Simultaneous Analysis of Enantiomeric Amino Acids Based on Derivatization with (*S*)-(+)-2-*t*-Butyl-2-methyl-1,3-benzodioxole-4-carboxylic Acid and High-Performance Liquid Chromatography

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The fluorescent chiral derivatizing agent in the title, (*S*)-TBMB carboxylic acid, was applied for highly sensitive HPLC analysis of enantiomeric amino acids. Enantiomeric amino acids derivatized with the agent could be separated as the diastereomers on ODS (C-18) column and determined in less than 0.05 picomole amounts owing to the strong fluorescent chromophore of the reagent ($\text{Ex}=310\text{ nm}$, $\text{Em}=380\text{ nm}$). Three types of HPLC conditions with different pH values were proposed for the group analysis of amino acids: pH=5.7 for the simultaneous analysis of all amino acids, pH=4.6 for the selective analysis of polar amino acid derivatives and pH=6.0 for that of less polar ones.

Keywords High-performance liquid chromatography, enantiomer analysis, amino acid analysis, chiral derivatizing agent, fluorescent agent

Development of a general method to determine the enantiomeric composition of amino acids has become an important subject in many fields dealing with medicines, foods and natural products since unusual D-amino acids have been widely found also as components of peptides and proteins.¹⁻³ Among chromatographic methods to discriminate between D- and L-amino acids^{4,5}, HPLC methods based on fluorescent chiral derivatization have provided promising ways⁶⁻¹¹ since they allow simple and highly sensitive determination. In our study on development of a new type of fluorescent chiral derivatizing agents with a 1,3-benzodioxole skeleton, we reported (*S*)-2-*t*-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid [(*S*)-TBMB carboxylic acid, Fig. 1] as a versatile reagent¹²⁻¹⁵ and applied it for the enantiomer analysis of glycerides¹⁶ and carbohydrates.¹⁷ Here, we wish to extend its application to amino acids and to propose a highly sensitive HPLC method to determine their enantiomers.

Fig. 1 Structure of (*S*)-TBMB carboxylic acid.

Precolumn derivatization of amino acids with (*S*)-TBMB-COCl

Free amino acids in a 0.1 M lithium carbonate buffer (100 μL , pH 9.0) were mixed with an excess amount of (*S*)-TBMB-COCl/ CH_3CN solution (100 μL) and kept for 30 min at room temperature. To the mixture was added 4 mM L-cysteic acid in lithium carbonate buffer (100 μL) to decompose the excess reagent. An aliquot (5 μL) of the solution was injected into an HPLC column.

HPLC analysis

HPLC analysis was conducted using CAPCELL PAK-UG120 (ODS column, $4.6\phi\times 150\text{ mm}$, Shiseido) at 20–22°C and a mixture of CH_3CN in 0.01 M sodium citrate buffer as the eluting solvents. The CH_3CN concentration and the pH values were changed under the optimized HPLC conditions (see text). Flow rate was fixed at 1 ml/min, and the eluents were monitored by fluorescent detection at Ex 310 nm and Em 380 nm.

Experimentals

Reagents

All solvents (CH_3CN and water) were distilled and filtered through a membrane before use. (*S*)-TBMB-COCl was prepared according to our previous method¹⁸, and its CH_3CN solution (1 mg/ml) was used for the precolumn derivatization of amino acids.

Preparation of analytical samples from a lactic drink

A lactic drink (1 ml, Yakult Mil-Mil, Yakult Co. Ltd., Tokyo) was treated with 3% sulfosalicylic acid (1 ml) and centrifuged (2000g, 10 min, 5°C) for deproteinization. The supernatant (1 ml) was mixed with a mixture of diethylether and hexane (1:1) and centrifuged (2000g, 10 min, 5°C). An aliquot (1 ml) of the aqueous layer was mounted on a cation exchange resin (Dowex 50W-X8, H⁺ form, 14 mmφ×50 mm). After the column was washed with water (30 ml), amino acids were eluted with 3 M NH₄OH. The eluent solution was evaporated *in vacuo*. The residue was diluted with a lithium carbonate buffer (pH 9.0, 2 ml), and an aliquot (100 μl) was used for the derivatization with (*S*)-TBMB-COCl and subjected to the HPLC analysis in the same manner as that described above.

Results and Discussion

Derivatization of amino acids with (*S*)-TBMB carboxylic acid

(*S*)-TBMB carboxylic acid was converted into (*S*)-TBMB carbonyl chloride after its treatment with thionyl chloride in benzene (60°C, 15 min).¹⁸ The activated agent, diluted with acetonitrile, was used for the derivatization of amino acids or could be kept in a refrigerator at 4°C without any racemization and decomposition for more than 6 months [HPLC analysis with (*S*)-phenylalanine]. Free amino acids in a 0.1 M lithium carbonate buffer (100 μl, pH 9.0) was mixed with an excess amount of the reagent solution (100 μl) at room temperature to afford *N*-(*S*)-TBMB carbonyl amino acid derivatives.

Concentration of the reagent solution necessary to complete the reaction within 15 min (room temperature, 15–20°C) was investigated. The reactions of three types of amino acids (100 μM) are summarized in Fig. 2 to show that higher concentration (*ca.* 3000 μM) was necessary to complete the reaction of acidic amino acids than was needed for neutral and basic amino acids (*ca.* 1000 μM). The reactions at higher temperatures between 30 and 55°C gave similar results.

Under the optimized reaction conditions described above or in the Experimental section, hydroxy groups of Ser and Thr and the secondary amino group of Pro were not derivatized, while Lys was doubly labeled to give an *N*(α),*N*(ε)-di-(*S*)-TBMB carbonyl derivative. Although tryptophan was also derivatized, the fluorescence was quenched (the response was *ca.* 1/400 of that of the other amino acid derivatives).

Reaction of equimolar amounts of amino acids followed by the HPLC analysis gave the results summarized in Table 1. These show the relative reactivity of amino acids with the reagent and the fluorescence intensity of the derivatives. The higher intensity of Lys indicated that both α- and ε-amino groups reacted with the agent. There was an unexpectedly large difference in the peak area between D-Met and L-Met and also one between D-

Fig. 2 Investigation of reagent concentrations necessary for the completion of the derivatization within 15 min at room temperature (concentration of amino acids=100 μM).

Table 1 Relative HPLC peak area of amino acids derivatized with (*S*)-TBMB carboxylic acid

	L	D		L	D
Arg	1.00	0.98	Val	1.00	1.01
His	0.76	0.98	Met	1.16	0.65
Ser		1.15 ^a	Ile	0.95	1.06
Glu		0.87 ^a	Leu	0.97	1.04
Asp	1.05	1.61	Phe	1.26	1.1
Thr	1.15	1.22	Cystine	0.97	0.60
Gly		0.83	<i>meso</i>		0.43
Tyr	0.96	0.96	Lys	1.93	2.03
Ala	1.51	1.25	Trp	0.0	0.0

a. Calculated as the half peak area of overlapping peaks of D- and L-isomers.

cystine and L-cystine with an -S- group. Simultaneous UV detection at 310 nm revealed that this difference did not stem from the reactivity, but from the difference of the fluorescence intensity between the two isomers, probably due to the effect of the -S- group.

A linear relation could be obtained between the concentration of amino acids and their HPLC peak area for every amino acid at least between 0.01 and 10 μM (*n*=3). This means that the quantitative analysis will also be possible. The detection limit of the derivatives was 0.01 pmol as the injection amount (*S/N*=3/1).

Separation of individual amino acid enantiomers on reversed-phase HPLC column

For the enantiomer separation on HPLC, we tested a C-18 (ODS) column using acetonitrile (CH₃CN) in a 0.01 M sodium citrate buffer (pH 4.0–6.0). The co-solvent CH₃CN was chosen after testing MeOH and tetrahydrofuran.¹⁵ The buffer solution was kept weakly acidic not only to keep the stability of the HPLC column but also to hold amino acid derivatives on the column to achieve better separation. Preliminary HPLC analysis using 15% CH₃CN indicated that His and Arg were most rapidly eluted (earlier than 15 min), while Lys was eluted

Table 2 HPLC separations of amino acid enantiomers as the (S)-TBMB carbonyl derivatives

Amino acids	Retention time (min) and k'^a		R_s^b
	L-Isomer	D-Isomer	
Polar group			
His	6.42 (1.57)	6.96 (1.78)	1.4
Arg	7.93 (2.17)	8.25 (2.30)	0.9
Ser	9.19 (2.68)	9.19 (2.68)	— ^c
Glu	9.60 (2.84)	9.60 (2.84)	— ^d
Asp	9.89 (2.96)	10.29 (3.12)	1.2
Thr	11.47 (3.59)	10.89 (3.36)	1.4
Ala	13.13 (4.25)	12.69 (4.08)	1.3
Tyr	13.37 (4.35)	14.25 (4.70)	2.0
Less polar group			
Val	12.08 (3.83)	10.46 (3.18)	2.3
Met	13.00 (4.20)	11.11 (3.44)	2.3
Ile	18.81 (6.52)	16.67 (5.67)	2.3
Leu	20.20 (7.08)	17.58 (6.03)	2.7
Phe	22.32 (7.93)	20.93 (7.37)	1.6
Cystine	29.18 (10.67)	28.13 (10.25)	1.4
Lys	37.81 (14.12)	38.41 (14.36)	1.0

a. k' in parenthesis=capacity factor.

b. R_s =resolution factor.

c. R_s =1.4 by isocratic elution with a CH₃CN–sodium citrate buffer (pH=5.7).

d. R_s <0.5 by isocratic elution with a CH₃CN–sodium citrate buffer (pH=5.7).

HPLC conditions: elution solvents, solvent gradient between 15% CH₃CN and 60% CH₃CN in 0.01 M sodium citrate buffers (pH=4.6 for polar group and pH=6.0 for less polar group); HPLC column, ODS column (4.6 mm×150 mm, particle size=5 μm); fluorescence detection at Ex 310 nm and Em 380 nm; flow rate=1.0 ml/min at ambient temperature.

after the longest delay (>120 min). With increasing either the CH₃CN ratio or the pH of the solvents, every amino acid was more rapidly eluted.

Enantiomers of each amino acid could be moderately to sufficiently separated under the conditions summarized in Table 2. There, amino acids are classified into two groups depending on their polarity. The resolution factor (R_s) was obtained for each group using a different elution condition, especially in the pH of the buffer as follows: pH=4.6 for polar amino acid derivatives and pH=6.0 for less polar ones. This was done in order to shorten the analytical times. Higher resolution (R_s >2.0) could be obtained commonly for neutral amino acids, while the separation was not satisfactory for Ser and Glu. For Ser, however, higher separation could be achieved by increasing the pH (5.7) of the buffer solution. Here, it should be noticed that the elution order of D- and L-isomers was reversed by the polarity of the side groups of amino acid derivatives; for acidic and basic amino acids (Asp, His, Arg), the L-isomer was eluted faster than the D-isomer, while for neutral amino acid derivatives (Thr, Ser, Val, Ala, Ile, Phe etc.) the D-isomer was eluted faster. For Lys, however, L-isomer was eluted faster than the D-isomer, although, the ε-

Fig. 3 Effects of pH of HPLC elution buffer on the retention times of amino acid derivatives and (S)-TBMB carboxylic acid.

amino group was also carbonylated by (S)-TBMB carboxylic acid into a less polar group.

Attempts for the simultaneous analyses of enantiomeric amino acids

Such HPLC analysis showed that D- and L-isomers of each amino acid were separable under the reversed-phase HPLC condition as the (S)-TBMB carbonyl derivatives. Here, we tried to establish an HPLC condition for the simultaneous analysis of enantiomeric amino acids. A variety of gradient modes was tested using 15% CH₃CN and 60% CH₃CN in a 0.01 M sodium citrate buffer (pH 5–6). During the optimization, another problem arose from the overlapping of a reagent peak on amino acids, especially on D- and L-Ser (Fig. 3). The reagent peak was, however, found to shift either forwards or backwards by changing the pH of buffer (between 5–6) while keeping similar separation of amino acids. After numerous trials, we selected an HPLC condition for the simultaneous analysis (pH=5.7) to give an HPLC chart given in Fig. 4. There, most of D-amino acids could be separated from the other peaks, except for D-Glu and D-Tyr (for D-Tyr, use of lower pH buffer gave good separation, as shown in Fig. 5).

Since the HPLC condition for the simultaneous analysis as described above has taken a long time (80 min) and the separation of polar amino acids was insufficient, we tried to propose supplemental HPLC conditions suitable for the group analysis of amino acids. For the analysis of polar amino acid derivatives, lower pH (4.6) was useful to move the reagent peak backwards (Fig. 5), while, for that of less polar ones, higher pH (6.0) was used to shift the reagent peak forwards (Fig. 6). In

Fig. 4 Simultaneous analysis of amino acid enantiomers. Column: CAPCELL PAK-UG120 (ODS, 4.6 mm×150 mm, Shiseido). Elution: 15–60% CH₃CN in 0.01 M sodium citrate buffer (pH=5.7). Fluorescence detection: excitation at 310 nm and emission at 380 nm (* unidentified peaks).

Fig. 5 Selective analysis of polar amino acid enantiomers. Elution: 15–30% CH₃CN in 0.01 M sodium citrate buffer (pH=4.6). The other HPLC conditions were the same as those for the simultaneous analysis in Fig. 4.

Fig. 6 Selective analysis of neutral amino acid enantiomers. Elution: 15–60% CH₃CN in 0.01 M sodium citrate buffer (pH=6.0). The other HPLC conditions were the same as those for the simultaneous analysis in Fig. 4.

the former, all polar amino acid derivatives appeared in front of the reagent, showing better separation compared with the condition for the simultaneous analysis. Even under this condition, however, D- and L-Glu still could not be separated. In the latter, all D- and L-isomers

could be separated in a much shorter time (40 min) while keeping similar separation to that of the simultaneous analysis (80 min).

Analysis of enantiomeric amino acids in a fermented milk drink

In order to demonstrate the utility of our method, we

Fig. 7 HPLC profile of polar amino acids in a fermented milk drink. Elution: 15–30% CH₃CN in 0.01 M sodium citrate buffer (pH=4.6). The other HPLC conditions were the same as those for the simultaneous analysis in Fig. 4.

tried to determine the enantiomer composition of amino acids in a fermented milk drink. The lactic drink was deproteinized, defatted and purified with a cation exchange resin in the manner described in the Experimental section to afford an analytical sample.

HPLC analyses of lactic drink samples were conducted in the manner described for the group analysis. The HPLC profile shown in Fig. 7 indicated that D-Ala was involved to a large extent [$29\% = 100 \times D/(D+L)$]. Although the L-Tyr peak was overlapped on L-Ala under the present HPLC condition, the D-Ala/L-Ala ratio could be calculated from the analysis using racemic TBMB carboxylic acid since (R)-TBMB carbonylated L-Tyr was separable from the L-Ala peak. Moreover, the result was confirmed using HPLC conditions for the simultaneous analysis. Since D-Ala has been widely found in microbially fermented foods and beverages^{19,20}, it should originate in the bacterial metabolism.²¹

In conclusion, we have applied (S)-TBMB carboxylic acid for the HPLC analysis of enantiomeric amino acids. Recently, similar HPLC methods using OPA^{6,8,10} and FLEC^{7,22} have been popularly used for the same purpose and also compared with other methods in reviews.^{4,5} Compared with these methods, the present method using (S)-TBMB carboxylic acid allows higher sensitive detection (less than 0.05 pmol on column). Although the separation of some amino acids under the proposed conditions could not be achieved, simple derivatization procedures and high stabilities of the derivatives will be advantageous for the practical purpose and for further efforts to establish a more efficient HPLC condition to

Fig. 8 HPLC profile of neutral amino acids in a fermented milk drink. Elution: 15–60% CH₃CN in 0.01 sodium citrate buffer (pH=6.0). The other HPLC conditions were the same as those for the simultaneous analysis in Fig. 4.

separate every enantiomeric amino acid.

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