Journal of Chromatography, 236 (1982) 151–156
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,347

DETERMINATION OF AMINO ACIDS BY MEANS OF GLASS CAPILLARY GAS–LIQUID CHROMATOGRAPHY WITH TEMPERATURE-PROGRAMMED ELECTRON-CAPTURE DETECTION

JASBIR CHAUHAN and ANDRÉ DARBRE*

Department of Biochemistry, University of London King's College, Strand, London WC2R 2LS (Great Britain)

(First received July 6th, 1981; revised manuscript received September 7th, 1981)

SUMMARY

Amino acids were separated as their heptafluorobutyryl isobutyl ester derivatives on a glass capillary support-coated open tubular column coated with OV-101 and Chromosorb R, using temperature programming and the electron-capture detector. The relative molar response values showed that some derivatives were detectable in much smaller amount than others. The method was applied to the determination of amino acids attached to tRNA.

INTRODUCTION

During the past twenty years there have been extensive developments in the separation and analysis of amino acids by gas–liquid chromatography (GLC). Amino acids, being multifunctional, require derivatization of reactive groups to obtain suitably volatile compounds. A large number of different derivatives and techniques for preparing derivatives have been employed (see extensive reviews, refs. 1–3). Separation of the amino acid derivatives was carried out usually with conventional GLC packed columns, but more recently capillary columns have been introduced to obtain improved resolution and sensitivity of detection[4–6]. With few exceptions workers have used the flame ionization detector (FID)[3]. The potential use of the electron-capture detector (ECD) for amino acids derivatives possessing suitable electronegative substituent groups was indicated[7,8]. However, the ECD was used normally under isothermal conditions and it was not possible to separate derivatives showing a wide range of volatilities, and reference was made to the difficulty of using the ECD with temperature programming[8]. Recently, support-coated open tubular (SCOT) capillary columns with the ECD and temperature programming of the column oven were used for trifluoroacetylhexafluoroisopropyl ester derivatives of homovanillic, isohomovanillic and vanillylmandelic acids[9] and N-heptafluorobutyryl (HFB) amino acid isobutyl ester derivatives[10]. We report here on the separation of the N(O)-HFB amino acid isobutyl ester derivatives on a glass capillary SCOT column with temperature programming and the ECD and show the application of the method to amino acids obtained after deacylation of silk-worm tRNA.

0021-9673/82/0000–0000/$02.75

EXPERIMENTAL

Gas chromatography was carried out with a Hewlett-Packard Model 7620A gas chromatograph fitted with a 2-mCi ^{63}Ni ECD. Peak areas were determined with a Hewlett-Packard integrator Model 3370A. A capillary column (25 m × 0.4 mm I.D.) was coated with 15% OV-101 and 5% Chromosorb R by a single-step method[11]. Direct injections onto the column with sample volumes up to 2.0 μl were made without inlet heater[12].

The N(O)-HFB amino acid isobutyl ester derivatives were prepared as previously described[13,14] using 10–20 nmol of each amino acid. Norleucine, pipecolinic acid and homoarginine were used as internal standards. Isooctane was the solvent for sample dilution and injection.

RESULTS AND DISCUSSION

Fig. 1. shows the response–concentration curves for N(O)-HFB amino acid isobutyl ester derivatives. The linear dynamic range applied over a range of 10-fold increase in concentration for each amino acid under the conditions given and with the instrument used. It was claimed that there was a linear response within the 10–400 pg

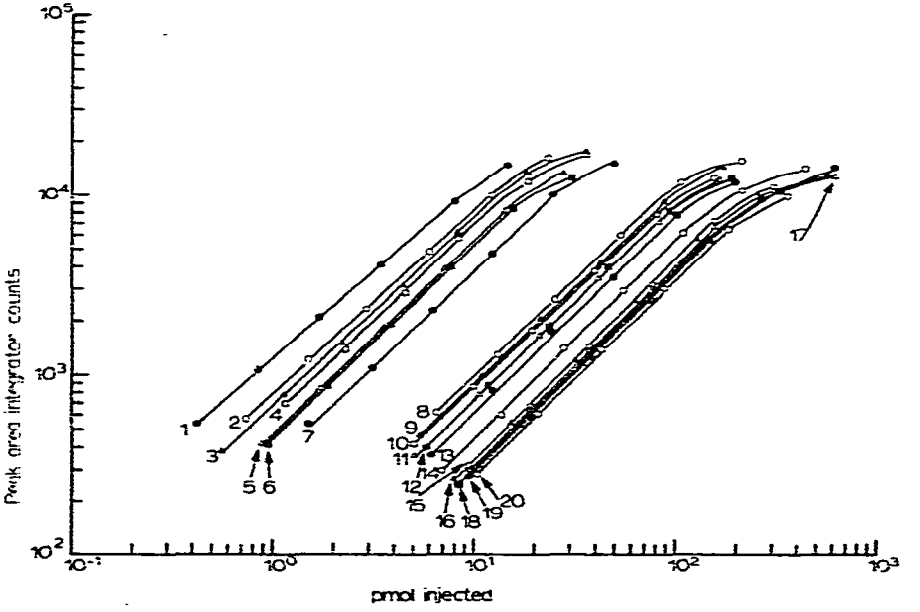

Fig. 1. Response-concentration curves for N(O)-HFB isobutyl ester derivatives of amino acids with the ECD. Preparation of sample: see Experimental. GLC conditions: 25 m × 0.4 mm I.D. coated with 5% Chromosorb R and 15% OV-101 SCOT column; carrier gas, hydrogen at a flow-rate of 3 ml/min; make-up gas argon–methane (90:10) flow-rate, 50 ml/min. Temperatures: detector, 320°C; no inlet heater block; column, 80°C programmed at 4°C/min. Pulse interval, 15 μsec; attenuation, 2 × 10^2; sample size, 1.0 μl. Curves: 1 = tyrosine; 2 = arginine; 3 = ornithine; 4 = threonine; 5 = lysine; 6 = serine; 7 = cystine; 8 = pipecolinic acid; 9 = glutamic acid; 10 = methionine; 11 = aspartic acid; 12 = proline; 13 = phenylalanine; 14 = alanine; 15 = leucine; 16 = 2-aminobutyric acid; 17 = glycine; 18 = norleucine; 19 = isoleucine; 20 = valine.

range for the same amino acid derivatives, but evidence was only presented for histidine[10]. Under our conditions the linear ranges for all the derivatives lay between *ca.* 1 and 100 pmol injected.

Increasing the pulse interval of the detector from 15 to 150 μsec showed a *ca.* 10-fold increase of sensitivity whilst still retaining a linear response over a limited range, as shown for alanine and lysine in Fig. 2. However, in practice it was best to work with a pulse interval of 15 μsec as previously reported[9]. Fig. 3 shows the separation of 27 N(O)-HFB amino acid isobutyl ester derivatives on a column at 80°C and programmed at 4°C/min to 230°C. Occasional "dips" in the baseline were seen. Increasing the pulse interval to gain increased sensitivity resulted in an increased size of the "dips", which made difficult the integration of peak areas. Some increase in sensitivity of detection was shown with an increase of pulse interval from 100 to 2000 μsec[15].

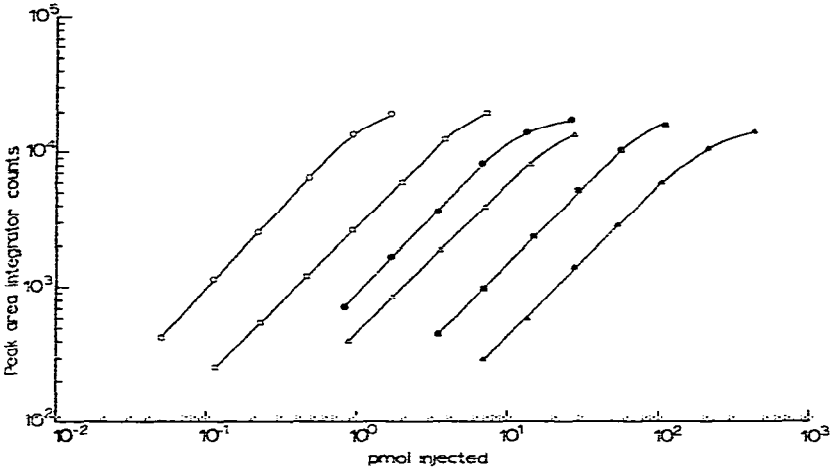

Fig. 2. Response–concentration curves for N(O)-HFB isobutyl ester derivatives of two amino acids with the ECD and different pulse intervals. Preparation of sample: see Experimental. GLC conditions as in Fig. 1. Pulse interval, 15 μsec; ▲ = alanine; △ = lysine. Pulse interval, 50 μsec; ■ = alanine; □ = lysine. Pulse interval, 150 μsec; ● = alanine; ○ = lysine.

Table I shows the relative molar response (RMR) values of HFB-amino acid isobutyl esters. The variation in RMR was *ca.* 36-fold (0.90 for valine to 32.7 for tyrosine). The reason for this wide range of RMR values cannot be fully explained. It is known that the ECD response varies with the type of electron-capturing group and the structure of the carrying molecule, but it is not proportional to the number of electron-capturing groups on a single molecule. Thus, because they carry two HFB groups, serine and threonine gave much higher RMR values than amino acids such as alanine, which carry only one HFB group. The very high value for tyrosine (32.7) was due not only to the presence of two HFB groups, but additionally to the effect of the aromatic ring. Thus, tyrosine showed a higher response than phenylalanine (Table I) as shown for the trifluoroacetyl (TFA) *n*-butyl ester derivatives[8]. Our results for the lower response given by 3,4-dihydroxyphenylalanine compared with that given by tyrosine cannot be explained.

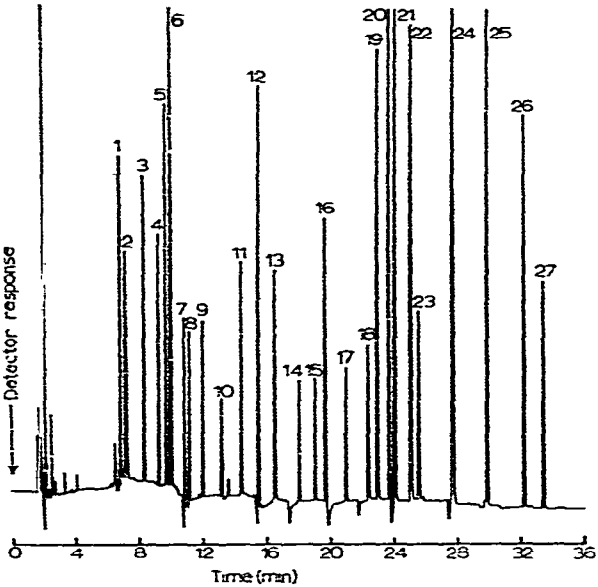

Fig. 3. Separation of N(O)-HFB isobutyl ester derivatives of amino acids with the ECD. Preparation of sample: see Experimental. GLC conditions: as in Fig. 1. The amounts of amino acids represented by individual peaks ranged from 1.4 pmol (tyrosine) to 34.0 pmol (valine). Peaks: 1 = alanine; 2 = glycine; 3 = 2-aminobutyric acid; 4 = valine; 5 = threonine; 6 = serine; 7 = leucine; 8 = isoleucine; 9 = norleucine; 10 = proline; 11 = pipecolinic acid; 12 = hydroxyproline; 13 = methionine; 14 = aspartic acid; 15 = phenylalanine; 16 = ornithine; 17 = glutamic acid; 18 = lysine; 19 = tyrosine; 20 = ε-monomethyllysine; 21 = 3,4-dihydroxyphenylalanine; 22 = arginine; 23 = carboxymethylcysteine; 24 = homoarginine; 25 = lanthionine; 26 = cystathionine; 27 = cystine.

Bengtsson *et al.*[10] rather surprisingly showed an ECD response for HFB-glycine isobutyl ester which was much greater than that for the corresponding lysine derivative, which carried two HFB groups. It was shown with N,O-bis-TFA threonine methyl ester that the ECD response was *ca.* 3500 times greater than the FID response, whilst N-TFA alanine, valine, isoleucine and leucine methyl esters gave an increased sensitivity of detection 100–200-fold over the FID[7]. HFB derivatives gave the best compromise between sensitivity and volatility, compared with TFA or pentafluoropropionyl derivatives[16] and N,O-bis-HFB tyrosine methyl ester could be detected down to *ca.* $0.5 \cdot 10^{-15}$ *M*[17].

The separation of N(O)-HFB-isobutyl ester derivatives of 27 amino acids on an OV-101 SCOT column with temperature programming from 80 to 230°C and the ECD set at 320°C is shown in Fig. 3. There were fewer extraneous peaks due to impurities than previously reported[10]. The small peak at 13.5 min following proline was due to cysteine which was derived from cystine. Histidine was not included in the mixture, because to ensure complete acylation of the amino acid it was recommended that on-column acylation should be used[14]. The excess acylating reagent affected the ECD response and this method was not applicable in our hands.

Widely differing concentrations of the amino acids were used because of their varying responses to the ECD. Thus there is a disadvantage in using the ECD for the determination of all the protein amino acids in a single sample. Different amounts of

TABLE I

RELATIVE RETENTION TIMES AND RELATIVE MOLAR RESPONSE VALUES OF N(O)-HFB AMINO ACID ISOBUTYL ESTER DERIVATIVES DETERMINED WITH THE ECD AGAINST NORLEUCINE (= 1.0).

GLC conditions as in Fig. 1. The retention time for norleucine in minutes is given in parentheses.

Amino acid	*Relative retention time*	*RMR ± S.D. (n = 8)*
Alanine	0.57	1.55 ± 0.03
Glycine	0.60	1.01 ± 0.03
2-Aminobutyric acid	0.69	1.06 ± 0.02
Valine	0.77	0.90 ± 0.03
Threonine	0.81	17.0 ± 0.31
Serine	0.83	15.1 ± 0.49
Leucine	0.90	1.14 ± 0.05
Isoleucine	0.93	0.95 ± 0.02
Norleucine	(11.90)	1.00
Proline	1.09	2.22 ± 0.08
Pipecolinic acid	1.21	3.05 ± 0.08
Hydroxyproline	1.30	24.3 ± 0.84
Methionine	1.38	2.46 ± 0.08
Aspartic acid	1.51	2.23 ± 0.05
Phenylalanine	1.59	2.02 ± 0.04
Ornithine	1.64	17.8 ± 0.97
Glutamic acid	1.76	2.50 ± 0.05
Lysine	1.87	15.2 ± 0.52
Tyrosine	1.92	32.7 ± 0.95
ε-Monomethyllysine	1.98	19.0 ± 0.73
3,4-Dihydroxyphenylalanine	2.01	25.4 ± 0.50
Arginine	2.09	21.8 ± 0.94
Carboxymethylcysteine	2.13	5.71 ± 0.15
Homoarginine	2.19	20.4 ± 0.87
Lanthionine	2.49	15.9 ± 0.43
Cystathionine	2.69	17.4 ± 0.77
Cystine	2.79	10.8 ± 0.53

the sample might require injecting onto the column for the determination of "low sensitivity" and "high sensitivity" electron-capturing derivatives.

It should be noted that when the ECD was set at 250°C a baseline rise was observed during the programme when the oven temperature exceeded *ca.* 180°C. The difficulty of using the ECD with temperature programming using conventional packed GLC columns was mentioned[8]. However, with capillary columns the carrier gas flow-rate (*ca.* 3 ml/min) is small compared with the 50 ml/min used for the make-up gas, and a steady baseline may be maintained as seen in Fig. 3.

The determination of amino acids attached to tRNA[18] was carried out in this study. Fig. 4a shows the separation of fourteen identifiable amino acids from tRNA detected with the ECD. The advantage of the use of the ECD over that of the FID is seen by comparison with Fig. 4b, where certain amino acids present in small amounts, such as proline, lysine and arginine, were barely detectable by the FID although they were readily determined with the ECD. In addition there were peaks of unknown composition with high electron-capturing properties which eluted after 5, 7, 30 and 32 min. The detection of these compounds was only possible with the ECD, and how they were derived from tRNA is now being investigated.

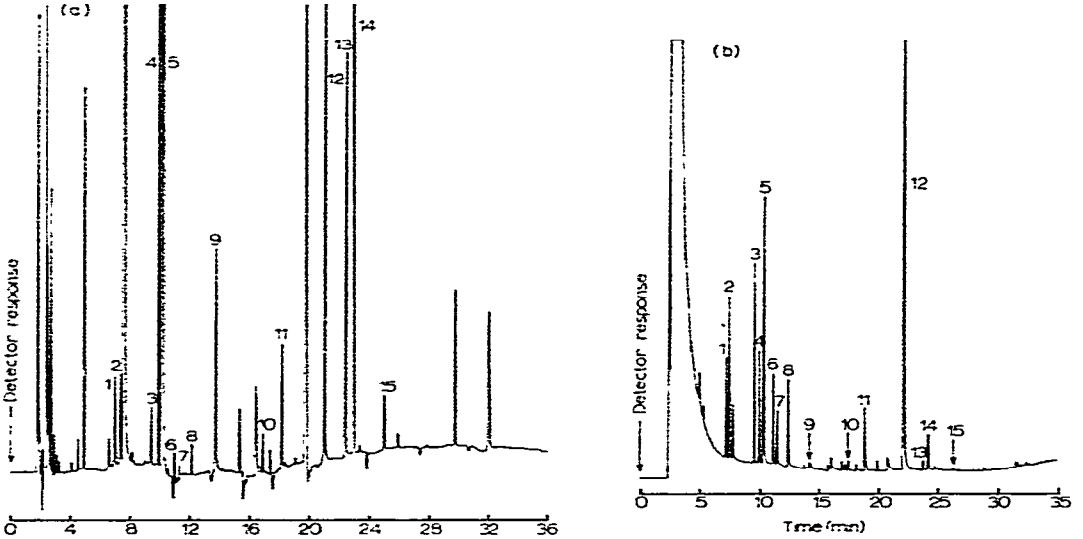

Fig. 4. Separation of N(O)-HFB amino acid isobutyl ester derivatives obtained from silk-worm tRNA after deacylation and analysed with (a) the ECD and (b) the FID. GLC conditions: (a) as in Fig. 1; (b) same conditions except make-up gas nitrogen flow-rate 30 ml/min; hydrogen 27 ml/min; air 350 ml/min; amount injected 20 times greater. Peaks: 1 = alanine; 2 = glycine; 3 = valine; 4 = threonine; 5 = serine; 6 = leucine; 7 = isoleucine; 8 = norleucine (I.S.); 9 = proline; 10 = methionine; 11 = aspartic acid; 12 = glutamic acid; 13 = lysine; 14 = tyrosine; 15 = arginine.

ACKNOWLEDGEMENTS

We thank the Ministry of Defence (A.M.T.E.) for financial support, Mr. Colin Chinnery for valuable technical assistance and Dr. G. Chavancy (Université Claude Bernard, Lyons, France) for silk-worm tRNA.

REFERENCES

1 K. Blau, in H. A. Szymanski (Editor), *Biomedical Applications of Gas Chromatography*, Vol. 2, Plenum Press, New York, 1968, p. 1.
2 B. Weinstein, *Methods Biochem. Anal.*, 14 (1966) 203.
3 P. Hušek and K. Macek, *J. Chromatogr.*, 113 (1975) 139.
4 R. F. Adams, F. L. Vandemark and G. J. Schmidt, *J. Chromatogr. Sci.*, 15 (1977) 63.
5 R. J. Pearce, *J. Chromatogr.*, 136 (1977) 113.
6 J. Desgres, D. Boisson and P. Padieu, *J. Chromatogr.*, 162 (1979) 133.
7 A. Islam and A. Darbre, *J. Chromatogr.*, 43 (1969) 11.
8 R. W. Zumwalt, K. Kuo and C. W. Gehrke, *J. Chromatogr.*, 57 (1971) 193.
9 J. Chauhan and A. Darbre, *J. Chromatogr.*, 183 (1980) 391.
10 G. Bengtsson, G. Odham and G. Westerdahl, *Anal. Biochem.*, 111 (1981) 163.
11 J. Chauhan and A. Darbre, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4 (1981) 11.
12 J. Chauhan and A. Darbre, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4 (1981) 260.
13 S. L. MacKenzie and D. Tenaschuk, *J. Chromatogr.*, 171 (1979) 195.
14 S. L. MacKenzie and D. Tenaschuk, *J. Chromatogr.*, 173 (1979) 53.
15 D. C. Fenimore and C. M. Davis, *J. Chromatogr. Sci.*, 8 (1970) 519.
16 G. S. King, B. R. Pettit, K. Blau and P. M. Lax, *Biomed. Mass. Spectrom.*, 6 (1979) 482.
17 K. Blau, in J. M. Rattenbury (Editor), *Amino Acid Analysis*, Ellis Horwood, Chichester, 1981, p. 48.
18 M. Butler, A. Darbre and H. R. V. Arnstein, *Biochem. J.*, 150 (1975) 419.