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CHIRAL SEPARATION OF ENANTIOMERS OF SUBSTITUTED α- AND β-ALANINE AND γ-AMINOBUTYRIC ACID ANALOGUES BY GAS CHROMATOGRAPHY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Gas chromatography (GC) with a chiral stationary phase, Chirasil-Val, has been used for separation of the enantiomers of several analogues of α- and β-alanine as their N-trifluoroacetyl isopropyl esters. The same chiral phase GC procedure has been applied to the enantiomeric separation of various substituted γ-aminobutyric acid analogues (GABA). Reversed-phase high-performance liquid chromatography (HPLC) with the chiral copper–L-proline complex allowed a clear resolution of all the α-amino acids in their underivatized forms. It yielded somewhat smaller separation coefficients for the substituted β-alanines and no resolution for the GABA analogues. The influence of the nature of the amino acid, α, β or γ, and the effects of the different substituents on the separation coefficients obtained by GC and HPLC are discussed.

INTRODUCTION

Many α- or β-substituted amines and amino acids have recently been synthesized as specific enzyme inhibitors[1–3]. In view of the stereoselectivity of the enzymes, it is believed that usually only one of the enantiomers or stereoisomers possesses biological activity[4,5]. It was therefore of prime importance to develop analytical procedures for control of the enantiomeric purity of the various isomers obtained either by enantioselective synthesis or separation techniques.

The development of appropriate gas chromatographic (GC) and high-performance liquid chromatographic (HPLC) procedures for the separation of enantiomers has generated considerable interest and has been the subject of several recent reviews[6–8]. Among the various chiral phases proposed for the GC separation, the Chirasil-Val phase, with L-valine-*tert.*-butylamide linked to a polysiloxane matrix, developed by Frank *et al.*[9], has found the widest application. It allows a clear separation of most of the common amino acids after appropriate derivatization[10–12]. Of HPLC procedures proposed for the separation of enantiomers of amino acids, the ligand-exchange chromatography with chiral copper complexes in the mobile phase pro-

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posed by Hare and co-workers[13,14] allows the resolution of most of the natural amino acids without derivatization.

We have used this Chirasil-Val phase for the GC resolution of a series of substituted α-, β- and γ-amino acids, *i.e.*, alanine, β-alanine and 4-aminobutyric acid (GABA) analogues. The different factors governing the separation, *i.e.*, nature of the derivatizing agents, size and position of the substituent, distance between the two asymmetric carbons if two chiral centres are present, will be discussed. The GC results will be compared to those obtained by the HPLC procedure using the chiral eluent, L-proline/copper, with a C_{18} reversed-phase column.

EXPERIMENTAL

Materials

DL- and L-alanine (1,2) and β-alanine (18) were from Sigma (St Louis, MO, U.S.A.), DL-3-aminobutyric acid (19), 4-aminobutyric acid (27, GABA), L-proline, trifluoroacetic (TFAA), pentafluoropropionic (PFPA) and heptafluorobutyric (HFBA) anhydrides from Aldrich (Steinheim, F.R.G.). 3,3,3-Trifluoro-DL-alanine (7), the reagents used and the HPLC grade solvents were from E. Merck (Darmstadt, F.R.G.). The D-enantiomer of 3-fluoroalanine (4)[15] was a gift from Merck Sharp & Dohme (Rahway, NJ, U.S.A.). All the other amino acids were synthesized at our Centre. 3-Fluoroalanine (3)[15], 3-chloroalanine (5)[16] and 3,3-difluoroalanine (6)[17] were obtained as described.

α-Methylalanine(2-aminoisobutyric acid) (Me-Ala, 8)[18], α-cyanomethylalanine, (9)[18], α-isopropylalanine (10)[18], α-methoxymethylalanine (11)[18], α-monofluoromethylalanine (MFM-Ala, 12)[19], α-difluoromethylalanine (DFM-Ala, 13)[20], α-chloromethylalanine (MCM-Ala, 14)[20] and α-chlorofluoromethylalanine (CFM-Ala, 15)[19] were obtained by the published procedures, as were α-allylalanine (allyl-Ala, 16)[21] and α-allenylalanine (allenyl-Ala, 17)[22].

The different β-alanine analogues, 3-amino-4-fluorobutyric acid (MFM-β-Ala, 20)[23], (±)-3-amino-4,4-difluorobutyric acid (DFM-β-Ala, 21) and its (−)-enantiomer (22)[24], 3-amino-4,4,4-trifluorobutyric acid (TFM-β-Ala, 23)[23], 3-amino-4-chloro-4-fluorobutyric acid (CFM-β-Ala, 24)[24], 3-amino-4-pentenoic acid (vinyl-β-Ala, 25)[23] and 3-amino-4-pentynoic acid (ethynyl-β-Ala, 26)[23] were prepared at our Centre.

The different 4-aminobutyric acid (GABA) analogues were obtained as follows. (±)-4-Aminopentanoic acid (Me-GABA, 28), its two enantiomers (*S*)-(−) (29) and (*R*)-(+) (30)[25], 4-amino-5-fluoropentanoic acid (MFM-GABA, 31), 4-amino-5,5-difluoropentanoic acid (DFM-GABA, 32) and 4-amino-5,5,5-trifluoropentanoic acid (TFM-GABA, 33)[23] were prepared by the described procedures, as were (±)-4-aminohex-5-enoic acid (vinyl-GABA, 34)[26] and its two enantiomers (*R*)-(−) (35) and (*S*)-(+), (36)[27], (±)-4-aminohex-5-ynoic acid (ethynyl-GABA, 37)[28], the corresponding enantiomers (*R*)-(−) (38) and (*S*)-(+), (39)[29], (±)-4-amino-5,6-heptadienoic acid (allenyl-GABA, 40) and the two antipodes (*R*)-(−) (41) and (*S*)-(+) (42)[22]. (*Z*)-4-Amino-6-fluoro-5-hexenoic acid (43), 4-amino-6,6-difluoro-5-hexenoic acid (44), 4-amino-5-fluoro-5-hexenoic acid (45) and 4-amino-5,6,6-trifluoro-5-hexenoic acid (46) were prepared as described[30].

Gas chromatographic analysis

Gas chromatography was performed on a Hewlett-Packard HP 5880 gas chromatograph equipped with a flame ionization detector and an automatic sample injector Model HP 7671. Helium was used as carrier gas, with an inlet pressure of 11 p.s.i. and splitting ratio 1:30. The temperature of the injector and detector was kept at 250°C.

The Chirasil-Val glass capillary columns OS 6411 (25 m × 0.25 mm I.D.) were supplied by C.G.C. Analytic (Mossingen, F.R.G.). Peak retention times and half-height widths were measured with a digital integrator HP-3388. Other conditions are given in the tables and figures.

Derivatization procedure. Typically, 0.5–2 mg of the amino acid was weighed in a 3-ml Reacti-Vial (Pierce Chemical Co., Rockford, IL, U.S.A.), sealed with a PTFE-lined septum. The different esters, *e.g.*, methyl, ethyl, isopropyl, *n*-propyl or pentafluoropropyl, were obtained by addition either of 1 ml of the appropriate alcohol followed by 100 μl of sulphonyl chloride with cooling in an ice-bath, or of 1 ml of a 80:20 (v/v) mixture of the alcohol and acetyl chloride[31]. The resulting solutions were then heated for 1 h at 65°C for the methyl, 75°C for the ethyl and 95°C for the isopropyl, *n*-propyl and pentafluoropropyl esters. For the sterically hindered carboxyl groups and/or those presenting a low pK_a, *e.g.*, α-substituted with one or several halogens, further heating up to 3 h and even a second esterification after evaporation of the reagent were necessary to increase the yields, which still remained fairly low. Nearly quantitative yields of esterification were, however, obtained by treating these amino acids with phosgene in tetrahydrofuran solution, leading to the formation of the corresponding N-carboxyanhydrides which were then treated with isopropyl alcohol saturated with hydrogen chloride[32]. This derivatization procedure was used for 3-chloroalanine (5), 3,3-difluoroalanine (6), 3,3,3-trifluoroalanine (7), α-difluoromethyl- (13), α-chloromethyl- (14) and α-chlorofluoromethylalanine (15). The excess of reagent was removed under a stream of nitrogen at room temperature or by moderate heating.

The amino acid esters were transformed into their fluoroacyl derivatives by the addition of 250 μl of the corresponding perfluoroanhydride, *i.e.*, TFAA, PFPA and HFBA at 0°C. The mixtures were then left at room temperature for 1 h or heated at 50°C for 10 min. The excess of reagent was removed under a stream of nitrogen at room temperature or at 35°C. For the relatively volatile N-acyl esters of the alanine and β-alanine analogues, care has to be taken to perform this step at room temperature under a gentle stream of nitrogen to reduce losses. The residue was finally dissolved in 0.2–1 ml of ethyl acetate and 0.5–2 μl aliquots were injected into the GC column.

HPLC analysis

The HPLC system consisted of a Model 6000A pump, a WISP 710 automatic injector and a Model 401 refractive index detector all from Waters (Milford, MA, U.S.A.). The derivatization system with *o*-phthalaldehyde (OPA) was similar to that previously described[33]. A piston mini pump from Milton Roy (Riviera Beach, FL, U.S.A.) was used for the post-column reagent, and the fluorescence detector was an Aminco Fluoromonitor (Silver Spring, MD, U.S.A.). The OPA reagent was prepared by addition per litre of a 0.5 *M* potassium borate solution (pH 10.5–10.6) of 3 ml of

TABLE I

RESOLUTION OF THE N-PERFLUOROACYL ESTERS OF DL-ALANINE AND (±)-β-3-FLUOROALANINE ON THE CHIRASIL-VAL COLUMN

The retention times are uncorrected with a hold-up time, t_m, of the column of 1.19 min and a column temperature of 75°C, isothermal. The separation coefficient is defined as $\alpha = \frac{t_2 - t_m}{t_1 - t_m}$. The resolution number is defined $R_N = 2\frac{(t_2 - t_1)}{W_1 + W_2}$, W_1, W_2 being the widths at half-height.

Derivative		DL-*Alanine (1)*				*(±)-3-Fluoroalanine (3)*			
Ester	*Acryl*	t_R *(min)*		α	R_N	t_R *(min)*		α	R_N
		D	*L*			*I**	*II*		
Methyl	TFA	3.12	3.61	1.254	6.90	3.87	4.19	1.119	3.23
	PFP	3.00	3.38	1.210	5.94	3.74	3.99	1.098	2.91
	HFB	3.64	4.12	1.196	5.71	4.61	4.92	1.091	2.79
Ethyl	TFA	4.06	4.89	1.289	7.55	5.02	5.53	1.133	3.72
	PFP	3.91	4.53	1.228	6.81	4.95	5.33	1.101	2.86
	HFB	4.81	5.60	1.218	6.75	6.19	6.65	1.092	2.86
Isopropyl	TFA	4.46	5.57	1.339	10.67	5.52	6.18	1.152	4.37
	PFP	4.33	5.15	1.261	8.91	5.47	5.93	1.107	3.36
	HFB	5.39	6.44	1.250	7.14	6.85	7.40	1.097	2.93

* The peak with the shortest retention time corresponds to the D-enantiomer as confirmed by a separate injection of D-3-fluoroalanine (4).

Brij 35, 2.5 ml of 2-mercaptoethanol and 800 mg of *o*-phthalaldehyde dissolved in 10 ml of ethanol.

The column was an Ultrasphere ion-pair column (150 mm × 4.6 mm, particle size 5 μm) from Beckman (Berkeley, CA, U.S.A.), in a jacket thermostatted at 20°C by circulated water. The chiral eluent consisted of an aqueous solution of $8 \cdot 10^{-3}$ *M* L-proline plus $4 \cdot 10^{-3}$ *M* copper(II) sulphate adjusted to pH 5.5 with 1 ml of 5 *M* sodium hydroxide per litre. The flow-rate of the mobile phase was 0.5 ml/min and that of the OPA reagent was 0.35 ml/min. A 3 m × 0.3 mm I.D. PTFE coil, immersed in a water-bath at 50°C, was used for the post-column derivatization. Some of the substituted amino acids failed to react with the OPA reagent and therefore these compounds were monitored with the refractive index detector.

RESULTS AND DISCUSSION

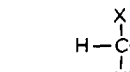
Resolution of alanine and its β-halogenated analogues

Chirasil-Val GC. We investigated first the effect of the various esters, methyl ethyl, isopropyl, and the combination with the different perfluoroacyl substituents on the retention times and the separation coefficient, α, for DL-Ala (1) and (±)-3-fluoroalanine (3). The results obtained, summarized in Table I, clearly show that the retention times increase with the chain length of the alcohol, as expected[11,34]. The

TABLE II

RESOLUTION OF ALANINE AND ITS β-HALOGENATED ANALOGUES BY GC ON CHIRASIL-VAL AS N-TRIFLUOROACETYL ISOPROPYL ESTERS AND BY HPLC AS FREE AMINO ACIDS WITH THE L-PROLINE/COPPER COMPLEX AS CHIRAL ELUENT

The GC column temperature was 75°C, other conditions and definitions being identical to those in Table I. The HPLC conditions were described in Experimental and the retention times are uncorrected. The void volume of the column was 1.75 ml.

		*GC*				*HPLC*		
		t_R *(min)*		α	R_N	t_R *(min)*		α
X		*I*	*II*			*I*	*II*	
CH_3 (DL-Alanine)	1	4.46	5.57	1.339	10.67	4.63	5.67	1.92
L-Alanine (*S*)-(+)	2		5.60				5.67	
(±)-CH_2F	3	5.52	6.18	1.152	4.37	4.82	6.61	2.36
(*S*)-(−)-CH_2F	4	5.45				4.8		
(±)-CH_2Cl	5	11.26	12.22	1.095	3.07	6.63	15.70	3.89
(±)-CHF_2	6	5.05	5.51	1.119	3.65	5.31	7.91	2.43
(±)-CF_3	7	2.55	2.83	1.206	4.75	5.34*	9.01*	2.99

* With refractive index detection.

retention times decrease also in the order HFB, TFA, PFP. The combination of isopropanol and TFA was found to give the largest separation coefficient and the greatest resolution for DL-Ala and for 3-fluoroalanine. These results are in overall agreement with those previously described[9,35]. Table II summarizes the chromatographic characteristics obtained with the Chirasil-Val column under isothermal conditions at 75°C. The resolution decreases from CH_3 as substituent (DL-Ala), to the CF_3 (7), the CH_2F (3) and the CHF_2 (6) analogues. The retention times increase from compound 1, to 6 and 3, whereas the CF_3 analogue, 7, has by far the shortest retention time. These results are in good agreement with the known volatilities of various fluorinated derivatives[36].

Chiral eluent HPLC. In Table II are listed the chromatographic characteristics obtained with the ligand-exchange HPLC procedure using L-proline/copper(II) as chiral eluent. An excellent separation was obtained for DL-Ala as already described[14]. We found that with L-Pro as chiral ligand in the eluent the separation coefficients obtained with the alanine analogues were larger than those obtained under identical conditions with N,N-di-*n*-propyl-L-alanine in the eluent[37,38]. Although the retention times of the first enantiomer to be eluted increases only slightly, the separation coefficient increases markedly with the degree of fluorine substitution, CH_2F, CHF_2, CF_3, as illustrated by the chromatograms shown in Fig. 1. DL-Ala (1), (±)-3-fluoroalanine (3) and (±)-3,3-difluoroalanine (6) were analyzed by fluorescence detection after OPA derivatization (Fig. 1A, B, C). 3,3,3-Trifluoroanaline (7), however, which does not react with OPA, was monitored by refractive index detection (Fig. 1D). It would appear that the separation mainly follows the chiral characteristics introduced by progressive fluorine substitution. These results show that the two diastereomeric

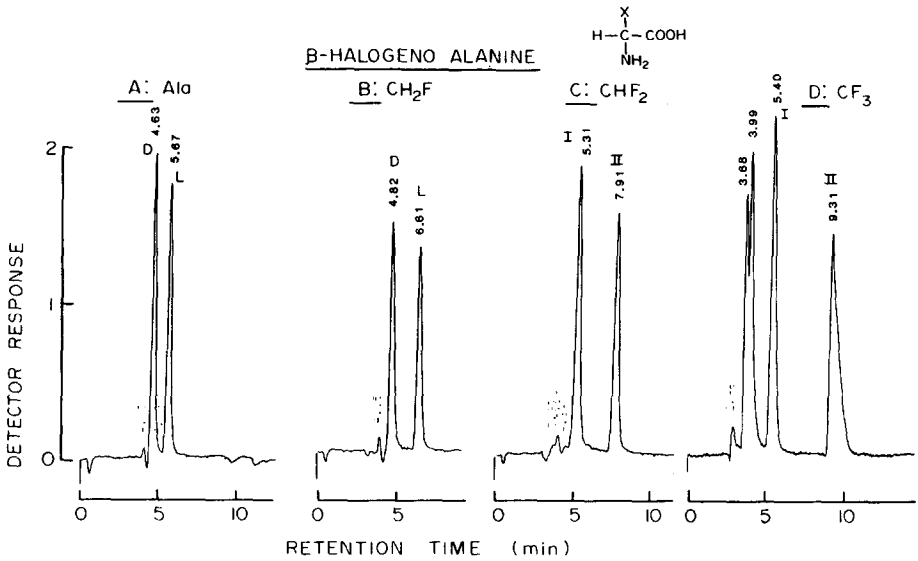


Fig. 1. Resolution of the enantiomers of underivatized DL-Ala and its fluorinated analogues by reversed-phase HPLC with L-proline–copper as chiral eluent: DL-Ala (1), 1.68 nmol (A); DL-3-fluoroalanine (3), 1.43 nmol (B); DL-3,3-difluoroanaline (6), 32.0 nmol (C); DL-3,3,3-trifluoroalanine (7), 740 nmol (D). Detection: (A)–(C), fluorescence after OPA derivatization; (D), refractive index. Other conditions as described in Experimental.

complexes formed by L-Pro, the copper ion and respectively the D- and L-alanine analogue present markedly increasing partition differences from the CH_3 to the CH_2F, CHF_2, CF_3 and the CH_2Cl groups. Apparently there is no correlation between the separation coefficients and the hydrophobic fragmental constants, *f*, as determined for aliphatic derivatives[39]. There seems, however, to be a good correlation with the fragmental constants of substituents of an aromatic ring, the *f* values increasing from 0.70 to 0.95, 1.08, 1.25 and 1.46 for CH_3, CH_2F, CHF_2, CF_3 and CH_2Cl respectively[39]. This increase in separation coefficient may also be related to the change in the basicity of the amino group whose pK_a decreases by about 1.4–1.6 units[40,41] for each fluorine substitution, from 9.9 for DL-Ala to 5.6 for 3,3,3-trifluoroalanine (7)[42].

In both chromatographic systems, the L-enantiomer of alanine possessing the (*S*)-(+)-configuration is eluted second. The (*S*)-(−)-enantiomer of 3-fluoroalanine (4) is, however, eluted first in both the GC and LC systems. The same order of elution should also hold for the other alanine analogues. The separation coefficients obtained with the halogenoalanine analogues make the chiral eluent HPLC procedure attractive for semipreparative resolution of the corresponding enantiomers.

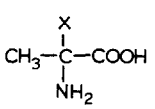
Resolution of the α-substituted alanine analogues

Chirasil-Val GC. The results obtained by GC analysis on the Chirasil-Val column for the N-trifluoroacetyl isopropyl esters of the different α-substituted analogues of alanine are summarized in Table III. For several compounds, although possessing an asymmetric carbon *e.g.*, the methoxymethyl (11), the chloromethyl (14) and also

TABLE III

RESOLUTION OF THE α-SUBSTITUTED ALANINE ANALOGUES BY GC WITH THE CHIRASIL-VAL COLUMN AND BY HPLC WITH THE CHIRAL ELUENT

The α-substituted alanine analogues were analyzed by GC as N-TFA isopropyl esters on Chirasil-Val; column temperature 75°C, isothermal. All other conditions identical to those in Tables I and II. HPLC conditions as described in the Experimental.

		*GC*			*HPLC*		
		t_R (min)		α	*t_R (min)*		α
X		*I*	*II*		*I*	*II*	
CH_3*	8	3.34			8.33		
(±)CH_2CN	9	3.29	3.88	1.278	8.95	13.91	1.91
(±)CH(CH_3)(CH_3)	10	5.91	6.33	1.089	27.16	67.27	2.69
(±)-CH_2OCH_3	11	5.08		1.000	14.08	21.37	1.67
(±)-CH_2F	12	3.70	3.75	1.020	9.58	10.24	1.11
(±)-CHF_2	13	3.58	3.64	1.025	12.20**	13.36**	1.13
(±)-CH_2Cl	14	6.44		1.000	14.99	23.00	1.70
(±)-CHFCl***	15	4.89	5.00	1.030	5.40§	6.24§	1.44
		7.91	8.05	1.021	5.40§	7.43§	2.07
(±)-$CH_2CH=CH_2$	16	5.27		1.000	22.78	54.45	2.64
(±)-$CH=C=CH_2$	17	8.71		1.000	21.95	47.09	2.36

* No asymmetric carbon.
** Refractive index detection.
*** Two asymmetric carbons and four stereoisomers.
§ Three peaks with respective areas 50:38:12.

the allyl (16) and the allenyl (17) analogues, no resolution was observed with the chiral GC phase. For the CH_2F (12) and CHF_2 (13) analogues, α is quite small compared to the values observed with the 3-fluorinated analogues of alanine, see Table II. The cyanomethyl (9) and isopropyl (10) analogues present appreciable separation coefficients. The value of 1.089 obtained for 10 is greater than that, 1.048, for α-methylvaline on a different chiral phase[43] or that, 1.030, of isovaline on Chirasil-Val[44]. The smaller resolution factors of these α-substituted alanine analogues are partly due to their lower chiral character and partly to the steric crowding, which leads to weaker interactions with the chiral phase. This steric hindrance is clearly illustrated by the low yields obtained for the esterification of some of the alanine analogues, especially difluoromethyl (13), chloromethyl (14), chlorofluoromethyl (15), using the classical esterification procedure. Nearly quantitative yields were, however, obtained by the reaction of the amino acid with phosgene and alcoholysis of the intermediate N-carboxyanhydride with isopropanol–hydrogen chloride[32].

Chiral eluent HPLC. The HPLC procedure with the L-proline copper complex as chiral eluent, was superior to the above procedure in that it allowed the resolution of all the enantiomers as underivatized amino acids. The separation coefficients listed in Table III are markedly smaller than those obtained for the corresponding β-halogenated analogues. The CH_2F, CHF_2 and CH_2Cl analogues have values of 1.11,

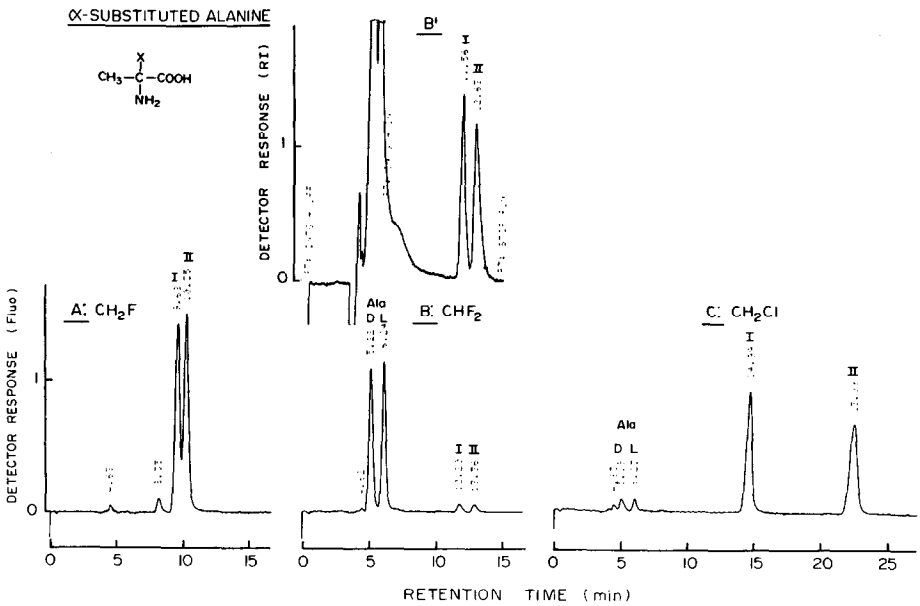


Fig. 2. Chiral eluent HPLC resolution of the α-halogenomethyl-substituted alanines: monofluoromethylalanine (12), 16.5 nmol (A); difluoromethylalanine (13), (B, 31.1 nmol, fluorescence detection; B', 537 nmol, RI detection); chloromethylalanine (14), 22.8 nmol (C). Other conditions as described in Experimental.

1.13 and 1.70 as compared to 2.36, 2.43 and 3.89 respectively for the corresponding β-halogenated analogues of alanine. The chromatograms in Fig. 2 clearly illustrate the differences in reactivity towards OPA of the different substituted α-substituted alanine analogues. The CHF_2 analogue (13) reacts poorly with OPA and therefore gives a weak response to fluorescence detection, whereas the 5% of DL-alanine present is clearly detected. The retention times steadily increase with the size and lipophilicity of the substituent, in the order CH_2F, CHF_2, CH_2Cl and in the series CH_2CN, CH_2OCH_3, $CH=C=CH_2$ and $CH_2CH=CH_2$. The chlorofluoromethyl analogue (15), has an unusually short retention time and gives only three peaks for the four stereoisomers present, the first peak corresponding to two diastereomers. This shortening of the retention time partly reflects the poor stability of the diastereomeric complex formed with L-proline and copper, due to the low pK_a of the amino group and to the steric hindrance. Nevertheless, the other resolutions obtained by the HPLC method are clearly superior to those of the GC method with the Chirasil-Val column.

β-Alanine analogues

Chirasil-Val GC. Table IV summarizes the GC results for the resolution of the β-alanine derivatives with the Chirasil-Val column. The PFP-ethyl esters yielded the best separation coefficient at a column temperature of 85°C. The separation coefficient and resolution numbers are quite satisfactory and increase in the order CH_3, CH_2F, CHF_2, CF_3. The resolution of the monofluoromethyl (20) and difluoromethyl (21) derivatives is illustrated by the chromatograms presented in Fig. 3A and B.

TABLE IV

RESOLUTION OF THE β-ALANINE ANALOGUES BY GC WITH THE CHIRASIL-VAL COLUMN AND BY HPLC WITH THE CHIRAL ELUENT

The β-alanine analogues were analyzed by GC as N-PFP ethyl esters on Chirasil-Val; column temperature 85°C, isothermal. All other conditions identical to those in Tables I–III. HPLC conditions as described in Experimental.

X (β-alanine)		GC				HPLC		
		t_R (min)		α	R_N	t_R (min)		α
X		I	II			I	II	
H (β-alanine)	18	5.78				3.95		
(±)-CH_3	19	5.61	5.81	1.045	1.33	4.16	4.38	1.33
(±)-CH_2F	20	8.89	9.32	1.056	1.95	5.78	5.78	1.00
(±)-CHF_2	21	7.98	8.53	1.081	2.86	6.98	7.40	1.12
(+)-CHF_2	22	7.99				7.00		
(±)-CF_3	23	5.78	6.62	1.183	6.10	11.21	12.07	1.11
(±)-CHFCl*	24	18.74	20.11	1.078	3.02	13.79**	15.89**	
		19.39	20.98	1.087	3.38			
(±)-CH=CH_2	25	7.79	8.28	1.074	2.62	7.15	8.33	1.32
(±)-C≡CH	26	8.63	9.28	1.087	2.50	7.05	9.02	1.55

* Two asymmetric carbons and four stereoisomers.

** The two peaks present respective areas of 82:18, peak I corresponding probably to three stereoisomers.

The chlorofluoromethyl analogue (24) which possesses two asymmetric carbons is separated into its four stereoisomers as shown by the chromatogram in Fig. 3C, peaks I, III and II, IV corresponding respectively to the two pairs of enantiomers. Concerning the order of elution, the (+)-enantiomer of the DFM-β-Ala (22), which is the biologically inactive enantiomer[24] and probably has the D-configuration, is eluted first as usually observed for the common amino acids. The absolute configuration, *R* or *S*, is as yet unknown.

Chiral eluent HPLC. The results obtained by the chiral eluent HPLC procedure, as listed in Table IV, show that the resolutions are quite low, monofluoromethyl-β-alanine (20) being unresolved. The other separation coefficients range between 1.1 and 1.55. This probably corresponds to the less favourable copper-chelating properties of the β-alanine analogues which form six-membered chelates instead of the five-membered ones formed by the α-amino acids. The logarithm of the stability constant of the bis(ligand)copper(II) complexes decreases, from 15.0 to 13.9[45], from α- to β-alanine. The formation of the mixed complex β-alanine–copper(II)–L-proline in the mobile phase is therefore less favourable, resulting in a decrease in α as compared to analogues of α-alanine (Table II) and the different α-substituted derivatives of α-alanine (Table III), except for the CHF_2 analogues (13 and 21) which have a similar resolution. The resolutions obtained by the chiral eluent HPLC procedure for the β-alanine analogues are therefore clearly inferior to those obtained by the GC procedure.

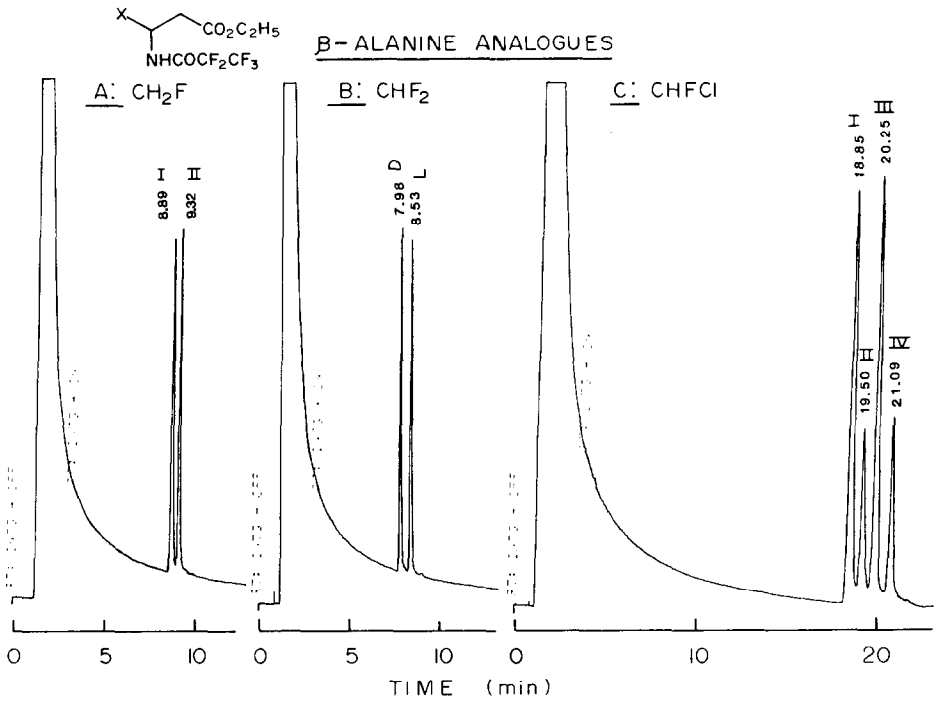


Fig. 3. GC separation of the enantiomers of β-alanine analogues as N-PFP ethyl esters with the Chirasil-Val column (amounts injected in parentheses): (A), monofluoromethyl-β-alanine (20) (6 nmol); (B), difluoromethyl-β-alanine (21) (5 nmol); (C), chlorofluoromethyl-β-alanine (22) (15 nmol). Column temperature: 85°C. Splitting ratio: 1:30. Other conditions as described in Experimental.

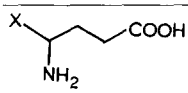
4-Aminobutyric acid (GABA) analogues

Chirasil-Val GC. We first investigated the effect on the separation coefficient and the resolution number for vinyl-GABA (34) of the various pairs of esters and perfluoroacyl derivatives. Among the different esters, methyl, ethyl, isopropyl and pentafluoropropyl, and the different perfluoroacyl, TFA, PFP and HFB derivatives, the N-PFP ethyl ester was found to yield the best resolution. Table V summarizes the properties of the different GABA analogues analyzed as PFP ethyl esters on the Chirasil-Val column under isothermal conditions (column temperature 105°C). It is interesting that Me-GABA (28) has a shorter retention time than unsubstituted GABA. On a normal-phase capillary column of the type CP-Sil 5 (polydimethylsiloxane phase), Me-GABA has, as expected, a longer retention time than GABA itself. This decrease in retention time apparently reflects the steric hindrance introduced by the methyl substituent. A slight decrease in retention time has also been observed with the corresponding β-alanine derivatives, see Table IV. The monofluoromethyl (31), difluoromethyl (32) and triluoromethyl (33) GABA derivatives have an analogous variation in retention times and a similar increase in α to those previously observed for the β-alanine (see Table IV) and in part the β-halogenated alanine analogues (see Table II). The increased resolution factor observed for the difluoro- and especially the trifluoromethyl analogues reflects a clear increase in the chiral

TABLE V

RESOLUTION OF THE GABA ANALOGUES AS N-PENTAFLUOROPROPIONYL ETHYL ESTERS ON CHIRASIL-VAL

Column temperature 105°C, isothermal. Other conditions and definitions were identical to those in Table I.

X ()		*Configuration and/or optical rotation*	t_R *(min)*		α	R_N
			I	*II*		
H (GABA)*	27		8.20			
CH_3	28	(±)	6.67	7.05	1.065	2.81
CH_3	29	*(S)*-(−)	6.60			
CH_3	30	*(R)*-(+)		7.05		
CH_2F	31	(±)	9.54	10.36	1.054	2.11
CHF_2	32	(±)	8.36	8.89	1.070	2.51
CF_3	33	(±)	5.33	5.82	1.106	3.55
$CH=CH_2$	34	(±)	9.71	10.36	1.073	2.64
$CH=CH_2$	35	*R*-(−)	9.94			
$CH=CH_2$	36	*S*-(+)		10.53		
$C\equiv CH$	37	(±)	10.55	11.10	1.056	2.02
$C\equiv CH$	38	*R*-(−)	10.73			
$C\equiv CH$	39	*S*-(+)		11.26		
$CH=C=CH_2$	40	(±)	17.72	18.78	1.061	2.18
$CH=C=CH_2$	41	*R*-(−)	17.83			
$CH=C=CH_2$	42	*S*-(+)		18.93		
$CH=CHF$	43	(±)	14.05	14.95	1.065	2.26
$CH=CF_2$	44	(±)	11.48	12.40	1.086	3.60
$CF=CH_2$	45	(±)	7.66	8.26	1.088	2.72
$CF=CF_2$	46	(±)	7.39	8.01	1.094	3.10

* No asymmetric carbon.

recognition associated with the CF_3 group. The basicity of the amino group strongly decreases with the fluorine substituents and pK_a decreases from 10.5 to 8.85, 7.40 and 5.80 in passing from Me-GABA (28), to MFM-GABA (31), DFM-GABA (32) and TFM-GABA (33), respectively[41]. These changes in basicity will therefore modify the interactions of the less basic nitrogen amide in the final PFP derivative with the carbonyl group of the valine amide function of the chiral stationary phase. Another factor which may account for the increased resolution of the CF_3 analogue originates probably from its greater volatility, which favours the specific interactions of the solute with the chiral diamide phase *versus* the general interactions with the polysiloxane backbone[12,46]. The increase in retention times observed for the vinyl-, ethynyl- and allenyl-GABA derivatives reflects the previously observed variation in volatility for these substituents, see Tables III and IV. The separation factors are all greater than 1.05 with resolutions greater than 2. The chromatogram in Fig. 4A illustrates the resolution obtained for the racemic mixture (*R,S*)-vinyl-GABA. Separate injections of the isolated enantiomers, see Fig. 4B and C, showed that the biologically active (*S*)-(+)-enantiomer (36)[27] has the longer retention time. This order of elution, (*R*)-enantiomer eluted before the (*S*), is the reverse of that observed for Me-GABA, whose (*S*)-(−)-enantiomer (29), is eluted before the (*R*)-(+) (30).

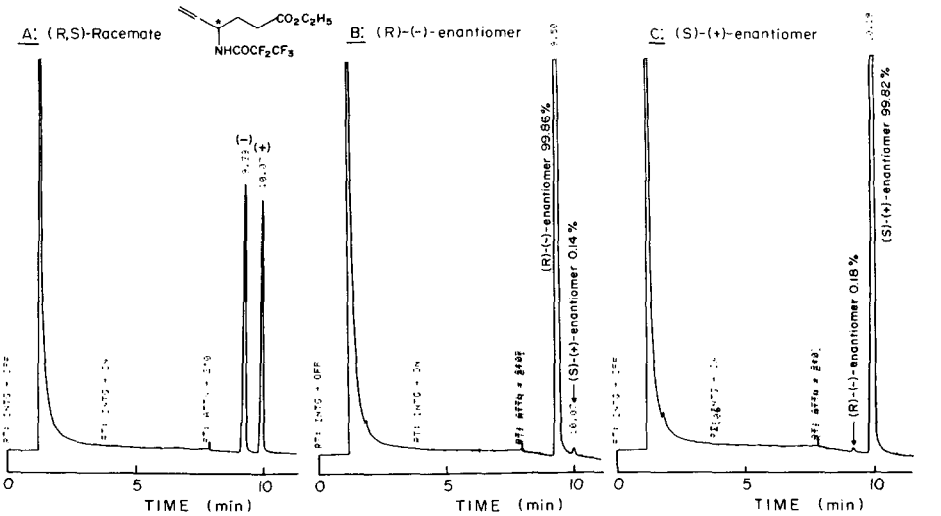


Fig. 4. GC separation of the enantiomers of (±)-vinyl-GABA as N-PFP ethyl esters with the Chirasil-Val column; (A), (±)-vinyl-GABA (34); (B), (*R*)-(−)-vinyl-GABA (35); (C), (*S*)-(+)-vinyl-GABA (36). The Chirasil-Val column, different from that used for the analyses in Table V, was kept at 100°C, isothermal. Amounts injected: 10 nmol. Splitting ratio: 1:30. At 8 min, the attenuation of the integrator was reduced by a factor of 4. Other conditions as described in Experimental.

This contradiction is only apparent because the absolute configuration is the same. The (*R*)-labelling of the (+)-CH_3 analogue eluted second originates from the Cahn–Ingold–Prelog notation. The CH_3 group is given a lower priority than the $-CH_2-CH_2-$ group whereas the carbon multiple bond is given a higher priority[47]. The (*R*)-(−)-enantiomers of ethynyl-GABA (38) and allenyl-GABA (41) are also eluted before the (*S*)-(+), as shown in Table V. The same order of elution should also apply to the other GABA analogues, MFM (31), DFM (32) and TFM (33) and the fluorine-substituted vinyl-GABA analogues (43–45). This chiral GC procedure has been routinely applied for the determination of the optical purity of the different resolved enantiomers, as illustrated in Fig. 4B and C for the enantiomers of vinyl-GABA with detection limits of around 0.05% of the contaminating antipode. Advantage has also been taken from the resolution obtained to study the pharmacokinetics of the two enantiomers of vinyl-GABA in human body fluids[48].

Chiral eluent HPLC. Different assays with the chiral eluent HPLC procedure using either L-proline or N,N-dipropyl-L-alanine on various reversed-phase columns failed to give any separation of the GABA analogues. The retention times obtained were fairly short, near to the void volume of the column. This failure to resolve the GABA analogues with ligand-exchange HPLC can easily be rationalized on the basis of the much lower stability constants for the two-ligand copper(II) complexes of the GABA analogues, the logarithms of their stability constants being less than 10 (ref. 41). The amount of the diastereomeric mixed-ligand complexes formed with L-proline and copper will therefore be too low to expect any resolution.

CONCLUSIONS

The different examples presented, α-, β- and γ-amino acid analogues, illustrate the potency and the limitations of the two chromatographic procedures. The GC resolution with the chiral stationary phase, Chirasil-Val, allows the analytical resolution of most of the different α- and β-alanine and GABA analogues studied, except some of the substituted α-alanine analogues possessing a greater steric crowding. The ligand-exchange reversed-phase HPLC procedure with L-proline/copper(II) as chiral complex gives greater separation coefficients for most of the α-amino acids studied. It does not need derivatization before analysis and the high sensitivity obtained with the *o*-phthalaldehyde derivatization procedure makes it the method of choice for the analysis of most of the α-amino acids studied. Moreover, it can readily be scaled up for the semipreparative resolution of mg amounts of the various amino acids[49]. However, it has some limitations, *i.e.*, relatively poor resolution for the β-amino acids and no resolution of the γ-amino acids. This limitation may eventually be overcome by the use of chiral ligands having a greater resolving power such as L-pipecolic acid[50] or chiral β-amino acids. The two methods discussed are essentially complementary and allow the resolution of nearly all the α-, β- and γ-amino acids studied.

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REFERENCES

1 A. L. Maycock, S. D. Aster and A. A. Patchett, in T. I. Kalman (Editor), *Drug Action and Design: Mechanism-Based Inhibitors*, Elsevier/North Holland, New York, Amsterdam, Oxford, 1979, pp. 115–129.
2 P. Bey, B. Metcalf, M. J. Jung, J. Fozard and J. Koch-Weser, in J. A. Keverling Buisman (Editor), *Strategy in Drug Research*, Elsevier, Amsterdam, 1982, pp. 89–106.
3 C. Walsh, *Tetrahedron*, 38 (1982) 871.
4 H. G. Floss and J. C. Vederas, in Ch. Tamm (Editor), *Stereochemistry*, Elsevier Biomedical Press, Amsterdam, 1982, pp. 161–199.
5 A. L. Maycock, S. D. Aster and A. A. Patchett, *Biochemistry*, 19 (1980) 709.
6 R. H. Liu and W. W. Ku, *J. Chromatogr.*, 271 (1983) 309.
7 V. Schurig, *Kontakte (Darmstadt)*, 1 (1986) 3.
8 V. A. Davankov, A. A. Kurganov and A. S. Bochkov, *Adv. Chromatogr. (N.Y.)*, (1983) 71–136.
9 H. Frank, G. J. Nicholson and E. Bayer, *J. Chromatogr. Sci.*, 15 (1977) 174.
10 H. Frank, G. J. Nicholson and E. Bayer, *Angew. Chem., Int. Ed. Engl.*, 17 (1978) 363.
11 I. Abe, K. Izumi, S. Kuramoto and S. Musha, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4 (1981) 549.
12 E. Bayer, *Z. Naturforsch., Teil B*, 38 (1983) 1281.
13 P. E. Hare and E. Gil-Av, *Science (Washington, D.C.)*, 204 (1979) 226.
14 E. Gil-Av, A. Tishbee and P. E. Hare, *J. Am. Chem. Soc.*, 102 (1980) 5115.
15 J. Kollonitsch, A. Patchett, S. Marburg, A. Maycock, L. Perkins, G. Doldouras, D. Dugan and S. Aster, *Nature (London)*, 274 (1978) 406.

16 J. L. Wood and V. Midlesworth, *J. Biol. Chem.*, 179 (1949) 529.
17 T. Tsushima and K. Kawada, *Tetrahedron Lett.*, 26 (1985) 2445.
18 P. Bey and J. P. Vevert, *Tetrahedron Lett.*, 17 (1977) 1455.
19 P. Bey, J. B. Ducep and D. Schirlin, *Tetrahedron Lett.*, 25 (1984) 5657.
20 P. Bey, J. P. Vevert, V. Van Dorsselaer and M. Kolb, *J. Org. Chem.*, 44 (1979) 2732.
21 P. Bey, unpublished results.
22 P. Casara, K. Jund and P. Bey, *Tetrahedron Lett.*, 25 (1984) 1981.
23 P. Bey, M. J. Jung, F. Gerhart, D. Schirlin, V. Van Dorsselaer and P. Casara, *J. Neurochem.*, 37 (1981) 1341.
24 D. Schirlin, S. Baltzer, J. G. Heydt and M. J. Jung, *J. Enzyme Inhibition*, (1987) in press.
25 E. Fischer and A. Groh, *Liebigs Ann. Chem.*, 383 (1911) 370.
26 B. Lippert, B. W. Metcalf, M. J. Jung and P. Casara, *Eur. J. Biochem.*, 74 (1977) 441.
27 C. Danzin and M. J. Jung, in A. E. Evangepoulos (Editor), *Chemical and Biological Aspects of Vitamin B_6 Catalysis*, Alan R. Liss, New York, 1984, Part A, pp. 377–385.
28 B. W. Metcalf and P. Casara, *Tetrahedron Lett.*, 38 (1975) 3337.
29 M. J. Jung, B. W. Metcalf, B. Lippert and P. Casara, *Biochemistry*, 17 (1978) 2628.
30 M. Kolb, J. Barth, J. G. Heydt and M. J. Jung, *J. Med. Chem.*, (1987) in press.
31 H. Frank, D. Bimboes and G. J. Nicholson, *Chromatographia*, 12 (1979) 168.
32 B. F. Erlanger and E. Brand, *J. Am. Chem. Soc.*, 73 (1951) 3508.
33 J. Wagner, N. Claverie and C. Danzin, *Anal. Biochem.*, 140 (1984) 108.
34 B. Koppenhoefer, H. Allmendinger, G. J. Nicholson and E. Bayer, *J. Chromatogr.*, 260 (1983) 63.
35 I. Abe, S. Kuramoto and S. Musha, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 6 (1983) 366.
36 M. Hudlicky, in *Chemistry of Organic Fluorine Compounds*, MacMillan, New York, 1962, pp. 288–313.
37 S. Weinstein, *Angew. Chem., Int. Ed. Engl.*, 21 (1982) 218.
38 S. Weinstein, M. H. Engel and P. E. Hare, *Anal. Biochem.*, 121 (1982) 370.
39 R. F. Rekker, in *The Hydrophobic Fragmental Constant*, Elsevier, Amsterdam, Oxford, New York, 1977, pp. 1–132.
40 D. D. Perrin, in S. H. Yalkowsky, A. A. Sinkula and S. C. Valvani (Editors), *Physical Properties of Drugs*, Marcel Dekker, New York, Basle, 1980, Ch. 1, pp. 1–48.
41 J. Wagner, unpublished results.
42 F. Weygand, W. Steglich, W. Oettmeier, A. Maierhofer and R. S. Loy, *Angew. Chem., Int. Ed. Engl.*, 5 (1966) 600.
43 R. Charles and E. Gil-Av, *J. Chromatogr.*, 195 (1980) 317.
44 H. Brückner, G. J. Nicholson, G. Jung, K. Kruse and W. A. König, *Chromatographia*, 13 (1980) 209.
45 D. D. Perrin, in *Stability Constants of Metal-Ion Complexes, Part B, Organic Ligands*, IUPAC Chemical Data Series No. 22, Pergamon, Oxford, New York, 1979.
46 B. Koppenhoefer and E. Bayer, *Chromatographia*, 19 (1984) 123.
47 R. S. Cahn, C. K. Ingold and V. Prelog, *Experientia*, 12 (1956) 81.
48 K. D. Haegele, J. Schoun, R. G. Alken and N. D. Huebert, *J. Chromatogr.*, 274 (1983) 103.
49 J. Wagner, C. Gaget, B. Heintzelmann and E. Wolf, *Anal. Biochem.*, (1987) in press.
50 J. Gübitz, F. Juffmann and W. Jellenz, *Chromatographia*, 103 (1982) 103.