Two-Dimensional High-Performance Liquid Chromatographic Determination of Chiral Amino Acids in Food Samples and Human Physiological Fluids Using Fluorescence Derivatization with 4-(N,N-Dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole

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Abstract
A two-dimensional high-performance liquid chromatographic (2D-HPLC) system using the pre-column derivatization with 4-(N,N-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) has been developed for the determination of alanine (Ala), aspartic acid (Asp) and serine (Ser) enantiomers. For the precise determination of trace amounts of D-amino acids in complex matrices, a highly sensitive and selective method is required. In the present study, amino acids were derivatized with DBD-F for the sensitive fluorescence detection and were analyzed by a selective 2D-HPLC system combining a reversed-phase column (Singularity RP18, 1.0 x 500 mm) and an enantioselective column (Singularity CSP-001S, 1.5 x 250 mm). The established system was successfully applied to the chiral amino acid analyses of Japanese traditional amber rice vinegar, human plasma and human urine samples. In the Japanese traditional amber rice vinegar and human urine, relatively high amounts of all the target D-amino acids were observed (%D=5.8-37.7), while trace amounts of D-Ser and D-Ala were found in human plasma (%D=0.5-2.1).

Keywords: D-Amino acids; Enantiomer separation; 2D-HPLC; 4-(N,N-Dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole

1. Introduction
D-Amino acids are counterparts of abundantly present L-amino acids in higher animals, and have been thought to be negligible compounds for a long time. With the recent progress of analytical techniques, however, various D-amino acids have been found in mammals including humans [1-3], and their physiological functions have been gradually elucidated [4,5]. For instance, D-serine (Ser) has a role as the neurotransmitter in the human brain [6,7] and is involved in the long-term potentiation [8]. D-Aspartic acid (Asp) is known to regulate the hormonal synthesis and secretion in the pineal gland and testis [9-11]. In the pituitary gland and pancreas, the localization of D-alanine (Ala) has been reported, and the relationship with the control of the blood glucose level is suggested [12,13]. The benefits of D-amino acids for beauty and health care, such as the skin barrier function of D-Ser [14], and the antioxidant effect of D-Asp [15], have also been reported, and foods and beverages containing D-amino acids are matters of interest. Furthermore, the alterations of the D-amino acid contents have been observed along with various diseases such as Alzheimer’s disease [16,17] and...
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chronic kidney disease [18,19]. Therefore, D-amino acids are now gathering attention as bio-functional molecules and candidates for novel biomarkers.

The D-amino acid contents in biological matrices are usually trace, and their determination is often disturbed by the presence of uncountable co-existing compounds. Therefore, for the precise determination of D-amino acids, a sensitive and selective analytical method is required. Until now, various analytical methods using gas chromatography (GC) [20], high-performance liquid chromatography (HPLC) [21,22] and capillary electrophoresis (CE) [23] have been developed for the chiral amino acid analysis. Among them, a two-dimensional (2D) HPLC system combining reversed-phase and enantioselective separations after fluorescence derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) is one of the most practically useful methods. The sample solution derivatized with NBD-F was injected into a reversed-phase column in the first dimension. The target amino acids were separated from co-existing compounds and were respectively fractionated into the sample trapping loops as mixtures of their D- and L-forms. The collected fractions were transferred to an enantioselective column in the second dimension, and the chiral separation of the target amino acids and enantioselective determination were performed. By using the 2D-HPLC system, various D-amino acids have been found in various real world samples including mammalian tissues/physiological fluids and also food/beverage samples [24-26]. In some matrices, however, interfering compounds, which were not separated in the first dimension, were eluted around the target amino acid enantiomers even in the second dimension. Thus, checking the results by changing the analytical conditions is desirable to obtain accurate quantitative values of the chiral amino acids, and the use of different reagents, columns and mobile phases is expected.

In the present study, 4-(N,N-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) was adopted as a new option of the derivatization reagent used for the 2D-HPLC analysis. DBD-F reacts with primary and secondary amines under basic conditions, and amino acids were converted into their fluorescence derivatives [27]. The novel 2D-HPLC system with DBD-derivatization has been developed for the determination of Ser, Asp and Ala enantiomers, and applied to the chiral amino acid analysis of the Japanese traditional amber rice vinegar, human plasma and human urine.

2. Experimental

2.1. Materials

D-Ala, L-Ala and D-Ser were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). D-Asp, L-Asp and L-Ser were obtained from Nacalai Tesque (Kyoto, Japan). The derivatizing reagent, DBD-F was a product of Tokyo Chemical Industry (Tokyo, Japan). Acetonitrile (MeCN) of HPLC grade was purchased from Nacalai Tesque. Methanol (MeOH) of HPLC grade, boric acid, formic acid (FA) and trifluoroacetic acid (TFA) were obtained from Fujifilm Wako Pure Chemical Corporation. Water was purified using a Milli-Q Integral 3 system (Merek, Darmstadt, Germany). All other reagents were of the highest reagent grade and were used without further purification.

2.2. Sample preparation

The Japanese traditional amber rice vinegar was obtained from Sakamoto Kurozu, Inc. (Kagoshima, Japan), and was diluted 1,000 times with water. To the diluted solution (10 µL), 10 µL of 400 mM sodium borate buffer (pH 9.3) and 5 µL of 40 mM DBD-F in MeCN were added. The mixture was heated at 70°C for 1 h, then the reaction was terminated by adding an aqueous 0.2% (v/v) TFA solution (75 µL). An aliquot (50 µL) of the reaction mixture was subjected to the 2D-HPLC system described in Section 2.3. The human physiological fluids (plasma and urine) were collected from healthy male volunteers (21-27 years old) from whom informed consent was obtained. This experiment was approved by the review board of the Clinical Research Network Fukuoka (No.14-E05). The volunteers were requested not to take foods/beverages containing high levels of D-amino acids, and the plasma and urine were collected from these volunteers at 9 a.m. after overnight fasting. The blood collected in a heparinized tube was centrifuged at 1,500 x g and 4°C for 5 min to obtain the plasma. Both the plasma and urine samples were stored at -30°C before use. To 10 µL of the plasma, 190 µL of MeOH was added and centrifuged at 12,000 x g for 5 min, then the obtained supernatant (10 µL) was evaporated to dryness under reduced pressure at 40°C. The residue was dissolved in 10 µL of water, then the amino acids in the solution were derivatized using the same procedure as that for the vinegar sample. The urine was diluted with 10 times the volume of water, and 10 µL of the solution was used for the derivatization. Derivatization of amino acids with DBD-F is shown in Fig. 1.

![Derivatization of amino acids with DBD-F](image)

**Fig. 1.** Derivatization of amino acids with DBD-F.
connection of two HPLC systems (Nanospace SI-2 series, Shiseido, Tokyo, Japan). The system for the first dimension was composed of three pumps (3101 and 3201), a 3010 degasser, a 3023 auto-sampler, a 3004 column oven and a 3213 fluorescence detector. An EZChorme Elite was used to operate the system and to process data. The system for the second dimension consisted of four pumps (3101 and 3201), a 3202 degasser, a 3033 auto-sampler, a 3014 column oven and a 3213 fluorescence detector. An EZChorme SI was utilized to operate the system and to process data. The flow diagram is shown in Fig. 2. In the first dimension, a reversed-phase column, Singularity RP18 (C18 bonded silica particle packed column, particle size 3 µm, 1.0 mm i.d. x 500 mm, originally designed/developed by the collaboration with KAGAMI, Osaka, Japan) was used at 45°C. As the mobile phase, an aqueous 20% MeCN 0.1% TFA solution was used at 30 µL/min. After reversed-phase separation in the first dimension, the fractions of DBD-Ser (4.5 min, 135 µL), DBD-Asp (4.5 min, 135 µL) and DBD-Ala (5.1 min, 153 µL) were collected in light-shielded tubes, and 100 µL of the fraction was introduced into an enantioselective column in the second dimension. For the enantiomer separations of the target DBD-amino acids, a Singularity CSP-001S column (N-3,5-dinitrophenylaminocarbonyl-L-leucine bonded aminopropylsilica particle packed column, particle size 5 µm, 1.5 mm i.d. x 250 mm, originally designed/developed by the collaboration with KAGAMI) was used at 25°C. The mobile phases for DBD-Ser, Asp and Ala were 0.035% FA in MeOH/MeCN (75/25, v/v), 0.10% FA in MeOH/MeCN (25/75, v/v) and 0.025% FA in MeOH/MeCN (25/75, v/v), respectively. The flow rate was 200 µL/min. DBD-amino acids were detected by their fluorescence emission at 540 nm with excitation at 420 nm.

3. Results and discussion

3.1. Development of a 2D-HPLC system for the determination of the DBD-Ser, Asp and Ala enantiomers

A 2D-HPLC system using pre-column derivatization with DBD-F has been developed for the determination of the Ser, Asp and Ala enantiomers. The D-forms of these three amino acids are frequently observed in food and biological matters of interest. Amino acids in the samples were derivatized with DBD-F, and an aliquot of the reaction mixture was injected to a reversed-phase column in the first dimension of the 2D-HPLC system. The target DBD-amino acids were separated from other amino acids and interfering compounds by the difference in their hydrophobicity, and were manually fractionated into light-shielded tubes as their D and L mixtures. An aliquot of the fraction was introduced into an enantioselective column in the second dimension, where the amino acid enantiomers were separated and determined.

In the first dimension, tandemly connected reversed-phase columns (Singularity RP18, 1.0 mm i.d. x 500 mm) were used, and the mobile phase conditions were investigated to separate the target DBD-amino acids from other proteinogenic amino acids. As the mobile phases, aqueous solutions containing 0.1% TFA and various concentrations of MeCN (15%, 17.5% and 20%) were tested. The obtained chromatograms are shown in Fig. 3. The retention times of the DBD-amino acids became shorter with the increasing concentration of MeCN. By using aqueous 15% and 17.5% MeCN 0.1% TFA solutions, DBD-Ser was not completely separated from other amino acids (Figs. 3B and 3C). On the other hand, all of the target DBD-amino acids were well separated from other amino acids within 90 min by using an aqueous 20% MeCN 0.1% TFA solution (Fig. 3A).

For the enantiomer separations, a Pirkle type enantioselective column, Singularity CSP-001S (1.5 mm i.d. x 250 mm) was used. The mixed solutions of MeOH and MeCN containing FA were selected as the mobile phases, and the FA concentration in addition to the composition of MeOH and MeCN were investigated. The results of separating the DBD-Ala enantiomers are shown in Fig. 4. Concerning the FA concentration, the solutions containing 0.10, 0.050 and 0.025% FA in MeOH/MeCN (50/50) were tested, and using the lower FA concentration resulted in the longer retention time and the better enantiomer separation. The ratio of MeOH and MeCN was investigated using 0.025% FA in MeOH/MeCN (25/75, 50/50 and 75/25), and the obtained resolution values were 2.95, 2.71 and 2.74, respectively.
respectively. As a result, 0.025% FA in MeOH/MeCN (25/75) was selected as the mobile phase for the enantiomer separation of DBD-Ala. The mobile phases for the DBD-Ser and Asp were also optimized, and the selected solutions were 0.035% FA in MeOH/MeCN (75/25) for DBD-Ser and 0.1% FA in MeOH/MeCN (25/75) for DBD-Asp. The resolution values of their enantiomers were 3.70 for DBD-Ser and 0.91 for DBD-Asp.

Until now, several 2D-HPLC systems have been developed for the determination of the Ser, Asp and Ala enantiomers using fluorescence derivatization with NBD-F and were applied to various biological matrices [26,28-31]. In the first dimension of these methods, a monolithic ODS column or a narrowbore ODS column was used for the reversed-phase separation, and aqueous solutions containing MeCN and TFA were used as the mobile phases. By using the monolithic ODS column (0.35 mm i.d. x 750-1000 mm), the retention times of NBD-Ser and Asp were 25-30 and 30-35 min, respectively, with 5-8% MeCN 0.05-0.06% TFA in H2O [28-31]. Concerning NBD-Ala, a gradient elution was used for the simultaneous determination with NBD-Ser, and the retention time was around 65 min using 5-15% MeCN 0.05% TFA in H2O [30]. By using the narrowbore ODS column (1.5 mm i.d. x 500 mm), NBD-Ser and Ala eluted at around 35 and 90 min, respectively, with 5-8% MeCN 0.05-0.06% TFA in H2O [28-31]. Concerning NBD-Ala, a gradient elution was used for the simultaneous determination with NBD-Ser, and the retention time was around 65 min using 5-15% MeCN 0.05% TFA in H2O [30].

DBD-amino acids were nicely separated from other amino acids and intrinsic substances, and the present reversed-phase separation is sufficient for practical use.

For the enantiomer separations of the NBD-amino acids, Pirkle-type enantioselective columns, such as a Sumichiral OA-2500S column (Sumika Chemical Analysis Service, Osaka, Japan) and a KSAACSP-001S column (Shiseido, Tokyo, Japan), were frequently used [26,28-31]. As the mobile phases, the mixtures of MeOH and MeCN containing citric acid or FA were utilized, and the reported separation factors (α values) of the enantiomers were 1.17-1.32 for NBD-Ser, 1.11 for NBD-Asp and 1.13-1.47 for NBD-Ala. In the present investigation, all of the target DBD-amino acid enantiomers were separated by a Singularity CSP-001S column using the mixed solution of MeOH and MeCN containing FA, and the obtained α values were 1.41 for DBD-Ser, 1.10 for DBD-Asp and 1.26 for DBD-Ala. The enantiomer separations of the DBD-amino acids obtained in the present study using a Singularity CSP-001S column were sufficient compared to those of the NBD-amino acids on the similar Pirkle type columns, indicating that the derivatization with DBD-F was practically useful for the chiral amino acid analysis in the biological matrices.

### 3.2. 2D-HPLC determination of DBD-Ser, Asp and Ala enantiomers in the vinegar sample and human physiological fluids

The 2D-HPLC system using pre-column derivatization with DBD-F designed/developed in section 3.1. was applied to the enantioselective analyses of Ser, Asp and Ala in the vinegar and human physiological fluids. The obtained chromatograms for the Japanese traditional amber rice vinegar, Kurozu, are shown in Fig. 5. In the first...
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The target DBD-amino acids were fractionated at the same retention times of the standard amino acids indicated by closed bars, and their fractions were transferred to the enantioselective column. In the second dimension, all of the target D-amino acids were clearly observed, and the %D values (D/(D+L) x 100) were 5.8 for Ser, 22.9 for Asp and 27.8 for Ala (Fig. 5A). These results were confirmed by using a Singularity CSP-001S column (having the opposite chiral center of the Singularity CSP-001S column). As shown in Fig. 5B, the retention orders of the enantiomers were reversed by using the 001R column; the L-forms eluted earlier than the D-forms. The obtained %D values were 6.3 for Ser, 21.1 for Asp and 27.9 for Ala, and these results were in good agreement with those obtained by the 001S column. As the further applications, human plasma and urine samples from healthy volunteers were analyzed. In the human plasma (Fig. 6), trace amounts of D-Ser and D-Ala were found, and the %D values were 2.1 and 0.5, respectively. On the other hand, relatively large amounts of D-Ser (%D=37.7), D-Asp (%D=11.5) and D-Ala (%D=17.4) were observed in the human urine (Fig. 7). These results were also confirmed by using the Singularity CSP-001R column. The obtained %D values in the human plasma were 2.1 for Ser and 1.6 for Ala, and those in the human urine were 39.1 for Ser, 11.2 for Asp and 19.6 for Ala. These results were almost consistent with those obtained by the 001S column.

Currently, D-amino acids in Kurozu, human plasma and human urine samples have been determined by various analytical methods using GC and LC frequently equipped with MS or MS/MS. In the Kurozu samples, certain amounts of several D-amino acids including Ser, Asp and Ala have been found [25,32-34], and their %D values were 1.7-5.5 for Ser, 14.4-35.3 for Asp and 18.5-29.2 for Ala. Concerning the human plasma, the reported %D values of Ser, Asp and Ala were 0.5-4.9, 0.0 (n.d.)-1.0 and 0.0 (n.d.)-1.2, respectively [35-40]. Human urine is known to contain relatively high levels of D-amino acids, and the %D values in previous reports were 19.0-56.5 for Ser, trace-8.1 for Asp and 5.7-39.4 for Ala [36,38,39,41-44]. In the present study, all of the target D-amino acids (Ser, Asp and Ala) were found in the Kurozu and human urine samples. The %D values of Ser, Asp and Ala were 5.8, 22.9 and 27.8 in the human plasma, trace amounts of D-Ser (%D=2.1) and D-Ala (%D=0.5) were found. These results were in good agreement with those previously reported, indicating that the developed method in the present study is applicable to the real-world samples. The DBD-amino acids can be analyzed by the same HPLC devices as those for NBD-amino acids, and the analyses...
using two derivatization reagents enable the precise determination of chiral amino acids in various real world samples.

4. Conclusion
In the present study, a 2D-HPLC system using the pre-column fluorescence derivatization with DBD-F has been developed for the determination of the Ser, Asp and Ala enantiomers. The target amino acid enantiomers were sufficiently separated as their DBD derivatives, and the developed 2D-HPLC system was applicable to the chiral amino acid analysis of Japanese traditional amber rice vinegar, human plasma and human urine. In all the tested matrices, the target amino acid enantiomers were successfully determined, indicating that the present 2D-HPLC system with DBD derivatization is a practically useful tool with high sensitivity and selectivity. The present concept is applicable to other amino acids and further studies are in progress.

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