Journal of Chromatography, 168 (1979) 255–259

CHROM. 11,341

Note

A new aspect of derivatisation in gas chromatography of micro-amounts of amino acids*

MIROSLAV MATUCHA

Institute for Research, Production and Application of Radioisotopes, 170 04 Prague 7 (Czechoslovakia)

and

EVA SMOLKOVÁ

Department of Analytical Chemistry, Faculty of Sciences, Charles University, 120 43 Prague 2 (Czechoslovakia)

(Received July 8th, 1978)

Of the many derivatives available for the GLC of amino acids, trifluoroacetylated *n*-butyl esters (TAB) have been widely used[1]. The technique of the determination has been reported in detail: sample preparation[2,3], derivative formation[2–12], separation[13–18], analysis of submicrogram amounts[15,19–22], problems of contamination[20–23] and purity of the reagents[2,3,20,21,23], applications to the analysis of biological substances[2,3,8,22,24,25], lunar samples[21,26] and of ^{14}C-labelled amino acids[12,27–30] have all been described. The physico-chemical constants[5] and the mass spectra of the TAB derivatives[31] have also been reported.

This procedure seemed to be sufficiently sophisticated for use in analysing carrier-free preparations of amino acids biosynthetically labelled with ^{14}C, for control of the radiochemical purity (usually 97–99%) and for the determination of concentration and specific activity of these substances. Normally, in establishing the procedure, one works with all, or at least with groups, of amino acids; however, we were obliged to analyse the individual compounds for impurities.

EXPERIMENTAL

Chemicals

Standards of non-radioactive amino acids were supplied by Calbiochem (Los Angeles, Calif., U.S.A.); ^{14}C-labelled amino acids originated from ÚVVVR (Prague, Czechoslovakia), and [^{14}C]glycine was also purchased from NEN (Dreieichenhain, G.F.R. *n*-Butanol, p.a., was obtained from Lachema (Brno, Czechoslovakia) and from Merck (Darmstadt, G.F.R.), trifluoroacetic anhydride (20% solution in dichloromethane) from Serva (Heidelberg, G.F.R.), acetonitrile, pyridine and dichloromethane, p.a., from Merck and 1,3-dichlorotetrafluoroacetone from Fluka (Buchs, Switzerland).

---

* Presented at the *Sixth International Symposium, Biomedical Applications of Chromatography*, May 1978, Hluboká, Czechoslovakia. The majority of the papers presented at this symposium will be published in *J. Chromatogr.*, Vol. 162, No. 2 (1979).

Derivatisation and gas chromatography

For preparation of the TAB derivatives, we used the procedure of Roach and Gehrke[10]; for the derivatisation of simple amino acids, condensation with 1,3-dichlorotetrafluoroacetone, as described by Hušek[32], was used.

The TAB derivatives, as well as oxazolidinones prepared from the amino acids, were analysed on a column (2 m × 2 mm I.D.) packed with 1% of the siloxane phase OV-225 on Chromosorb G HP (100–120 mesh); a Packard chromatograph (model 7409) equipped with dual flame ionisation detection was used, with argon (30 ml/min) as carrier gas and temperature-programming from 70° to 220° at 5°/min.

The concomitant determination of radioactivity was accomplished by combustion (at 650°) over copper oxide of substances in the split carrier-gas stream in a Packard Tricarb furnace (model 325); water was absorbed in a tube filled with magnesium perchlorate, and the residual gas was mixed (1:1) with methane for counting by means of a 2-ml gas flow-proportional counter. The counting unit consisted of a Berthold LB 2007 pre-amplifier, a 242 K high-voltage supplier, an amplifier, a dual-channel rate-meter, an LB 2520 scaler–timer and a Goerz RE 520 dual-pen recorder.

RESULTS AND DISCUSSION

After successful use with 1-mg amounts of carriers for carrier-free preparations of ^{14}C-labelled amino acids analysed as TAB derivatives, difficulties arose during extension of the method to the micro- and submicro-gram range. At temperatures higher than those corresponding to the retention times of the TAB derivatives of simple amino acids, several extraneous peaks regularly appeared; an example is shown in Fig. 1, the radiochromatogram of [^{14}C]glycine. This fact could not be attributed to lower radiochemical purity of the compounds, to the presence of higher alcohols in the *n*-butanol used in the esterification step, to incomplete acylation nor to any similar reason. The intensity of these peaks increased with decreasing sample size.

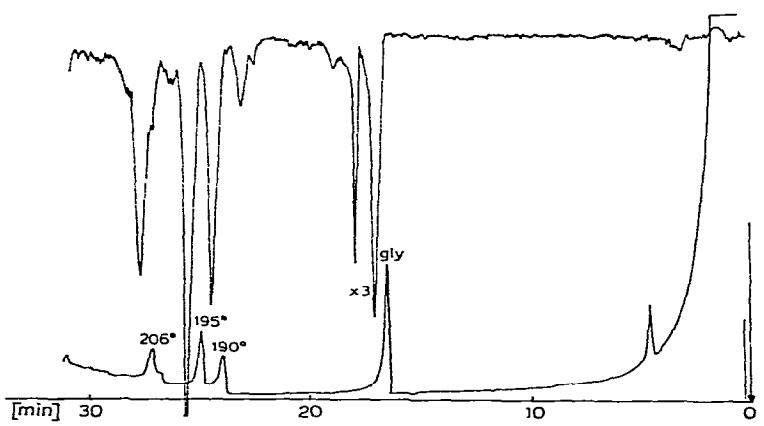

Fig. 1. Radio-gas chromatogram of carrier-free [^{14}C]glycine after butylation and trifluoroacetylation: upper curve, radioactivity trace; lower curve, mass trace of the injected portion of derivatised glycine (10 μCi).

In at least one reported analysis of submicrogram amounts of amino acids[19], the presence of extraneous peaks at high retention temperatures was also observed; the authors did not comment on this fact.

Gehrke and his co-workers[23] have drawn attention to possible sources of sample contamination by, *e.g.*, skin, dust, fingerprints, hair and reagents. They recommended that chromatographic-reagent blank values should be determined and that the performance of authentic amino acids in concentrations similar to those present in the sample should be studied[20]. Our reagent blank was, however, satisfactory (see Fig. 2), and other sources of contamination were virtually excluded. Cancalon and Klingman[12] attributed the presence of extraneous peaks to the low purity of *n*-butanol and explained the formation of these substances by polymerisation of carbonyl compounds (present in *n*-butanol) at the high temperature of esterification. The presence of radioactivity in the extraneous peaks, however, makes this explanation unlikely.

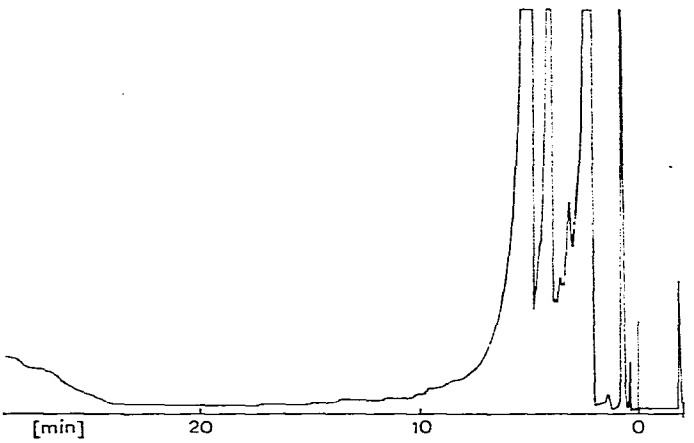

Fig. 2. Chromatographic-reagent blank of the procedure (100 μl of *n*-butanol used for esterification; acylation with 100 μl of trifluoroacetic anhydride).

The evident side-reactions of amino acids could be caused by the presence of more reactive impurities in the *n*-butanol, as the relative content of by-products of the derivatisation could be lowered by addition of the carrier and the impurities were formed during butylation (see Fig. 3). The addition of less than 0.1 % of butyraldehyde to *n*-butanol gave rise to many peaks on the chromatogram.

We offer the following explanation. The content (*ca.* 1 to 10 ppm) of carbonyl compounds, *e.g.*, butyraldehyde, in *n*-butanol is sufficient to react more readily and quantitatively with the NH_2-group of an amino acid during butylation, so that only the rest of the amino acid *n*-butyl ester is acylated by trifluoroacetic anhydride when the TAB derivative is formed. This "rest" is naturally proportional to the amount of amino acid originally present and is enhanced by using *n*-butanol of high purity. The critical factor of the derivatisation procedure is thus carbonyl-free *n*-butanol (which can be prepared by a known procedure[12]). Its purity cannot be established only by the chromatographic-reagent blank; the by-products of butylation are obviously not only polymers of carbonyl compounds.

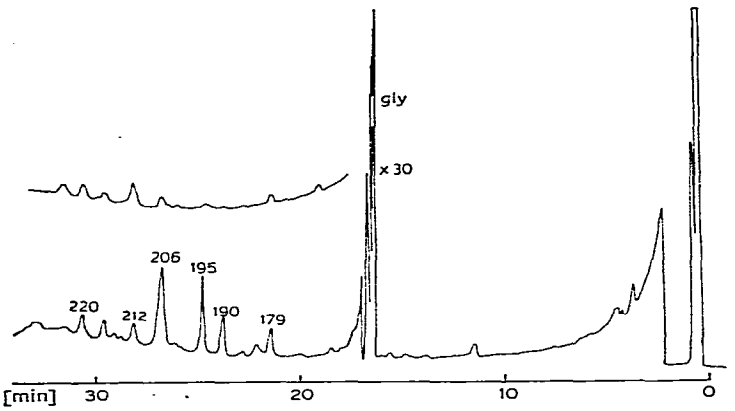

Fig. 3. Gas chromatogram of 10 μg of glycine after butylation (upper curve) and after butylation and acylation (lower curve).

Therefore, we suggest a further criterion to establish the suitability of reagents for the determination of micro- and submicro-amounts of amino acids as TAB derivatives. This is the derivatisation of a representative amino acid in an amount similar to that expected in the sample; the gas chromatogram of this product should contain no extraneous peaks indicative of the formation of by-products.

As an alternative to the formation of TAB derivatives, the use of oxazolidinones[32] (see Fig. 4) can be recommended; the GLC of amino acids as oxazolidinones is more convenient with respect to the problems described above. However, despite many possible difficulties associated with the use of TAB derivatives, there is no doubt as to the usefulness of this method in general, but, in determing micro-amounts of amino acids, quantitative formation of a well-defined volatile derivative is essential.

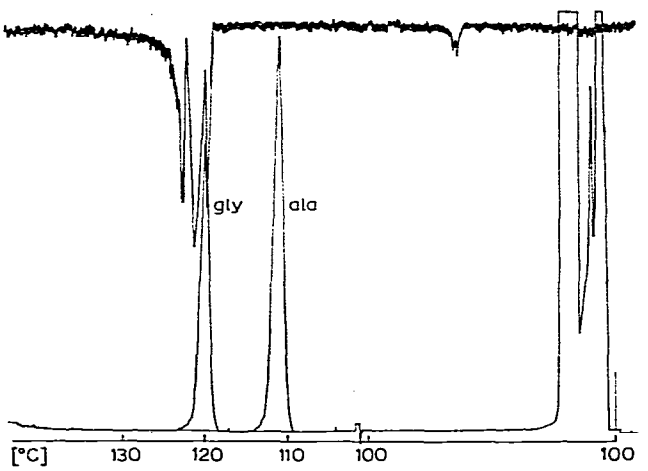

Fig. 4. Radio-gas chromatogram of [^{14}C]glycine containing non-radioactive alanine as internal standard in the form of bis(chlorodifluoromethyl)-1,3-oxazolidin-5-one. The sensitivity of the radioactivity trace of the [^{14}C] glycine peak was decreased by a factor of 10.

It can also be concluded that the use of labelled compounds in chromatography permits monitoring of the compounds during derivatisation as well as during the chromatographic process.

REFERENCES

1 P. Hušek and K. Macek, *J. Chromatogr.*, 113 (1975) 139.
2 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: Macro, Semimicro and Micro Methods*", Jackson Anal. Biochem. Lab., Columbia, Mo., 1968.
3 F. E. Kaiser, C. W. Gehrke, R. W. Zumwalt and K. C. Kuo, *J. Chromatogr.*, 94 (1974) 113.
4 C. Zomzely, G. Marco and E. Emery, *Anal. Chem.*, 34 (1962) 1414.
5 W. M. Lamkin and C. W. Gehrke, *Anal. Chem.*, 37 (1965) 383.
6 C. W. Gehrke, W. M. Lamkin, D. L. Stalling and F. Shahrokhi, *Biochem. Biophys. Res. Commun.*, 19 (1965) 328.
7 D. L. Stalling and C. W. Gehrke, *Biochem. Biophys. Res. Commun.*, 22 (1966) 329.
8 C. W. Gehrke and D. L. Stalling, *Separ. Sci.*, 2 (1967) 101.
9 D. Roach, C. W. Gehrke and R. W. Zumwalt, *J. Chromatogr.*, 43 (1969) 311.
10 D. Roach and C. W. Gehrke, *J. Chromatogr.*, 44 (1969) 269.
11 C. W. Gehrke and K. Leimer, *J. Chromatogr.*, 53 (1970) 195.
12 P. Cancalon and J. D. Klingman, *J. Chromatogr. Sci.*, 12 (1974) 349.
13 C. W. Gehrke and F. Shahrokhi, *Anal. Biochem.*, 15 (1966) 97.
14 M. Stefanovic and B. L. Walker, *Anal. Chem.*, 39 (1967) 710.
15 C. W. Gehrke, R. W. Zumwalt and L. L. Wall, *J. Chromatogr.*, 37 (1968) 398.
16 W. J. Mc Bride, Jr., and J. D. Klingman, *Anal. Biochem.*, 25 (1968) 109.
17 D. Roach and C. W. Gehrke, *J. Chromatogr.*, 43 (1969) 303.
18 C. W. Gehrke and H. Takeda, *J. Chromatogr.*, 76 (1973) 63.
19 R. W. Zumwalt, K. Kuo and C. W. Gehrke, *J. Chromatogr.*, 55 (1971) 267.
20 R. W. Zumwalt, K. Kuo and C. W. Gehrke, *J. Chromatogr.*, 57 (1971) 193.
21 C. W. Gehrke, R. W. Zumwalt, D. L. Stalling, D. Roach, W. A. Aue, C. Ponnamperuma and K. A. Kvenvolden, *J. Chromatogr.*, 59 (1971) 305.
22 W. Frick, D. Chang, K. Folkers and G. D. Daves, Jr., *Anal. Chem.*, 49 (1977) 1241.
23 J. J. Rash, C. W. Gehrke, R. W. Zumwalt, K. C. Kuo, K. A. Kvenvolden and D. L. Stalling, *J. Chromatogr. Sci.*, 10 (1972) 444.
24 D. J. Casagrande, *J. Chromatogr.*, 49 (1970) 537.
25 R. W. Zumwalt, D. Roach and C. W. Gehrke, *J. Chromatogr.*, 53 (1970) 171.
26 C. W. Gehrke, *Space Life Sci.*, 3 (1972) 342.
27 W. J. Mc Bride and J. D. Klingman, *J. Neurochem.*, 19 (1972) 865.
28 A. Del Favero, A. Darbre and M. Waterfield, *J. Chromatogr.*, 40 (1969) 213.
29 M. Matucha, V. Svoboda and E. Smolková, *J. Chromatogr.*, 91 (1974) 497.
30 A. I. Kunakovskij and V. I. Mišin, *Proc. Conf. Organičeskije sojedinĕnija mĕčennyje radioaktivnymi isotopami, Mariánské Lázně, 1976*, ČSKAE, Prague, 1977, p. 216.
31 K. R. Leimer, R. H. Rice and C. W. Gehrke, *J. Chromatogr.*, 141 (1977) 121.
32 P. Hušek, *J. Chromatogr.*, 91 (1974) 475.