Enantiomeric Separation of 2-Hydroxyglutarate Using Chiral Mobile Phase Additives

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Abstract
2-Hydroxyglutarate (2-HG) was enantiomerically separated using copper(II) acetate and N,N-dimethyl-L-phenylalanine as chiral additives. These compounds formed diastereomeric complexes with 2-HG, which were successfully separated on an achiral (ODS) column. Several parameters, such as additive concentration, the type of organic modifier, and column temperature, were optimized. Using the optimal mobile phase (1 mM copper(II) acetate and 2 mM N,N-dimethyl-L-phenylalanine in a 10% aqueous methanol solution), 2-HG enantiomers were successfully separated in 15 min with a resolution of 1.93.

Keywords: Liquid chromatography; Chiral selector; Copper(II) acetate; Diastereomeric complex

1. Introduction
Enantiomeric separation is becoming increasingly important in various fields such as medical and agricultural disciplines [1,2]. For example, some drugs are optically active, where one enantiomer exhibits medicinal effects, while the other induces side effects. Unfortunately, it is difficult to achieve the separation of enantiomers because they have the same physical properties.

Liquid chromatography (LC) is widely used for enantiomeric separation. There are two types of LC methods: indirect and direct. In the case of indirect methods, diastereomers are produced by the reaction of an analyte with an optically active derivatization reagent, enabling them to be separated on an achiral column. This strategy can be applied to numerous analytes bearing various functional groups. However, derivatization procedures may cause unintended side reactions, decomposition, and racemization. In addition, the derivatization reagents must be highly enantiomerically pure, which is sometimes difficult to ensure.

Direct methods, e.g., those involving the use of a chiral stationary phase, enable the analysis of enantiomers without derivatization. Such a method allows for easy sample preparation and analysis, and is appropriate for preparative-scale chromatography. Another type of direct method employs chiral mobile phase additives, which interact with enantiomers to form diastereomers. The diastereomers can then be separated on an achiral column (usually, a reverse-phase column). To date, several chiral mobile phase additives, such as macrocyclic antibiotics, cyclodextrins, and ligand exchangers, have been reported [3]. Macrocyclic antibiotics such as vancomycin and norvancomycin interact with analytes at various chiral centers and functional groups, and the resultant structural differences induced by the additives contribute to enantiomeric separation [4,5]. Cyclodextrins form host-guest complexes with analytes, which differ depending on the enantiomer. For example, chlorothalidone, naproxen, and thalidomide enantiomers have been separated by using β-cyclodextrin and its substituted derivatives [6-8]. Ligand exchangers are composed of metal ions such as Cu²⁺ and a chiral selector ligand, and they form diastereomeric complexes with the enantiomers of target analytes; the differences in the stabilities of these complexes contribute to their separation. This method has been applied to amino acids [9-11] and α-hydroxylic acids [12,13].

In this study, we focused on 2-hydroxyglutarate (2-HG, Fig. 1), which is a chiral compound related to cancer
Several methods for the separation of 2-HG enantiomers have been reported. For indirect methods, two derivatization reagents have been used: diacetyl-L-tartaric acid (DATAN) [17] and N-(p-toluenesulfonfyl)-L-phenylalanyl chloride (TSPC) [18], both of which reacted with 2-HG at its hydroxyl group to form diastereomers. The DATAN-derivatized 2-HG enantiomers were separated in 5 min on an ODS column, while approximately 300-fold higher sensitivity was observed for TSPC-derivatized 2-HG than for the underivatized 2-HG. However, these methods have some disadvantages. For instance, the derivatization reagent must be enantiomerically pure, and ensuring the same is often difficult. Moreover, derivatization may cause isomerization of 2-HG. Chiral stationary phases have also been applied to the separation of 2-HG enantiomers. For example, Calderón et al. used a Chiralpak QD-AX column with immobilized tert-butylcarbamoyl-quinidine (a chiral anion exchanger) to separate 2-HG enantiomers [19]. We recently succeeded in the enantiomeric separation of 2-HG derivatized with 4-nitro-7-piperazino-2,1,3-benzoxadiazole using a CHIRALPAK IC, cellulose tris(3,5-dichlorophenylcarbamate) immobilized column [20]. The developed method was used to quantify 2-HG enantiomers in cancer cell samples. However, several chiral columns, which are usually expensive, are needed to decide the appropriate column. To resolve the disadvantages of indirect methods and chiral stationary phase methods, chiral mobile phase additives is used for enantiomeric separation of 2-HG.

In this study, a direct method to separate 2-HG enantiomers was investigated. 2-HG is a small molecule with polar functional groups; thus, a ligand exchanger that successfully enabled the enantiomeric separation of amino acids was applied. Since both amino acids and 2-HG have carboxylic groups, the formation of diastereomeric complexes observed for the former should also be observed for the latter. N,N-Dimethyl-L-phenylalanine and copper(II) acetate were used as chiral mobile phase additives. The concentrations of copper(II) acetate and N,N-dimethyl-L-phenylalanine are the basic factors for enantiomeric separation, as they would form diastereomeric complexes with 2-HG. Thus, their concentrations would affect the separation. First, copper(II) acetate concentrations of 0.1, 0.5, 1, and 2 mM were evaluated, with the remainder of the mobile phase comprising 2 mM N,N-dimethyl-L-phenylalanine in water/methanol (90/10, v/v). The mobile phase was used after filtration. The flow rate was 1.0 mL/min, and the column temperature was 20 °C. UV detection was performed at a wavelength of 220 nm.

3. Results and discussion

3.1. Optimization of copper(II) acetate and N,N-dimethyl-L-phenylalanine concentrations

Copper(II) acetate and N,N-dimethyl-L-phenylalanine are the basic factors for enantiomeric separation, as they would form diastereomeric complexes with 2-HG. Thus, their concentrations would affect the separation. First, copper(II) acetate concentrations of 0.1, 0.5, 1, and 2 mM were evaluated, with the remainder of the mobile phase comprising 2 mM N,N-dimethyl-L-phenylalanine in H2O/methanol (90/10). When the concentration of copper(II) acetate was below 0.5 mM, enantiomeric separation was not observed. D- and L-2-HG were successfully separated using > 1 mM copper(II) acetate; however, these higher concentrations decreased the capacity factors of the 2-HG enantiomers (1 mM: k’ = 5.0 for D-2-HG and 6.3 for L-2-HG, 2 mM: k’ = 4.3, D-2-HG and 5.4 for L-2-HG), which worsened the resolution (1 mM: Rs = 1.82, 2 mM: Rs = 1.18). Accordingly, 1 mM was chosen as the optimal concentration of copper(II) acetate.

Next, the N,N-dimethyl-L-phenylalanine concentration was optimized. As shown in Fig. 2, the resolution of D-2-HG and L-2-HG is the highest when 2 mM of N,N-dimethyl-L-phenylalanine is used. Hence, this concentration was selected as the optimal value.

3.2. Optimization of organic modifier
Organic modifiers also affect enantiomeric separation; hence, we evaluated the effect of two different modifiers (methanol and acetonitrile) on the enantiomeric separation. When 10% acetonitrile was added to the mobile phase instead of methanol, 2-HG was eluted faster, and its enantiomeric separation was not observed. For greater retention, the acetonitrile concentration was decreased, and enantiomeric separation was achieved with 5% acetonitrile. However, the resolution when using acetonitrile (0.91) was lower than that obtained when using methanol (1.82), as shown in Fig. 3. Hence, methanol was chosen as the optimal organic modifier.

Next, the methanol content (5%, 10%, and 20%) was investigated. When 20% methanol was used, D- and L-2-HG were not separated. When less than 10% methanol was used, D-2-HG and L-2-HG were successfully separated. A lower concentration of methanol contributed to a longer retention time (5%: $k' = 7.14$ for D-2-HG and 9.42 for L-2-HG; 10%: $k' = 5.04$ for D-2-HG and 6.28 for L-2-HG). The resolution of D/L-2-HG using 10% methanol was higher than that using 5% methanol (1.82 vs. 1.27, respectively). Hence, the optimal methanol concentration was considered to be 10%.

### 3.3. Optimization of column temperature

Column temperature is an important factor influencing analyte retention and separation efficiency. Here, temperatures of 10, 20, 30, 40, and 50 °C were investigated for the enantiomeric separation of 2-HG. The capacity factors of D-2-HG and L-2-HG and the resolutions between D/L-2-HG are shown in Fig. 4. Higher temperatures contributed to shorter retention times of the 2-HG enantiomers, as expected. However, the resolution was the best at 20 °C. Hence, 20 °C was chosen as the optimal column temperature.

### 3.4. Optimization of stationary phase

As mentioned above, the mobile phases were optimized on an ODS column; however, since the stationary phase can also influence the enantiomeric separation of 2-HG, a C8 column was also examined using the optimized mobile phase. The chromatograms obtained from both the columns are shown in Fig. 5. The capacity factors and resolution on the ODS column were higher than those on the C8 column (ODS column: $k' = 5.28$ for D-2-HG and 6.74 for L-2-HG, $R_s = 1.93$; C8 column: $k' = 3.56$ for D-2-HG and 4.36 for L-2-HG, $R_s = 1.54$). Accordingly, the ODS column was deemed appropriate for the separation of the 2-HG enantiomers.

### 3.5. Possible structures of the diastereomeric complexes

The 2-HG enantiomers were successfully separated under the optimized conditions mentioned above, which could be due to the diastereomeric complexes that 2-HG formed with copper(II) acetate and N,N-dimethyl-L-phenylalanine. The two carboxyl and hydroxyl groups of 2-HG have the potential to coordinate with copper(II) ions, but only two of the three groups would interact. The complexes formed with D-2-HG and L-2-HG differed in terms of their structures and stability, which likely contributed to the separation of D-2-HG and L-2-HG. Considering these diastereomeric structures, dicarboxylic acids and α-hydroxy acids can likely be enantiomerically separated using the same chiral additives.
4. Conclusion

In this study, the enantiomeric separation of 2-HG was achieved using chiral mobile phase additives. Baseline separation was accomplished with a mobile phase of 1 mM copper(II) acetate and 2 mM N,N-dimethyl-l-phenylalanine in a 10% aqueous methanol solution on an ODS column at 20 °C. The chiral additives formed diastereomeric complexes with 2-HG, which contributed to the separation of the 2-HG enantiomers. This direct method is rapid and does not require derivatization nor chiral stationary phase columns, thus being superior to indirect methods. Considering the resultant diastereomeric complexes, the chiral additives used here are likely applicable to other enantiomeric dicarboxylic acids and α-hydroxy acids.

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