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Original Paper

Development of a Highly-Sensitive Two-Dimensional HPLC System with Narrowbore Reversed-Phase and Microbore Enantioselective Columns and Application to the Chiral Amino Acid Analysis of the Mammalian Brain

Aogu FURUSHO¹, Reiko KOGA¹², Takeyuki AKITA¹, Yurika MIYOSHI³, Masashi MITA³, Kenji HAMASE*¹

¹Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
²Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan
³Shiseido Co., Ltd., 1-6-2 Higashi-shimbashi, Minato-ku, Tokyo 105-8310, Japan

Abstract
A highly-sensitive two-dimensional high-performance liquid chromatographic (2D-HPLC) system with narrowbore reversed-phase and microbore enantioselective columns has been developed for the determination of alanine (Ala) and serine (Ser) enantiomers. The amounts of D-amino acids in biological samples are extremely low in most cases and improvement in the sensitivity is still required. For a highly-sensitive analysis, a narrowbore reversed-phase column, KSAARP (1.5 mm x 500 mm, having a wider ID than the microbore column) was used in the first dimension, and a microbore enantioselective column, KSAACSP-001S (1.0 mm x 250 mm, having a narrower ID than the frequently reported enantioselective columns) was used in the second dimension. The amino acids were derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole and detected by the fluorescence detectors. The 2D-HPLC system was validated using a mouse cerebrum in addition to the standard amino acids, and satisfactory calibration lines and precision results were obtained. In the mouse cerebrum, a large amount of D-Ser and a small amount of D-Ala were clearly observed. These results indicated that the present method is applicable to the determination of the trace amounts of D-amino acids in tissue samples.

Keywords: D-Amino acids; Enantiomer separation; Two-dimensional HPLC

1. Introduction
Amino acids are essential molecules for living creatures as the components of proteins and peptides. All proteinogenic amino acids except for glycine (Gly) have a chiral center at the α-carbon having a hydrogen, a carboxy group, an amino group and a side chain, thus D- and L-enantiomers are present. Among these enantiomers, L-amino acids are predominant over the D-amino acids in the higher animals including humans, and it has been considered for a long time that animals can utilize only the L-amino acids as biofunctional molecules like neurotransmitters and metabolism-related substances.

However, the recent progress in analytical technologies has enabled the screening of the trace amounts of D-amino acids, and various D-forms have been found in mammalian tissues and physiological fluids [1,2]. In addition, their origins, physiological functions and relations to diseases have also been gradually unveiled. Especially, D-serine (Ser) and D-alanine (Ala) are well known for their binding activities to the glutamate receptor in vitro [3], and various studies to elucidate their distributions and functions in vivo have been carried out [4,5]. D-Ser is localized in the cerebral cortex and the hippocampus [4,6], and regulates neurotransmission by binding to the Gly site of the N-methyl-D-aspartate

*Corresponding author: Kenji HAMASE
Tel: +81-92-642-6598; Fax: +81-92-642-6598
E-mail: hamase@phar.kyushu-u.ac.jp

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receptors [7-9]. D-Ala is localized in the adrenocorticotropic hormone secreting cells of the anterior pituitary gland [10] and in the β-cells of the pancreas [11], suggesting that D-Ala is involved in the control of the blood glucose level. Regarding the relations to diseases, the changes in the amounts of both D-Ser and D-Ala have been reported in patients with various diseases including Alzheimer's disease [12-14] and with renal disorders [15-17]. Thus, these D-amino acids are now expected as novel drug candidates and biomarkers.

For the analysis of D-amino acids in biological samples, various analytical methods using capillary electrophoresis (CE), gas chromatography (GC) and HPLC have been reported [5,18-21]. CE methods using a buffer containing a chiral selector, such as β-cyclodextrin, are used as highly sensitive methods because the injection volumes of the samples are extremely small [19,22]. Chiral GC methods using an enantiomeric column, such as the Chirasil-L-valine, capillary column are useful for the chiral analysis of most of the proteinogenic amino acids with acceptable short analysis time [6,20,23]. Concerning HPLC, reversed-phase HPLC methods using chiral derivatization reagents have been reported [5,21]. For the derivatization, a variety of chiral reagents, such as o-phthalaldehyde plus chiral thiol compounds [24,25], 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide [26,27] and (+)-1-(9-fluorenyl)ethyl chloroformate [28,29] have been used. In addition, reversed-phase HPLC methods in combination with tandem mass spectrometry (MS/MS) have also been developed for the selective determination of D-amino acids. As the MS compatible diastereomer, forming reagents, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(N, N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole [30], N-(4-nitrophenoxycarbonyl)-l-phenylalanine-2-methoxethyl ester [31] and some others are used. Chiral LC-MS/MS methods using enantioselective columns as the separation media were also reported [32,33]. These HPLC methods are widely used for the determination of D-amino acids in tissues and physiological fluids, for example, D-Ser in the brain and D-Asp in the endocrine tissues. However, these CE, GC and HPLC methods have only one-dimensional separation column for the chemical isolation/purification of molecules, and the determination of trace levels of D-amino acids is frequently disturbed by known/unknown substances present in the tissues and physiological fluids.

As more highly selective methods, 2D-HPLC methods combining reversed-phase separation and enantiomer separation have been developed [34-36]. In the system, amino acids are separated/fractionated from interfering compounds in a reversed-phase column, and amino acid enantiomers in the collected fractions are separated using an enantioselective column. Until now, several research studies have been published that described the distribution and origin of various D-amino acids in mammals using these 2D-HPLC methods [37-42]. In some cases, however, the determination of extremely trace amounts of the D-forms is difficult due to the insufficient sensitivity. Thus, improvement in the sensitivity for chiral amino acid analysis is continuously expected.

In the present study, a 2D-HPLC method combining a narrowbore reversed-phase column and a microbore enantioselective column has been designed and developed for the highly-selective analysis of the Ala and Ser enantiomers. The internal diameter of the reversed-phase column (first dimension) is wider than that of the enantioselective column used in the second dimension, and the equipped narrowbore reversed-phase column (1.5 mm ID) could accept higher amounts (loading capacity) of the samples. On the other hand, the internal diameter of the enantioselective column is narrower than those of the reversed-phase column (first dimension) and also the previously reported enantioselective columns, and the newly equipped microbore enantioselective column (1.0 mm ID) enables the more sensitive analysis of the D-amino acids. This type of column combination is normally difficult for the 2D-HPLC analysis with the whole fraction transfer concept due to the mobile phase incompatibility. The newly designed/developed "reversed ID type" 2D-HPLC system was evaluated using standard amino acids and was applied to the determination of the Ala and Ser enantiomers in the mouse cerebrum.

2. Experimental

2.1. Materials

D-Ala, D-Ser, DL-Ser, L-Ala, L-asparagine (Asn), L-aspartic acid (Asp), L-glutamine (Gln), L-histidine (His) and L-Ser were obtained from Nacalai Tesque (Kyoto, Japan). DL-Ala, L-arginine (Arg), L-glutamic acid (Glu) and L-threonine (Thr) were obtained from Wako Pure Chemical Industries (Osaka, Japan). L-allo-Threonine (allo-Thr) was purchased from Tokyo Chemical Industry (Tokyo, Japan) and Gly was from Sigma-Aldrich (St. Louis, MO, USA)). The derivatizing reagent, 4-fluoro-2-nitro-2,1,3-benzoxadiazole (NBD-F) was a product of the Tokyo Chemical Industry. Methanol (MeOH) of HPLC grade, boric acid, formic acid (FA) and trifluoroacetic acid (TFA) were obtained from Wako Pure Chemical Industries. Acetonitrile (MeCN) of HPLC grade was purchased from Nacalai Tesque. Water was purified using a Milli-Q Integral 3 system (Merck Millipore, Darmstadt, Germany). All other reagents were of the highest reagent grade and used without further purification.

2.2. Animals

The male C57BL/6J mice (6 weeks of age, SPF) were purchased from Charles River Laboratories Japan, Inc.
The cerebrum was excised and homogenized at 3,500 rpm for 2 min in 20 x volumes of MeOH using a micro-homogenizing apparatus (Micro Smash™ MS-100R, TOMY SEIKO, Tokyo, Japan) at 4°C. The MeOH homogenate was centrifuged at 12,000 x g for 10 min, and the supernatant was collected. The supernatant (10 μL) of the cerebrum was evaporated to dryness under reduced pressure at 40°C. To the residue, 10 μL of water was added, and the amino acids in the solution were derivatized using the procedure as shown in Section 2.3. All animal experiments were performed with permission from the Animal Care and use Committee of Kyushu University (A29-037-0).

2.3. Sample preparation
To 10 μL of the amino acid solution (standard solution and aqueous solution obtained from the tissue sample), 10 μL of 400 mM sodium borate buffer (pH 8.0) and 5 μL of 40 mM NBD-F in dry MeCN were added. The mixed solution was heated at 60°C for 2 min and 75 μL of 0.2% (v/v) TFA in water was added. The obtained reaction mixture was further diluted with 0.2% (v/v) TFA in water, then 10 μL of the solution was injected into the two-dimensional HPLC system described in Section 2.4.

2.4. 2D-HPLC system for the determination of the Ala and Ser enantiomers as their NBD derivatives
The HPLC system was constructed using the NANOSPACE SI-2 series units (Shiseido, Tokyo, Japan) with a KSAA valve-controlling system (designed by the collaboration with Shiseido). The system consisted of a 3202 degasser, four 3101 pumps, a 3033 auto sampler, two column ovens (3004 and 3014), two 3013 fluorescence detectors, a 3012 high pressure valve and a 9986 multi-loop valve (having loops of 500 μL). An EZChrom SI system was used for the data processing. The flow diagram is shown in Fig. 1. The analytical column for the reversed-phase separation was a KSAARP column (1.5 mm i.d. x 500 mm) maintained at 25°C. The mobile phase for NBD-Ser was 0.075% formic acid in a mixed solution of MeOH-MeCN (25/75, v/v) and that for NBD-Ala was 0.03% formic acid in a mixed solution of MeOH-Ala (90/10, v/v) at the flow rate of 100 μL/min. Fluorescence detection of the NBD-amino acids was carried out at 530 nm with excitation at 470 nm.

3. Results and discussion
3.1. Reversed-phase and enantioselective separations of Ala and Ser as their NBD derivatives
The newly designed 2D-HPLC system consisted of a narrowbore ODS column for the reversed-phase separation and a microbore enantioselective column for the chiral separation. As the first step for the development of the 2D-HPLC system, the mobile phase conditions for both dimensions were investigated using standard amino acids. The enantiomers of Ala and Ser were selected as the target amino acids because the amounts of D-Ala and D-Ser in mammalian bodies are the subjects of high interest considering their biological functions and relations to diseases.

For the reversed-phase separation of NBD-Ala and NBD-Ser from other amino acids, a KSAARP column (1.5 mm i.d. x 500 mm) was used. The analytes were 11 amino acids including Ala, Ser and 9 other hydrophilic amino acids which are frequently required to be separated in the first dimension. As the mobile phases, aqueous solutions containing MeCN and TFA were used and the effect of the MeCN concentration was investigated. As shown in Fig. 2, the retention times of the NBD-amino acids became longer by decreasing the concentration of MeCN, and the target amino acids (Ala and Ser) were well separated from other amino acids within 90 min using MeCN/TFA/water (15/0.05/85, v/v/v).

For the enantiomer separations of NBD-Ala and NBD-Ser, a Pirkle-type enantioselective column, KSAACSP-001S (1.0 mm i.d. x 250 mm) was used. The separation conditions were optimized using mixed solutions of MeOH-MeCN containing various concentrations of FA.

![Flow diagram of the 2D-HPLC system for the determination of Ala and Ser enantiomers as their NBD derivatives.](image)
The typical chromatograms are shown in Fig. 3. By decreasing the FA concentration in the mobile phase, the retention times of the NBD-amino acids became longer, and higher resolution values were obtained. As a result, the enantiomers of NBD-Ala and NBD-Ser were nicely separated within 40 min using a mixed solution of MeOH-MeCN (80/20, v/v) containing 0.1% FA. The separation factor of NBD-Ala was 1.32 and that of NBD-Ser was 1.47. The resolution of NBD-Ala was 3.18 and that of NBD-Ser was 4.59.

Until now, several HPLC systems have been reported for the reversed-phase separation of NBD-Ser and Ala. The hydrophobicity of Ala is much higher than that of Ser, therefore, different mobile phases are normally needed for the separation of these 2 amino acids [43]. By a monolithic ODS column (0.53 mm i.d. x 750 mm), an aqueous solution including 5% MeCN and 0.05% TFA was used for the separation of NBD-Ser with the retention time of around 30 min. For the separation of NBD-Ala, 15% MeCN and 0.05% TFA in water was used (retention time is around 20 min). To simultaneously separate NBD-Ser and Ala, the gradient elution is frequently used. Using aqueous solutions including 5-20% MeCN and 0.05% TFA, NBD-Ser and Ala were separated by a monolithic ODS column (0.53 mm i.d. x 750 mm), and the retention times were around 25 and 65 min, respectively [44]. However, the gradient elution needs two pumps and the system is more complicated than the isocratic elution system. In the present investigation, NBD-Ser and Ala were well separated by a KSAARP column (1.5 mm i.d. x 500 mm) with the isocratic elution of the aqueous solution containing 15% MeCN and 0.05% TFA. The retention times of NBD-Ser and Ala were at 35 and 86 min, respectively, and also nicely separated from the other amino acids.

Concerning the enantiomer separations of NBD-Ala and Ser, Sumichiral OA series columns (Sumika Chemical Analysis Service, Osaka, Japan) have been used in several studies. By using the OA-3100S and OA-4700SR columns, the NBA-Ala and Ser enantiomers were separated using 5 mM citric acid in MeOH [45]. The reported separation factors of the NBD-Ala enantiomers were 1.23-1.31, and those of the NBD-Ser enantiomers were 1.25-1.33. An OA-2500S column has also been used with mixed solutions of MeOH-MeCN containing citric acid as the mobile phase [43]. Both the NBD-Ala and Ser enantiomers were separated in 10-15 min, and the reported separation factor of NBD-Ala was 1.23 and those of NBD-Ser was 1.22. In the present investigation, the NBD-Ala and Ser enantiomers were nicely separated within 30 min on a KSAASCP-001S column using a single mobile phase, i.e., MeOH-MeCN (80/20) containing 0.1% FA. Concerning the separation factor and the resolution, sufficient values were obtained (Ala; $\alpha=1.32$, $R_s=3.18$, Ser; $\alpha=1.47$, $R_s=4.59$), and various applications using the present KSAACSP column are expected.

3.2. Development of the 2D-HPLC system with narrowbore-ODS and microbore-enantioselective columns and validation of the method

Combining a narrowbore-ODS column and a microbore-enantioselective column using the conditions described in Section 3.1, an online 2D-HPLC system has been developed. The outline of the flow diagram of the 2D-HPLC system was shown in Fig. 1. In the first dimension, target amino acids were separated as their NBD-derivatives from other compounds and were collected into the multi-loop valve. The fractions were then...
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automatically introduced into the enantioselective column in the second dimension. The mobile phases were an aqueous solution containing 15% MeCN and 0.05% TFA for the first dimension and a mixed solution of MeOH-MeCN (80/20, v/v) containing 0.1% FA for the second dimension. As shown in Fig. 4, the volume of the fraction of NBD-Ala became relatively large, and the enantiomers were not sufficiently separated in the second dimension because of the incompatibility of the mobile phases used for the first dimension injected together with the NBD-Ala to the second dimension and that used for chiral separation in the second dimension. In addition, NBD-Gly was not completely separated from a reagent peak in the first dimension. Therefore, the conditions for both dimensions were further investigated, and all hydrophilic NBD-amino acids were separated from each other and also from interferences derived from reagents using a 10% MeCN aqueous solution containing 0.04% TFA in the first dimension. In the second dimension, a mixed solution of MeOH-MeCN (90/10, v/v) containing 0.03% FA was selected as a mobile phase for NBD-Ala enantiomers. Concerning NBD-Ser enantiomers, their retention times were close to those of NBD-Gln enantiomers in both first and second dimensions and it is likely that NBD-Gln enantiomers interfere with the determination of NBD-Ser enantiomers. Thus, the mobile phase conditions were investigated in order to accomplish the simultaneous separation of NBD-Ser and NBD-Gln enantiomers in the second dimension. As a result, NBD-Ser and NBD-Gln enantiomers were nicely separated using a mixed solution of MeOH-MeCN (25/75, v/v) containing 0.075% FA as shown in Fig. 5. The enantiomer separations of NBD-Ala and NBD-Ser were sufficient and the obtained resolution values were 4.34 for NBD-Ala enantiomers and 7.24 for NBD-Ser enantiomers.

The established 2D-HPLC system was validated by checking the calibration lines, intra-day precision and inter-day precision using standard amino acids. The precision and the accuracy were also investigated using the mouse cerebrum. The sample preparation procedure for the mouse cerebrum is described in Sections 2.2 and 2.3. The calibration lines of all the standard target amino acids were linear with correlation coefficients higher than 0.9999, and the RSD values of the intra-day precision were 1.17-2.27%, while those of the inter-day precision were 1.79-3.43% (n=4). Concerning the mouse cerebrum sample, the RSD values of the intra-day precision were lower than 3.50% and those of the inter-day precision were lower than 8.30% (n=4). The accuracy of the method was evaluated by adding the target amino acid enantiomers to the mouse cerebrum sample and the recovery values were 93.7-110.0% (Table 1). These results indicated that the present 2D-HPLC system is applicable for the determination of the Ala and Ser enantiomers in the mouse cerebrum.

Currently, several 2D-HPLC systems have been developed for the d-amino acid analysis of biological samples and food samples [2,5,46]. In most cases, the internal diameter of the reversed-phase column (first dimension) was smaller than that of the enantioselective column (second dimension); for example, a reversed-phase column of 0.53 mm i.d. and an enantioselective column of 1.5 mm i.d. were frequently connected [36,38,40,41,43,44]. These combinations are suitable to establish the 2D-HPLC system using the different mobile phases in both dimensions. However, the loading capacity of the first dimension is limited because of the small inner diameter. In addition, the sensitivity in the second dimension is not high because...
of the sample diffusion in the second column. To precisely determine the trace amounts of D-amino acids in biological samples, an analytical method with a higher sensitivity is required. In the present 2D-HPLC system, a reversed-phase column of 1.5 mm i.d. was used in the first dimension. Compared with the column of 0.53 mm i.d., the internal diameter was about 2.8 times wider, and the cross-section was about 8 times larger. Therefore, the reversed-phase column used in the present study could accept about ten times higher amounts of the samples, which is suitable for the analysis of the samples with low concentrations. In the second dimension, the enantioselective column of 1.0 mm i.d. was used. Compared with the column of 1.5 mm i.d., the internal diameter was 2/3 times narrower, and the sensitivity was about twice higher. In total, the sensitivity of the present system is in principle about 20 times higher than that of the previous system and wide applications using various biological samples are expected.

3.3. Determination of Ala and Ser enantiomers in the mouse cerebrum

The results described in Section 3.2 suggest that quantitative and reproducible analysis of the Ala and Ser enantiomers could be performed by the present 2D-HPLC system. In Section 3.3, therefore, the amounts of D-Ala and D-Ser were determined in the mouse cerebrum to demonstrate the applicability to the real biological samples. The obtained chromatograms are shown in Fig. 6. In the second dimension, a large amount of D-Ser and a trace amount of D-Ala were clearly observed. The amount of D-Ala was $3.3 \pm 0.4$ (mean $\pm$ SE of 4 mice) nmol/g wet tissue, and that of D-Ser was $190.7 \pm 7.9$ nmol/g wet tissue. To confirm these values, the same cerebrum samples were also analyzed by the 2D-HPLC system equipped with a KSAACSP-001R column which is an opposite enantioselective column to KSAACSP-001S. The obtained values were $4.3 \pm 2.1$ nmol/g wet tissue (D-Ala) and $196.6 \pm 0.3$ nmol/g wet tissue (D-Ser), and these results were in good agreement with those obtained by the 2D-HPLC system using a 001S column.

The amounts of D-Ala and D-Ser in the mouse cerebrum have been determined using various analytical methods. With the reversed-phase HPLC method using chiral derivatization with $o$-phthalaldehyde and $N$-tert-butyloxy carbonyl-L-cysteine, the reported amount of D-Ala was n.d.-12.4 nmol/g wet tissue and that of D-Ser was 310-423.2 nmol/g wet tissue [47,48]. Nagata et al. derivatized amino acids with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide and analyzed by preparative two-dimensional TLC followed by the reversed-phase HPLC determination [49,50]. In the report, the amounts of D-Ala and D-Ser were determined to be 3.5 nmol/g wet tissue and 263-353 nmol/g wet tissue, respectively. A 2D-HPLC method equipped with a widely used combination of microbore-ODS (0.53 mm ID) and narrowbore-enantioselective (1.5 mm ID) columns was also reported with NBD-F as the derivatization reagent, and the amounts of D-Ala and D-Ser were about 5 nmol/g wet tissue and about 300 nmol/g wet tissue, respectively [43]. In the present investigation, using the "reversed-ID type" 2D-HPLC system equipped with narrowbore-ODS and microbore-enantioselective columns, the amount of D-Ala in the mouse cerebrum was $3.3 \pm 0.4$ nmol/g wet tissue and that of D-Ser was $190.7 \pm 7.9$ nmol/g wet tissue, and these values are quite consistent with those previously reported. These results indicated that the present method is successfully applicable for the determination of D-amino acids in the tissue samples, and is thought to be a powerful

### Table 1. Validation of the method for the determination of Ala and Ser enantiomers.

| Amino acids | Calibration line | Precision (RSD, %) | Mouse cerebrum |
|-------------|-----------------|--------------------|----------------|
|             | Calibration range (pmol) | Equation | Correlation coefficient | Intra-day | Inter-day | Precision (RSD, %) | Equation | Correlation coefficient | Intra-day | Inter-day | Accuracy (%) |
| D-Ala       | 0.001-1         | $y = 20.6x - 0.01$ | 1.00000         | 2.05 | 2.03 | 2.14 | 5.76 | 98.6 |
| L-Ala       | 0.05-10         | $y = 14.5x - 0.25$ | 0.99999         | 2.27 | 2.45 | 3.01 | 8.30 | 93.7 |
| D-Ser       | 0.001-1         | $y = 28.5x - 0.02$ | 0.99999         | 1.17 | 1.79 | 2.76 | 6.58 | 109.9 |
| L-Ser       | 0.05-10         | $y = 16.3x - 0.35$ | 0.99998         | 1.40 | 3.43 | 3.50 | 6.74 | 110.0 |

**Fig. 6.** 2D-HPLC separations of Ser and Ala enantiomers in the mouse cerebrum as their NBD derivatives. The detailed separation conditions are the same as shown in Fig. 5.
tool for the trace analysis of D-amino acids in real world samples.

4. Conclusion
In the present study, a newly designed "reversed ID type" 2D-HPLC system has been developed for the simultaneous determination of Ala and Ser enantiomers as their NBD derivatives. The system was validated using standard amino acids, and successfully applied to a mouse cerebrum sample. The 2D-HPLC method proposed in the present paper, having a wider ID column for larger sample loading in the first dimension and narrower ID column for higher sensitive detection in the second dimension, is suitable for the trace analysis of D-amino acids in complicated biological samples. The present system would surely be a powerful tool for chiral amino acid analysis of real world samples, and further studies using various clinical samples, animal samples and foods/beverages are now in progress.

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