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Rapid analysis of amino acid enantiomers by chiral-phase capillary gas chromatography

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Abstract

Amino acids are derivatized with ethyl chloroformate or 2,2,2-trifluoroethyl chloroformate in a reaction medium containing different alcohols into a wide variety of N(O)-alkoxycarbonyl alkyl esters. The derivatives are produced to be used in a rapid separation of enantiomeric isomers by capillary GC with L-valine-*tert.*-butylamide-modified polydimethylsiloxane as chiral stationary phase. Among the various amino acid derivatives prepared, the N(O)-2,2,2-trifluoroethoxycarbonyl 2′,2′,2′-trifluoroethyl ester gave fairly stable compounds and attained almost complete separation of all enantiomeric pairs, except for Pro, within 31 min. The derivatization method was found to be suitable for enantiomer separation rather than quantitative analysis.

1. Introduction

Chiral stationary phases in capillary gas chromatography (GC) have undergone exciting developments over the last two decades [1,2]. Especially for the analysis of amino acid enantiomers, the method is well documented and known as a reliable technique for the determination of optical purities. GC methods for the analysis of amino acids have some inherent advantages over HPLC methods. GC still gives shorter analysis times and needs less expensive equipment and maintenance. For the application of GC as a technique for the analysis of amino acids, e.g. drugs, the optical purity of the amino acids has to be investigated during the different steps and the optical yield during the asymmetric synthesis of amino acids has to be determined. Furthermore, the technique has been applied to follow racemization during peptide or protein hydrolysis prior to individual amino acid analysis–using GC–MS by the deuterium labeling method [3,4]–and to estimate the age of archaeological material from the extent of Asp racemization [5,6]. For the separation of amino acid enantiomers, L-valine-*tert.*-butylamide-linked polydimethylsiloxane (Chirasil-Val) [7], L-valine-*S*-1-(α-phenylethyl)-amide-linked polydimethylsiloxane [8], and octakis(3 - O - butyryl-2,6-di-O-pentyl)-γ-cyclodextrin [9] are known as commercially available stationary phases suitable for the coating of capillaries.

However, a common problem in GC is the volatility of the sample. In contrast to the procedure used in HPLC, in GC the amino acids must be converted to volatile derivatives that are

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suitable for separation and elute within a reasonable time. Therefore, the development of suitable derivatization techniques for amino acids is considered to be a prerequisite to obtain successful separations.

Enantiomer separation of amino acids plays a role in one of the classical applications of chiral-phase GC, pioneered by Gil-Av et al. [10], who demonstrated the direct separation of amino acids derivatized into N-trifluoroacetyl (TFA) methyl esters on N-TFA-L-isoleucine lauryl ester and N-TFA-L-phenylalanine lauryl ester as chiral stationary phases. Since then, only the N(O)-perfluoroacyl methyl, isopropyl, and *n*-propyl ester derivatives of amino acids have been adopted. In order to derivatize amino acids into N(O)-perfluoroacyl alkyl esters, laborious multi-step processes were required: complete removal of the water from the sample, esterification with heating, removal of the excess alcohol, acylation with heating, and removal of the excess reagents. These processes take at least 1 h in total and prevent speeding up of the derivatization. Some other derivatization methods have been introduced using isocyanates [11] and phosgene [12] as derivatizing reagents. However, these two types of derivatives could not be prepared with omittance of the first step, the removal of water. Moreover, because of their toxicity, these compounds are difficult to handle safely in the laboratory. Makita et al. [13] reported a unique method for the derivatization of amino acids into N(O)-isobutyloxycarbonyl methyl esters for quantitative analysis. In this method the first step –the removal of water– was eliminated, but the subsequent processes seem to be complicated.

In previous papers [14,15], we have described a fast method for the derivatization of amino acids for enantiomeric analysis that was first introduced by Husek and co-workers [16–18]. They applied this method to quantitative analysis on normal achiral phases. The method has been reported to be able to derivatize all amino acids in a single step, with alkyl chloroformates at room temperature, within 1 min. Later, Wang et al. [19] reported an extended application of chloroformates, using different alcohols in the reaction medium to obtain a variety of different esters.

In the present study, we have investigated a more extended approach for the derivatization of amino acid enantiomers, using the new reagent 2,2,2-trifluoroethyl chloroformate besides ethyl chloroformate, and we studied the formation of the wide variety of derivatives in combination with various alcohols to be esterified. 2,2,2-Trifluoroethyl chloroformate was found to give more volatile derivatives than ethyl chloroformate; moreover, the former 2,2,2-trifluoroethyl ester derivatives gave fairly faster elution than the chloroformate derivatives.

2. Experimental

2.1. Materials

All amino acids were purchased from Sigma (St. Louis, MO, USA). They were dissolved as a mixture or individually to a concentration of 2.5 μmol/ml of each in 0.1 *M* HCl as a standard stock solution. Ethyl chloroformate (ECF), methanol (MeOH), ethanol (EtOH), 2,2,2-trifluoroethanol (TFEtOH), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIPrOH), and pyridine were obtained from Wako Chemicals (Osaka, Japan). 2,2,3,3,3-Pentafluoro-1-propanol (PFPrOH), chloroform (ethanol free), and triphosgene were from Tokyo Kasei (Tokyo, Japan).

2.2. Preparation of 2,2,2-trifluoroethyl chloroformate

Triphosgene (25 g, 84 m*M*) was dissolved in about 200 ml of freshly distilled toluene in a three-necked flask equipped with a mechanical stirrer, thermometer, and dropping funnel. The solution was ice-cooled, and 20.4 ml (250 m*M*) of pyridine were added dropwise to the solution with stirring over a period of 2 h while keeping the temperature of the solution below 5°C. After completion of the addition, the solution was stirred for another hour, and cooled with ice. Then, TFEtOH (15 ml, 200 m*M*) was added dropwise to the solution in 30 min at a temperature below 5°C under continuous stirring. After the addition of TFEtOH, the ice-bath was removed and the solution was left stirred over-

night. A white precipitate was formed, which was filtered off, and the filtrate distilled twice yielding 7.2 g of 2,2,2-trifluoroethyl chloroformate (TFECF) (b.p. 67–69°C). ^{1}H NMR spectroscopy (270 MHz, $CDCl_3$): $\delta = 4.62$ (2H, q, $J = 7.93$ Hz).

2.3. Preparation of amino acid derivatives

Standard amino acid stock solution (100 μl) was transferred into a Reacti-Vial (1 ml volume, Pierce, IL, USA), and 50 μl of alcohol–pyridine mixture (3:1, v/v) were added. To this solution, 10 μl of alkyl chloroformate was added, the vial was capped tightly, and shaken vigorously for 10 s. Immediately amino acid derivatives of N(O)-alkoxycarbonyl alkyl esters were formed. Then 30 μl of chloroform were added to the vial and the vial was shaken again for extraction of the derivatives into the organic layer. Finally, about 1 μl of the organic phase was withdrawn by a microliter syringe and injected directly onto the gas chromatographic system.

2.4. Preparation of chiral stationary phases

L-Valine-*tert.*-butylamide modified polydimethylsiloxane (Chirasil-Val) was used as stationary phase for the separation of enantiomers. Chirasil-Val was prepared according to the procedure described in Ref. [20] and contained anchored chiral side-chains in the ratio of 1:4, relative to unsubstituted dimethylsiloxane units [20].

2.5. Preparation of glass capillary columns

A glass capillary (0.8 mm O.D., 0.25 mm I.D.) was drawn from borosilicate glass tube (Pyrex). The capillary was leached with 6 *M* HCl, dehydrated in vacuo, and deactivated with diphenyltetramethyldisilazane [21]. The deactivated capillary was coated with a 0.3% solution of the stationary phase in *n*-pentane by a static method. The capillary was installed in a gas chromatograph and conditioned by programming to 230°C at 1°C/min and left overnight before use.

2.6. Apparatus

A Shimadzu GC-9AM gas chromatograph (Kyoto, Japan) equipped with a flame ionization detector was used throughout this study. Helium was used as carrier gas, and split injection mode (split ratio 1:40) was used. A Shimadzu C-R7A was used for data processing.

3. Results and discussion

ECF and TFECF, each combined with various alcohols, gave a wide variety of amino acid derivatives. Fig. 1 shows the reaction mechanism of an amino acid with alkyl chloroformate in the reaction medium containing various alcohols (described detailed in Ref. [19]). However, depending on the choice of the combination of alkyl chloroformate and alcohol, sometimes a by-product was formed which prevented rapid identification of the signal and correct determination of the peak area. For complete suppression of by-product formation the electronegativity of R′ must be larger than that of R″ (see Fig. 1). All derivatives prepared in this study were stable compounds, except when the TFECF-HFIPrOH combination was used, which for some amino acids did not give a sufficient peak signal. In most cases, neutral amino acids with one carboxyl and one amino group gave fully resolved peaks, while amino acids with a hydroxy group gave only a small peak which occasionally was difficult to identify, especially for the derivatives of the TFECF-HFIPrOH combination. Table 1 shows the separation factors and resolutions of all derivatives obtained on the Chirasil-Val capillary column. Baseline separation was obtained when the resolution was higher than 1.25. The enantiomeric pairs of Ala, Val, *allo*-Ile, Ile, Leu, Glu, Ser, Met, Phe, and Orn could be separated completely in all their derivative forms. However, Pro did not show any indication of separation at all, and His, Tyr, and Trp were not completely baseline separated. Comparing the separation factors and resolutions of the six derivatives, no substantial differences were observed. At the start of this study, the derivatives of the TFECF-HFIPrOH combina-

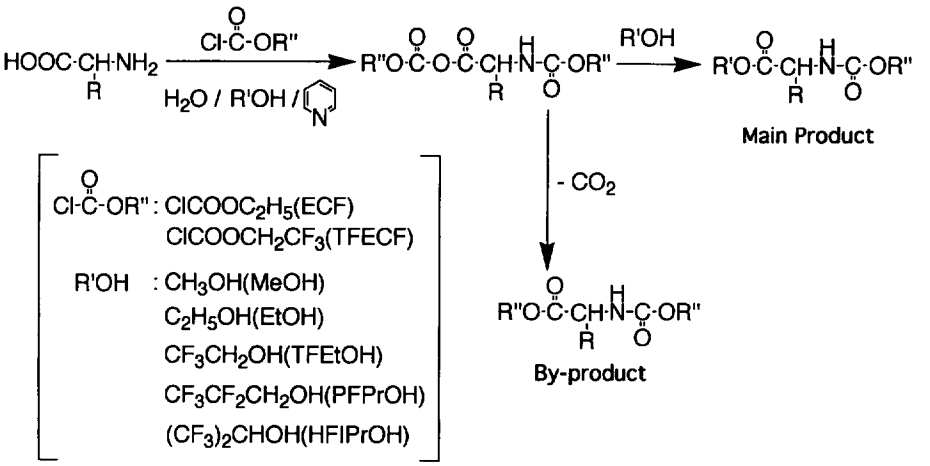

Fig. 1. Reaction mechanism of amino acids with alkyl chloroformates in a reaction medium containing different types of alcohols.

Table 1
Separation factors and resolutions of amino acid derivatives

Amino acid	Derivative						Column temp. (°C)
	ECF-EtOH	ECF-MeOH	ECF-TFEtOH	ECF-PFPrOH	TFECF-TFEtOH	TFECF-HFIPrOH	
Ala	1.098 (4.79)	1.077 (3.28)	1.106 (4.06)	1.104 (5.20)	1.095 (3.52)	1.103 (3.04)	100
Val	1.078 (4.04)	1.062 (2.77)	1.101 (4.28)	1.098 (5.21)	1.079 (2.96)	1.106 (3.86)	100
Pro	ns	ns	ns	ns	ns	ns	100
allo-Ile	1.074 (3.91)	1.062 (2.93)	1.078 (2.72)	1.078 (3.58)	1.079 (2.98)	1.080 (2.73)	120
Ile	1.060 (3.23)	1.049 (2.27)	1.065 (1.94)	1.064 (2.86)	1.059 (2.10)	1.063 (1.98)	120
Leu	1.104 (4.34)	1.084 (2.89)	1.108 (4.53)	1.107 (4.30)	1.098 (4.52)	1.103 (3.96)	120
Asp	1.034 (1.76)	1.029 (0.85)	1.034 (1.47)	1.034 (1.51)	1.030 (1.26)	1.022 (0.61)	140
Thr	1.049 (2.06)	1.030 (1.10)	1.028 (1.45)	1.028 (1.19)	1.065 (2.22)	nd	140
Glu	1.071 (3.48)	1.058 (2.65)	1.083 (4.38)	1.086 (3.86)	1.078 (3.13)	1.080 (4.00)	150
Ser	1.055 (2.46)	1.045 (1.84)	1.035 (1.94)	1.038 (1.78)	1.042 (1.98)	nd	150
Met	1.068 (3.11)	1.054 (1.63)	1.071 (2.79)	1.069 (3.27)	1.065 (2.95)	1.061 (2.17)	150
Phe	1.052 (2.11)	1.044 (1.40)	1.048 (1.80)	1.047 (1.98)	1.045 (1.81)	1.041 (1.77)	150
Orn	1.036 (1.70)	1.033 (1.39)	1.029 (1.61)	1.029 (1.48)	1.029 (1.32)	nd	210
His	1.025 (1.12)	1.023 (0.79)	1.023 (0.91)	1.021 (0.88)	1.020 (0.53)	nd	210
Lys	1.030 (1.50)	1.026 (1.33)	1.024 (1.32)	1.024 (1.30)	1.022 (1.02)	nd	210
Tyr	1.025 (1.03)	1.019 (0.65)	1.020 (0.63)	1.022 (0.81)	1.018 (0.61)	1.018[a] (0.80)	210
Trp	1.027 (0.94)	1.023 (0.79)	1.022 (1.04)	1.022 (1.13)	1.020 (0.75)	1.018 (0.89)	210

The data represent the separation factor; data in parenthesis represent resolution.
ns: not separated.
nd: not detected.
[a] 200°C.
ECF-EtOH: N(O)-ethoxycarbonyl ethyl ester, ECF-MeOH: N(O)-ethoxycarbonyl methyl ester, ECF-TFEtOH: N(O)-ethoxycarbonyl 2,2,2-trifluoroethyl ester, ECF-PFPrOH: N(O)-ethoxycarbonyl 2,2,3,3,3-pentafluoro-1-propyl ester, TFECF-TFEtOH: N(O)-2,2,2-trifluoroethoxycarbonyl 2′,2′,2′-trifluoroethyl ester, TFECF-HFIPrOH: N(O)-2,2,2-trifluoroethoxycarbonyl 1,1,1,3,3,3-hexafluoro-2-propyl ester.

tion were expected to have a large separation factor due to the steric effect of the bulky hexafluoroisopropyl group, and because favorable results were obtained with N(O)-TFA isopropyl esters of amino acids on the same phase. However, the enantioselectivity of the derivatives toward Chirasil-Val was unexpectedly not very high, and the derivatives were too unstable to obtain chromatographic data for some amino acids. Table 2 shows the retention times and the relative retentions of all amino acid derivatives of the L-form. Relative retentions are calculated with the retention times of the ECF-EtOH derivatives taken as 1.00. The retention times of all amino acids decreased with substituting ethyl ester for methyl ester, and with the introduction of fluorine atoms into the derivatives. The higher the content of fluorine atoms, the shorter the retention time, except for Ala, Gly, Thr, which, on the contrary, showed increased retention times, and for Val, Glu, which showed a small change in retention times. This decrease in retention time caused by using fluorine-containing derivatizing reagents was generally remarkable in amino acids with three reactive functional groups. Pro was an exceptional case, in which, most remarkably, the retention time reduced with increasing fluorine content. No marked decrease in separation factor for amino acids derivatized with fluorine-containing reagents was observed. The decrease in retention time when using fluorine-containing derivatives of amino acids might allow operating at a lower column oven temperature, which further enhances enantiomer separation. Fig. 2 shows a typical chromatogram of an amino acid enantiomeric mixture derivatized with the TFECF-TFEtOH combination. Pro showed no separation, as described earlier, but the components eluted in the first half–from Ala to Phe–are almost completely separated. Fig. 3 shows the racemization test chromatogram for derivatives obtained with the

Table 2
Retention times and relative retentions of L-amino acid derivatives

Amino acid	Derivative						Column temp. (°C)
	ECF-EtOH	ECF-MeOH	ECF-TFEtOH	ECF-PFPrOH	TFECF-TFEtOH	TFECF-HFIPrOH	
Ala	10.60	7.59 (0.72)	8.15 (0.77)	8.73 (0.82)	5.90 (0.56)	4.56 (0.43)	100
Gly	11.29	8.39 (0.74)	9.23 (0.82)	9.92 (0.88)	7.99 (0.71)	6.43 (0.57)	100
Val	18.44	13.12 (0.71)	12.63 (0.68)	12.81 (0.69)	7.99 (0.43)	5.34 (0.29)	100
Pro	23.74	17.23 (0.73)	12.47 (0.53)	12.67 (0.53)	6.32 (0.27)	4.13 (0.17)	100
allo-Ile	11.94	9.19 (0.77)	7.91 (0.66)	7.79 (0.65)	5.17 (0.43)	3.48 (0.29)	120
Ile	12.85	9.85 (0.77)	8.79 (0.68)	8.59 (0.67)	6.64 (0.52)	3.83 (0.30)	120
Leu	14.78	11.33 (0.77)	10.30 (0.70)	10.14 (0.69)	7.25 (0.49)	4.95 (0.33)	120
Asp	15.27	9.57 (0.63)	7.91 (0.52)	7.15 (0.47)	6.20 (0.41)	3.55 (0.23)	140
Thr	20.64	16.24 (0.79)	16.89 (0.82)	16.73 (0.81)	5.44 (0.26)	nd	140
Glu	15.13	11.62 (0.77)	11.83 (0.78)	11.42 (0.75)	10.65 (0.70)	6.07 (0.40)	150
Ser	15.36	11.92 (0.78)	10.42 (0.68)	10.10 (0.66)	6.17 (0.40)	nd	150
Met	17.26	14.29 (0.83)	12.99 (0.75)	12.10 (0.70)	9.10 (0.53)	5.79 (0.34)	150
Phe	24.41	19.83 (0.81)	16.69 (0.68)	15.20 (0.62)	10.74 (0.44)	6.72 (0.28)	150
Orn	12.75	11.25 (0.88)	9.68 (0.76)	8.56 (0.67)	5.97 (0.47)	nd	210
His	15.03	13.07 (0.87)	9.30 (0.62)	8.08 (0.54)	4.34 (0.29)	nd	210
Lys	16.93	14.77 (0.87)	12.30 (0.73)	10.79 (0.64)	7.06 (0.42)	nd	210
Tyr	21.16	18.66 (0.88)	15.23 (0.72)	13.09 (0.62)	7.15 (0.34)	4.72 (0.22)	210
Trp	57.20	50.15 (0.88)	37.46 (0.65)	31.83 (0.56)	22.06 (0.39)	12.58 (0.22)	210

The data represent retention time (min); data in parenthesis represent relative retention time in the case where the retention time of ECF-EtOH is taken as 1.00.
nd: not detected.
For more details, see Table 1.

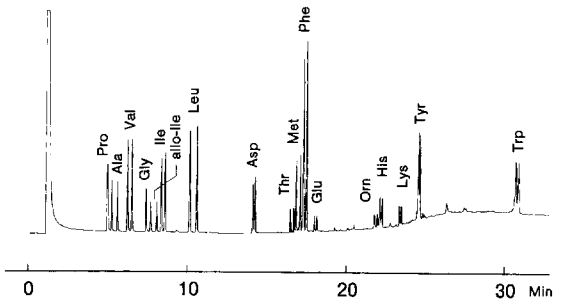

Fig. 2. Gas chromatogram of N(O)-2,2,2-trifluoroethoxycarbonyl 2′,2′,2′-trifluoroethyl esters of an amino acid mixture prepared from the D- and L-forms. Column, Chirasil-Val (20 m × 0.25 mm I.D. glass capillary, film thickness, 0.16 μm); column temperature, 95°C, 2 min hold, and then programmed at 3°C/min to 120°C, 7°C/min to 225°C; carrier gas, helium; column inlet pressure, 1.2 kg/cm^2; split ratio, 1:40.

TFECF-TFEtOH combination. The chromatogram shows the amino acid derivatives prepared from an L-amino acid mixture. As easily recognized from the GC, no racemization has occurred during sample treatment. For all amino acid derivatives, the D-enantiomers eluted faster, only in the case of Glu the L-enantiomer eluted fast. It is considered that Glu converted to a different type of derivative than the other amino acids. The TFECF-TFEtOH derivatives of the amino acids were found to give the best separation properties among the six derivatives prepared in this study, and enabled a faster programming rate of the column temperature, resulting in elution of all amino acids within 31 min. The elution time is almost the same as found for the N(O)-perfluoroacyl alkyl esters on the same phase. Fig. 4 shows the GC peak-area ratios of

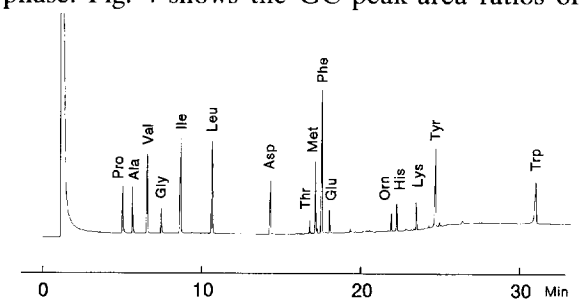

Fig. 3. Gas chromatogram of N(O)-2,2,2-trifluoroethoxycarbonyl 2′,2′,2′-trifluoroethyl esters of an amino acid mixture prepared from the L-form. Conditions as in Fig. 2.

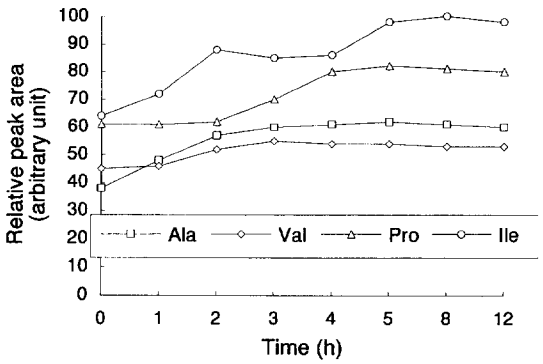

Fig. 4. Relationships between relative peak area and reaction time in derivatization of amino acids into N(O)-ethoxycarbonyl ethyl esters.

Ala, Val, Ile, and Pro relative to the internal standard methyl laurate, for derivatization with the ECF-EtOH combination, versus time after addition of the ECF and the extraction solvent chloroform, followed by GC injection. The reaction mixture was continuously shaken with a mechanical shaker. The four amino acids show a gradually increasing peak signal with the lapse of time, and a plateau was reached after approximately 5 h. Fig. 4 proves that the reaction of amino acids with chloroformates is not instantaneous or that the extraction of the derivatives with chloroform is not rapidly completed, as described in the literature [17]. The reaction mechanism of amino acids with alkyl chloroformates and alcohols might be more complex or slower in some processes than pointed out in the literature [17,19]. It can be assumed that the derivatization method is more suited to qualitative analysis, such as enantiomer separation, than to quantitative analysis.

4. Conclusions

Derivatives formed by the one-step derivatization of amino acids with alkyl chloroformates in combination with various alcohols have been used in enantiomer separation by chiral-phase capillary GC. Use of derivatives of TFECF combined with TFEtOH has resulted in a considerable reduction in retention times without a

decrease in the separation factors, which made the GC enantiomeric separation of amino acids fast, not only in the derivatization step but also in the GC run. The first half of the eluted amino acids were completely separated into their enantiomeric pairs, except for Pro, but still the second half of the eluted components were not completely resolved.

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