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GAS–LIQUID CHROMATOGRAPHY OF AMINO ACID DERIVATIVES

THE EFFECT OF SUBSTITUENTS*

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SUMMARY

The effect of the substituent groups in amino acid derivatives on their behavior in gas–liquid chromatography has been further investigated. As in our previous studies on glycine derivatives, in this work leucine and alanine were used as standard units. Acyl esters of leucine and alanine have been synthesized and chromatographed on both polar and non-polar columns at isothermal temperatures. The additive effects of the substituent groups on the N and C terminals of the amino acid were confirmed in the leucine series, which showed that the sum of the slopes of N-acylleucine methyl esters and of N-acetylleucine alkyl esters equaled the slope of N-acylleucine alkyl esters in graphs of the logarithm of the retention time *versus* the number of carbon atoms in the substituent. The same effects were observed for the alanine series.

In variations of the substituent R of the amino acid with the substitutions at N and C terminals of the amino acid derivatives, the sum of the slopes of the three monosubstituted acylamino acid esters: acylalanine methyl esters, acetylalanine alkyl esters and acetylamino acid methyl esters, equals the slope of the trisubstituted acylamino acid alkyl esters. The result appears to confirm further the additive effect of the substituting groups of amino acid derivatives.

An alternative expression of the effect of the substituent groups is the differential of the logarithm of the retention times of any two adjacent members of a series, $\log C_{x+1} - \log C_x = \Delta C$, where ΔC is a constant. ΔC is affected little by the nature of the column or by other factors under the experimental conditions used, with the exception of the column temperature.

INTRODUCTION

In a recent communication[1], we reported the gas–liquid chromatography (GLC) of N-acylamino acid alkyl esters, showing that the elution patterns of twenty-

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four amino acids on polar and non-polar columns are similar but not identical. It was found that the effect on the retention time of the N-acyl group and that of the alkyl ester are nearly identical, although the alkyl ester groups appeared to have slightly greater effects than the acyl groups. The plot of the logarithm of the retention time *versus* the number of carbon atoms in the substituent was found to be linear. The sum of the slope of the graph for N-acylglycine methyl esters and N-acetylglycine alkyl esters equals the slope of that for N-acylglycine alkyl esters, which demonstrates that the effects of the substituents on amino acid derivatives in GLC are additive.

The basic structure of the amino acid derivatives studied is as follows:

$$X_1\text{-CONH}\underset{\displaystyle R}{\underset{|}{C}}\text{HCOO-}X_2$$

For the glycine series studied, R = H and $X_1 = X_2$. It was not known, however, whether our observation was only of an isolated case for the glycine series or a more general phenomenon applicable to all amino acid series. We consequently chose leucine for the present investigation. Additionally, the chromatographic behavior of the N-acylalanine alkyl ester series was studied. The results appeared to be consistent with our earlier observations, and confirm our findings for the glycine series.

With these affirmative results, it became pertinent to study also molecules with three substituting groups. As shown in the above formula, R was varied with both the N and C terminal substitutions, *i.e.*, $R = X_1 = X_2$. Amino acid derivatives such as acetylalanine methyl ester ($R = X_1 = X_2 = CH_3$) and its homologs would fulfil the requirements. The identical substituents on all three terminals of the molecule by design would ensure simplicity in evaluating and calculating the effect on the chromatogram.

EXPERIMENTAL

Amino acids were of pure grade purchased from Takara Kohsan Co., Ltd., Tokyo, Japan, and from Sigma Co., St. Louis, Mo., U.S.A. They were all of the L-form and contained no other amino acid or nucleic acid detectable chromatographically in a 30-μg sample. Their purity was confirmed in the laboratory by paper chromatography in two solvent systems: water–formic acid–2-butanol (150:30:20) and 2-butanol–3 % ammonium hydroxide (150:60).

Compressed gases were obtained from Hong Kong Oxygen & Acetylene Co., Ltd., Hong Kong: N_2, 99.9991 %; H_2, 99.5 %; and air with oil and hydrocarbons filtered out.

The gas chromatograph was an F & M Model 5750 (Hewlett-Packard Co.) with dual coil columns, dual flame ionization detectors and a dual-pen recorder. Column A was a 1.83 m (6 ft.) × 3.2 mm ($^1/_8$ in.) O.D. stainless-steel tube, with 10 % Carbowax 20M on Chromosorb W, 60–70 mesh, acid washed and dimethylchlorosilane treated, pre-conditioned for 10 h at 225°. Column B was a 1.83 m (6 ft.) × 3.2 mm (1/8 in.) O.D. stainless-steel tube, with 1 % silicone gum GE XE-60 on Chromosorb W, 60–70 mesh, acid washed and dimethylchlorosilane treated, pre-conditioned as for column A. The column was later coated with hexamethyldisilazane by injecting 10 μl of 10 % of the silylation reagent in anhydrous hexane and again pre-conditioned

at 225° for 1 h. The gas flow-rates were 28, 32 and 280 ml/min (20, 30 and 100 mm on the gauges of the instrument) for H_2, N_2 and air, respectively.

The acylamino acid was made by acylating the amino acid with either the acid anhydride or acid chloride. Esterification was accomplished by treating the acylamino acid with the appropriate alcohol in dry benzene solution in the presence of Amberlite IR-120 (H)[1]. The amino acid was derivatized in 0.5–1-mg amounts. The N-acylamino acid ester was dissolved in 1 ml of either dry benzene or the alcohol in a calibrated volumetric tube (0.5–1.0 $\mu g/\mu l$), and was used immediately for chromatographic studies. A 5-μl Hamilton syringe was used and 0.5–2.0 μl of the solution was injected into the columns for measurements. The amount of the amino acid in the form of acyl alkyl ester was in the range 0.5–2.0 μg per determination. When required, the solution of N-acylamino acid ester was diluted to 5–30-fold for measurements at various concentrations; at no time was less than 0.5 μl used. The N-acylamino acid esters were chromatographed both individually and as a mixture under isothermal conditions.

For the column temperatures of 200° and less, the injection port and detector were set at 230° and 210°, respectively. The injection port and detector temperatures were set at 265° and 240°, respectively, when the column was operated at 225°.

RESULTS AND DISCUSSION

Commercially packed columns were chosen for this investigation on account of their availability and minimum variation in column characteristics owing to packing, composition of stationary and liquid phases, etc.

The aliphatic amino acids such as glycine, alanine, butyrine*[1–3], norvaline and norleucine were chosen on account of the increasing length of the side-chain in the amino acid derivatives. Symmetrical substitutions, $X_1 = X_2$ on the N and C terminals of the amino acid, were made; for example, the CH_3 of the acetyl and the CH_3 of the methyl ester in the N-acetyl amino acid methyl ester, which straddled the $-CONHCHCOO-$ unit, assumed a form of symmetry. The same symmetry applied to the C_2H_5 of *n*-propionyl and the C_2H_5 of the ethyl ester in N-*n*-propionylamino acid ethyl ester. The N-acylating groups varied from acetyl to enanthyl and the esters from methyl to *n*-hexyl. As column B was coated with hexamethyldisilazane and as the amount of the acylamino acid esters used was very small, no asymmetry of peaks was observed in the chromatograms as found occasionally with a polar compound on a non-polar liquid phase column[4]. The process of a solute entering a solvent in GLC has been discussed[5,6].

The logarithm of the retention time of the acylamino acid esters was plotted against the number of carbon atoms in the substituting chains. As a linear plot was obtained, the slope of each series was thus obtained graphically. Alternately, a linear regression analysis was made on an IBM 1130 computer. The slope and the intersection were obtained with a correlation coefficient that was at or above 0.970 in all of the cases studied. If the first point of any series of measurements was

* The name butyrine is used for α-amino-*n*-butyric acid in this paper.

excluded, the correlation coefficient was 0.990 and above. The values obtained between the graphical method and the regression analysis were within a range no greater than 3%.

The leucine and alanine series

The GLC characteristics of N-acyl and alkyl esters of glycine were studied[1]. To replace the glycine, leucine was chosen as the standard unit in the present studies. N-Acylleucine methyl esters, N-acetylleucine alkyl esters and N-acylleucine alkyl esters were prepared and chromatographed on both the polar and non-polar columns at isothermal temperatures. The effect of substitutions on the N and C terminals of the amino acids was demonstrated in the plots of the logarithm of the retention times *versus* the number of carbon atoms in the substituted chains. By varying the acyl ester groups of acylleucine alkyl esters from one to six carbon atoms, the retention time was systematically prolonged in geometrical proportion to the number of carbon atoms. The effect of the alkyl ester appeared consistently to be slightly greater than that of the acyl, as observed in the glycine series. However, in linear regression analysis, the *T*-test for comparison of the two sets of slopes showed that the difference was not significant at the 0.05 level. The effects of N-acyl derivatives and alkyl esters of leucine on the retention times are shown in Tables I, II and III. The slopes of the graphs for N-acylleucine methyl esters plus the slopes of the graphs for N-acetylleucine alkyl esters equaled the slopes of the graphs for N-acylleucine alkyl esters at all temperatures, as shown in Table IV, which demonstrated again that the effects of the substituents X_1 and X_2 are additive.

TABLE I

RETENTION TIMES (min) OF N-ACYLLEUCINE METHYL ESTERS

N-Acyl group	*Column A*			*Column B*			*Ester group*
	165°	*200°*	*225°*	*150°*	*165°*	*200°*	
Acetyl	25.2	8.2	3.0	4.4	2.4	0.9	Methyl
Propionyl	25.2	8.2	3.0	5.0	2.8	1.1	Methyl
n-Butyryl	32.6	10.0	3.6	7.4	3.8	1.3	Methyl
n-Valeryl	46.6	13.4	4.6	11.6	5.6	1.7	Methyl
n-Caproyl	67.6	18.2	6.0	18.4	8.2	2.2	Methyl
Enanthyl	100.0	25.2	7.8	31.6	13.0	3.0	Methyl

TABLE II

RETENTION TIMES (min) OF N-ACETYLLEUCINE ALKYL ESTERS

N-Acyl group	*Column A*			*Column B*			*Ester group*
	165°	*200°*	*225°*	*150°*	*165°*	*200°*	
Acetyl	25.2	8.2	3.0	4.4	2.4	0.9	Methyl
Acetyl	25.4	8.1	3.1	5.0	2.8	1.1	Ethyl
Acetyl	34.0	10.4	3.7	7.8	3.9	1.4	*n*-Propyl
Acetyl	47.6	13.7	4.6	11.8	5.5	1.8	*n*-Butyl
Acetyl	68.2	18.6	6.1	18.8	8.2	2.5	*n*-Amyl
Acetyl	99.6	25.6	8.0	29.6	12.0	3.6	*n*-Hexyl

TABLE III

RETENTION TIMES (min) OF N-ACYLLEUCINE ALKYL ESTERS

N-Acyl group	*Column A*			*Column B*			*Ester group*
	165°	*200°*	*225°*	*150°*	*165°*	*200°*	
Acetyl	26.2	8.2	3.0	4.4	2.4	0.9	Methyl
Propionyl	30.2	9.4	3.1	5.5	2.5	1.1	Ethyl
n-Butyryl	61.2	15.4	4.4	10.1	4.8	1.4	*n*-Propyl
n-Valeryl	106.0	25.3	7.1	25.2	8.8	2.2	*n*-Butyl
n-Caproyl		47.1	12.0	62.0	17.6	3.8	*n*-Amyl
Enanthyl		91.4	20.4	164.0	35.2	7.0	*n*-Hexyl

TABLE IV

SLOPES CALCULATED FROM THE LOGARITHM OF THE RETENTION TIME AGAINST THE NUMBER OF CARBON ATOMS IN THE SUBSTITUENTS

Esters	*Column A*			*Column B*		
	165°	*200°*	*225°*	*150°*	*165°*	*200°*
Acyl Gly methyl esters	0.152	0.138	0.123	0.160	0.150	0.120
Acetyl Gly alkyl esters	0.168	0.142	0.123	0.162	0.155	0.140
Acyl Gly alkyl esters	0.329	0.268	0.240	0.332	0.310	0.265
Acyl Ala methyl esters	0.158	0.135	0.111	0.154	0.141	0.115
Acetyl Ala alkyl esters	0.163	0.140	0.112	0.166	0.152	0.122
Acyl Ala alkyl esters	0.322	0.283	0.240	0.336	0.312	0.256
Acyl Leu methyl esters	0.152	0.128	0.118	0.196	0.150	0.130
Acetyl Leu alkyl esters	0.162	0.132	0.118	0.196	0.160	0.137
Acyl Leu alkyl esters	0.313	0.262	0.234	0.397	0.308	0.264

The present investigation of the leucine derivatives has substantiated our previous observation on the glycine series. The results invariably showed that the effects of substitutions on the N and C terminals of the amino acid are additive, as in the glycine series, and that the effect of the alkyl ester group is consistently larger than that of the acyl group, indicating slightly higher polarity, although the difference is insignificant statistically. If the slopes of the graphs for the leucine series are compared in juxtaposition with those of the glycine series, as shown in Table IV, the additive nature of the slopes at various temperatures for both the glycine and leucine series is apparent. At a particular temperature, namely 165° for both columns, the slopes in both series are also comparable. The only exception to this pattern is column B at 150°, for which the reason is not yet established.

In Table IV are also shown the slopes of the graphs for acylalanine alkyl esters, obtained from Tables V, VI and VII. The additive nature of the effects of the substituting groups on both the N and C terminals confirms further our observations on the glycine series reported earlier[1] and those of the leucine series discussed above. At one particular temperature, the slopes seem to be confined in a very narrow range for all of the monosubstituted acyl esters of glycine, alanine and leucine. For

TABLE V

RETENTION TIMES (min) OF N-ACYLALANINE METHYL ESTERS

N-Acyl group	*Column A*			*Column B*			*Ester group*
	165°	*200°*	*225°*	*150°*	*165°*	*200°*	
Acetyl	15.2	5.0	2.4	1.6	1.2	0.8	Methyl
Propionyl	16.2	5.3	2.5	1.9	1.4	0.8	Methyl
n-Butyryl	21.0	6.6	2.9	2.5	1.7	1.0	Methyl
n-Valeryl	30.6	9.1	3.9	3.6	2.5	1.3	Methyl
n-Caproyl	45.6	12.6	5.1	5.2	3.5	1.7	Methyl
Enanthyl	67.7	17.4	6.8	7.8	5.0	2.3	Methyl

TABLE VI

RETENTION TIMES (min) OF N-ACETYLALANINE ALKYL ESTERS

N-Acyl group	*Column A*			*Column B*			*Ester group*
	165°	*200°*	*225°*	*150°*	*165°*	*200°*	
Acetyl	15.2	5.0	2.4	1.6	1.2	0.8	Methyl
Acetyl	16.6	5.4	2.5	1.9	1.3	0.8	Ethyl
Acetyl	23.4	7.1	3.1	2.9	1.9	1.1	*n*-Propyl
Acetyl	34.7	9.8	3.9	4.1	2.6	1.4	*n*-Butyl
Acetyl	50.0	13.2	5.1	6.0	3.7	1.9	*n*-Amyl
Acetyl	73.8	18.6	6.8	8.8	5.2	2.5	*n*-Hexyl

TABLE VII

RETENTION TIMES (min) OF N-ACYLALANINE ALKYL ESTERS

N-Acyl group	*Column A*			*Column B*			*Ester group*
	165°	*200°*	*225°*	*150°*	*165°*	*200°*	
Acetyl	15.2	5.0	2.4	1.6	1.2	0.8	Methyl
n-Propionyl	18.4	5.7	2.4	2.4	1.7	1.0	Ethyl
n-Butyryl	32.3	9.1	3.6	4.2	2.8	1.4	*n*-Propyl
n-Valeryl	67.6	17.8	6.2	9.2	5.5	2.6	*n*-Butyl
n-Caproyl	140.0	32.5	11.0	19.4	11.2	4.7	*n*-Amyl
Enanthyl	295.0	62.6	19.6	42.6	23.2	8.6	*n*-Hexyl

example, at 165° in column A, the slopes of the graphs for all of the monosubstituted acylamino acid esters are around 0.160, while under the same conditions in column B the value is 0.153. Any effect due to the polarity of the columns is very small. The average slopes of the graphs for the disubstituted derivatives of glycine, alanine and leucine are 0.320 and 0.310 for columns A and B, respectively. The slopes on both columns decrease in the same quantitative manner as the column temperature rises. For example, the slopes of the graphs for acylleucine alkyl esters given in Table IV are 0.313, 0.262 and 0.234 for 165°, 200° and 225°, respectively. Fig. 1 illustrates further that the slopes of the graphs for acetylleucine alkyl esters in both columns A and B are parallel at 165° as well as at 200°. That is, for the

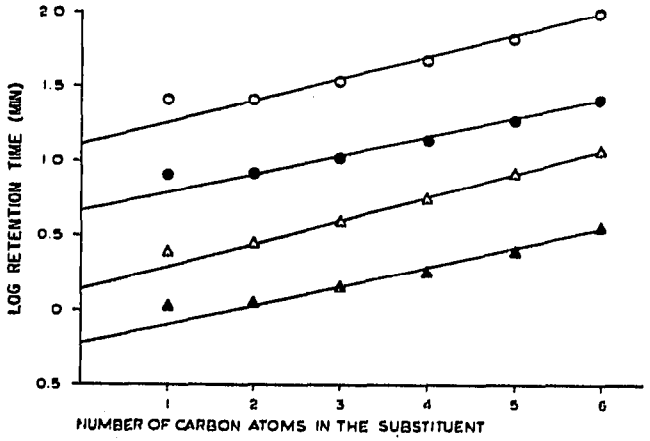


Fig. 1. Gas–liquid chromatograms of acetylleucine alkyl esters. At 165°, ○—○ and △—△ for columns A and B, respectively. At 200°, ●—● and ▲—▲ for columns A and B, respectively

same series of acylamino acid esters, the slopes are in parallel in both columns at a given temperature, which seems to indicate that the relative effect of a particular substituting group is constant, irrespective of the nature of the columns. The intercepts on the axis by the parallel slopes, however, are markedly distinct, as the plot for acetylleucine alkyl esters at 165° intersects at 1.11 on column A and at 0.13 on column B. The intercept can be regarded as an expression of the nature of the column. If the column and temperature are made constant, such as on column A at 165°, the graphs for the acetyl alkyl esters of glycine, alanine and leucine have intercepts on the axis of 1.12, 0.89 and 1.10, respectively.

The trisubstituted amino acid series

The results for the glycine, alanine and leucine derivatives studied have demonstrated that the chain-length on both N and C terminal substitutions of an amino acid dictates the retention time in a quantitatively additive manner. All of the amino acid derivatives examined so far have the type of molecular structure shown in the introduction, where R is H, CH_3 and $(CH_3)_2CHCH_2$ for the glycine, alanine and leucine series, respectively. It was of interest to examine whether the variations in R would result in the same additive property shown by the disubstituted derivatives if R were varied so that the substituents at the N and C terminals are the same. The alanine series would provide the required structural variation. The variation of R with the substitution at the N and C terminals of the amino acid derivatives would assure simplicity in evaluating and calculating the effects on the chromatogram, as $R = X_1 = X_2$. The substituents would all be methyl, ethyl, *n*-propyl, etc. The trisubstituted derivatives were synthesized and their chromatographic pattern and retention times determined as shown in Table VIII. The chromatograms of the acyl group and the ester group, varied independently, were obtained as shown in Tables V and VI, from which the slopes of the graphs were calculated (Table IV). By variation of R in the amino acid, the acetyl methyl esters of glycine, alanine, butyrine, norvaline, norleucine, α-aminoenanthic acid and α-amino-*n*-caprylic acid were synthesized and their chromatographic patterns were measured, as shown

TABLE VIII

RETENTION TIMES (min) OF N-ACYLAMINO ACID ALKYL ESTERS

N-Acyl group	*Amino acid*	*Ester group*	*Column A*			*Column B*
			165°	*200°*	*225°*	*165°*
Acetyl	Ala	Methyl	15.2	5.0	2.4	1.2
Propionyl	But	Ethyl	20.0	6.8	2.8	1.9
n-Butyryl	norVal	*n*-Propyl	51.2	13.0	4.9	4.4
n-Valeryl	norLeu	*n*-Butyl	137.6	29.0	9.9	11.4
n-Caproyl	α-Aminoenanthic	*n*-Amyl	370.0	71.0	21.8	28.2
Enanthyl	α-Aminocaprylic	*n*-Hexyl		183.2	49.4	73.6

TABLE IX

RETENTION TIMES (min) OF N-ACETYLAMINO ACID METHYL ESTERS

Amino acid	*Column A*			*Column B*
	165°	*200°*	*225°*	*165°*
Gly	28.8	8.8	2.4	1.5
Ala	15.2	5.0	2.8	1.2
But	18.0	5.7	3.4	1.4
norVal	25.3	7.4	3.8	1.8
norLeu	35.6	9.8	4.3	2.6
α-Aminoenanthic	52.1	13.4	5.6	3.7
α-Aminocaprylic	78.2	18.6	7.5	5.4

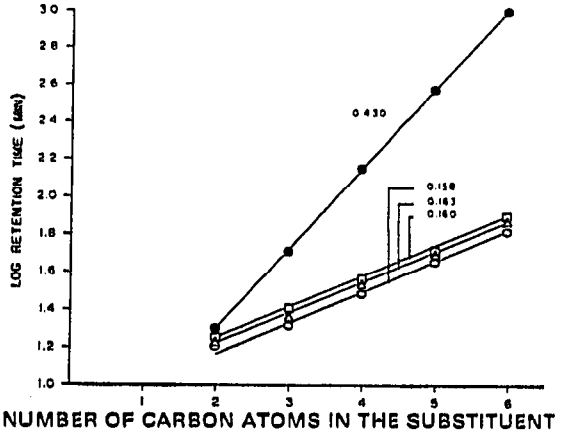
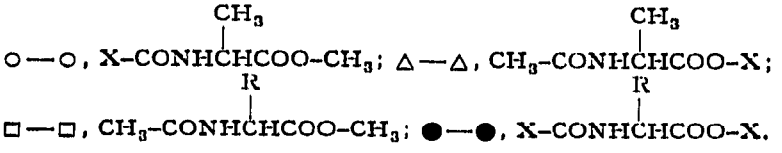


Fig. 2. Logarithm of retention time *versus* number of carbon atoms in the substituent on column A at 165°.



in Table IX. Variation in R produced slopes of the graphs at different temperatures that are comparable with those of all the other monosubstituted derivatives investigated so far. To illustrate the relationship between the monosubstituted and the trisubstituted amino acid derivatives, an example is given in Fig. 2 for the four series studied in column A at 165°. The sum of the slopes of the graphs for the acylalanine methyl esters (0.158), acetylalanine alkyl esters (0.161) and acetylamino acid methyl esters (0.160) is 0.479. The slope of the graph for the acylamino acid alkyl esters (the trisubstituted series) is found to be 0.430 under identical experimental conditions. This result appears to confirm further the additive nature of the substituting groups on amino acid derivatives.

Alternately, the effect of the substitutions can be expressed in terms of the retention time of two adjacent members of a given series of amino acid derivatives, *i.e.*, $\log C_{x+1} - \log C_x = \Delta C$, where ΔC is a constant. For example, at 165° on column A, the enanthylleucine methyl ester and *n*-caproylleucine methyl ester of the acylleucine methyl ester series (Table I) gave a ΔC value of 0.160, which actually is the slope given in Table IV. Thus, $C_{x+1}/C_x = 1.45$. The higher the column temperature, the smaller is the ratio. For the same pair of leucine derivatives, the ratios are 1.36 and 1.31 at 200° and 225°, respectively. That is, the contribution per carbon–carbon bond to the retention time in GLC is a constant under a given set of conditions for any set of amino acid derivatives. The contribution of a carbon–carbon bond of the substituting group is affected little by the nature of the column or the nature of the amino acid. The most prominent factor that affects this constant is the column temperature.

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