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Development of an online two-dimensional high-performance liquid chromatographic system in combination with tandem mass spectrometric detection for enantiomeric analysis of free amino acids in human physiological fluid

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ABSTRACT

An automated two-dimensional HPLC-MS/MS system was designed and developed for the highly selective determination of trace levels of D-amino acids. As the targets, frequently observed ones in mammalian physiological fluids, Ala, Asp, Glu, Leu, Pro and Ser, were selected because these D-amino acids are the potential biomarkers for the early and sensitive diagnoses of various diseases. The target analytes were derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), then isolated by a reversed-phase column in the first dimension. The NBD-amino acid fractions were automatically collected into the multi-loop device, and introduced into the enantioselective column in the second dimension to separate the D- and L-forms followed by detection by an MS/MS. The obtained resolution values of the enantiomers were 1.87–5.17 and the calibration lines, precision and accuracy were practically sufficient. By using the present 2D HPLC-MS/MS system, trace levels of D-amino acids in complex biological matrices were determined without disturbance by the intrinsic interfering compounds, and successfully applied to the analysis of the human clinical samples (plasma and urine).

1. Introduction

Since all proteinogenic amino acids, except for glycine, have a chiral center at least at the alpha carbon, these amino acids have mirror-image isomers called the D- and L forms. Although the L-amino acids are thought to be the predominant compounds for living beings on the Earth, the presence of D-amino acids has been revealed both in the free form and also in the protein bound form along with the recent progress in analytical techniques [1–3]. In particular, D-aspartate (Asp) and D-serine (Ser) in their free forms were frequently found in mammals including humans and their localization and physiological functions have been intensively studied. D-Asp is observed in endocrine tissues and has association with the synthesis/secretion of hormones such as melatonin and testosterone [4–6]. D-Ser exists as a co-agonist of the *N*-methyl-D-aspartate subtype of glutamate (Glu) receptors in the frontal brain area and modulates the neurotransmission [7–9]. Recently, not only the above-mentioned D-Asp and D-Ser, but various D-amino acids (namely, D-alanine (Ala) [10–13], D-Glu [14,15], D-leucine (Leu) [11,16] and D-proline (Pro) [10,17,18]) have also been found in the tissues and physiological fluids of mammals. For instance, D-Ala and D-Pro were observed in the plasma of patients with a renal disorder and thought to have an association with the disease because the amounts of these D-amino acids have a positive correlation with the serum creatinine level [19–21]. D-Ser was also clarified to increase in the spinal cord of animals with amyotrophic lateral sclerosis which is a progressive neurodegenerative disorder [22]. D-Glu accumulates in mouse hearts lacking a specific protein, 9030617O03Rik, which is reduced during heart failure [15]. Therefore, various D-amino acids are the focus as new potential biomarkers for the early and sensitive diagnoses of diseases.

Until now, several approaches for amino acid analysis with discrimination of the D and L forms have been published. One of the simple methods is enzymatic analysis. D-Amino acid oxidase and D-aspartate oxidase are frequently employed and by the enzymatic reaction, the α-keto acids are produced from the

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D-amino acids [23,24]. These keto-acids are usually converted into the respective hydrazones by the reaction with hydrazine, and are specifically detected by absorbance at 445 nm [25,26]. As methods using chromatography, gas chromatography (GC) and high-performance liquid chromatography (HPLC) are widely adopted with diastereomer formation or the use of chiral stationary phases. D(+)-2-Butanol is utilized for the esterification of the amino acids to obtain diastereomers, and the derivatives are analyzed by achiral GC columns [27]. As the chiral stationary phase, Chirasil-L-valine is commonly used for the enantioselective GC analysis [28]. Concerning HPLC, chiral derivatizing reagents, such as *o*-phthalaldehyde (OPA) with chiral thiols [29,30], 1-fluoro-2,4-dinitrophenyl-5-L-Ala amide (FDAA) [31] and (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) [32], are widely used and the amino acid enantiomers are converted into diastereomers followed by separation utilizing reversed-phase columns. The chiral stationary phases are also frequently used coupled with the derivatization of D- and L-amino acids with highly fluorescent reagents [11–18,33–35]. A variety of LC–MS/MS methods with and without derivatization have also been reported [36–39]. Among the already reported methods, the combination of reversed-phase separation and enantioselective separation, namely the two-dimensional HPLC concept, is an outstanding strategy because of its high sensitivity and selectivity. However, the determination of trace levels of amino acid enantiomers tends to be disturbed by the co-elution of various unknown and/or known intrinsic substances in biological matrices even when 2D-HPLC (and also 1D LC–MS/MS) is used. In order to overcome this problem, the improvement in the selectivity of the analytical methods is still strongly desired, and one of the effective and straight-forward approaches is to increase the analytical dimension.

In the present study, an online two-dimensional HPLC-MS/MS system composed of reversed-phase separation (first dimension), chiral separation (second dimension) and MS/MS detection (third and fourth dimensions) has been developed for the determination of free amino acid enantiomers in complicated biological matrices. By combining the MS/MS detection, most of the intrinsic substances having different MS and MS/MS profiles can be distinguished. As the target amino acids, Ala, Asp, Glu, Leu, Pro and Ser were selected because the D-forms of these amino acids were frequently observed in mammalian tissues and physiological fluids [1,25]. The present system was applied to human urine and plasma samples because these two are most commonly used as clinically obtained matrices.

2. Experimental

2.1. Materials

L-Ala, D- and L-Asp, L-Leu, L-Ser were obtained from Nacalai Tesque (Kyoto, Japan). The other D- and L-amino acids were purchased from Wako Pure Chemical Industries (Osaka, Japan). The derivatizing reagent, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), was from Tokyo Chemical Industry (Tokyo, Japan). Acetonitrile (MeCN) and methanol (MeOH) of hypergrade for LC–MS were purchased from Merck Millipore (Darmstadt, Germany). Boric acid, trifluoroacetic acid (TFA) and formic acid (FA) were products of Wako. Water was purified by a Milli-Q Integral 3 system from Merck Millipore. All other reagents were of the highest grade and used without further purification.

2.2. Preparation of purified NBD-amino acids for the optimization of MS/MS conditions

To 40 μL of 1 mM L-amino acids in water, 40 μL of 400 mM sodium borate buffer (pH 8.0) and 20 μL of 40 mM NBD-F in MeCN were added. After the mixture was heated at 60 °C for 2 min, 100 μL of an aqueous 0.5% (v/v) TFA solution was added. The resulting solution (50 μL) was subjected to the HPLC system (consisting of a PU-980 pump, a DG-980-50 degasser, an LG-980-02 gradient unit, a UV-2075Plus detector and an 807-IT integrator, JASCO, Tokyo, Japan) and the NBD-amino acid was isolated from other reagent peaks and side-products by an enantioselective column (Sumichiral OA-2500S, 1.5 mm i.d. x 250 mm, 25 °C). A mixture of MeCN/MeOH (50/50, v/v) containing 2% FA was used as the mobile phase at a flow rate of 200 μL/min. Detection of the NBD-amino acids was carried out by the absorbance at 470 nm. The purified NBD-amino acid solutions were used for the optimization of the MS/MS conditions.

2.3. Pre-column derivatization of amino acids in human physiological fluids

The plasma and urine were collected from healthy male volunteers who were 21–27 years old from whom informed consent was obtained. The experiments were approved by the review board of the Clinical Research Network Fukuoka (No. 14-E05). The plasma and urine were obtained from these volunteers at 9 AM under a fasting condition. The volunteers had been forbidden to have a meal containing D-amino acids abundantly and allowed to drink only tea and/or water during the sampling. After collected in a heparinized tube, the blood was centrifuged at 1,500 x *g* and 4 °C for 5 min, then the plasma was obtained. These clinical samples (plasma and urine) were stored at -80 °C until used. For 10 μL of plasma, 190 μL of MeOH was added and centrifuged at 12,000 x *g* for 5 min. The supernatants (50 μL) obtained from the plasma-MeOH suspension were evaporated to dryness under reduced pressure at 40 °C. The residue was dissolved in 10 μL of water and mixed with 10 μL of 400 mM sodium borate buffer (pH 8.0). To the mixture, 5 μL of 40 mM NBD-F in MeCN was added and heated at 60 °C for 2 min. The reaction was terminated by adding 75 μL of an aqueous 0.2% (v/v) TFA solution. The urine was diluted 5 times with water and the diluted solution (10 μL) was derivatized in the same manner as that for plasma. Aliquots (10 μL) of these reaction mixtures were subjected to the 2D HPLC-MS/MS system described in Section 2.4.

2.4. Two-dimensional (multiple heartcutting) HPLC-MS/MS system

To design and develop the multiple heartcutting 2D-HPLC system, NANOSPACE SI-2 instruments (Shiseido, Tokyo, Japan) were utilized. The present system consisted of a 3202 degasser, 3101 and 3201 pumps, a 3023 auto sampler, 3004 and 3014 column ovens, a 3012 high pressure valve, a 9986 multi-loop unit with 6 loops (the volume of each loop is 650 μL), a 3002 UV detector, a 3013 fluorescence detector and a tandem mass spectrometer (Triple Quad™ 5500, Sciex, Framingham, MA, USA). As the reversed-phase column for the first dimension of the system, a KSAARP column (1.0 mm i.d. x 500 mm, an original ODS column designed by collaboration with Shiseido) was adopted and maintained at 45 °C. For the reversed-phase separation of the target amino acids, the stepwise gradient elution using aqueous 10% (0–180 min), 20% (180–240 min) and 35% (240–360 min) MeCN solutions containing 0.05% TFA was adopted. The flow rate was 25 μL/min. The elution of the NBD-amino acids was monitored by the absorbance at 470 nm. For the enantiomer separations in the second dimension, a KSAACSP-001S column (1.5 mm i.d. x 250 mm, an original Pirkle-type column produced by collaboration with Shiseido) was used at 25 °C. The mobile phase for the separation of NBD-D- and L-Asp was a mixture of MeCN-MeOH (75/25, v/v) containing 0.5% FA. NBD-D- and L-Glu were separated using a mixture of MeCN-MeOH (80/20, v/v) containing 0.3% FA. As the mobile phase for the enantioselective separation of NBD-Ala, NBD-Leu, NBD-Pro and NBD-Ser, a

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