SEPARATION OF AMINO ACIDS AS THEIR N-TRIFLUOROACETYL-*n*-BUTYL ESTERS BY GAS CHROMATOGRAPHY

F. MARCUCCI, E. MUSSINI, F. POY* AND P. GAGLIARDI*
Istituto di Ricerche Farmacologiche "Mario Negri", Milan (Italy)
(Received October 15th, 1964)

Gas–liquid chromatography techniques may lead to rapid analysis of quantities of amino acids smaller than those detectable by classic ion-exchange column chromatography[1–3]. For example HUNTER *et al.*[4] and ZLATKIS *et al.*[5] used gas chromatography for the separation of aliphatic amino acids which had been converted to the corresponding aldehydes by means of ninhydrin. BIER AND TEITELBAUM[6] separated the amines obtained from decarboxylation of the corresponding amino acids. BAYER *et al.*[7,8] reported the separation of some aliphatic amino acids as methyl esters. By using nitrous acid LIBERTI[10] transformed the amino acids to α-hydroxy acids which were methyl-esterified and then separated by gas–liquid chromatography.

A gas-chromatographic separation has also been reported for methyl esters of α-chloro acids obtained from some aliphatic amino acids[11].

More recently several attempts of separation were made by combining the esterification of the carboxyl group with an acetylation of the amino group. Thus different authors have preferred one or other of the following transformations: N-acetyl-*n*-propyl[18], *n*-butyl[9], or *n*-amyl[14] esters; or N-trifluoroacetyl methyl[12,13], *n*-butyl[17], or *n*-amyl[22] esters.

Amino acids have also been separated by gas chromatography after their esterification with trimethyl-silane[15]. Finally HORNING *et al.*[16], using a different approach, separated the phenylthiohydantoin and dinitrophenyl derivatives of amino acids.

The present study concerns a method for the preparation of the N-trifluoroacetyl-*n*-butyl esters of eleven amino acids and the determination of the best conditions for their separation by gas chromatography.

MATERIALS

A Fractovap model C analytical unit P.AID/f (Carlo Erba, Milan) with hydrogen flame detector and automatic temperature programmer was used. Two meter stainless steel coiled columns (internal diameter 2 mm, external diameter 4 mm) were packed with 3 different stationary phases (obtained from Applied Science Laboratories Inc.): (a) neopentyl-glycol succinate, 1 %; (b) EGSS-X 1 %; (c) Carbowax 20 M, 1 %. The solid supports were Gas Chrom P (80–100 mesh) or Chromosorb (100–120 mesh).

The L-amino acids, chromatographically pure, were obtained from Mann Research Laboratories Inc., New York, N.Y., isoleucine was obtained from Hoffman

* Of D.A.S.-C. Erba, Milan.

La Roche, Basel. The *n*-butanol, methylene chloride and trifluoroacetic anhydride, gas-chromatographically pure, were obtained from Fluka, Switzerland.

A cationic resin, Dowex 50Wx4, from Down Co., was used after separation to a uniform size of about 200 mesh.

Thin-layer chromatography was made on glass plates (20 × 20 cm) by stratification of silica gel (Kieselgel G, Merck) with an automatic apparatus[23].

PREPARATION OF THE N-TRIFLUOROACETYLAMINO ACID *n*-BUTYL ESTERS

The hydrochlorides of the amino acids (total amount from 2.5 to 5 mg) were esterified in 10 ml of *n*-butanol together with 100 to 200 mg of dry Dowex 50Wx4, H^+ form* (200 mesh) as catalyst[19, 20]. After 3 h at 130° the butanol was decanted and the resin washed twice with 10 ml butanol. Ten ml of a citrate buffer (semi-saturated solution) at pH 6.95 and 20 ml of methylene chloride were then added to the washed resin. The effect of pH on the percentage of butyl esters eluted from the resin is shown in Fig. 1.

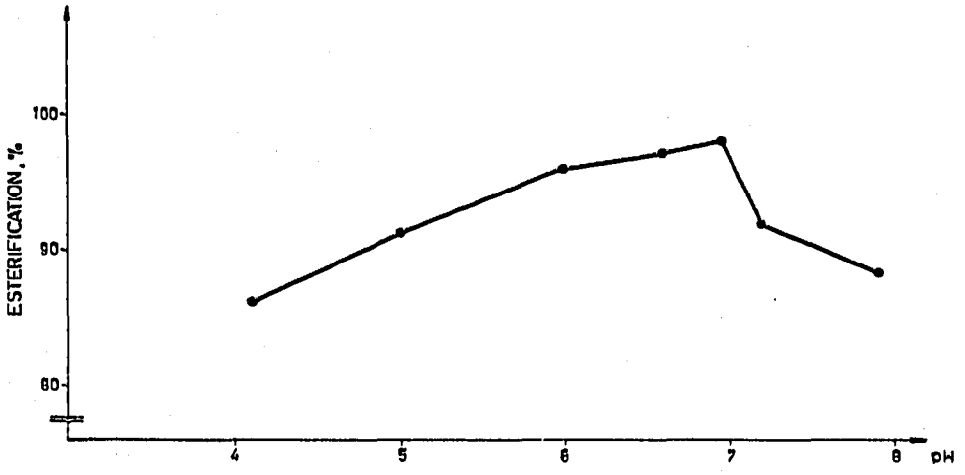

Fig. 1. Per cent esters eluted at different pH values.

After shaking for 5 min, the aqueous phase was discarded while the organic layer containing the esters of the amino acids was concentrated, under vacuum (15 mm Hg) in an ice bath, to a volume of 10 ml.

The butyl esters were then mixed, with continuous stirring, with 5 ml of a solution of trifluoroacetyl anhydride 1.5–2 % in methylene chloride. The excess of the reagent was removed under vacuum (15 mm Hg) whilst in an ice bath.

The residue was dissolved in 0.5 ml of methylene chloride and 3–5 μl of this were introduced into the chromatographic column.

With this procedure it was possible to prepare also the N-trifluoroacetyl, *n*-amyl or methyl esters. However, we selected the *n*-butyl ester because, being less volatile than the methyl esters, it could be manipulated with a minimal loss. Furthermore, under our experimental conditions, the *n*-butyl esters could be separated in a shorter time than the *n*-amyl esters.

* The use of resins with a higher degree of cross-linking (Dowex 50Wx8 or x12 or x20) or of carboxylic resins (Amberlite IRC50) does not improve the esterification.

QUANTITATIVE ESTIMATIONS

To determine whether the esterification of the amino acids with *n*-butanol was complete the reactivity to ninhydrin (0.2 % in *n*-butanol) after migration on a thin-layer chromatoplate was studied. With the solvent used (benzene–*n*-butanol (75:25, v/v)) the butyl esters showed various R_F's, after a 90-min run, while unmodified amino acids do not migrate from the point of application (see Fig. 2). Under these conditions methylene chloride extract did not show any trace of unmodified amino acids.

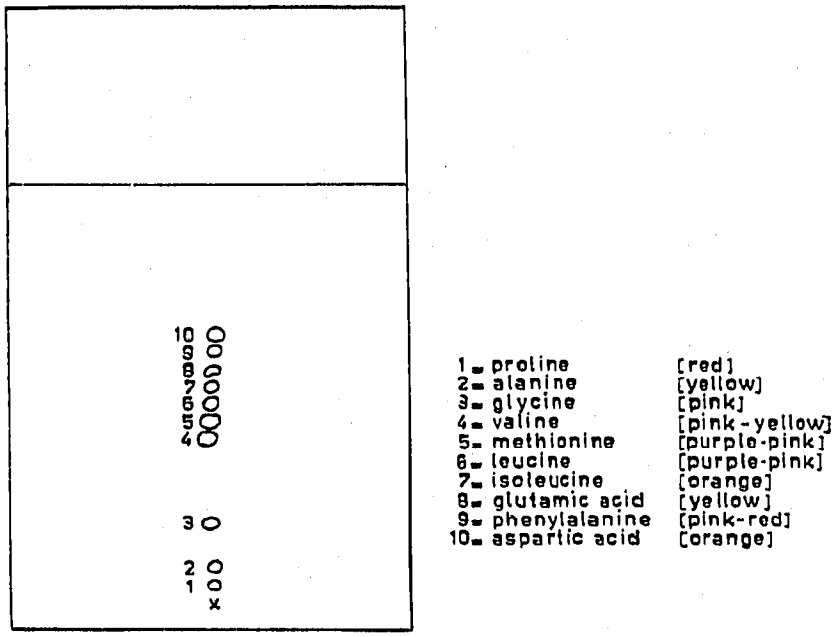

Fig. 2. Separation of a mixture of ten amino acid butyl esters by thin-layer chromatography. Spot identification by means of ninhydrin reagent (colours in parentheses).

Acidic hydrolysis of the amino acid *n*-butyl esters performed with 6 *N* HCl for 24 h and subsequent reaction with ninhydrin[21] confirmed that the esters represented up to 98 % of the amino acids passed through the process. After trifluoroacetylation the reactivity to ninhydrin was completely negative.

Further studies are in progress to obtain esterification of other amino acids and quantitative results after gas chromatography. The solution of these problems might permit the application of the procedure for the complete analysis of protein hydrolysates.

GAS-CHROMATOGRAPHIC SEPARATION

The column temperature was programmed from 90° to 200° with a linear increase of 2.5°/min. This temperature program was selected after various attempts as the most suitable for obtaining the separation of the 11 amino acid derivatives used (alanine, valine, isoleucine, leucine, glycine, proline, threonine, methionine, aspartic acid, phenylalanine, and glutamic acid). The flow rate of the carrier gas nitrogen was 14 c.c./min and the volume/min, as measured through a by-pass, was 5 c.c. Among

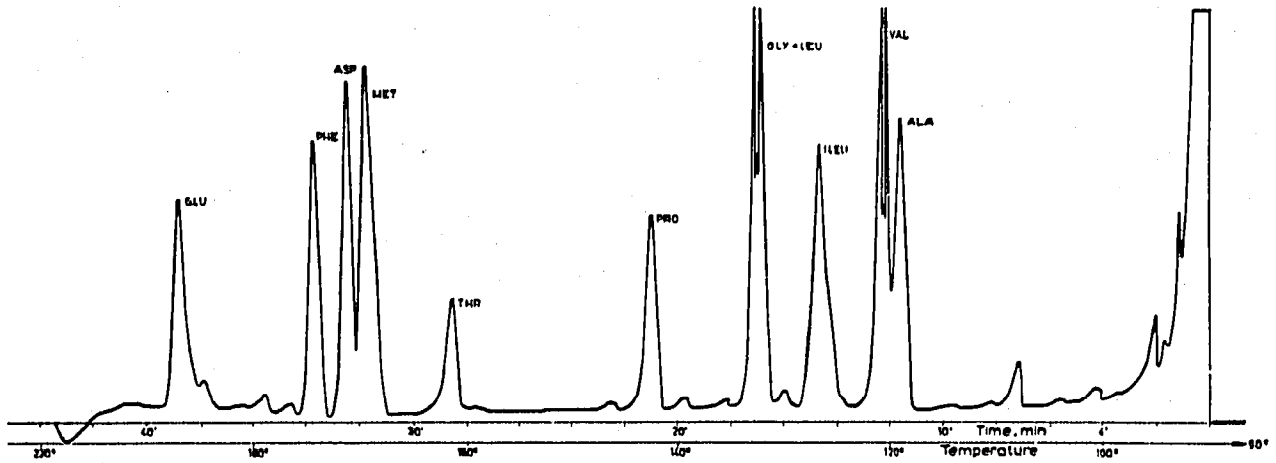

Fig. 3. Gas chromatogram of a mixture of 11 amino acids. NeoPGS 1 %. Carrier N_2 14 cc/min, H_2 23 cc/min, air 300 cc/min. Program 2.5°/min.

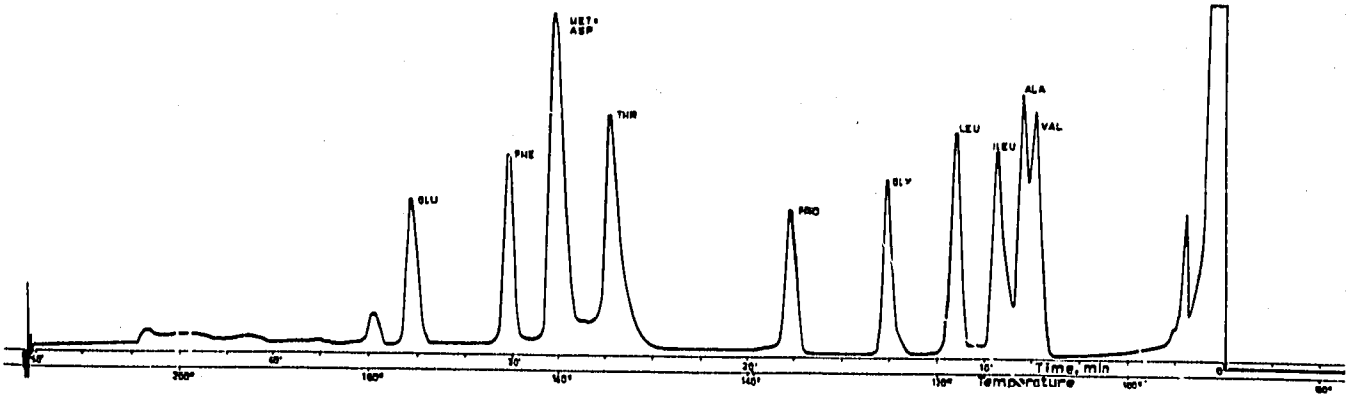

Fig. 4. Gas chromatogram of the same amino acids mixture. EGSS-X 1 %. Carrier N_2 14 cc/min H_2 23 cc/min, air 300 cc/min. Program 2.5°/min.

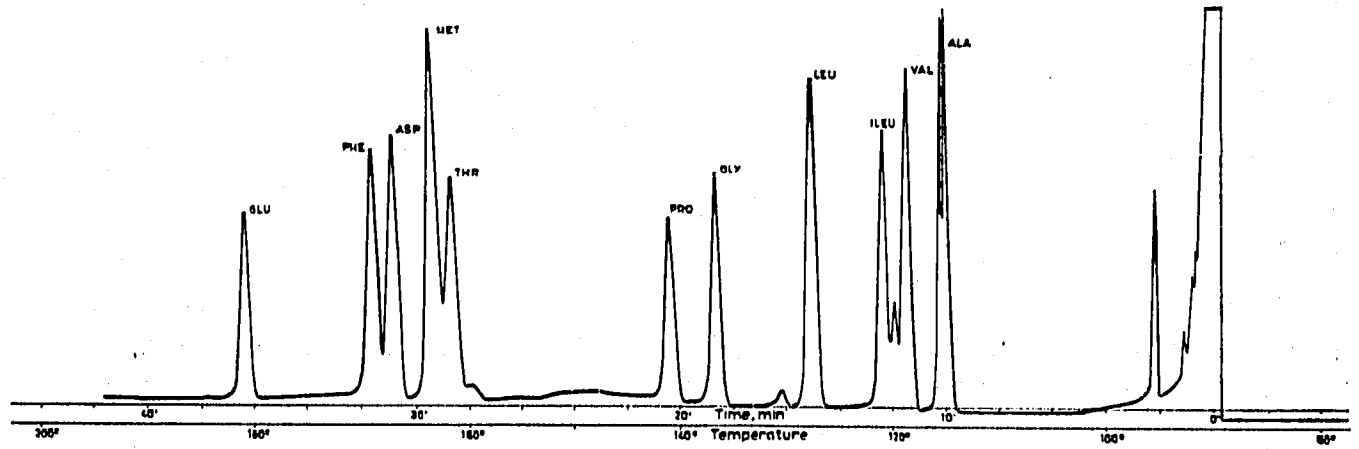

Fig. 5. Gas chromatogram of the same amino acids mixture. Carbowax 20 M 1 %. Chromosorb 80/100 mesh. Carrier N_2 14 cc/min, H_2 23 cc/min, air 300 cc/min. Program 2.5°/min.

the stationary phases used, only the neopentyl-glycol succinate has been reported in the literature[14,17] for separation of amino acids. EGSS-X and Carbowax 20 M, not previously employed for this type of analysis, gave more satisfactory results. Neopentyl-glycol succinate does not permit the separation of glycine from leucine. With EGSS-X it was possible to separate these two amino acids, but methionine and aspartic acid emerged in a single peak. Only when working with Carbowax 20 M was it possible to obtain a fair separation of all the 11 amino acids.

The peak pertaining to isoleucine includes also allo-isoleucine, contained as impurity in the sample of isoleucine.

The two amino acids are resolved only with Carbowax 20 M.

Figs. 3, 4 and 5 show typical chromatograms of amino acid derivatives after separation with the 3 stationary phases.

Furthermore, neopentyl-glycol succinate under our experimental conditions can only be used for a very limited number of analyses, while EGSS-X and Carbowax 20 M have the advantage that they permit up to one hundred separations.

SUMMARY

Eleven amino acids (alanine, valine, isoleucine, leucine, glycine, proline, threonine, methionine, aspartic acid, phenylalanine, and glutamic acid) were esterified in the carboxyl group with *n*-butanol in the presence of a catalyst resin and then transformed into their N-trifluoroacetyl derivatives.

The amino acid derivatives were satisfactorily separated by means of gas chromatography using Carbowax 20 M as stationary phase.

The experimental conditions necessary to obtain the best results are described.

REFERENCES

1 S. Moore and W. H. Stein, *J. Biol. Chem.*, 211 (1954) 893.
2 S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, 30 (1958) 1185.
3 K. A. Piez and L. Morris, *Anal. Biochem.*, 1 (1960) 187.
4 I. R. Hunter, K. P. Dimick and J. W. Corse, *Chem. Ind. (London)*, (1956) 294.
5 A. Zlatkis, J. F. Oro and A. P. Kimball, *Anal. Chem.*, 32 (1960) 162.
6 M. Bier and P. Teitelbaum, *Ann. N.Y. Acad. Sci.*, 72 (1959) 641.
7 E. Bayer, in D. H. Desty (Editor), *Gas Chromatography*, Academic Press, New York, 1958, p. 833.
8 E. Bayer, K. H. Reuther and F. Born, *Angew. Chem.*, 69 (1957) 640.
9 G. C. Youngs, *Anal. Chem.*, 31 (1959) 1019.
10 A. Liberti, in D. H. Desty (Editor), *Gas Chromatography*, Academic Press, New York, 1958, p. 341.
11 M. Melamed and M. Renard, *J. Chromatog.*, 4 (1960) 339.
12 F. Weygand and R. Geiger, *Chem. Ber.*, 89 (1956) 647, 1543.
13 H. A. Saroff and A. Karmen, *Anal. Biochem.*, 1 (1960) 344.
14 D. E. Johnson, S. J. Scott and A. Meister, *Anal. Chem.*, 33 (1961) 669.
15 K. Ruhlmann and W. Giesecke, *Angew. Chem.*, 73 (1961) 133.
16 J. J. Pisano, W. J. A. VandenHeuvel and E. C. Horning, *Biochem. Biophys. Res. Commun.*, 7 (1962) 82.
17 C. Zomzely, G. Marco and E. Mery, *Anal. Chem.*, 34 (1962) 1414.
18 I. Graff, J. P. Wein and M. Winitz, *Federation Proc.*, 22 (1963) 244.
19 S. Sussman, *Ind. Eng. Chem.*, 38 (1946) 1228.
20 P. J. Mill and W. R. C. Crimmin, *Biochim. Biophys. Acta*, 23 (1957) 432.
21 S. Moore and W. H. Stein, *J. Biol. Chem.*, 211 (1954) 907.
22 K. Blau and A. Darbre, *Biochem. J.*, 88 (1963) 8P.
23 F. Marcucci and E. Mussini, *J. Chromatog.*, 11 (1963) 270.