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Enantioselective determination of citrulline and ornithine in the urine of D-amino acid oxidase deficient mice using a two-dimensional high-performance liquid chromatographic system

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ABSTRACT

Two-dimensional high-performance liquid chromatographic (2D-HPLC) and 2D-HPLC-mass spectrometric (2D-HPLC–MS) systems have been designed and developed for the determination of the citrulline (Cit) and ornithine (Orn) enantiomers. Several D-amino acids have already been identified as novel physiologically active molecules and biomarkers, and the enantioselective evaluation of the amounts, distributions and metabolisms of non-proteinogenic amino acids gain as well increasing interest. In the present study, highly selective analytical methods were developed using a capillary monolithic ODS column (0.53 mm i.d. x 1000 mm) for the reversed-phase separation of the target analytes from the matrix compounds in the first dimension, and a narrowbore-Pirkle type enantioselective column, KSAACSP–105 S (1.5 mm i.d. x 250 mm), was used for the enantiomer separation in the second dimension. The amino acids were analyzed after pre-column derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) and detected by the fluorescence detector and MS. The systems were applied to the urine of D-amino acid oxidase (DAO) deficient B6DAO$^-$ mice and control C57BL mice to evaluate the presence and metabolism of the Cit and Orn enantiomers in mammals. As a result, all of the 4 target enantiomers (D-Cit, L-Cit, D-Orn, L-Orn) were found in the urine of both strains. The %D value of Cit (D-Cit/Cit × 100) increased about 3-fold in the urine of the DAO deficient mice and that of Orn also tended to increase with the DAO deficiency. These results were definitely confirmed by a 2D-HPLC–MS detection system. Further investigations about the biological significance of these D-isomers are currently ongoing.

1. Introduction

D-Amino acids, the enantiomers of L-amino acids, have been gradually revealed to exist in higher animals including human beings [1–3]. As for the proteinogenic amino acids, various D-enantiomers, such as D-serine (D-Ser) [4,5], D-aspartic acid (D-Asp) [6,7], D-alanine (D-Ala) [8,9], D-proline (D-Pro) [10,11] and D-leucine (D-Leu) [12,13], have been found in mammals. Especially among them, the origins, distributions and functions of D-Ser and D-Asp have been well-studied due to their relatively high intrinsic amounts. D-Ser is highly localized in the cerebral cortex and hippocampus of mammals [4,5]. It modulates the neurotransmission of the *N*-methyl D-aspartate (NMDA) receptor and it is also reported to play significant roles in memory acquisition and study efficiency [14–16]. D-Asp was found to be present in the endocrine tissues, such as the adrenal gland and the anterior pituitary gland, and it is indicated to be involved in the synthesis and secretion of various hormones [17,18]. It has also been elucidated that intrinsic amounts of several D-amino acids changed in renal and neurological disorders [19–21], and these D-amino acids are now considered as physiologically active substances and biomarkers.

Besides the proteinogenic amino acids, many different metabolic-related amino acids are present in mammals with various functions [22–25]. Some of these metabolic-related amino acids have stereo-isomers, and both D- and L-forms chemically exist. However, the precise determination of these non-proteinogenic

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amino acids is difficult in many cases, because their intrinsic amounts are often extremely low. As for the proteinogenic amino acids, it has been clarified that the enantiomers have different biological functions and their synthetic and metabolic pathways are also different [26–28]. Therefore, the enantioselective analysis of these non-proteinoginic amino acids and the clarification of their existence, function and metabolism is of importance. In the present study, citrulline (Cit) and ornithine (Orn), well-known non-proteinogenic amino acids and found in the urea cycle, were selected as the target amino acids. The amounts of Cit and Orn have been used as biomarkers of urea cycle disorders but without discriminating the enantiomers. The L-forms of Cit and Orn are also used as supplements [29–31]. However, their enantioselective distributions, functions and metabolic pathways have scarcely been reported due to the lack of an appropriate highly sensitive and selective analytical method to enable their enantioselective determination in biological matrices.

Several methods for the enantiomer separation of standard amino acids including Cit and Orn have been reported [32–34]. By using reversed-phase HPLC, the urethane-protected amino acid *N*-carboxyanhydrades derivatized Orn was separated [32]. Underivatized Cit and Orn were separated with the polysaccharide-based column [33], and also the enantiomer separations of these two amino acids were achieved using a capillary electrochromatography (CEC) [34]. Concerning the intrinsic amounts of Cit and Orn, their amounts (D plus L forms) in urine of mammals have been reported using several analytical methods. Both Cit and Orn have been measured in human urine by gas chromatography-mass spectrometry (GC–MS) and LC methods but without discrimination of the D and L forms [23,24]. As for the enantioselective determination, only the Orn enantiomers were reported to exist in urine of rodents using a GC–MS method [35]. The reported intrinsic amounts of D-Orn were trace levels which led us to the development of highly-selective and sensitive HPLC analytical methods for the simultaneous and precise determination of the Cit and Orn enantiomers in biological matrices. A 2D-HPLC system combining reversed-phase and enantiomer separations in combination with fluorescence and/or MS based detection is considered to be one of the most effective tools.

In the present investigation we describe a 2D-HPLC-fluorescence- and a 2D-HPLC–MS-detection system and their application for the determination of the Cit and Orn enantiomers in mouse urine. In order to clarify the enantioselective metabolism of these amino acids, C57BL mice having normal D-amino acid oxidase (DAO) activity and DAO deficient B6DAO$^-$ mice were investigated. DAO is an enzyme metabolizing neutral and basic D-amino acids in mammals and is reported to have a relation with various diseases such as schizophrenia and amyotrophic lateral sclerosis [21,36]. In the present study, the Cit and Orn enantiomers were determined in the urine of C57BL and B6DAO$^-$ mice as the first step to clarify their enantioselective metabolic pathways, and also to evaluate them as possible biomarkers of the DAO deficiency.

2. Experimental

2.1. Materials

The enantiomers of Cit and Orn were obtained from Tokyo Chemical Industry (Tokyo, Japan), and the standard amino acids, Type H, were from Wako Pure Chemicals (Osaka, Japan). The fluorescence derivatization reagent, NBD-F, was purchased from Tokyo Chemical Industry, and boric acid, formic acid and trifluoroacetic acid (TFA) were from Wako Pure Chemicals. Methanol (MeOH) and acetonitrile (MeCN) of HPLC grade were obtained from Wako Pure Chemicals and Nacalai Tesque (Kyoto, Japan), respectively. Water was purified using a Milli-Q Integral 3 system (Merck Millipore, Darmstadt, Germany). All other reagents were of the highest grade and used without further purification.

2.2. Animals

Male C57BL and B6DAO$^-$ mice (7 weeks of age) were selected for the present study. The C57BL mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan) as the control mice. The DAO deficient mice, B6DAO$^-$ mice, were bred at the Center of Biomedical Research, Graduate School of Medical Sciences, Kyushu University (Fukuoka, Japan) and kept under a 12 h light/12 h dark cycle (lights on at 8:00 a.m.). They were given free access to food (CLEA Rodent Diet CA-1, CLEA Japan, Tokyo, Japan) and water. All experiments were performed with the permission of the Animal Care and Use Committee of Kyushu University (A27-061-0).

2.3. Sample preparation

The sample preparation procedure was designed basically followed by the validated one [26]. Briefly, the mice were anesthetized and euthanized by exsanguination from the abdominal aorta. The urine samples were stored at −80 °C after collected from their bladders. To 10 μL of 10 times water-diluted urine, 10 μL of 400 mM sodium borate buffer (pH 8.0) and 5 μL of 40 mM NBD-F in MeCN were added, then the mixture was heated at 60 °C for 2 min. To stop the derivatization, 75 μL of an aqueous 0.1% TFA solution was added to the reaction mixture and 2 μL was injected into the 2D-HPLC system described in Section 2.4.

2.4. 2D-HPLC-fluorescence- and 2D-HPLC–MS-detection systems for the determination of Cit and Orn enantiomers

The 2D-HPLC system was established using a NANOSPACE SI-2 semi-micro HPLC apparatus (Shiseido, Tokyo, Japan) with a KSAA valve-controlling system (originally designed by collaboration with Shiseido). The EZChrom SI system was used for the data processing of the 2D-HPLC system and MassLynx4.1 was used for the QDa detector. 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, 3213 fluorescence detectors and a 9986 multi-loop unit (having 2 loops of 200 μL). A capillary-monolithic ODS column, ML-1000 (0.53 mm i.d. × 1000 mm, originally designed by collaboration with Shiseido), was used for the reversed-phase separation in the 1st dimension and mobile phases A and B for gradient elution were aqueous 5% and 25% MeCN containing 0.05% TFA, respectively. The gradient elution of NBD-amino acids was carried out as follows: from 0 to 120 min, linear gradient from 100% A to 100% B and from 120 to 180 min, 100% B. The column was kept under 45 °C and the flow rate of the mobile phase was 25 μL/min. After the reversed-phased separation, the NBD-derivatized Cit and Orn were automatically fractionated in the multi-loops and introduced to the narrowbore-enantioselective column in the 2nd dimension. For the enantiomer separations of NBD-Cit and Orn, a KSAACSP-105S column (1.5 mm i.d. × 250 mm, originally designed by collaboration with Shiseido) was used at 25 °C. The mobile phase was 0.1% formic acid in a mixed solution of MeOH-MeCN (90:10, v/v) for the separation of the Cit enantiomers and 0.5% formic acid in a mixed solution of MeOH-MeCN (50:50, v/v) for the Orn enantiomers. The flow rate was 200 μL/ min. The NBD-amino acids were detected either by their fluorescence emission at 530 nm with excitation at 470 nm or by MS. For the MS detection, the Acquity QDa detector (Waters Corporation, Milford, MA, USA) was used. Conditions for the QDa detector were as follows: probe temperature, 300 °C; ESI capillary voltage, 0.8 V; cone voltage, 5.0 V. The negative-ion mode was selected and