Journal of Chromatography, 75 (1973) 13–18

CHROM. 6321

GAS–LIQUID CHROMATOGRAPHIC RESOLUTION OF SEVERAL PROTEIN AMINO ACID ENANTIOMERS ON A PACKED COLUMN

F. RAULIN* AND B. N. KHARE

Laboratory for Planetary Studies, Center for Radiophysics and Space Research, Cornell University, Ithaca, N.Y. 14850 (U.S.A.)

(Received August 28th, 1972)

SUMMARY

The simultaneous gas–liquid chromatographic resolution of several protein amino acid enantiomers has been accomplished on a column packed with ethylene glycol adipate coated on Chromosorb G, using the N-trifluoroacetyl 2-butyl ester diastereomeric derivatives.

INTRODUCTION

Many investigations have been successfully carried out on the gas–liquid chromatographic (GLC) resolution of racemic amino acids on capillary columns. Two different principles are employed. One method consists of adding an asymmetrical center to the gas chromatograph column, while using an optically active stationary phase, such as N-trifluoroacetyl (N-TFA)-L-valyl-L-valine cyclohexyl ester[1–3], or N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester[4,5]. In the other method, the asymmetry arises from a diastereomeric derivative of the amino acid, such as the N-TFA 2-butyl ester[8–14], N-TFA 2-octyl ester[9], or N-TFA L-methyl ester[15].

The GLC separation of amino acid enantiomers on a column packed with N-TFA-L-valyl-L-valine cyclohexyl ester has permitted only the separation of D- and L-alanine[6,7]. But in the case where diastereomeric derivatives are used, the separation, on a packed column, of several amino acid enantiomers, as their N-TFA L-methyl esters[15], N-TFA S-propyl esters[16], or N-α-chloroacyl methyl esters[17] has been reported.

Independently of the foregoing investigations, many studies have been developed on the GLC of the amino acids. Since 1967, several papers have been published by GEHRKE *et al.*[18–21] on the GLC separation of the twenty protein amino acids, describing techniques for the derivatization of the amino acids to their N-TFA *n*-butyl esters, and the GLC separation on a dual column system ethylene glycol adipate (EGA)/OV-17. While using this method, we discovered a doublet for the isoleucine peak. After some investigation, it appeared that this double peak was due to the separation of alloisoleucine from isoleucine, which has two asymmetric centers. From the four stereomeric combinations DL, DD, LD, and LL of a diastereomer, the enantiomorphic pairs have been deduced to be gas chromatographically unseparable[10]; but DD is

* Present address: Laboratoire d'Énergétique Biochimique, Université Paris–Val de Marne, Avenue du Général de Gaulle, –94– Créteil, France.

separable from DL, and LD from LL. Hence, the separation of alloisoleucine from isoleucine on an EGA column[22] suggested the possibility of using packed column techniques for the resolution of diastereomeric derivatives of amino acids. It seemed important to us to connect this resolution with the separation of racemic amino acids which is now well-known and very widely utilized.

This paper describes the simultaneous resolution of several protein amino acid diastereomers, on a packed column, using the process of derivatization[19-21] (but with 2-butanol in place of *n*-butanol), and the EGA columns described by GEHRKE *et al.*[18-20].

EXPERIMENTAL

Apparatus

Pyrex glass reaction vials, with Teflon-lined screw caps, were obtained from Analytical Biochemistry Laboratories, Columbia, Mo., U.S.A. The sand-bath was constructed in our laboratory. The ultrasonic cleaner was a Heat Systems Ultrasonics apparatus. The gas chromatograph was a Hewlett-Packard Model 5750, with two hydrogen flame detectors, one differential electrometer, and a linear temperature programmer.

Reagents

Trifluoroacetic anhydride (TFAA), was "Eastman Grade", from Distillation Product Industries. The solution of 3 *N* HCl in 2-butanol, was made in a glass–Teflon system, with HCl from a Matheson Company compressed gas cylinder. HCl was passed through a glass wool–silica gel filter and a sulfuric acid trap, before bubbling through 2-butanol. The normality of HCl was determined by weighing a known volume of solution. Methylene chloride was "certified A.C.S. spectranalyzed" from Fisher and used without other distillation. All solvents used for the derivatization and for making the columns were gas chromatographically analyzed in our laboratory, on a Porapak Q column. They showed less than 0.01 % water.

Standard solutions, containing one or several protein amino acids, each 2.5 mmoles/l in 3 *N* HCl were used for the calibrations.

Derivatization

The derivatization was done at the microgram level, as described by ROACH AND GEHRKE[19], while using 2-butanol in place of *n*-butanol. 1 ml of sample is evaporated in a micro-reaction vial, and placed in a sand-bath. Then, 0.2 ml of methylene chloride is added to remove azeotropically the last trace of water.

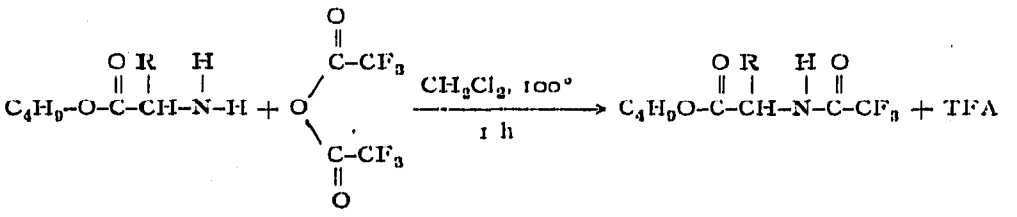
Esterification:

$$NH_2\text{–}CH(R)\text{–}C(=O)\text{–}OH + HOC_4H_9 \xrightarrow[15\ min]{HCl,\ 100^\circ} NH_2\text{–}CH(R)\text{–}C(=O)\text{–}OC_4H_9 + H_2O$$

For the direct butylation of the amino acids, 0.2 ml of 2-butanol, in 3 *N* HCl, is added. The reaction vial is firmly closed, and, after 30 sec ultrasonic mixing and manual shaking, is heated for 15 min at 100°, in the sand-bath. Then the 2-butanol

is evaporated, and 0.2 ml of methylene chloride is again added to remove azeotropically the last trace of water.

Acylation:



0.2 ml of methylene chloride and 0.05 ml of TFAA are added, and the closed vial, after 30 sec ultrasonic mixing and manual shaking, is heated in the sand-bath for 1 h at 100°. When the vial is cool, 5 μl of the sample, after manual shaking, is injected into the gas chromatograph.

Gas–liquid chromatographic conditions

Three different EGA dual column systems were used. These glass columns, made in our laboratory according to the prescription of GEHRKE *et al.*[18,20], were the following:

(1) One 6 ft. long × 4 mm I.D., packed with 0.325 wt. % of EGA stabilized grade, coated on 80–100 mesh AW HT, Chromosorb G; (2) a second identical column, but packed with 0.650 wt. % of EGA; and (3) an 11 ft. long × 2 mm I.D. glass column, packed with 0.325 wt. % EGA stabilized grade, coated on 80–100 mesh AW HT, Chromosorb G.

For all columns, the support material was heated in the laboratory for 15 h at 550°.

Temperature of the injector: 210°; temperature of the detector (FID): 260°.

Flow-rates: carrier, nitrogen, 50 ml/min; hydrogen, 40 ml/min; air, 500 ml/min.

RESULTS AND DISCUSSION

The separation of fourteen amino acids, as their N-TFA 2-butyl esters, on the second column, with a temperature rate of 2°/min is shown in Fig. 1. The relative position of each amino acid is the same as when using the N-TFA *n*-butyl esters, except for the inversion between the phenylalanine and aspartic acid peaks. The peaks of alanine, valine, leucine, threonine, serine, cysteine, phenylalanine, and tyrosine exhibit doublets, indicating partial resolution of the enantiomorph pairs of these amino acids.

As checked with the isoleucine–alloisoleucine pair, the peak of the doublet with the smallest retention time corresponds to the unseparable pairs DD + LL; the second peak, to the pairs DL + LD. Under these conditions, glycine and DD/LL-isoleucine are coeluted, and there is no separation between methionine and aspartic acid.

By using a smaller temperature rate, 1°/min, the resolution of the diastereomeric pairs is better, as shown in Fig. 2; particularly, the tryptophan peak displays partial resolution. But the D,L-glycine derivative and the DD/LL-isoleucine derivative are always coeluted, and the separation between methionine and aspartic acid derivatives continues to be poor.

While using column (1), containing a smaller (0.325 %) concentration in EGA, all fourteen amino acids are gas chromatographically separated (Fig. 3). The resolution of the diastereomeric pairs in this case is about the same, for the same temperature rate (here, 2°/min), as with the column coated with 0.65 % EGA (Fig. 1).

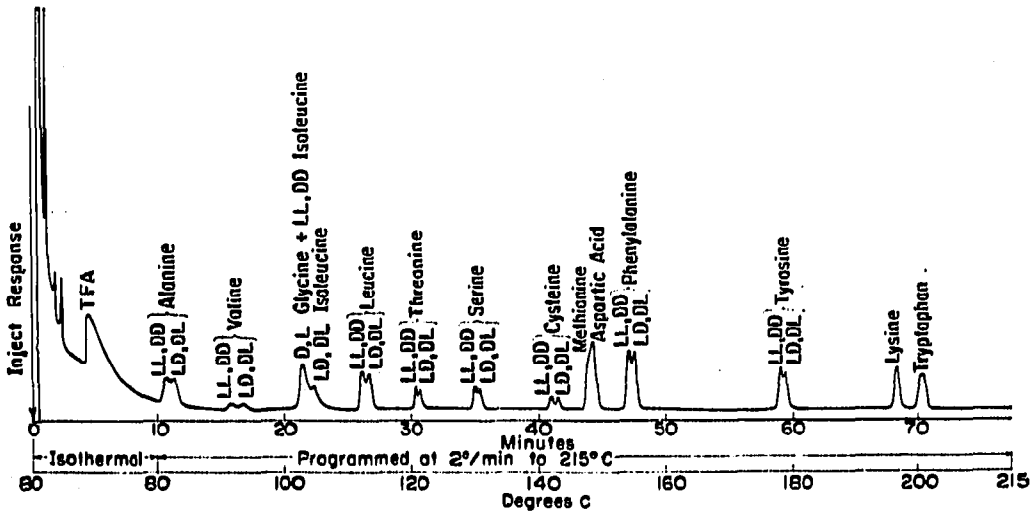


Fig. 1. Gas chromatogram of fourteen N-TFA-amino acid 2-butyl esters. 6 ft. × 4 mm I.D. glass column packed with 0.65 wt. % EGA coated on 80–100 mesh AW HT, Chromosorb G. Programmed temperature: isothermal for 10 min at 80°, 2°/min up to 215°. Sensitivity: 4×10^{-9} a.f.s. Each peak corresponds to about 25 nmoles of D,L-amino acid.

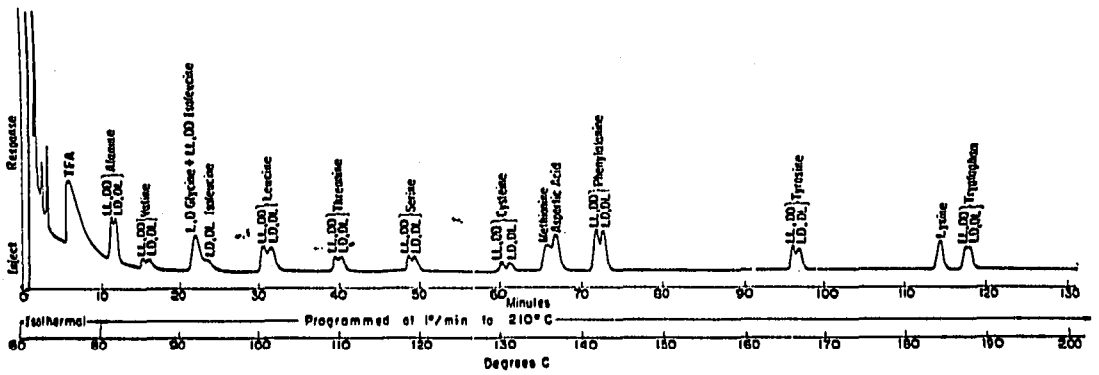


Fig. 2. Gas chromatogram of fourteen N-TFA-amino acid 2-butyl esters. Same conditions as for Fig. 1, except: programmed temperature: isothermal for 10 min at 80°, programmed at 1°/min up to 210°. Sensitivity: 2×10^{-9} a.f.s.

GLC of seventeen protein amino acids, as their N-TFA 2-butyl esters, on the longer column (3) is shown in Fig. 4. All the amino acids derivatives, except for methionine and hydroxyproline, are gas chromatographically separated, while using 2°/min for the programmed temperature rate. The peaks for glycine, of which the derivative has only one asymmetric center, and lysine do not present any doublet. But all the other protein amino acids diastereomers are partially resolved. The DL/LD-methionine derivative is coeluted with the DD/LL-hydroxyproline derivative.

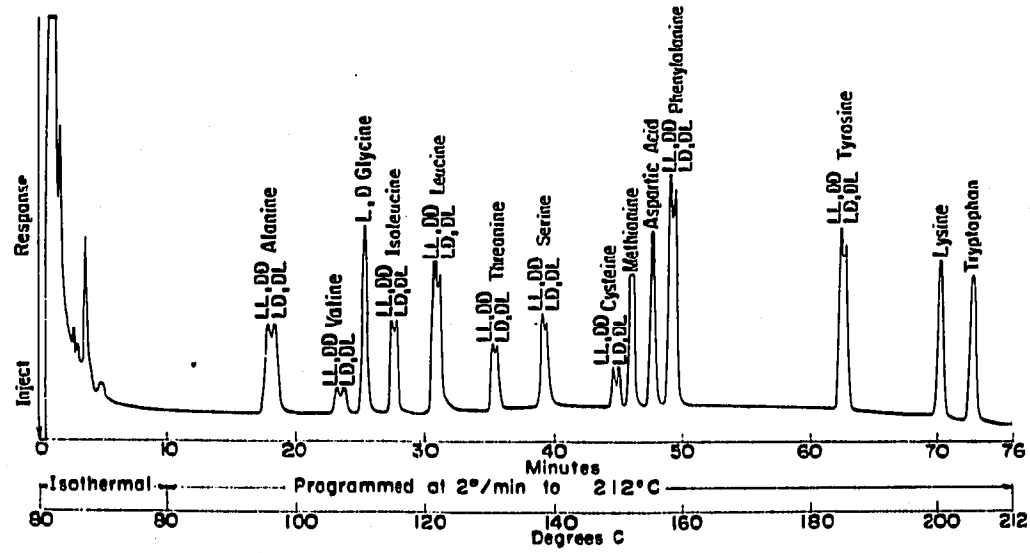


Fig. 3. Gas chromatogram of fourteen N-TFA-amino acid 2-butyl esters. Same conditions as for Fig. 1, except: 6 ft. × 4 mm I.D. glass column coated with 0.325 wt. % EGA on 80–100 mesh AW HT, Chromosorb G. Sensitivity: 1×10^{-9} a.f.s.

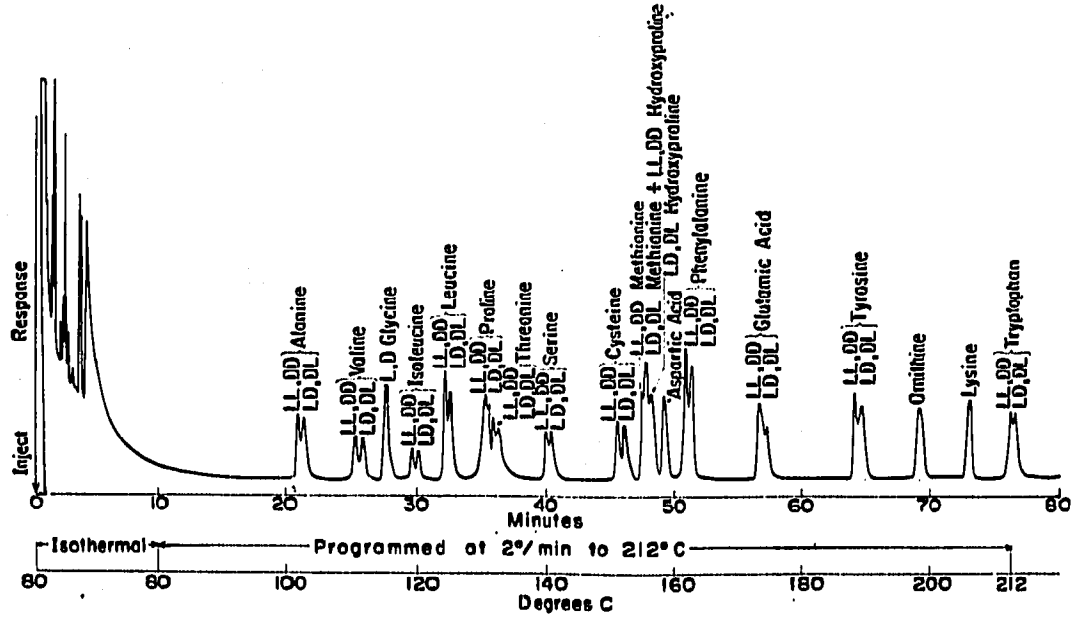


Fig. 4. Gas chromatogram of seventeen N-TFA-amino acid 2-butyl esters. Same conditions as for Fig. 1, except: 11 ft. × 2 mm I.D. glass column, coated with 0.325 wt. % EGA on 80–100 mesh AW HT, Chromosorb G. Sensitivity: 1×10^{-9} a.f.s.

The best results for the GLC separation of diastereomers have been obtained with this last column. The separation, on this column, of the seventeen protein amino acids, as their N-TFA *n*-butyl esters, gives the same relative position of each peak,

as with the 6 ft. × 4 mm I.D. glass column[22]. But using a column with such a small inner diameter limits the injected volume to only 5 μl.

ACKNOWLEDGEMENTS

We are grateful to Professor CARL SAGAN for his suggestions in this work. This was supported in part by N.A.S.A. Grant NGR 33-010-101.

REFERENCES

1 E. GIL-AV, B. FEIBUSH AND R. CHARLES-SIGLER, in A. B. LITTLEWOOD (Editor), *Gas Chromatography 1966*, Institute of Petroleum, London, 1966, p. 227.
2 S. NAKAPARKSIN, P. BIRRELL, J. ORO AND E. GIL-AV, *J. Chromatogr. Sci.*, 8 (1970) 177.
3 E. BAYER, E. GIL-AV, W. KOENIG, S. NAKAPARKSIN, J. ORO AND W. PARR, *J. Amer. Chem. Soc.*, 92 (1970) 1738.
4 W. KOENIG, W. PARR, H. LICHTENSTEIN, E. BAYER AND J. ORO, *J. Chromatogr. Sci.*, 8 (1970) 183.
5 W. PARR, C. YANG, J. PLETERSKI AND E. BAYER, *J. Chromatogr.*, 50 (1970) 510.
6 E. GIL-AV AND B. FEIBUSH, *Tetrahedron Lett.*, (1967) 3345.
7 F. RAULIN, B. KHARE AND C. SAGAN, to be published.
8 R. CHARLES, G. FISHER AND E. GIL-AV, *Isr. J. Chem.*, 1 (1963) 234.
9 E. GIL-AV, R. CHARLES AND G. FISHER, *J. Chromatogr.*, 17 (1965) 408.
10 G. E. POLLOCK, V. I. OYAMA AND R. D. JOHNSON, *J. Gas Chromatogr.*, 3 (1965) 174.
11 G. E. POLLOCK AND V. I. OYAMA, *J. Gas Chromatogr.*, 4 (1966) 126.
12 K. KVENVOLDEN, E. PETERSON, AND F. BROWN, *Science*, 169 (1970) 1079.
13 K. KVENVOLDEN, J. LAWLESS, K. PERING, E. PETERSON, J. FLORES, C. PONNAMPERUMA, I. R. KAPLAN AND C. MOORE, *Nature*, 228 (1970) 923.
14 J. LAWLESS, K. KVENVOLDEN, E. PETERSON, C. PONNAMPERUMA AND C. MOORE, *Science*, 173 (1971) 626.
15 S. V. VITT, H. B. SAPOROVSKAYA, I. P. GUDKOVA AND V. M. BELIKOV, *Tetrahedron Lett.*, 30 (1965) 2575.
16 J. W. WESTLEY AND B. HALPERN, in C. L. A. HARBOURN (Editor), *Gas Chromatography 1968*, Institute of Petroleum, London, pp. 119–128.
17 B. HALPERN AND J. M. WESTLEY, *Chem. Commun.*, 12 (1965) 246.
18 C. W. GEHRKE, D. ROACH, R. W. ZUMWALT, D. L. STALLING AND L. L. WALL, *Quantitative Gas–Liquid Chromatography of Amino Acids in Proteins and Biological Substances*, Report of Analytical Biochemistry Laboratories, 1968.
19 D. ROACH AND C. W. GEHRKE, *J. Chromatogr.*, 44 (1969) 269.
20 R. W. ZUMWALT, K. KUO AND C. W. GEHRKE, *J. Chromatogr.*, 55 (1971) 267.
21 C. W. GEHRKE, K. KUO AND R. W. ZUMWALT, *J. Chromatogr.*, 57 (1971) 209.
22 F. RAULIN, P. SHAPSHAK AND B. N. KHARE, *J. Chromatogr.*, 73 (1972) 35.