Development of a three-dimensional HPLC system for the simultaneous determination of lactate and 3-hydroxybutyrate enantiomers and the applications to various food and clinical samples

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論　文　名	Development of a three-dimensional HPLC system for the simultaneous determination of lactate and 3-hydroxybutyrate enantiomers and the applications to various food and clinical samples（乳酸および 3-ヒドロキシ酪酸鏡像異性体を対象とした三次元 HPLC 法開発と食品・臨床サンプルへの適用）
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論 文 審 査 の 結 果 の 要 旨

Due to the homochirality features, the chiral properties of hydroxy acid enantiomers including lactate (LA) and 3-hydroxybutyrate (3HB) were ignored. However, with the progress of analytical technology, the minor forms (D-LA and L-3HB) were found in mammals including human beings and their biological meanings are gradually elucidated. It is found that the levels of the enantiomers change in various diseases such as diabetic mellitus and these enantiomers are considered as novel biomarkers. To accurately determine the trace amounts of enantiomers, multi-dimensional high-performance liquid chromatography (HPLC) system is thought as one of the powerful methods. A three-dimensional HPLC (3D-HPLC) system has been developed and applied to the determination of LA and 3HB enantiomers in mammalian samples. However, the interfering substances are different between matrices and the selectivity is still a matter of concern. Therefore, the applicant designed/developed the highly selective 3D-HPLC systems by thoroughly investigating the conditions in all dimensions with the utilization of human urine as a real matrix. Besides, the method has been applied to various food/beverages and clinical disease samples.

The samples of human plasma/urine and food/beverages were properly diluted or homogenized, and derivatized with NBD-PZ and DMT-MM. The reaction mixtures were injected to the 3D-HPLC systems composing of Singularity RP18 columns (1.0 x 500 mm) (1D), Singularity MX-005 columns (1.0 x 500 mm) (2D) and Chiralpak IG/AD-H columns (2.0 x 500 mm) (3D). The target hydroxy acids were detected by the fluorescence (Ex. 470 nm, Em. 530 nm).

To separate the main interfering substances in the real sample, the conditions in 1D and 2D were investigated with the utilization of human urine as a real matrix. In 1D, various reversed-phase stationary phases and mobile phases were tested. As a result, tandemly connected Singularity RP18 columns (1.0 mm x 500 mm) were selected and used at 40°C. The mobile phase was 15% ethanol (EtOH) 0.05% TFA aqueous solution with the flow rate at 25 μL/min. In 2D, several mixed-mode stationary phases were designed and synthesized. Using the Singularity MX-005 column (1.0 mm x 250 mm), 3HB could be well separated from the main interfering substance at 25°C with EtOH as a mobile phase (Rs = 1.7). In 3D, different immobilized type stationary phases were checked and the Chiralpak IG column (1.0 mm x 250 mm) could separate both LA and 3HB enantiomers with the Rs higher than 4.1 using MeOH as mobile phases. The method was fully validated by the calibration curves, precision and accuracy using the standard hydroxy

acid solutions, human plasma and urine, and sufficient values were obtained. Using this method, the target chiral hydroxy acids, LA and 3HB enantiomers, could be successfully analyzed without the interference of other intrinsic compounds in the human plasma and urine. The amounts of LA and 3HB enantiomers were quantified and the values were consistent with the other reports. These results indicate that the present 3D-HPLC system is useful for the determination of LA and 3HB enantiomers in human plasma and urine.

To evaluate the values of chiral hydroxy acids as the possible biomarkers for diseases, the applicant considered that the effect of dietary intake on the levels of them in human plasma should be checked. Determining LA enantiomers in various fermented product may help to study the presence of them in our daily life/environment and ingesting the high LA-containing food could elucidate the effect of dietary intake. Therefore, the 3D-HPLC system was applied to the determination of LA enantiomers in fermented products including vinegars. Among the food/beverages, the Japanese traditional amber rice vinegar (SK) contained high amounts of both D-LA and L-LA (13.1 and 22.8 μmol/mL, respectively). Developmental changes of the Japanese traditional amber rice vinegars were also checked. The levels of D- and L-LA reached to the highest concentrations after 1 month of fermentation and gradually decreased to certain concentrations (D-LA and L-LA were 9.4 ± 1.0 and 19.4 ± 1.8 μmol/mL, respectively). The effect of dietary intake was investigated by ingesting the Japanese traditional amber rice vinegars, and significant differences between the concentrations of both D- and L-form LA were observed before and after ingestion. A healthy volunteer was recruited and the time course of 24 h after ingesting the Japanese traditional amber rice vinegars was studied. The results indicate that LA enantiomers are applicable as the biomarkers of diseases with the consideration of mealtime.

Furthermore, the applicant checked the relationships between chronic kidney disease (CKD) and LA/3HB enantiomers. Because CKD has high risk to death and is difficult to be observed in its early stage, prevention and early diagnose are required. In addition, it has been found that LA and 3HB are related to kidney disease. Therefore, the levels of LA and 3HB enantiomers in the non-CKD and CKD subjects are worthy studying. As a result, the significant differences were found in the total LA and D-LA between the non-CKD and CKD subjects. By plotting the ROC curves of a newly proposed formula containing D-LA and eGFR, the best AUC was obtained with a highly specific cut-off value. Therefore, it is thought that D-LA is strongly related with CKD, and it is meaningful to analyze LA with chiral insight. Furthermore, the combination of D-LA and eGFR would become a possible biomarker for the early diagnose of the CKD patients even having high eGFR values.

In the present thesis, a 3D-HPLC system with high selectivity has been designed/developed for the simultaneous chiral analysis of LA and 3HB, and it is applicable to various matrices including food/beverages and clinical samples. The presence of the target hydroxy acids, the effect of dietary intake and their relationships to disease were revealed. These results are helpful to elucidate the biological meanings of LA and 3HB enantiomers in the health science. Further studies for the determination of LA and 3HB with chiral insight in the clinical applications are expected.

以上の通り、本研究の学問的価値は極めて高く、申請者は博士（創薬科学）の学位に値すると認める。