medium (DMEM), heat-inactivated horse serum, fetal bovine serum (FBS), dialyzed FBS (dFBS) and other materials used in cell culture were from Gibco-BRL (New York, USA).

2.3. Cell culture

MPT1 cells were maintained on a polystyrene plate at 37°C in 5% CO_2 and 100% humidity in complete DMEM containing 10% heat-inactivated horse serum, 5% heat inactivated FBS, and 100 units/ml of penicillin and 100 μg/ml of streptomycin. The cells were then inoculated onto 6-well plates in complete DMEM and cultured for 1 day, after which the medium was replaced with DMEM with 5% dFBS. The culture was continued for another day. The culturing medium was collected and the cells were rinsed twice with 1 ml of phosphate-buffered saline (PBS). Cells and the culture media were both used for D- and L-Asp determination. The cells were also cultured in DMEM with 5% dFBS in the presence of 5 m*M* LCSA.

2.4. Sample preparation

To 200 μl of medium sample, 10 μl of 100% trichloroacetic acid was added and vortex-mixed. After centrifugation at 10 000 *g* for 10 min, 200 μl of the supernatant was placed into a new Eppendorf tube, neutralized with 50 μl of 1 *M* NaOH and 125 μl of 200 m*M* borate buffer (pH 9.5) and water was added to a final volume of 500 μl. Fluorescent derivatization was carried out by adding 30 μl of 50 m*M* NBD-F in acetonitrile to 100 μl of the resulting sample solution and heating the mixture at 60°C for 5 min. The reaction was stopped by adding 170 μl of 1% trifluoroacetic acid, after which the mixture was filtered through a 0.45 μm filter (Millex-LH, Millipore, Bedford, MA, USA). Finally, 10 μl of the filtrate was applied to the HPLC system.

Culture media (DMEM with 5% dFBS) spiked with D- and L-Asp at the concentrations of 0, 12.5, 25, 50, 100, 150, 200, 250 n*M* were employed for the recovery and linearity experiments. To determine D- and L-Asp levels in cultured MPT1 cells, cellular amino acids were extracted by methanol in a final volume of 2 ml. An aliquot (100 μl) of the extract was evaporated to dryness under reduced pressure, after which the residue was dissolved in 40 μl of 50 m*M* borate buffer (pH 9.5). Then 30 μl of 50 m*M* NBD-F in acetonitrile was added and the mixture was heated at 60°C for 5 min. The reaction was terminated with 930 μl of 1% acetic acid in methanol. The resulting solution was filtered and applied to the HPLC system described above.

2.5. Chromatographic conditions

Fig. 1 shows a flow diagram of the automated column-switching HPLC system. The injected sample was first separated on an octyl silica column (RPC) with a mobile phase of 50 m*M* sodium acetate, pH5.1 (M1) for 5 min. The fraction eluted from the RPC column with the retention time between 2.2 and 3.3 min (NBD-Asp fraction) was introduced into an ODS cartridge column (TC) by switching the valve (V) to position B. This trapped the NBD-Asp in the TC column. When the valve was set back to position A, the trapped analytes were backward eluted and introduced into the chiral column (CSPC). The enantioseparation of NBD-D- and -L-Asp was carried out on Pirkle-type chiral columns (CSPC), OA3100 or OA2500S, with a mobile phase of 7 m*M* citric acid in methanol. In the meantime, the RPC column was regenerated for the next sample injection by a 5-min washing with M2, a mixture of M1 and acetonitrile (30:70, v/v), followed by another 5 min washing with methanol

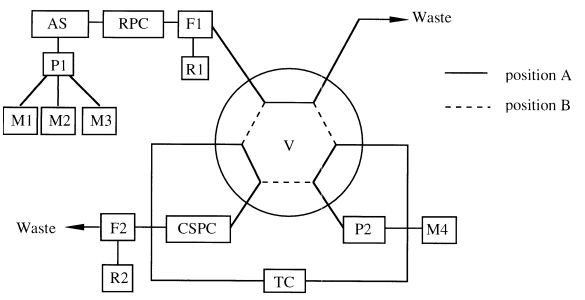

Fig. 1. Flow diagram of the column-switching HPLC system. When the switching valve is set at position A, the sample is primarily separated on an octyl silica (C8) column (RPC). The NBD-Asp-containing fraction eluted with the retention time between 2.2 and 3.3 min is introduced into the C_{18} trap column (TC) by switching the valve to position B. When the valve is set back to position A, NBD-Asp trapped in the TC column is then backward eluted and introduced into the analytical column (CSPC). In the meantime, the RPC column is regenerated for the next sample injection. For further details, see text. M1–4: mobile phase, P1,2: pump, AS: autosampler, F1,2: fluorescence detector, R1,2: integrator, V: switching valve.

(M3) and re-conditioning with the initial mobile phase (M1). Analytical columns (RPC and CSPC) were maintained at 40°C and the trap column at room temperature.

3. Results and discussion

3.1. Column-switching HPLC system

In this study, we developed a new HPLC system in which two separation steps, namely, a step where individual amino acids are isolated, followed by enantioseparation of each amino acid, are linked together by an automated column-switching system. A flow diagram illustrating the relevant points of this system is shown in Fig. 1 and details of the procedure are described in Section 2. In this system, an octyl silica (C8) column (RPC) is used for the reserve phase separation of NBD-Asp from other amino acid derivatives, while an octadecyl silica (C_{18}) column (TC) is used for the quantitative trapping of NBD-Asp. As the hydrophobicity of C_{18} column is much higher than that of C8, NBD-Asp (or other NBD-amino acids) is quantitatively retained in the C_{18} trap column (TC). The reduced size of the trap column made it possible to minimize the volume of aqueous mobile phase (M1) which is unavoidably introduced into the normal-phase column (CSPC). This protects the CSPC column from possible damage caused by the aqueous mobile phase and consequently increases the durability of the column. In the method we reported earlier [5], the NBD-Asp-containing fraction from the reverse phase column was manually collected and concentrated by evaporation prior to injection into the chiral column. The method described here is thus more efficient as these tedious manual operations are eliminated altogether. The reliability and sensitivity are also improved by avoiding the potential sample loss that could occur during these manual operations.

Since the pK_{a1} and pK_{a2} of Asp are 1.88 and 3.65, respectively, two carboxyl groups of NBD-Asp can be reasonably ionized at weak acidic pH. Therefore it can be expected that NBD-Asp is well separated from other neutral and basic amino acid derivatives with a weak acidic mobile phase in reverse phase mode. We used sodium acetate buffer, pH 5.1, as a mobile phase (M1) for the reverse phase separation on the C8 column (RPC). Under these conditions, NBD-Asp was completely isolated from the other amino acid derivatives. Its retention time was approximately 2.68 min with a peak width at 5% peak height being 0.3 min (Fig. 2a). Thus, the fraction eluted from the RPC column with the retention time between 2.2 and 3.3 min was introduced into the trap column (TC) by switching the valve. Direct injection of standard NBD-D- and -L-Asp into the chiral column (CSPC) and injection into this column via the switching HPLC system resulted in almost identical chromatograms (data not shown). This indicates that NBD-Asp is quantitatively trapped in and recovered from the TC column, and that the enantioseparation on the chiral column is not disturbed by our switching HPLC system. Fig. 2b shows the typical chromatogram of NBD-D- and -L-Asp purified by our HPLC system.

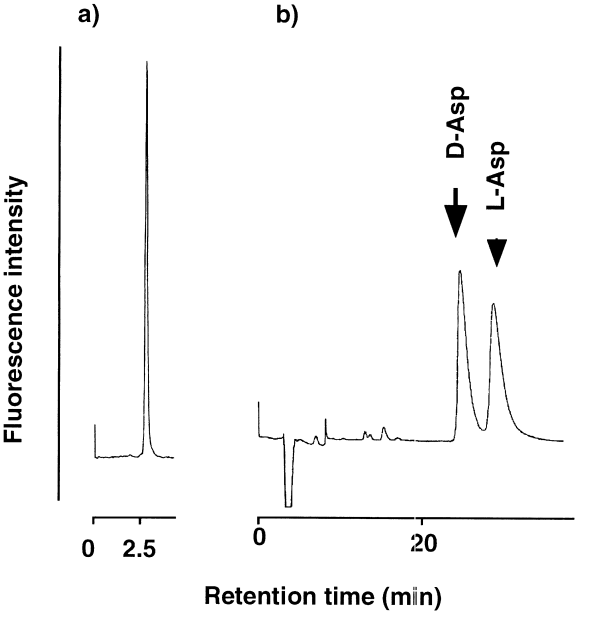

Fig. 2. Chromatograms of NBD-Asp standards. (a) Chromatogram of an NBD-Asp standard separated on the octyl silica column (RPC) (1 pmol of NBD-Asp was injected). (b) Chromatogram of standard NBD-D- and -L-Asp separated by the column-switching HPLC system (500 fmol of NBD-D- and -L-Asp was injected). The Sumika OA3100 column was used for the chiral separation.

3.2. Extraction and derivatization of amino acids

In the method that we reported previously [5], methanol was used to extract the amino acids and to deproteinize the samples. This meant that prior to amino acid derivatization, the methanol had to be evaporated and the residue then had to be redissolved. In the method described here, we employed trichloroacetic acid instead of methanol, as described in Section 2. This makes the new method more efficient, as it eliminates the evaporation and redissolution steps and the consequent sample loss that can occur during these steps.

NBD-F has been reported to label amino groups under alkali circumstances [26], and we found that amino acids were derivatized at pH 8.0 in the method we published earlier. However, the fluorescent intensity of the amino acid derivatives varied between samples, especially when the samples were in culturing medium. This variability may be because of the low buffering capacity of borate buffer, pH 8.0, and/or from unknown interfering components present in the culturing medium. Consequently, we examined the derivatization efficiency for Asp-spiked medium samples that have pHs ranging between 8.0 and 10.5. The fluorescent intensities of NBD-Asp derivatized at pH 9.0 to pH 10.5 were greater compared with that derivatized at pH 8.0, and the variation between the samples was diminished. We thus employed borate buffer, pH 9.5, in this study.

3.3. Linearity and recovery

The linearity and recovery for D- and L-Asp were evaluated according to the method described in Section 2. Fig. 3a depicts the chromatogram of blank medium (DMEM with 5% dFBS), in which no D-Asp peak appears. When this medium is spiked with 12.5 n*M* D- and L-Asp, the D-Asp peak emerges at the retention time identical to that of standard D-Asp, and L-Asp increases correspondingly (Fig. 3b). The detection limit for D-Asp in the medium sample is improved, as concentrations as low as 5 n*M* can be detected ($S/N=3$). The correlation between peak heights and concentrations ranging from 12.5 to 250 n*M* was good for both D- and L-Asp (correlation coefficients of 0.999 and 0.998, respectively). The recovery from medium samples spiked with 50 n*M* D- and L-Asp was 95.6 ± 2.4 and $111\pm4.5\%$, respectively, and with 200 n*M* D- and L-Asp, 98.7 ± 6.0 and $105.9\pm5.2\%$ ($n=3$), respectively.

3.4. Application of the method to biological samples

In order to demonstrate the utility of our improved HPLC method in detecting amino acids in biological samples, D- and L-Asp levels in the culturing medium of the MPT1 cell line were determined. The D-Asp level in the culturing medium was below the detection limit but 4.66 ± 0.24 μ*M* of L-Asp could be detected (Fig. 3c). However, when LCSA, a strong inhibitor for the L-Glu transporter that has an affinity for D-Asp as well as for L-Glu and L-Asp [13], was included in the culturing medium, the levels of D- and L-Asp in the culturing medium were observed to be increased to 220 ± 47 n*M* and 26.3 ± 4.4 μ*M*, respectively (Fig. 3d). D- and L-Asp in the medium are presumed to be ordinarily taken up into the cells through GLAST, a subtype of L-Glu transporter which is expressed in the cells and has an affinity for D- and L-Asp in addition to L-Glu. When LCSA, an inhibitor of the transporter was added in the medium, the cellular uptake of D- and L-Asp was presumably suppressed and this suppression resulted in the increase of D- and L-Asp levels in the medium.

We also applied our method to blood samples, as shown in Fig. 4. Here, the levels of D- and L-Asp in the blood of an 8-week-old Sprague–Dawley rat were found to be 1.7 and 57.3 nmol/ml, respectively. These results are consistent with the previously reported values of 2.2 ± 0.3 and 48.9 ± 5.4 nmol/ml, respectively [20]. In addition, we applied our method to determine the levels of D- and L-Asp within MPT1 cells. The levels detected by our improved method agree with those observed when we used our previously reported method [5] (Table 1).

There have been some reports which apply column-switching HPLC to the enantioseparation of the protein amino acids. Tapuchi et al. [27] carried out enantioseparation of dansyl-amino acids by combining reverse phase column with a subsequent chiral ligand-exchange system, in which a chiral selector (L-propyl-*n*-dodecylamide-Ni(II)) was used as a mo-

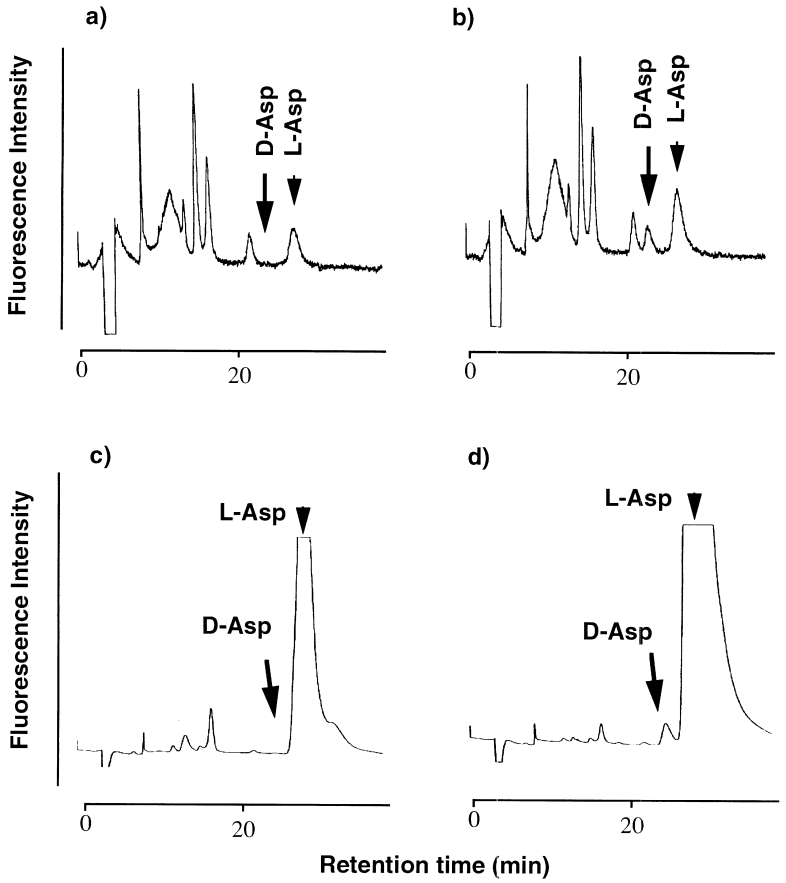

Fig. 3. Chromatograms of culturing media. (a) Blank medium (DMEM with 5% dFBS), (b) the medium spiked with 12.5 n*M* D- and L-Asp, (c) the medium in which MPT1 cells were cultured for 1 day, and (d) the medium in which MPT1 cells were cultured for 1 day with 5 m*M* of LCSA, a L-Glu transporter inhibitor. Arrows: D-Asp; arrowheads: L-Asp. The Sumika OA3100 column was used for the chiral separation.

bile phase additive. Rizzi et al. [28] also monitored dansyl-D-Glu and -D-Ala levels in protein hydrolyzates by a similar method where β-cyclodextrin was employed instead. In these methods however, only a portion of the eluate from the reverse phase column was introduced into a subsequent chiral column. Chen and Wu [29] coupled a solid-phase extraction column to a chiral ligand-exchange column by column-switching technique to separate D- and L-Asp. In the latter chiral column, the ligand on the column and Asp form complexes with Cu^{2+} ion. Recently Inoue et al. [30] reported a column-switching system to determine D-Leu contents in rat brain. In this system, the eluted fraction from a micro reverse phase column was pooled in a transfer loop and then introduced into a conventional size of chiral column.

In the method described in this report, organic mobile phase is employed for the enantioseparation of Asp, which enhances fluorescence intensities of the analytes and thus increases the sensitivity. In addition, NBD-Asp separated by the reverse phase column is quantitatively retained in the trap column and introduced into the chiral column. Moreover, the reduced size of the trap column minimizes the volume of aqueous mobile phase which is unavoidably introduced into the normal phase chiral column and then increases the durability of the chiral column. The method described here allows the separation and determination of D- and L-Asp, not

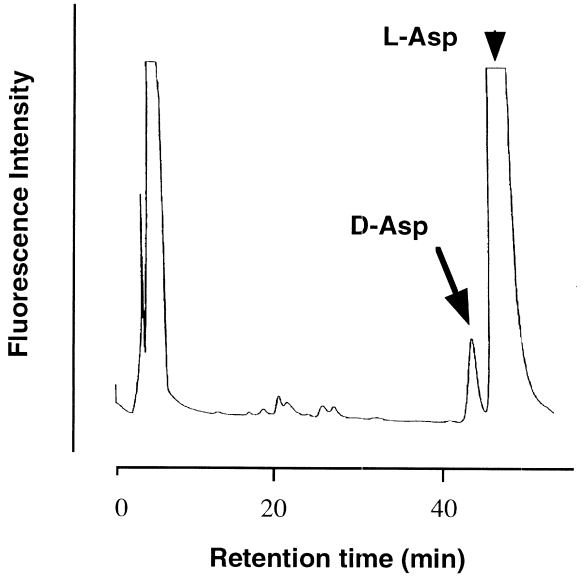

Fig. 4. Chromatogram of rat blood sample. A blood sample was taken from the abdominal aorta of a male Sprague–Dawley rat (8 weeks of age). Then 200 μl of blood was mixed with an equal volume of 10% trichloroacetic acid and centrifuged (5000 *g*, 10 min, 4°C) to remove protein and cell debris. Finally, 200 μl of the supernatant was treated as described in Section 2. The Sumika OA2500S column was used for the chiral separation.

overall separations of amino acid enantiomers. However, the method can be adapted for the quantification of other amino acid enantiomers after minor modification(s).

4. Conclusions

The study reported here describes a very convenient and effective HPLC method that we have developed to determine D- and L-Asp levels present in complex biological samples. The introduction of an automated column-switching system and optimization of sample pretreatment made it possible to omit the tedious manual operations involved in sample preparation, which thus saves time. The reliability and sensitivity of this method are also improved compared with our previously reported method [5]. The HPLC system that we have described here can also be employed for the analysis of other amino acids after minor modification(s).

Table 1
Comparison of the column-switching method with the method reported previously [5] for determination of cellular D- and L-Asp levels (mean±SD, $n=3$)

	D-Asp (nmol/well)	L-Asp (nmol/well)
Present method	0.597±0.02	9.94±0.62
Previous method	0.565±0.03	9.33±0.15

5. Nomenclature

NMDA	*N*-methyl-D-aspartic acid
NBD-F	4-fluoro-7-nitro-2,1,3-benzoxadiazole
LCSA	L-cysteine sulfinic acid
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
dFBS	dialyzed FBS.

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