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SEPARATION OF ENANTIOMERS ON PACKED COLUMNS CONTAINING OPTICALLY ACTIVE DIAMIDE PHASES

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SUMMARY

Chromatography of fourteen protein amino acids has been studied on 2–4-m long packed columns containing chiral diamide phases. Twelve of the thirteen optically active compounds examined could be resolved, with $R \geqslant 1$ in many instances. Two of the phases, N-docosanoyl-L-valine *tert.*-butylamide and N-lauroyl-L-valine 2-methyl-2-heptadecylamide, can be employed at temperatures as high as 190° and 180°, respectively, without losing their efficiency, even after prolonged use. The problem of peak overlap in the analysis of mixtures of different amino acids was examined and partially solved.

INTRODUCTION

The resolution of optically active compounds and, in particular, of α-amino acids by gas chromatography on capillary columns coated with chiral phases is a well established procedure. For some time we have carried out further work on this approach with the object of employing the more readily handled packed columns for the same purpose. Much progress has been made towards solving the problems involved, as described in some preliminary communications[1,2]. We wish now to report more fully on the status of this research.

It should be recalled that in 1967 some of the present authors[3] demonstrated on alanine that the dipeptidic phase, N-TFA-L-valyl-L-valine cyclohexyl ester (where TFA = trifluoroacetyl), is efficient enough to bring about resolution on a 2-m long column, provided that the N-TFA-O-*tert.*-butyl ester is used; unfortunately, such esters of the amino acids are not readily prepared.

Details of a highly efficient diamide phase, N-lauroyl-L-valine *tert.*-butylamide (I), were published by Feibush in 1971[4]. Subsequently, a study on the influence of structural factors on the selectivity of chiral solvents of formula RCONHCH(iPr)CONHR′ (where iPr = isopropyl) was carried out by Beitler and Feibush[7]. The high selectivity of these phases and the large number of plates that can be obtained when they are used seemed to make them suitable for resolution on packed columns. The above two workers showed that the overall highest selectivity is obtained for diamides that have an unbranched R group and a tertiary R′ radical. In fact, the first chiral diamide phase (I) reported in 1971[4] was found to be more efficient than any of those synthesized

subsequently. However, I suffers from relatively high column bleeding, which limits the working temperature to 130–140°. In order to overcome this disadvantage, we prepared the following higher homologues of I: N-docosanoyl-L-valine *tert.*-butylamide (II) and N-lauroyl-L-valine 2-methyl-2-heptadecylamide (III). These two materials show no loss of weight by thermogravimetric analysis (TGA) until 190° and 180°, respectively. Their melting points are also convenient, being 85–86° and 47–48°, respectively. We have examined the behaviour of 14 protein amino acids on short columns (2–4 m) containing the diamides I–III.

EXPERIMENTAL

Materials

The N-TFA-amino acid esters were synthesized as described previously[5]. Phase I was supplied by Miles-Yeda, Rehovot, Israel; the other phases were prepared according to the procedures given below.

N-Docosanoyl-L-valine tert.-butylamide (II). Docosanoic (behenic) acid (10.2 g) in 100 ml of dry dioxane was cooled in an ice–water bath; N-hydroxysuccinimide (3.45 g) and N,N'-dicyclohexylcarbodiimide (6.18 g) were added successively and the mixture was stirred for 48 h (ref. 6). The reaction mass was then filtered, the dioxane distilled off and the residue dissolved in diethyl ether and re-filtered. Evaporation of the ether left a white solid, which was stirred overnight with light petroleum (b.p. 40–60°). The docosanoate of N-hydroxysuccinimide (3.5 g) was obtained in 27% yield; m.p. 91–94°; the nuclear magnetic resonance (NMR) spectrum agreed with the expected structure.

The above active ester (2.5 g) was condensed with L-valine *tert.*-butyalmide[7] (1 g) in the presence of triethylamine (0.63 g) in 100 ml of dry chloroform[6]. The mixture was first stirred in an ice–water bath and then at room temperature for 48 h, treated with active charcoal, filtered and the solvent distilled off. The remaining solid was dissolved in diethyl ether and filtered. The ether solution was washed successively with 2% hydrochloric acid, water, 5% sodium hydrogencarbonate solution, again with water, dried over sodium sulphate and filtered. Evaporation of the ether left a solid (4.5 g), which was chromatographed on silica gel (containing 6% of water) with ethyl acetate–*n*-hexane (15:85) as the eluent. The resulting N-docosanoyl-L-valine *tert.*-butylamide (II) (0.9 g) had an NMR spectrum that agreed with the expected structure; m.p. 85–86°. Elemental analysis: found: C, 74.94; H, 12.48; N, 6.07%; calculated for $C_{31}H_{62}N_2O_2$: C, 75.24; H, 12.63; N, 5.66%. TGA showed that II does not lose any material at temperatures up to 190°.

The optical purity of II was found to be 89% by hydrolysis, derivatization of the valine formed and gas chromatography on a chiral phase.

N-Lauroyl-L-valine 2-methyl-2-heptadecylamide (III). 2-Methyl-2-heptadecanol was obtained by the action of methylmagnesium iodide on methyl palmitate; yield 90%; m.p. 36–38° [ref. 8, 36°]. The alcohol (45 g) was refluxed for 3 h with an excess of acetyl chloride (20 ml). The 2-chloro-2-methylheptadecane obtained was washed with 90 ml of 5% sodium hydrogen carbonate solution; yield 93%; b.p. 118–122°/0.2 mm. To a solution of the chloroalkane (44 g) in 400 ml of absolute ethanol, 60 ml of liquid ammonia was added in an autoclave. The mixture was stirred at 120° for 1 week. The product was filtered, the ethanol distilled off in a rotatory evaporator,

the residue dissolved in diethyl ether, filtered, the solid re-dissolved in chloroform and refiltered. Evaporation of the chloroform gave 2-methyl-2-heptadecylamine hydrochloride (3.5 g); m.p. 106–110°; yield 7%; 34 g of the chloroalkane were recovered unchanged.

In the final step of the synthesis[6], the hydrochloride (3.5 g) in 300 ml of dry chloroform was cooled in an ice–water bath and a solution of the N-lauroyl-L-valine derivative of N-hydroxysuccinimide[7] in 150 ml of chloroform and triethylamine (2.44 g) was added, the reaction mixture being stirred for 48 h. The product was treated with active charcoal, filtered and the chloroform distilled off. The remaining solid was worked up as for II, followed by chromatography on silica gel (containing 6% of water) with ethyl acetate–*n*-hexane (1:9) as the eluent. The final product, N-lauroyl-L-valine 2-methyl-2-heptadecylamide (III), had m.p. 47–48° and its infrared (IR) and NMR spectra were in agreement with the expected structure. Elementary analysis: found: C, 76.11; H, 12.30; N, 4.96%; calculated for $C_{35}H_{70}N_2O_2$: C, 76.3; H, 12.81; N, 5.09%. TGA showed that III could be used up to 180° as a gas chromatographic stationary phase. By hydrolysis of III and enantiomeric analysis of the valine formed, the optically purity of the synthetic product was found to be 83%.

Chromatographic conditions

A Hewlett-Packard 7626A research chromatograph equipped with a dual flame ionization detector was used. Aluminium tubing of length 1.9–4 m and I.D. 2 mm was used for the columns, which were filled with Chromosorb W AW or P AW DMCS, coated with 10% of phase. Helium pressure was 60 p.s.i. More details of the chromatographic conditions are given in the tables and the legends of the figures.

RESULTS AND DISCUSSION

The very high selectivity of I–III for most of the N-TFA-α-amino acid methyl and isopropyl esters is shown in Tables I and II. The order of emergence is the same as found for all other diamide[7] and dipeptide[5,9] phases, namely $r_{L/D} \geq 1$, where $r_{L/D}$ is the ratio of the corrected retention volume of the L-isomer to that of the D-isomer. It should be noted that the actual figures for the selectivity of the pure chiral solvents are higher than those listed, because of the occurrence of some inversion during their synthesis (see Experimental)[7]. It is remarkable that, as for the dipeptide type of phases[5], racemization on the column is very slow, and a noticeable decrease in efficiency was observed (for phase III) only after more than 18 months of extensive use (decrease to 60% optical purity).

The separation of the enantiomers of aspartic acid and proline on packed columns is difficult. Whereas the former can be partially resolved at relatively low temperatures, requiring long retention times, at best only a shoulder could be observed for proline. Low resolution factors for these two amino acids have also been reported for the dipeptide type of phases using capillary columns[5]. The lack of an NH group in the N-TFA proline ester and the presence of the additional possibility of association with the solvent via the β-carboxylic group of aspartic acid permits an interpretation of this behaviour. A detailed explanation of the mechanistic reasons for the low coefficient of resolution for the aspartic acid derivatives on diamides will be presented elsewhere[7].

TABLE I

SEPARATION OF THE ENANTIOMERS OF N-TFA METHYL ESTERS OF PROTEIN AMINO ACIDS

Retention times and resolution factors for N-trifluoroacetyl methyl esters on packed columns containing chiral diamide phases.

N-TFA methyl ester of	*Enantiomer*	*Column I**			*Column II***			*Column III****		
		Temperature (°C)	*Corr. retention time (min)*	$r_{L/D}$	*Temperature (°C)*	*Corr. retention time (min)*	$r_{L/D}$	*Temperature (°C)*	*Corr. retention time (min)*	$r_{L/D}$
Ala	D	110	9.05	1.173	120	4.80	1.183	130	8.32	1.096
	L		11.15			5.68			9.12	
Thr	D		17.28	1.102		7.95	1.126		11.55	1.077
	L		19.04			8.96			12.45	
Val	D		14.32	1.145		7.76	1.148		11.84	1.071
	L		16.40			8.91			12.69	
Gly			18.80	–		9.04	–		17.36	–
*allo*Ile	D		22.91	1.165		12.56	1.167		18.08	1.076
	L		(26.68)§			14.67			19.45	
Ile	D		(25.12)§	1.143		13.55	1.145		19.45	1.081
	L		28.72			15.52			21.04	

Ser	D		40.88	1.095		16.96	1.108		—	
	L		44.76			18.80				
Leu	D		39.68	1.256		18.75	1.269		26.24	1.167
	L		49.84			23.80			30.64	
Pro	D		32.88	1.039		20.73	1.000		40.80	1.000
	L		34.17	(shoulder)						
Asp	D	140[§§]	27.04	1.038	180	7.52	1.000	170	17.46	1.000
	L		28.08	(shoulder)						
Met	D		71.44	1.135		17.52	1.079		38.67	1.070
	L		81.15			18.91			41.39	
Glu	D		70.00	1.107		16.06	1.064		38.67	1.044
	L		77.52			17.10			40.37	
Phe	D		100.16	1.124		24.75	1.064		52.96	1.052
	L		112.40			26.35			55.76	

[*] Column I: 4 m × 2 mm I.D., containing 60–80 mesh Chromosorb W AW, coated with 10% N-lauroyl-L-valine *tert.*-butylamide (I).

[**] Column II: 3 m × 2 mm I.D., containing 80–100 mesh Chromosorb P AW DMCS, coated with 10% N-docosanoyl-L-valine *tert.*-butylamide (II).

[***] Column III: 3 m × 2 mm I.D., containing 80–100 mesh Chromosorb P AW DMCS, coated with 10% N-lauroyl-L-valine 2-methyl-2-heptadecylamide (III).

[§] These two peaks overlapped partially.

[§§] Maximum permissible temperature.

TABLE II

SEPARATION OF THE ENANTIOMERS OF N-TFA ISOPROPYL ESTERS OF PROTEIN AMINO ACIDS

Retention times and resolution factors for N-trifluoroacetyl isopropyl esters on packed columns containing chiral diamide phases. Columns as in Table I.

N-TFA isopropyl ester of	*Enantiomer*	*Column I*			*Column II*			*Column III*		
		Temperature (°C)	*Corr. retention time (min)*	$r_{L/D}$	*Temperature (°C)*	*Corr. retention time (min)*	$r_{L/D}$	*Temperature (°C)*	*Corr. retention time (min)*	$r_{L/D}$
Ala	D	120	11.52	1.187	130	6.69	1.196	130	12.05	1.138
	L		13.68			8.00			13.71	
Thr	D		18.35	1.133		8.86	1.128		16.00	1.102
	L		20.80			10.00			17.63	
Val	D		17.41	1.162		10.80	1.167		18.27	1.096
	L		20.24			12.60			20.03	
Gly	–		24.32	–		12.16	–		23.52	–
*allo*Ile	D		25.75	1.174		16.26	1.176		26.75	1.098
	L		30.24			19.12			29.36	

Leu	D		39.76	1.257		22.08	1.273		36.01	1.212
	L		50.00			28.11			43.65	
Pro	D		39.44	1.022		28.16	1.000		54.88	1.000
	L		40.32	(shoulder)						
Asp	D	140	46.96	1.037	190	11.36	1.000	180	23.20	1.000
	L		48.72							
Met	D		75.68	1.160		17.15	1.077		35.16	1.084
	L		87.84			18.48			38.14	
Glu	D		104.56	1.132		21.92	1.065		46.08	1.066
	L		125.60			23.36			49.12	
Phe	D		110.98	1.147		24.91	1.066		50.72	1.056
	L		127.36			26.56			53.57	
Orn	D					77.52	1.098		170.72	1.103
	L					85.12			184.40	
Lys	D					108.88	1.080		244.80	1.083
	L					117.60			265.28	

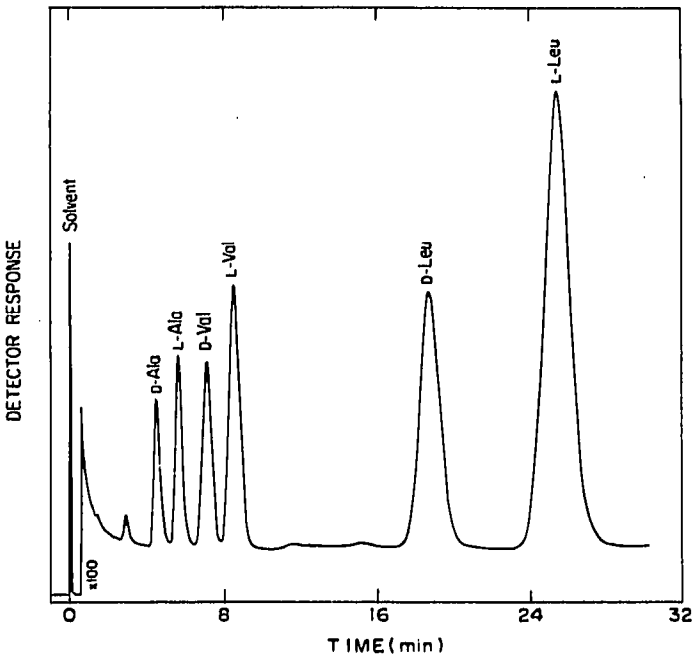

Fig. 1. Chromatogram of the N-TFA methyl esters of alanine, valine and leucine (enriched in the L-isomer). Chromatographic conditions: column, 1.9 m × 4 mm I.D., containing 60–80 mesh Chromosorb W AW coated with 10% of phase I; temperature, 110°.

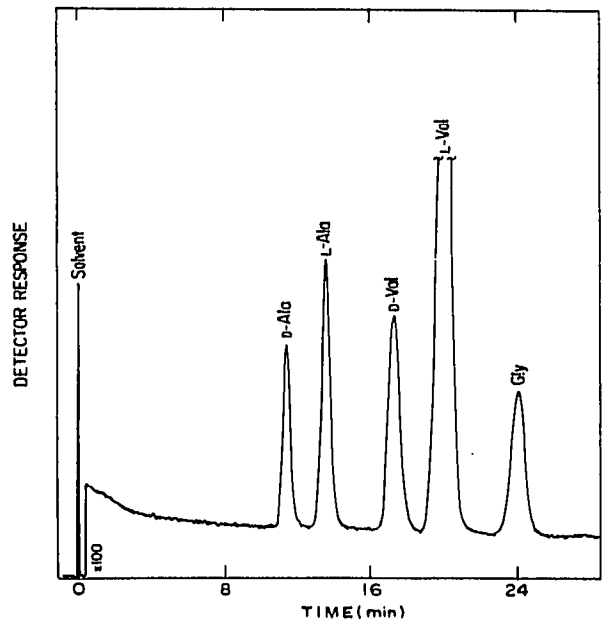

Fig. 2. Chromatogram of the N-TFA isopropyl esters of alanine, valine (enriched in the L-isomer) and of glycine. Chromatographic conditions: column, 4 m × 2 mm I.D., containing 60–80 mesh Chromosorb W AW coated with 10% of phase I; temperature, 120°.

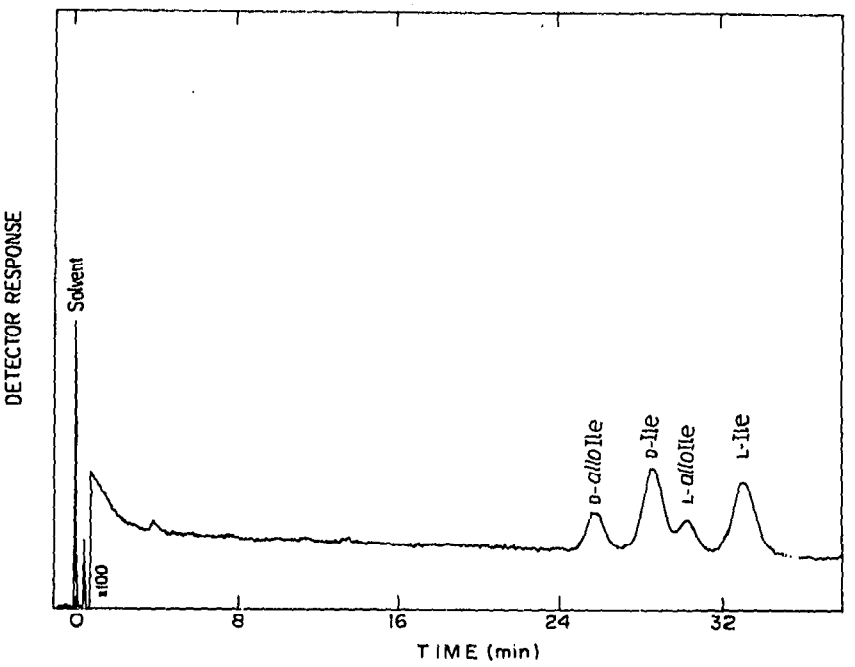

Fig. 3. Chromatogram of the N-TFA isopropyl esters of *allo*isoleucine and isoleucine. Chromatographic conditions as in Fig. 2.

*N-Lauroyl-*L*-valine tert.-butylamide (I)*

On phase I, alanine, valine, glycine, *allo*isoleucine, isoleucine and leucine can be separated from each other and simultaneously resolved, both in the form of their N-TFA-O-methyl and of their N-TFA-O-isopropyl esters (see Tables I and II). The examples given in Figs. 1–3 show the surprisingly good peak resolution that can be obtained for most of these compounds. Only *allo*isoleucine and isoleucine do not show baseline resolution for two of the peaks (Fig. 3). The N,O-di-TFA-threonine methyl esters are well resolved ($r_{L/D}$ = 1.101 at 110°), but overlap with the L-valine and glycine derivatives. Replacement of methyl with isopropyl increases the resolution factor ($r_{L/D}$ = 1.133 at 110°), but there is still overlap with the corresponding valine esters. Di-TFA-D-serine-O-methyl ester overlaps with the methyl ester of D-leucine. Other overlaps are those of the (non-resolved) N-TFA-proline and D-leucine isopropyl esters, of the methyl esters of methionine and glutamic acid, and of the isopropyl derivatives of glutamic acid and phenylalanine.

The N-TFA isopropyl ester of aspartic acid was partially resolved (R = 0.93), when operating at 130° with a flow-rate of helium of 30 ml/min); corrected retention times were 82.2 and 86.5 min for the D- and the L-isomer, respectively*. Phase I cannot be used above *ca.* 140°.

*N-Docosanoyl-*L*-valine tert.-butylamide (II)*

There is considerable analogy between the behaviour of phases I and II, but

* We are indebted to Dr. T. Hobo, University of Maryland, College Park, Md., U.S.A., for this determination on a 3 m × 2 mm I.D. glass column filled with 100–120 mesh Chromosorb W AW coated with 10% of I.

the occurrence of peak overlap has a different pattern. Thus, there is a larger difference between the retention times of the isopropyl esters of threonine and of valine, whereas glycine and L-valine derivatives overlap considerably. It should be noted that the temperatures at which phase II was examined were different than those for I, particularly for methionine, glutamic acid and phenylalanine. An important property of II is its low volatility, which permits operation at high temperature, and thus the ready resolution of derivatives with very long retention times, such as the di-TFA esters of ornithine and lysine (Fig. 4) ($r_{L/D}$ values of 1.098 and 1.080, respectively; Table II, 190°).

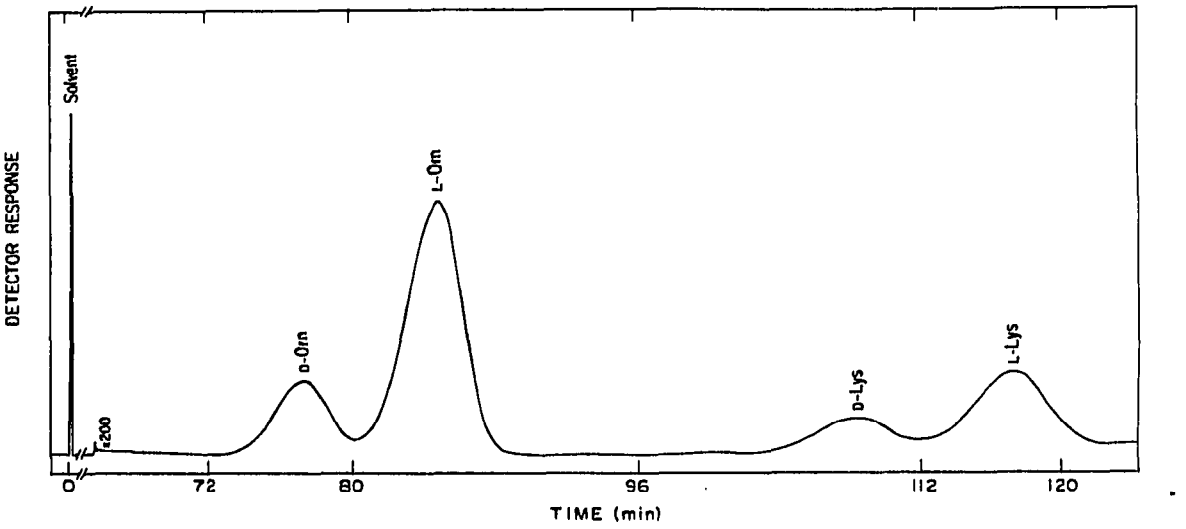

Fig. 4. Chromatogram of the di-TFA isopropyl esters of ornithine and lysine. Chromatographic conditions: column, 3 m × 2 mm I.D., containing 80–100 mesh Chromosorb P AW DMCS coated with phase II; temperature, 190°.

*N-Lauroyl-*L*-valine-2-methyl-2-heptadecylamide (III)*

As for II, the main feature of phase III is the extension of the maximum permissible operating temperature (to about 180°). For the more volatile amino acids, it can be seen in Tables I and II that glycine is well separated from valine, and proline (non-resolved) from leucine, in both the methyl and the isopropyl ester series. The *allo*isoleucine–isoleucine group shows, however, only three peaks and the threonine enantiomers overlap with those of valine.

For the less volatile amino acids, the methyl esters of D-methionine and D-glutamic acid have identical retention times at 130° (Table I), whereas in the isopropyl ester series overlap occurs between L-glutamic acid and D-phenylalanine.

CONCLUSIONS

The diamide phases I–III permit the resolution on packed columns of twelve of the thirteen optically active protein amino acids studied; however, the separation of the enantiomers of proline still requires the use of capillaries.

The problem of peak overlap with different amino acids can be tackled in several ways. Chromatography of various derivatives on the same phase is one possibility. Thus, on phase III the isopropyl esters of D,L-methionine, D-glutamic acid and L-phenylalanine are completely resolved, while L-glutamic acid and D-phenylalanine overlap. The relative amounts of these overlapping peaks can be calculated by chromatographing, in addition, the methyl esters for which the enantiomers of phenylalanine are completely separated from those of the other two amino acids (see Table I).

The use of derivatives such as the esters of 3-pentanol or acylation with perfluorinated homologues of acetic anhydride might solve other problems of overlapping in an analogous way. Another possibility is to employ two or more different columns. Thus, on phase II, the *allo*isoleucine and isoleucine isopropyl esters are resolved into four peaks, but there overlapping occurs between L-valine and glycine. On the other

TABLE III

SEPARATION OF THE N-TRIFLUOROACETYL ISOPROPYL ESTERS OF PROTEIN AMINO ACIDS ON DUAL PACKED COLUMNS CONTAINING A CHIRAL AND AN ACHIRAL PHASE

The columns consisted of column III (see Table I, footnote), followed by a 3 m × 2 mm I.D. column containing 80–100 mesh Chromosorb P AW DMCS, coated with 10% SE-30.

N-TFA isopropyl ester of	*Enantiomer*	*Temperature (°C)*	*Corr. retention time (min)*
Ala	D	130	23.40
	L		25.12
Thr	D		34.00
	L		35.60
Val	D		38.32
	L		41.04
Gly	—		39.36
*allo*Ile	D		52.00
	L		60.00
Ile	D		55.20
	L		62.64
Leu	D		70.24
	L		78.24
Pro	D, L		97.92
Asp	D, L	180	39.20
Met	D		59.97
	L		63.49
Glu	D		76.27
	L		80.19
Phe	D		85.97
	L		90.43

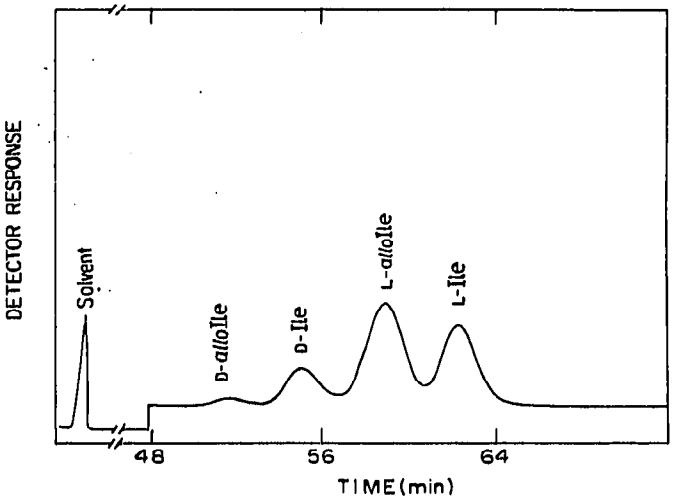

Fig. 5. Chromatogram of the N-TFA isopropyl esters of *allo*isoleucine and isoleucine. Chromatographic conditions: dual column consisting of (1) 3 m × 2 mm I.D. tube containing 80–100 mesh Chromosorb P AW DMCS coated with 10% phase III, and (2) 3 m × 2 mm I.D. tube containing 80–100 mesh Chromosorb P AW DMCS coated with 10% SE-30; temperature, 130°.

hand, this same mixture, when chromatographed on phase III, gives an excellent separation between valine and glycine isopropyl esters, but incomplete resolution of the *allo*isoleucine–isoleucine group of peaks (see Table II).

A further approach is the use of dual columns, the first of which contains the chiral phase III and the second the achiral phase SE-30. Such symmetric phases have been used successfully for the gas chromatography of amino acids (without enantiomer separation) by various workers, particularly Gehrke *et al.*[10]. This combina-

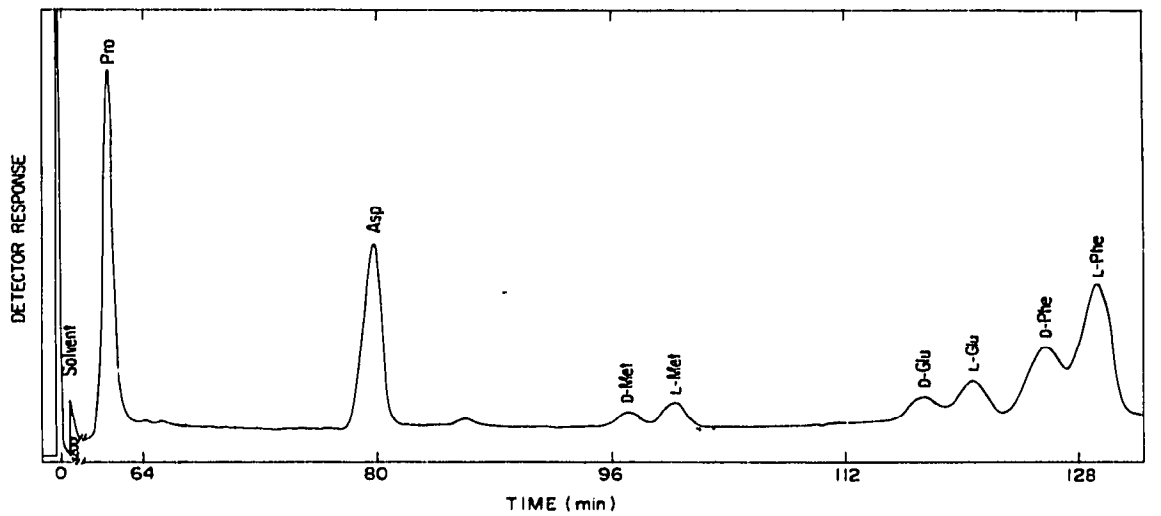

Fig. 6. Chromatogram of the N-TFA isopropyl esters of proline, aspartic acid, methionine, glutamic acid and phenylalanine (enriched in the L-isomer). Chromatographic conditions: columns as in Fig. 5; temperature, isothermal at 130° for 30 min, programmed at 1°/min up to 150° and then at 2°/min up to 180° (final temperature). Neither proline nor aspartic acid show any resolution under these conditions.

tion solved a number of problems of overlapping. Inspection of Table III shows that all of the compounds listed, with the exception of the L-valine–glycine pair, were readily separated from each other and all, except for proline and aspartic acid, were resolved. The good separation achieved for the *allo*isoleucine and isoleucine enantiomers is shown in Fig. 5. The degree of peak resolution obtained for methionine, glutamic acid and phenylalanine can be seen in Fig. 6. An even better resolution of these three amino acids can be achieved by modifying the rate of temperature programming[2].

The methods developed have already been used in our laboratory for the study of the configuration of the amino acids in a number of synthetic di- and tripeptides[11]. We have also supplied phase I to other laboratories, which have reported highly satisfactory results for the resolution of single amino acids[12,13].

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