Development of a Three-Dimensional HPLC System for the Simultaneous Determination of Lactate and 3-Hydroxybutyrate Enantiomers in Mammalian Urine

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Abstract
For the highly chemoselective separations of lactate (LA) and 3-hydroxybutyrate (3HB) enantiomers in mammalian urine, an online three-dimensional HPLC system (3D-HPLC) composed of reversed-phase (KSAARP, 1.0 mm i.d. x 250 mm), mixed-mode (KSAAMX, 1.5 mm i.d. x 250 mm) and enantioselective (Chiralpak AD-H, 2.0 mm i.d. x 250 mm) columns has been developed. Following the pre-column derivatization with 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ), NBD-LA and 3HB were separated in the first and second dimensions as their scalar D plus L mixtures and the enantiomer separations were performed in the third dimension with high separation factors (α = 1.70 for LA; α = 1.43 for 3HB). The present system was applied to the urine of mice and humans. For the mice urine, the D and L-forms of NBD-LA and 3HB were well separated without visible interfering compounds, and trace levels of D-LA (less than 5% of total LA) and non-negligible amounts of L-3HB (34.4% of the total 3HB) were clearly observed. Trace levels of the LA and 3HB enantiomers were detected for the human urine samples, although the existence of a few interfering peaks were observed.

Keywords: Enantiomer separation; Lactate; 3-Hydroxybutyrate; Three-dimensional HPLC

1. Introduction
Lactate (LA) and 3-hydroxybutyrate (3HB) are the typical chiral hydroxy acids in the metabolic pathways. LA is the product derived from pyruvate that is converted from glucose via glycolysis without oxygen requirement [1,2]. 3HB is the most abundant ketone body in the circulation, which is produced from fatty acids in the hepatocytes via ketogenesis during fasting or intense exercise [3]. Both of them are considered as signaling metabolites [4-7] and energy sources which are preferred to be taken up into the tissues under certain disease conditions, for example, the human brain utilizes blood LA during hyperlactatemia [8], and the human heart utilizes ketone bodies under diabetic conditions [9]. It is reported that LA and 3HB are related to several diseases, for instance, the concentrations of LA and 3HB increase in the human brain during fasting-induced ketosis [10]. Several studies have indicated that LA and 3HB help tumor growth and metastasis [11-13]. For the neurodegenerative diseases, the concentration of LA in the cerebrospinal fluid elevates in patients with Alzheimer’s disease (AD) [14] and Parkinson’s disease (PD) [15]. The cognition of AD...
patients is improved by the administration of 3HB through a ketone diet [16]. It is thought that the concentrations of LA and 3HB influence the homeostasis in our bodies and their balance is an issue of concern.

Having one asymmetric carbon, two enantiomers, i.e. the D-form and L-form, chemically exist for both LA and 3HB. In the past years, it has been considered that only one counterpart of the enantiomers of LA and 3HB (L-LA and D-3HB) are present in the mammalian tissues and physiological fluids. Recently, due to the progress in analytical technologies, the minor forms of the LA and 3HB enantiomers (D-LA and L-3HB) have been found in biological fluids. Besides, it is reported that the metabolisms of these enantiomers are different [1,17]. The biological significance of these minor enantiomers is getting more interest for several diseases conditions. The concentration of D-LA increases in the plasma of diabetes mellitus [18,19] and diabetic ketoacidosis [20]. The administration of D-3HB protects the PD mice from a deficit in mitochondria respiration [21]. D-LA acidosis, an uncommon form of metabolic acidosis, occurs in patients of short bowel syndrome with an elevated D-LA level [22]. In addition, the plasma and urinary levels of D-LA increase in infants with necrotizing enterocolitis [23,24]. These reports indicated that distinguishing the levels of the enantiomers of LA and 3HB is one of the keys to determining the new biomarkers and to develop an effective diagnostic method for various diseases.

The aim of the present study is to develop a highly selective three-dimensional high-performance liquid chromatographic (3D-HPLC) method for the simultaneous determination of LA and 3HB enantiomers in mammalian urine. Until now, the presence of LA and 3HB enantiomers in living beings has been elucidated using various techniques such as capillary electrophoresis [25,26], gas chromatography [27], liquid chromatography-mass spectrometry (LC-MS) [28-31] and 2D-HPLC [32-39]. Focusing on the applications in mammalian physiological fluids, LC is the most widely used method. For example, an LC-MS method with a chiral derivatization reagent has been reported for the chiral analysis of LA in human plasma and urine [31]; the LC-MS methods with chiral stationary phases for the enantioselective determination of LA in human urine have also been published [28-30]. Concerning 2D-HPLC, heart cutting methods with partial fraction transfer are reported for the detection of LA enantiomers in rat plasma [32], rat urine [35], human serum [33] and human urine [37], and for the detection of 3HB enantiomers in rat serum [34,36]. The online 2D-HPLC methods with whole fraction transfer are developed for the simultaneous determination of LA and 3HB enantiomers in human plasma and human urine [38,39]. As a non-invasive specimen, urine is more preferable than plasma. Although the approaches mentioned above are useful, the presence of numerous interferences in mammalian urine is always the crucial problem for the trace analysis of chiral hydroxy acids even using the 2D-HPLC methods. In addition, to the best of our knowledge there is no study available for the chiral analysis of 3HB in rodent urine. Therefore, a method with a higher selectivity is required for the simultaneous determination of LA and 3HB enantiomers in both rodent urine and human urine.

In the present study, therefore, a 3D-HPLC system combining reversed-phase, mixed-mode and enantioselective columns has been developed. Using the 3D-HPLC method, the peaks of the LA and 3HB enantiomers could be separated from other interferences due to the three different separation modes. Applications of the present method for the determination of the trace levels of the LA and 3HB enantiomers in the mice urine and human urine have also been reported.

2. Experimental

2.1. Materials

A racemic mixture of sodium LA and its enantiomers (D- and L-forms) were purchased from Sigma Aldrich (St. Louis, MO, USA). The racemic mixture of sodium 3HB and its D-enantiomer were also obtained from Sigma Aldrich. Sodium L-3HB was a product of Santa Cruz (Dallas, TX, USA). Acetonitrile (MeCN) of HPLC grade was acquired from Nacalai Tesque (Kyoto, Japan). Methanol (MeOH) and ethanol (EtOH) of HPLC grade and trifluoroacetic acid (TFA) were purchased from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan). 4-Nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ), 2,2′-dipyridyl disulfide (DPDS) and triphenylphosphine (TPP) were obtained from Tokyo Chemical Industry (Tokyo, Japan). Water was purified by a Milli-Q Integral 3 system (Merck, Darmstadt, Germany). All other reagents were of the highest reagent grade and were used without further purification.

2.2. Sample preparation of the urine samples

The female C57BL/6j mice (six weeks of age, SPF) were purchased from the National Laboratory Animal Center (Taipei, Taiwan). They were housed in a controlled-room (12 h light / 12 h dark, light on at 7 a.m.) with free access to food (Fwusow Industry Co. Ltd., Taichung, Taiwan) and water. The urine of mice was collected using a metabolic cage for 12 hours and stored at -80°C until used. The experiment was performed with permission from The Institutional Animal Care and Use Committee (approval number LAC-2015-0239). The human urine samples were collected from five volunteers (male, 21-27 years of age), following the approval from the review board of the Clinical Research Network Fukuoka (approval number...
These volunteers were informed to avoid a high amount of lactic acid intake before the collection (for 12h). Their urine samples were obtained at 9 a.m. without eating breakfast and stored at -80°C. Before derivatization, the samples were diluted to the proper concentrations with water (x2 - x10 times). To 10 μL of the solution containing LA and 3HB (standard solutions or urine samples), 5 μL of water and 85 μL of MeCN were added.

The mixture was centrifuged at 800 x g at 4°C for 10 min to obtain the supernatant. To 10 μL of the supernatant, 20 μL of an MeCN solution containing 5 mM NBD-PZ, 50 mM of DPDS and 50 mM of TPP was added. After storing at 25°C for 60 min, 220 μL of an aqueous 0.1% TFA solution was added and 100 μL of the reaction mixture was injected into the 2D-HPLC or 3D-HPLC system.

**2.3. 3D-HPLC system for the enantioselective analysis of LA and 3HB as their NBD derivatives**

The 3D-HPLC analysis was performed using Nanospace SI-2 instruments (Shiseido, Tokyo, Japan) operated by an EZChrom SI software and a KSAA valve controlling system. The flow diagram of the 3D-HPLC system is shown in Fig. 1. The first dimension of the 3D-HPLC system was comprised of a pump (3101), an auto sampler (3033), a column oven (3004) and a fluorescence detector (3013).

A KSAARP column (a C18 bonded reversed-phase column, particle size 3 μm, 1.0 mm i.d. x 250 mm, designed by the collaboration with Shiseido) was used at 40°C. An aqueous solution containing 15% MeCN 0.05% TFA was used as the mobile phase at the flow rate of 50 μL/min. In this dimension, LA and 3HB were fractionated as their scalemic d plus l NBD-derivatives by a high pressure valve (3012) coupled with a multi-loop unit (equipped with two loops of 500 μL). These entire fractions were transferred to the second dimension automatically. The second dimension was composed of a degasser (3202), a pump (3101), a column oven (3014) and a fluorescence detector (3013). NBD-LA and NBD-3HB were isolated from the other compounds by a mixed-mode column, KSAAMX (1.5 mm i.d. x 250 mm, an originally designed column by the collaboration with Shiseido having N-3,5-dinitrophenylaminocarbonyl-Gly as the selector, particle size 5 μm), at 25°C. As the mobile phase, ethanol was selected for both NBD-LA and NBD-3HB at the flow rate of 100 μL/min. The peaks of NBD-LA and NBD-3HB were again separately collected to a loop of 600 μL and introduced to the final (third) dimension by switching the high pressure valve. The third dimension was composed of a degasser (3202), a pump (3101), a column oven (3014) and a fluorescence detector (3013). For the enantiomer separation, a polysaccharide-coated column, Chiralpak AD-H (2.0 mm i.d. x 250 mm, Daicel, Osaka, Japan), was used at 25°C. The enantiomers of NBD-LA and NBD-3HB were separated into the D-form and L-form using EtOH as the mobile phase. The flow rate was 200 μL/min. The fluorescence detections of NBD-LA and NBD-3HB were carried out at 530 nm with excitation at 470 nm.

**Fig. 1.** Flow diagram of the 3D-HPLC system. P, pump; AS, auto sampler; CO, column oven; FLD, fluorescence detector; HPV, high pressure valve; W, waste. The column for 1D is a KSAARP (1.0 x 250 mm); a KSAAMX (1.5 x 250 mm) for 2D; a Chiralpak AD-H column (2.0 x 250 mm) for 3D.

**Fig. 2.** 2D-HPLC separations of NBD-LA and NBD-3HB enantiomers in the mice urine. In 1D, a Capcell Pak C18 ACR column (1.5 x 250 mm) was used at 40°C. The mobile phase was a 25% MeOH 0.01% TFA in H2O and the flow rate was 100 μL/min. In 2D, a Chiralpak AD-H column (2.0 x 250 mm) was used at 25°C. The mobile phase for NBD-LA was MeOH/MeCN (95/5, v/v) and for NBD-3HB was EtOH/MeCN (95/5, v/v). The flow rate was 200 μL/min. Detection was performed at 530 nm (excitation at 470 nm).
3. Results and discussion

3.1. Development of a 3D-HPLC system for the simultaneous determination of the LA and 3HB enantiomers

To determine the trace amounts of the LA and 3HB enantiomers in various biological matrices, the applicability of an already reported 2D-HPLC system was examined. The chromatograms obtained using mouse urine are shown in Fig. 2. In the first dimension, a Capcell Pak C18 ACR column (1.5 mm x 250 mm) was used and the target NBD-LA and NBD-3HB were fractionated to the multi-loop device and transferred into the second dimension. In the second dimension, a Chiralpak AD-H column (2.0 mm x 250 mm) was used and their enantiomers were separated. However, due to the large number of interfering substances, the determination of D-LA and L-3HB was difficult. Therefore, a 3D-HPLC system with a higher selectivity was developed in the present study.

In the first dimension of the 3D-HPLC system, an ODS-type RP column (KSAARP, 1.0 mm x 250 mm) was used to separate the hydroxy acids (LA and 3HB) as their NBD-derivatives based on their hydrophobicity. The mobile phase conditions were investigated using different concentrations of MeCN ranging from 10 to 20% in 0.05% TFA aqueous solutions. NBD-LA and NBD-3HB were nicely separated with appropriate retention times (39.7 and 46.7 min, respectively) when an aqueous solution containing 15% MeCN 0.05% TFA was used at 40°C (Fig. 3). After RP separation, the peaks of NBD-LA and NBD-3HB were fractionated automatically into the multi-loop unit and transferred to the second dimension. In the second dimension, a mixed-mode column (KSAAMX, 1.5 mm i.d. x 250 mm) was used. For the mobile phase, various organic solvents including MeCN, MeOH, EtOH and their mixtures (50/50, v/v) were tested. As shown in Fig. 4, the retention profiles of NBD-LA and NBD-3HB have similar trends. The retention times were the shortest when using MeCN as the mobile phase, and the retention times were the longest when using EtOH as the mobile phase. The mixtures containing 50% MeCN showed relatively short retention times; the values were almost the same as those obtained by pure MeCN. To have higher peak capacities separating NBD-LA and NBD-3HB from other intrinsic compounds, EtOH was selected as the mobile phase in the second dimension. Using EtOH, the retention time of NBD-LA was 22.7 min, and the peak capacity was 18.9. Concerning NBD-3HB, the retention time was 17.6 min and the peak capacity was 17.5.

After fractionating the peaks of NBD-LA and NBD-3HB, the collected eluents were introduced to the third dimension. In the third dimension, the enantioselective separations of both NBD-LA and 3HB were performed by a polysaccharide bonded column, Chiralpak AD-H (2.0 mm x 250 mm). Mixed solutions of polar organic solvents with different ratios of MeCN/MeOH and MeCN/EtOH were used to separate the LA and 3HB enantiomers as their NBD-derivatives (Fig. 5, the fractions of NBD-LA and NBD-3HB collected from the second dimension were on-line injected into the third dimension). As a result, both the NBD-LA and NBD-3HB enantiomers were well separated using pure EtOH. The separation factor (α) and resolution (Rs) values of the NBD-LA enantiomers were 1.70 and 3.35, respectively. Those of the NBD-3HB enantiomers were 1.43 and 2.01, respectively.

Combining these three dimensions, an on-line connected 3D-HPLC system was developed. The chromatograms obtained using the standard LA and 3HB enantiomers are shown in Fig. 6. In the first dimension, NBD-LA and NBD-3HB were eluted at around 40 and 47 min, respectively, and introduced to the second dimension by switching the multi-loop valve. In the second dimension, the NBD-derivatives of LA eluted at 23 min, while NBD-3HB eluted at 18 min. These fractions were collected again and transferred to the third dimension.

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**Fig. 3.** Reversed-phase separations of NBD-LA and NBD-3HB by a KSAARP column (1.0 mm i.d. x 250 mm) using 0.05% TFA aqueous solutions containing 10-20% MeCN.

**Fig. 4.** Separation of (A) NBD-LA and (B) NBD-3HB by a mixed-mode column, KSAAMX (1.5 mm i.d. x 250 mm), using various mobile phase conditions.
where their enantiomers were separated. The retention times of D-LA and L-LA were 19 and 32 min, respectively, and those of L-3HB and D-3HB were 16 and 23 min, respectively.

In the past 10-20 years, various researchers have developed 2D-HPLC systems for the chiral analyses of trace levels of intrinsic compounds in order to obtain high sensitivity and high selectivity. Actually, a large number of reports using 2D-HPLC was published for the trace analysis of enantiomers including amino acids [40-44], dipeptides [45] and hydroxy acids [32,34,35,38,39]. Concerning the hydroxy acids, 2D-HPLC methods for the LA and 3HB enantiomers have been reported including heart-cutting 2D-HPLC [32,34,35] and whole-fraction transfer 2D-HPLC [38,39]. Heart-cutting column-switching HPLC methods were reported for determining the LA enantiomer in rat plasma [32] and rat urine [35]. In the first dimension, a TSK gel ODS-80Ts column (4.6 mm x 150 mm) was used in both studies, and the center fraction of the peak of LA was fractionated/transferred to the second dimension. Using a phenylcarbamoylated β-cyclodextrin immobilized column (4.6 mm x 250 mm) [32] or a polysaccharide-coated Chiralpak AD-RH column (4.6 mm x 150 mm) [35] as the stationary phase, the LA enantiomers were separated into the D-form and L-form. The reported α and Rs values were 1.34 and 3.39 [32], or 1.43 and 2.63 [35], respectively. For the 3HB enantiomers, a heart-cutting column-switching system was also reported utilizing the same reversed-phase column (a TSK gel ODS-80Ts column, 4.6 mm x 150 mm) and two Chiralcel OD-RH columns in tandem form (4.6 mm x 150 mm, total length 300 mm) [34]. The obtained α and Rs values were 1.09 and 1.60, respectively. Liu et al. reported an on-line 2D-HPLC method using a reversed-phase capillary monolithic ODS column (0.53 mm x 1000 mm) in the first dimension, and the whole fractions of LA and 3HB were transferred to the second dimension. In the second dimension, two columns were needed, i.e., a Chiralpak QD-AX column (1.5 mm x 150 mm) for separation of the LA enantiomers and an KSAAASP-001S column (1.5 mm x 250 mm) for the 3HB enantiomers [38]. The α values of the LA and 3HB enantiomers were 1.14 and 1.08, respectively. As a simpler 2D-HPLC system in which the LA and 3HB enantiomers were simultaneously separated by a single enantioselective column, a Chiralpak AD-H column (2.0 mm x 250 mm) was used in the second dimension [39]. A Capcell Pak C18 ACR column (1.5 mm x 250 mm) was used in the first dimension. Concerning the enantiomer separation, high α and Rs values (1.82 and 7.06 for LA; 1.53 and 4.27 for 3HB, respectively) were obtained. These on-line 2D-HPLC systems are useful for the enantioselective analyses of LA and 3HB in a variety of real world matrices. However, in some cases, especially in the urine samples, the determination is disturbed by the large amounts of co-eluting compounds due to the insufficient selectivity of the reported methods. Therefore, a 3D-HPLC system, which has an additional dimension to the

**Fig. 5.** Enantioselective separations of (A) NBD-LA and (B) NBD-3HB by a Chiralpak AD-H column (2.0 mm i.d. x 250 mm) using various mobile phase conditions.

**Fig. 6.** 3D-HPLC separations of NBD-LA and NBD-3HB enantiomers. In 1D, a KSAAARP column (1.0 mm i.d. x 250 mm) was used at 40°C. The mobile phase was 15% MeCN 0.05% TFA aqueous solution and the flow rate was 50 μL/min. In 2D, a KSAAAMX column (1.5 mm i.d. x 250 mm) was used at 25°C. The mobile phase was EtOH and the flow rate was 100 μL/min. In 3D, a Chiralpak AD-H column (2.0 mm i.d. x 250 mm) was used at 25°C. The mobile phase was EtOH and the flow rate was 200 μL/min. NBD-LA and NBD-3HB were detected by fluorescence with excitation at 470 nm and emission at 530 nm.
2D-HPLC, is designed and utilized in the present study. The retention properties of both NBD-LA and 3HB were practically sufficient in all 3 dimensions. Concerning the enantiomer separations, the α and Rs values were also acceptable, although there is always a problem of mobile phase incompatibility in the multi-dimensional analyses. Therefore, the present 3D-HPLC system was used for the analyses of human and mouse urine samples in Section 3.2.

### 3.2. Determination of LA and 3HB enantiomers in the mammalian urine

Using the 3D-HPLC system described in Section 3.1., the LA and 3HB enantiomers in the mouse urine and human urine were determined. Figure 7 shows the chromatograms when analyzing the mouse urine. Although various co-eluting peaks were observed in the first dimension, NBD-LA and NBD-3HB were fractionated according to the retention times of the standard NBD-LA and NBD-3HB (indicated by the closed bars). In the second dimension, NBD-LA and NBD-3HB were separated again from interfering compounds. After the separations in the first and second dimensions, the NBD-LA and NBD-3HB enantiomers were nicely separated into their D- and L-forms without interferences in the third dimension.

In the mice urine, the percentage of D-LA (%D, D/(D+L) x 100) was trace (less than 5.0%) and the percentage of L-3HB (%L, L/(D+L) x 100) was 34.4%. The repeatability of the present system was checked by duplicate analyses and almost the same results were obtained. Using the same 3D-HPLC system, the human urine sample was also analyzed. The obtained chromatograms are shown in Fig. 8. Similar to the mouse urine, LA and 3HB were separated/fractionated from each other as their NBD-derivatives in the first/second dimensions, and introduced to the third dimension. Since the human urine was more complicated than mouse urine, the isolation in the first and second dimensions was not sufficient. Although non-negligible co-eluting compounds appeared in the third dimension, a trace level of D-LA was observed in the human urine. Concerning 3HB, the amount of the L-form was almost the same as that of the D-form.

Until now, a variety of 2D-HPLC methods had been developed and applied for the determination of the LA and 3HB enantiomers in real world samples including physiological fluids [32-39]. Among them, some methods were applied to mammalian urine [35,37-39]. For the rat urine, the reported concentration of D-LA was much lower than that of the L-form [35] and to the best of our
knowledge there is no report describing the values of the 3HB enantiomers in rodent urine. The present study is consistent with the reported results that D-LA is the minor form. In addition, the present study is the first report showing the D/L ratio of 3HB in mice urine. For healthy human urine, the observed values of D-LA and L-LA were 0.6 and 2.5 μM [38], and 3.7 and 15.4 μM [39], respectively. In the present study, the level of D-LA was much lower than that of L-LA, which is quite similar to the previous studies. For 3HB in human urine, the determined values were 0.1 and 0.2 μM [38], and 2.3 and 3.3 μM [39], respectively. In the present study, the level of the L-form was almost the same as that of the D-form, which is similar to previous reports. These results indicated that the present method is useful for the simultaneous determination of the LA and 3HB enantiomers in the urine samples and wider applications are expected.

4. Conclusion

In the present study, an online 3D-HPLC system has been designed and developed for the simultaneous analysis of LA and 3HB enantiomers in mammalian urine. Using this method, interfering peaks eluted around the target hydroxy acids were drastically decreased, and the presence of trace amounts of D-LA and D/L-3HB were demonstrated in the urine of the mice and humans. These results indicate that the present online 3D-HPLC system is a powerful and useful tool for the analysis of trace levels of hydroxy acids in complicated matrices including urine, and the stereoselective research studies of LA and 3HB in various diseases are ongoing.

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