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Resolution of Racemic Amino Acids by Gas Chromatography. I. N-Trifluoroacetyl-L-prolyl Derivatives

Hiroshi Iwase and Asao Murai

Central Research Laboratories, Ajinomoto Co., Inc.[1]

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The L- and D-amino acids could be resolved by coupling N-trifluoroacetyl-prolyl chloride (L-TPC) as the resolving agent by gas chromatography. The reaction conditions were studied in detail, the solvents, the amount of resolving agent and reaction time for quantitative derivatization. The 22 stationary phases were studied for chromatographic separation. Relative retention times and separation factors were determined for each enantiomeric pair of 15 amino acids and glycine. Relation between separation factors and structure of amino acids were also discussed.

Gas chromatography has been proved to be useful for the analysis of amino acids in biological substances because of its rapidity, accuracy, simplicity and sensitivity. As amino acids are not volatile to permit direct analysis, they must be converted into suitable derivatives prior to gas chromatography. Since Hunter, *et al.*[2] used oxidation with ninhydrin to prepare volatile aldehydes from aliphatic amino acids, a number of methods of derivatization for gas chromatography have been investigated by many workers.[3]

Amino acids are known to racemize under various conditions.[4] The occurence of D-amino acids in some bacteria, cell-wall materials, insects and antibiotics[5] is well known. Separation of optical isomers of amino acids is very important to know the presence of contents of D-amino acids in the synthetic or naturally occurring substances.

Since the optical resolution of camphor was made by Casanova and Corey[6] in 1961, the separation of optical isomers by gas chromatography has been carried out by many investigators. Two different methods have been adapted; 1) derivatization of the enantiomeric amino acids with optically active reagents to form diastereoisomers and chromatography on optically inactive stationary phases;[7] 2) derivatization of the enantiomeric amino acids with optically inactive reagents and chromatography on optically active stationary phases.[8] Halpern and Westley[9] resolved the racemic amino acids which were converted to their diastereomeric N-trifluoroacetyl-L-prolyl amino acid derivatives. L-Proline was used as resolving agent because it does not racemize during the acylation and the peptide bond formation.

1) Location: *1-1 Suzuki-cho, Kawasaki-ku, Kawasaki-city, Kanagawa.*
2) I.R. Hunter, K.P. Dimick and J.W. Corze, *Chem. & Ind.* (London), **1956**, 294.
3) C.W. Gehrke and D.L. Stalling, *Separ. Sci.*, **2**, 101 (1967); C.W. Gehrke, K. Kuo and R.W. Zumwalt, *J. Chromatog.*, **57**, 209 (1971); R.W. Zumwalt, K. Kuo and C.W. Gehrke, *J. Chromatog.*, **57**, 193 (1971); C.W. Gehrke, H. Nakamoto and R.W. Zumwalt, *J. Chromatog.*, **45**, 24 (1969); C.W. Gehrke and K.R. Leimer, *J. Chromatog.*, **57**, 219 (1971).
4) K. Toi, Y. Izumi and S. Akabori, *Bull. Chem. Soc. Japan*, **35**, 1422 (1962); S. Nakaparksin, E. Gil-Av and J. Oro', *Anal. Biochem.*, **33**, 374 (1970).
5) J.J. Corrigan, *Science*, **64**, 142 (1969); A. Meister, "Biochemistry of the Amino Acids," 2nd ed., Academic Press, New York, 1965, p. 113.
6) J. Casanova and E.L. Corey, *Chem. & Ind.* (London), **1961**, 1664.
7) R. Charles, G. Fischer and E. Gil-Av, *Israel J. Chem.*, **1**, 234 (1963); E. Gil-Av, R. Charles and G. Fischer, *J. Chromatog.*, **17**, 408 (1965); G.E. Pollock and V.I. Oyama, *J. Gas Chromatog.*, **4**, 126 (1966).
8) W.A. Koenig, W. Parr, H.A. Lichtenstein, E. Bayer and J. Oro', *J. Chromatog. Sci.*, **8**, 183 (1970); S. Nakaparksin, P. Birrell, E. Gil-Av and J. Oro', *J. Chromatog. Sci.*, **8**, 183 (1970).
9) B. Halpern and J.W. Westley, *Tetrahedron Letters*, **1966**, 2283; B. Halpern and J.W. Westley, *Biochem. Biophys. Res. Commun.*, **19**, 361 (1965).

However, the reaction conditions, the column conditions and the relation between the separation factors and the structure of the racemic amino acids for the resolution of amino acid enantiomers were not reported in detail and basic amino acids were not studied. In this study the experimental conditions for the resolution of enantiomers which were converted to their diastereomeric derivatives using L-TPC[10] as the resolving agent for monoaminocarboxylic acids, monoaminodicarboxylic acids, diaminocarboxylic acids and glycine were investigated in detail.

Experimental

Apparatus and Condition——Apparatus: A Hewlett-Packard Model 402 gas chromatograph equipped with dual flame ionization detector was used. Two glass column of 5.5 ft×1/4 liter in. O.D. and 4 ft×1/4 liter in. O.D. were used. The column packings were 5% OV-1 on 100—120 mesh Supercoport for monoaminocarboxylic acids and diaminocarboxylic acids and 2% PEGA, stabilized grade, for monoaminodicarboxylic acids. Helium was used as the carrier gas at 60 ml/min.

Reagents and Materials——All solvents used in this study were reagent grade. Amino acids were obtained from Ajinomoto Co., Sigma Chemical Co., and Tokyo Kasei Co. L-Proline was twice recrystallized from absolute ethanol-diethylether. Thionyl chloride was distilled according to the method described elsewhere.[11] BSTFA, TMCS and acetonitrile were obtained from Pierce Chemical Co. The stationary phase, OV-1 and PEGA, were obtained from Analabs Inc. The support materials, 100—120 mesh Supelcoport, was obtained from Supelco. Inc. L-TPC was prepared according to the method of Dabrowiak.[12] Hypovial was purchased from Pierce Chemical Co.

Preparation of Amino Acid Derivatives——TPAM were prepared according to the method of Halpern[9] and Dabrowiak[12] with some modification as follows: the neutral amino acid mixture (alanine, valine, leucine and proline) containing each 5 mg was esterified with 5 ml of thionyl chloride-methanol (1+9) for 2 hr, and excess of the reagent and the solvent were removed *in vacuo*. To the residue, a 2 ml portion of L-TPC containing 0.017 mole in 100 ml chloroform was added and the mixture was neutralized with 0.1 ml of triethylamine and allowed to stand for 15 min at room temperature. The mixture was washed with 2—3 ml of distilled water for 30 sec in a 10 ml separating funnel and dried over anhydrous sodium sulfate. A 3 μl portion of this solution was injected into gas chromatograph.

After triethylamine and L-TPC were added to the hydroxy amino acid methyl ester (threonine), the solvent was removed *in vacuo* and followed by addition 1 ml of acetonitrile, 0.5 ml of BSTFA and 5—6 drops of TMCS. After standing it for 15 min at room temperature, a 3 μl portion of this solution was injected into gas chromatograph.

Acidic and basic amino acids were treated in the same manner as those with the neutral amino acids.

Result and Discussion

The Neutral Amino Acids (Alanine, Valine, Leucine and Proline)

Each 50 mg of racemic amino acid was esterified and then the solvent was removed *in vacuo*. The residue was dissolved in 20 ml of methanol containing *n*-eicosane as an internal standard. Each 2 ml of this methanol solution was concentrated and submitted to the preparation of TPAM.

Solvent

Triethylamine, pyridine and N,N-dimethylaniline were tested as a solvent. A 0.1 ml of each organic basic solvent and 1 ml of L-TPC were added to the amino acid methyl esters, and the products were checked by gas chromatography. When the non-basic solvent was

10) Abbreviations L-TPC: N-trifluoroacetyl-prolyl chloride, TPAM: N-trifluoroacetyl prolyl amino acid methyl esters, BSTFA: N,O-bis-(trimethylsilyl) trifluoroacetamide, BSA: N,O-bis-(trimethylsilyl) acetamide, MSTFA: N-methyl-N-trimethylsilyltrifluoroacetamide, MSA: N-methyl-N-trimethylsilylacetamide, TMSDEA: N-trimethylsilyldiethylamine, TMSDMA: N-trimethylsilyldimethylamine, TSIM: N-trimethylsilylimidazole, TMCS: trimethylchlorosilane, PEGA: polyethylene glycol adipate, Scot: support coated open tubular.

11) L.F. Fieser "Experiments in Organic Chemistry," 3rd ed., Heath, Boston, 1957, p. 345.

12) J.C. Dabrowiak and D.W. Cooke, *Anal. Chem.*, 43, 791 (1971).

used, each peak, especially alanine and proline was too small to confirm the identity. The use of pyridine resulted in unsatisfactory yield of peptide bond formation. When triethylamine and N,N-dimethylaniline were used, large peaks were obtained. As N,N-dimethylaniline caused another unknown peak, the use of triethylamine as solvent was employed.

Triethylamine and L-TPC

Evaluation of the optimum condition for the formation of TPAM with triethylamine and L-TPC was made by comparing the peak height of L-leucine, D-proline relative to that of *n*-eicosane. The results are given in Tables I and II. As is seen in Tables I and II, when

TABLE I. Effect of the Amount of the L-TPC on Peak Height Ratio

L-TPC added	Peak height ratio	
	L-leucine/I.S.[a]	D-proline/I.S.
1ml	0.98	0.73
2	1.61	0.87
3	1.61	0.87

a) I.S. represents internal standard (*n*-eicosane).

TABLE II. Effect of the Amount of the Triethylamine on Peak Height Ratio

Triethylamine added	Peak height ratio	
	L-leucine/I.S.[a]	D-proline/I.S.
0.1ml	1.61	0.87
0.3	1.61	0.87

a) I.S. represents internal standard (*n*-eicosane).

0.1 ml of triethylamine and 2 ml of L-TPC were added to the mixture of amino acid esters and *n*-eicosane, the suitable peaks were obtained. After the successive injection of the mixtures for several times into gas chromatograph, an abnormal peak was often observed. After washing this solution with 2—3 ml of distilled water in a 10 ml separating funnel for 30 sec, the abnormal peak disappeared and clear peaks without tailing were obtained.

TABLE III. Effect of Reaction Time on the Formation of the L-TPC Derivatives

Reaction time	Peak height ratio (amino acid/I.S.)[a]							
	alanine		valine		leucine		proline	
	D-	L-	D-	L-	D-	L-	D-	L-
15min	3.30	3.09	2.58	2.31	2.00	1.91	1.21	1.10
30	3.28	3.13	2.59	2.31	1.98	1.92	1.21	1.09
60	3.25	3.11	2.62	2.34	2.00	1.92	1.20	1.10
90	3.25	3.11	2.62	2.31	1.99	1.91	1.20	1.11
180	3.31	3.11	2.66	2.31	1.99	1.91	1.20	1.10
300	3.33	3.11	2.56	2.28	1.99	1.89	1.18	1.07

a) I.S. represents internal standard (*n*-eicosane).

Reaction Time

Reaction time was tested for the formation of TPAM. Evaluation of the optimum reaction time was made by comparing the peak height of alanine, valine, leucine and proline relative to that of *n*-eicosane. The results are shown in Table III. As is seen in Table III,

the reaction immediately completes. The sample was allowed to stand for 15 min at room temperature and each sample was washed with 2—3 ml of distilled water and dried over anhydrous sodium sulfate and injected into gas chromatograph.

Hydroxy Amino Acid (Threonine)

Racemic threonine was derivatized as the same manner as alanine, valine, leucine and proline. But N-trifluoroacetyl prolyl threonine methyl ester could not be separated. Threonine derivatives could be, however, separated after the trimethylsilylation of the hydroxy group. Many silylating methods have been used for the analysis of amino acids, nucleic acids, steroids, carbohydrates and organic acids. Eight silylating reagents were tested for the silylation of hydroxy group of threonine. Acetonitrile was used as the solvent for trimethylsilylation. The reagents such as MSA, TMSDMA, TMSDEA caused unknown peaks and TSIM caused tailing. Therefore, BSA, BSTFA and MSTFA were studied in detail to prepare the trimethylsilyl derivatives. Evaluation of the optimum condition for the silylation was made by comparing the relative peak height of threonone to *n*-eicosane. The results are given in Table IV. Table IV shows that BSTFA is superior to BSA and MSTFA and that

Table IV. Effect of Reaction Time on the Formation of L-TPC Derivatives

Reaction time	Peak height ratio (threonine/I.S.[a])					
	BSTFA		BSA		MSTFA	
	D-	L-	D-	L-	D-	L-
15min	1.85	1.79	1.82	1.75	1.73	1.73
60	1.84	1.78	1.83	1.81	1.73	1.73

a) I.S. represents internal standard (*n*-eicosane).

the silylation reaction completes within 15 min at room temperature. When racemic threonine methyl ester was trimethylsilylated followed by addition of L–TPC according to the method of Dabrowiak,[12] sharp peak with sufficient intensity was not obtained. The trimethylsilylation must be done after L–TPC is added to the racemic threonine methyl ester.

Basic Amino Acid (Lysine)

Lysine derivative was prepared as the same manner as the neutral amino acids. *n*-Octacosane was used as an internal standard. Evaluation of the optimum reaction time was made by comparing the peak height of lysine relative to that of *n*-octacosane. The results are shown in Table V.

Table V. Effect of Reaction Time on the Formation of L-TPC Derivatives

Reaction time	Peak height ratio	
	L-lysine/I.S.[a]	D-lysine/I.S.
15min	1.13	0.94
60	1.14	0.93
180	1.11	0.92

a) I.S. represents internal standard (*n*-octacosane).

As can be seen in Table V, the sample is allowed to stand for 15 min at room temperature for the complete silylation.

Stationary Phases

Various stationary phases of packed and scot column were tested for the separation of racemic amino acids.

Alanine, valine, leucine, isoleucine, threonine, serine, norvaline, norleucine, proline, methionine, phenylalanine, ornithine and lysine were separated on non-polar stationary phases. Proline, isoleucine, threonine, serine, methionine, phenylalanine, aspartic acid and glutamic acid were separated on polar stationary phases. For example, alanine, valine, leucine and proline were separated completely on OV-1 (dimethylsilicone), but aspartic acid and glutamic acid were not separated on it. On the other hand, alanine, valine and leucine were not separated on PEGA, but proline, aspartic acid and glutamic acid were separated on it. The separation of L-valine and D-leucine is more difficult with increasing in the amount of polar groups of silicone stationary phases, for example, L-valine and D-leucine were not separated completely on OV-17 (50% phenyl methyl silicone), OV-22 (65% phenyl methyl silicone), OV-25 (75% phenyl methyl silicone), OV-210 (50% trifluoropropyl methyl silicone) and OV-225 (25% cyanopropyl 25% phenyl methyl silicone). OV-1 and PEGA were used for the resolution of racemic amino acids. Some of the gas chromatograms are shown in Fig. 1 and Fig. 2.

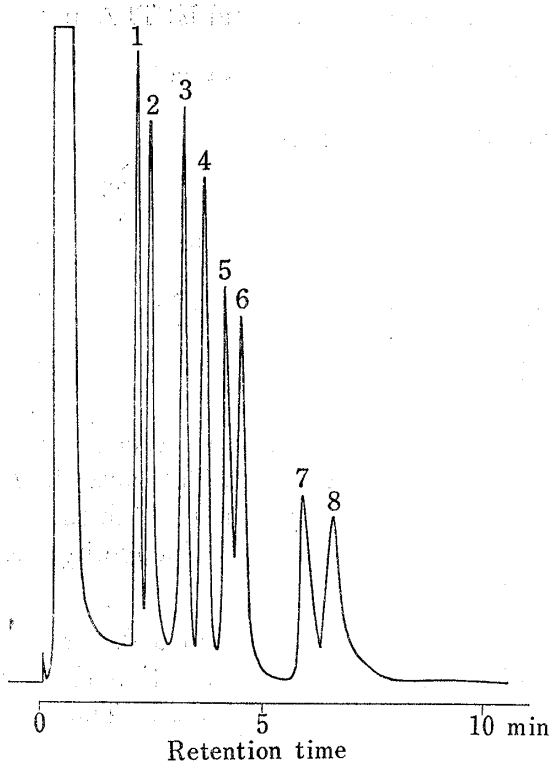

Fig. 1. Gas Chromatogram of Diastereomeric Esters of Amino Acids on OV-1

1: D-alanine, 2: L-alanine, 3: D-valine, 4: L-valine, 5: D-leucine, 6: L-leucine, 7: D-proline, 8: L-proline

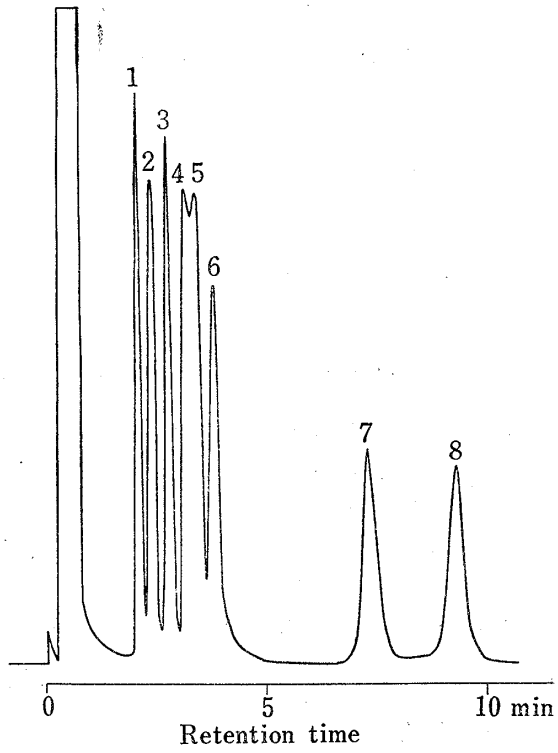

Fig. 2. Gas Chromatogram of Diastereomeric Esters of Amino Acids on OV-25

1: D-alanine, 2: L-alanine, 3: D-valine, 4: L-valine, 5: D-leucine, 6: L-leucine, 7: D-proline, 8: L-proline

Scot column (SE-30) was not useful for the separation of diastereomeric amino acid derivatives because of the tailed peaks. A glass column was superior to a stainless steel one in which tailed peak was obtained.

Relation between Separation Factors and Structure of Amino Acids

Relative retention time and separation factors, α, (rL/D) of TPAM are given in Tables VI, VII and VIII. Separation factors were calculated from the definition as follows,

$$\alpha = t_{R_2} - t_a / t_{R_1} - t_a$$

where t_{R_1} and t_{R2} are the retention time (from injection) of the first and the second components, respectively, and t_a is the retention time of non-adsorbed species (methane).

TABLE VI. Gas Chromatographic Data for Racemic Amino Acids as Their N-Trifluoroacetyl L-Prolyl Peptide Methyl Esters with 5% OV-1 on Supelcoport at 180°

Amino acid	Enantiomer	RRT[a]	r L/D
Alanine	D	0.428	1.156
	L	0.494	
Glycine	—	0.517	—
Valine	D	0.678	1.172
	L	0.794	
Norvaline	D	0.806	1.103
	L	0.889	
Leucine	D	0.917	1.109
	L	1.000	
Isoleucine	D	0.967	1.132
	L	1.094	
Norleucine	D	1.139	1.098
	L	1.250	
Serine	D	1.128	1.104
	L	1.417	
Threonine	D	1.339	1.158
	L	1.550	
Proline	D	1.406	1.115
	L	1.567	
Methionine	D	2.672	1.075
	L	2.878	
Phenylalanine	D	3.956	1.077
	L	4.439	

a) Relative retention time, reference compound is N-TFA-L-leucine prolyl peptide methylester. t_R=4.73 min

TABLE VII. Gas Chromatographic Data for Racemic Amino Acids as Their N'-Trifluoroacetyl L-Prolyl Peptide Methyl Esters with 2% PEGA on Supelcoport at 205°

Amino acid	Enantiomer	RRT[a]	r L/D
Threonine	D	0.192	1.262
	L	0.243	
Isoleucine	D	0.231	1.246
	L	0.287	
Serine	D	0.239	1.223
	L	0.293	
Proline	D	0.558	1.248
	L	0.697	
Aspartic acid	D	1.000	1.069
	L	1.069	
Methionine	D	1.483	1.143
	L	1.694	
Glutamic acid	D	1.694	1.126
	L	1.909	
Phenylalanine	D	2.111	1.057
	L	2.231	

a) RRT, reference compound is N-TFA-D-aspartic acid prolyl peptide methylester. t_R=7.92 min

There are a number of reports concerning the elution of racemic amino acids in gas chromatography. Vitt, *et al.*[13] showed that L-amino acids had shorter retention times than D-forms which were converted to N-trifluoroacetyl-amino acid 1-menthyl esters on PEGA as a stationary phase. Koenig, *et al.*[8] showed that D-amino acids had shorter retention times

13) S.V. Vitt, M.B. Saporowskaya, I.P. GudKova and V.M. Belikov, *Tetrahedron Letters*, **1965**, 2575.

TABLE VIII. Gas Chromatographic Data for Racemic Amino Acids as Their N-Trifluoroacetyl L-Prolyl Peptide Methyl Esters with 5% OV-1 on Supelcoport at 270°

Amino acid	Enantiomer	RRT[a]	r L/D
Ornithine	D	0.860	0.916
	L	0.788	
Lysine	D	1.106	0.904
	L	1.000	

a) RRT, reference compound is N-TFA-L-lysineprolyl peptide methyl ester. t_R=6.05 min

than L-amino acids which were converted to N-trifluoroacetyl amino acid isopropyl esters using optically active stationary phase (N-trifluoroacetyl-L-phenylalanyl-L-leucine cyclohexyl ester or N-trifluoroacetyl-L-valyl-L-valine cyclohexyl ester). Though Halpern, *et al.*[9] reported the elution of racemic amino acids which were converted to TPAM, we also found that D-amino acids except basic amino acids had shorter retention times than L-amino acids using L–TPC as the resolving agent on all stationary phases investigated. The L-amino acids of basic amino acids were shorter retention times than D-amino acids. This may due to that basic amino acids have two amino groups which are masked by L–TPC.

Relation between separation factors and structure of amino acids were studied from Tables VI, VII and VIII. Derivatives of DL-alanine (R=$-CH_3$), norvaline (R=$-(CH_2)_2CH_3$), norleucine (R=$-(CH_2)_3CH_3$), valine (R=$-CH\langle^{CH_3}_{CH_3}$), leucine (R=$-CH_2CH\langle^{CH_3}_{CH_3}$), isoleucine (R=$-CH\langle^{CH_3}_{CH_2CH_3}$), methionine (R=$-CH_2CH_2SCH_3$), proline (R=$-(CH_2)_3-$), aspartic acid (R=$-CH_2COOH$), glutamic acid (R=$-CH_2CH_2COOH$), ornithine (R=$-(CH_2)_3NH_2$) and lysine (R=$-(CH_2)_4NH_2$) were chosen for investigation. Table VI shows that an increase in methylene groups was accompained by a decrease in the separation factors for alanine, norvaline and norleucine. This was true for valine, leucine and isoleucine. On the other hand, for acidic amino acids, Table VII shows that an increase in methylene group was accompained by an increase in separation factors. For basic amino acids, Table VIII shows that an increase in methylene groups was accompained by a decrease in the separation factors. Comparing norleucine with methionine in which the δ methylene group of norleucine is substituted by a sulfur atom, separation factor of methionine was smaller than that of norleucine on OV-1, but was larger on PEGA. Norleucine was not resolved on PEGA. Comparing isoleucine with leucine and valine, isoleucine could be resolved on both stationary phases, but leucine and valine were not resolved on PEGA. This may due to the difference of chain-branching of amino acids.

The method here established is useful for the resolution of each L- and D-amino acid, but it is difficult to resolve the complex mixture of L- and/or D-amino acids because some of these peaks may be overlapped each other.