*Journal of Chromatography*, 355 (1986) 273–280
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 299

# THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION AND GAS–LIQUID CHROMATOGRAPHIC SEPARATION OF SEVEN AMINOBUTYRIC ACIDS IN THE PRESENCE OF PROTEIN AND NON-PROTEIN AMINO ACIDS

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(Received September 30th, 1985)

## SUMMARY

Gas–liquid chromatographic separation of the following aminobutyric acids as their trifluoroacetyl *n*-propyl esters was achieved on 0.65% ethylene glycol adipate coated on Chromosorb W AW: α-aminoisobutyric; α-aminobutyric; β-aminoisobutyric; β-aminobutyric γ-aminobutyric; γ-amino-β-hydroxybutyric and diaminobutyric acid. Only the two isomers of 3-aminobutyrate were not completely separated. Additionally, the positions of the aminobutyric acids and some other amino acids occurring in bacterial fermentation broths on a cellulose thin-layer plate after two-dimensional separation are given.

In the fermentation broth of *Clostridium oncolyticum* M 55, only α-aminobutyric and γ-aminobutyric acid were identified as excretion products. In addition to these, *Clostridium sporogenes* produced additionally β-aminobutyric acid in quantities equalling γ-aminobutyric acid production. α-Aminoisobutyric and β-aminoisobutyric acid were completely absent, and γ-amino-β-hydroxybutyric and diaminobutyric acid were detected in low concentrations throughout the entire fermentation.

## INTRODUCTION

Several aminobutyric acids have been shown to be intermediates or end-products of metabolic pathways in various organisms. α-Aminobutyric acid (α-ABA) has been observed as an endproduct of various *Clostridium* spp.[1]. α-Aminoisobutyric acid (α-AIBA) cannot be metabolized and is therefore suited to transport studies.

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0021-9673/86/$03.50 

β-Aminobutyric acid (β-ABA) has been identified as an intermediate, at least as its Coenzyme A thioester, in the lysine degradation of various *Clostridia*[2]. β-Aminoisobutyric acid (β-AIBA) is a well-known end-product of thymidine degradation in many organisms. The inherited deficiency of the enzyme β-aminoisobutyrate transaminase in humans leads to the excretion of this particular amino acid in the urine of patients[3]. The function of gamma-aminobutyric acid (GABA) as a neurotransmitter is well established.

Diaminobutyric acid (DABA) has been shown to compete with GABA during synaptonemal uptake[4]. It could be detected as a constituent of the cell wall of some plant pathogenic Corynebacteria[5]. Mouse fibroma cells can be damaged by incubation with DABA and antitumour activity was also demonstrated *in vivo*[6]. DABA is also a constituent of the antibiotic polymyxin[7]. In summary, there is very little knowledge concerning the biological significance and function of many of these compounds.

Although many methods for amino acid determination include some of the compounds listed above, no report has focused on the separation and quantitation of this particular group of compounds. Of these, only the clinically important α-ABA, β-AIBA and GABA, if any, are included in the classical ion-exchange chromatographic methods[8]. Although high-performance liquid chromatography (HPLC) of amino acids has become well established in the last few years, the methods published to date include only α-ABA[9–12] and occasionally GABA[13].

Many of the methods based on gas–liquid chromatographic (GLC) separation of acylalkyl esters of amino acids allow the determination of several of these compounds. Adams reported the chromatographic data of α-ABA, α-AIBA, β-AIBA and GABA, derivatized to their N-acetyl-*n*-propyl esters[14]. Most of these co-eluted with protein amino acids.

Determination of these compounds, as well as DABA, has been described by Amico *et al.*, who analysed the amino acids as their N-trifluoroacetyl (TFA-) *n*-butyl esters either on OV-17[15] or on ethylene glycol adipate (EGA)[16] as the stationary phase. Simultaneously, Siezen and Mague published the chromatographic data of these five compounds and that of γ-amino-β-hydroxybutyric acid (GABOB) and of β-ABA after preparing the heptafluorobutyryl (HFB-) isobutyl esters[17]. They used OV-101 as the stationary phase for separation. Again, the aminobutyric acids were separated only poorly or not at all from at least one other amino acid when using this system. Later, the separation of α-ABA, β-AIBA and GABA from the protein amino acids as their HFB-isobutyl derivatives was reported[18].

None of the methods previously described allows the determination of all seven compounds in a single run when other amino acids commonly found in biological fluids are present.

The authors recently reported the excretion of α-ABA and GABA in considerable amounts by *Clostridium oncolyticum* M 55[19]. Because some unidentified peaks occurred in the chromatograms, we investigated the chromatographic behaviour of the aminobutyric acids not previously included in our amino acid determination method[20]. Herewith, we describe the separation and quantitation of seven amino butyric acids on 0.65% EGA after derivatization to their TFA-*n*-propyl esters.

## MATERIALS AND METHODS

Acetyl chloride and methylene chloride (gold label quality) were obtained from Aldrich (Beerse, Belgium), trifluoroacetic anhydride (reagent grade) was purchased from Pierce (Rotterdam, The Netherlands), and α-Aminocaprylic acid (α-ACA) (B-grade) was purchased from Calbiochem (San Diego, CA, U.S.A.). The amino acids were chromatographically pure and obtained from Serva (Heidelberg, F.R.G.). Dowex 50W-X8 was obtained from BioRad (Vienna, Austria). All other chemicals were analytical grade from Merck (Darmstadt, F.R.G.). Standard solutions (2.5 m*M*) were prepared in 0.1 *M* hydrochloric acid and stored at 4°C.

Chromatographic materials were purchased from Supelco (Crans, Switzerland). Chromatography was performed with a Hewlett-Packard HP 5880A gas chromatograph with BASIC-capability equipped with flame ionization detectors and an automatic sampler HP 7672A.

The bacterial strains used were *Clostridium oncolyticum* M 55 (ATCC 13732) and *Clostridium sporogenes* NCIB 10696, respectively. Cultivation of the clostridium species will be described elsewhere (G. F. X. Zuder and H. Brantner, unpublished results). The bacteria were cultivated on complex media containing: (1) 4% (w/v) casein peptone, 0.5% (w/v) meat extract and 0.5% (w/v) sodium chloride in potable water (pH = 7.35); (2) Trypticase Soy Broth; (3) 4% (w/v) meat extract in potable water (pH = 7.35).

After pretreatment with sulphosalicylic acid, the amino acids were isolated from the medium by cation exchange with Dowex 50W-X8 and derivatized to their N(O,S)-TFA-*n*-propyl esters as described previously[21]. The derivatives were analysed on a 6 ft. × 2 mm I.D. glass column filled with 0.65% EGA coated on Chromosorb W AW. Other supports resulted in a significant loss of separation capability. The carrier gas was helium at a flow-rate of 25 ml/min. The flame ionization detector was supplied with 450 ml of air and 30 ml of hydrogen per min. Due to the differences in size and polarity of the amino acid derivatives, a temperature programme is necessary. The oven temperature began at 110°C for 1 min, was then increased to 220°C by 20°C/min, and then held constant until a total analysis time of 15 min was completed.

Quantitation is best performed by internal standardization. In this investigation, α-ACA was used for standardization, because no co-elution with any other amino acid was observed, whereas the norleucine derivative co-elutes with pipecolic acid.

For TLC identification of the amino acids, the method described by Kraffczyk *et al.*[22] was employed. The amino acids are separated in the first dimension by a basic solvent. After removing the first solvent, the plates are developed in a second dimension with an acidic solvent. In contrast to the procedure suggested by Kraffczyk *et al.*, samples of the fermentation broths were analysed without addition of a standard solution.

## RESULTS

When the derivatization method described by Gamerith[21] is employed for the preparation of the TFA-*n*-propyl esters of the amino acids, the responses listed in

TABLE I

RELATIVE MOLAR RESPONSES (RMR) CALCULATED TO NORLEUCINE

Values are the means of five determinations, except for the two 3-aminobutyric acids ($n$ = 3).

| *Amino acid* | *RMR* | *Standard deviation* | *Relative standard deviation (%)* |
|---|---|---|---|
| α-AIBA | 0.75 | 0.026 | 3.4 |
| α-ABA | 0.76 | 0.010 | 1.3 |
| β-AIBA | 0.73 | 0.007 | 1.0 |
| β-ABA | 0.78 | 0.012 | 1.6 |
| GABA | 0.73 | 0.009 | 1.3 |
| GABOB | 0.82 | 0.021 | 2.6 |
| DABA | 0.82 | 0.030 | 3.7 |

Table I, result. The standard deviations and relative standard deviation are also given. The responses relative to norleucine suggest that the reaction is almost complete. The standard deviations are comparable to those described in earlier investigations[21].

Very similar responses were found for the monoaminobutyric acids. Investigation of the derivatization reactions was therefore considered to be unnecessary.

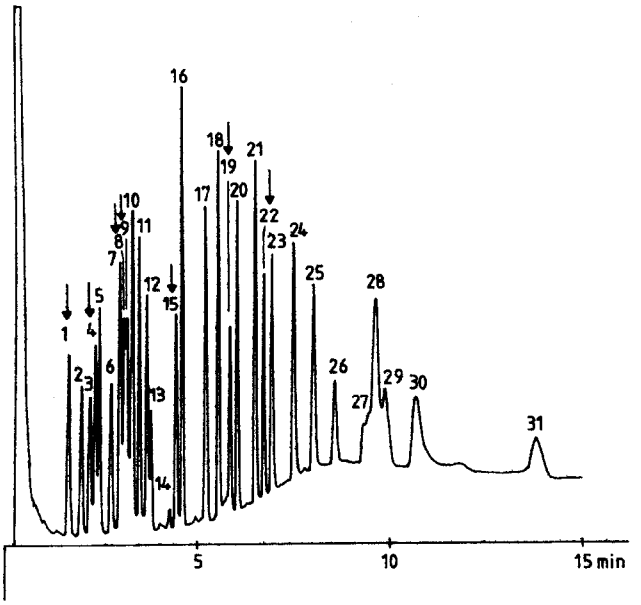


Fig. 1. Separation of seven aminobutyric acids from other amino acids. Chromatographic parameters are given in the text. Chart speed 1 cm/min, attenuation 32. The standard solution contained *ca.* 0.25 μmol/ml except for Hyl (0.14 m*M*) and His (0.75 m*M*); 3 μl were injected. Peak labelling: 1 = α-AIBA; 2 = Ala; 3 = sarcosine, 4 = α-ABA, 5 = Val, 6 = Gly, 7 = Ile, 8 = β-AIBA, 9 = β-ABA, 10 = β Ala + Leu, 11 = norleucine, 12 = Pro, 13 = Thr, 14 = Ser, 15 = GABA, 16 = α-ACA, 17 = Asx, 18 = Phe, 19 = GABOB, 20 = Glx, 21 = α-amino adipic acid, 22 = DABA, 23 = α-APA, 24 = Orn, 25 = Lys, 26 = Trp, 27 = Hyl, 28 = DAPA, 29 = methionine sulphone, 30 = His, 31 = cystathionine. The aminobutyric acids are marked by arrows.

Separation of the aminobutyric acids from the other amino acids is shown in Fig. 1. With the single-step temperature programme employed, the last aminobutyric acid derivative elutes after less than 7 min. The derivatives of β-ABA and β-AIBA are not separated completely, but coefficients of variation are as low as for other amino acids.

Application of this method to the analysis of a fermentation broth obtained after 30 h of *Clostridium sporogenes* growth is shown in Fig. 2. The derivatives of α-ABA, β-ABA and GABA were present. The identity of β-ABA was verified by loading a sample with this amino acid. The spiked peak revealed exactly the same retention time as when injecting the unloaded sample. GABOB and DABA were present in minor quantities. Fluctuation of their concentrations (50 ± 18 and 36 ± 11 mg/l) did not correlate with bacterial growth. When *Clostridium onocolyticum* M 55 is cultivated using the same experimental approach, a different spectrum of aminobutyric acids is found. The concentrations of the aminobutyric acids found in various media after cultivation of either *Clostridium sporogenes* or *Clostridium oncolyticum* M 55 are given in Table II.

The pattern of the excreted aminobutyric acids depends largely on the medium used for bacterial cultivation. *Clostridium oncolyticum* M 55 produced equal quantities of α-ABA and GABA when grown on a medium containing Trypticase Soy Broth. However, when a 4% meat extract was used as nutrient broth, very little GABA was found. All media resulted in a similar measure of growth.

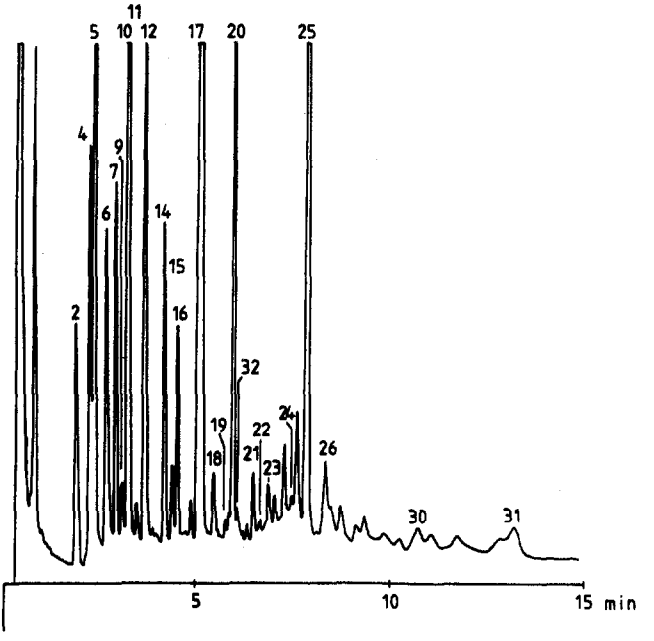


Fig. 2. Analysis of fermentation broth of *Clostridium sporogenes*. Cultivation in 4% casein peptone–0.5% meat extract for 30 h. Chromatography is described in the text. Attenuation was 64, chart speed 1 cm/min. Peak labelling as in Fig. 1 except that peak 11 represents pipecolic acid rather than norleucine, peak 17 = 5-aminovaleric acid + Asx (the separation is not visible in the chromatogram, but recognized by the integrator) and peak 32 = 5-aminolevulinic acid.

TABLE II

EXCRETION OF AMINOBUTYRIC ACIDS

| | *Amino acid (mg/l)* | | |
|---|---|---|---|
| | *α-ABA* | *β-ABA* | *GABA* |
| *C. sporogenes*, 30 h cultivated on 4% casein peptone–0.5% meat extract | 423 | 98 | 99 |
| *C. oncolyticum*, 24 h cultivated on 4% casein peptone–0.5% meat extract | 652 | – | 76 |
| *C. oncolyticum*, 48 h cultivated on 4% casein peptone–0.5% meat extract | 706 | – | 161 |
| *C. oncolyticum*, 48 h cultivated on 4% meat extract | 9 | – | 66 |
| *C. oncolyticum*, 48 h cultivated on Trypticase Soy broth | 664 | – | 595 |

TLC was used to verify the results obtained by GLC. In Fig. 3, the hatched spots indicate the positions of several amino acids not included in the original work of Kraffczyk *et al.*[22]. The GLC results were confirmed by the thin-layer chromatograms obtained with 2 μl of the fermentation broth. With this technique, the spots of α-ABA, GABA, GABOB, 5-aminovaleric acid (5-AVA). homoserine (HSer), β-ABA, alpha-aminopimelic acid (α-APA) and diaminopimelic acid (DAPA) become

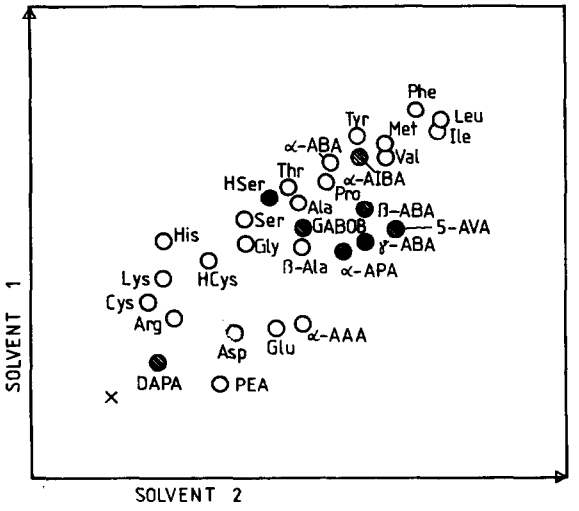


Fig. 3. TLC identification on a cellulose thin-layer plate. Solvent I: pyridine–dioxane–25% ammonia–water (35:35:15:15), solvent II: *n*-butanol–acetone–acetic acid–water (35:35:7:23). Precoated cellulose TLC plates (10 × 10 cm, Merck) were used. The plates were developed twice in both directions, then sprayed with ninhydrin solution and heated to 80°C.

TABLE III

RESPONSES OF THE AMINOBUTYRIC ACID DERIVATIVES RELATIVE TO THE GABA DERIVATIVE

| *Amino acid* | *TFA-n-propyl esters* | *TFA-n-butyl esters*[15,16] | *HFB isobutyl esters*[17] | *HFB isobutyl esters*[18] |
|---|---|---|---|---|
| α-AIBA | 1.03 | 0.79/0.92 | 0.6 | – |
| α-ABA | 1.04 | 0.73/0.85 | 0.94 | 1.41 |
| β-AIBA | 1.00 | 0.65/0.76 | 0.94 | 1.50 |
| β-ABA | 1.07 | – | 0.90 | – |
| GABA | 1.00 | 1.00 | 1.00 | 1.00 |
| GABOB | 1.12 | – | 1.12 | – |
| DABA | 1.12 | 0.53/0.61 | 1.00 | – |

visible after heating the plate to 80°C, whereas β-AIBA and α-AIBA are only detected upon heating to 110°C for several min. DABA was not detectable because of extreme tailing in the first dimension. The two β-aminobutyric isomers are obviously not separated by this technique. No spot was found for delta-aminolevulinic acid, which can be determined by GLC (G. Gamerith, unpublished results).

## DISCUSSION

Compounds with the same molecular weight and the same functional groups, as is the case with the monoaminobutyric acids, may be expected to have a very similar response when detected by flame ionization. The responses of six aminobutyric acid derivatives relative to that of GABA are listed in Table III. The values are very similar for the monoaminobutyric acids found in our experiments. Table III also includes values obtained from previously published methods. There, great differences in the responses of the aminobutyric acids point to incomplete derivatization or other experimental errors. Only the results of Siezen *et al.*[17] are comparable, except for the low response of α-AIBA, which perhaps results from losses during preparation.

Although several methods described previously include some or all compounds of this group, none permits the separation of all aminobutyric acids from the other amino acids. With the method presented here, the seven amino butyric acids can be separated within 7 min. The time for sample preparation and derivatization was minimized by working up ten samples simultaneously. Thus, the overall expenditure of time and money is far less than with ion-exchange methods.

The quantities of the excreted aminobutyric acids varied with the cultivated species and the medium. The most striking difference between the two *Clostridium* spp. is the production of β-ABA by *Clostridium sporogenes*, while *Clostridium oncolyticum* excretes only traces of this compound, if any.

In addition to its usefulness in investigations dealing with the excretion of organic acids, the quantitative determination of metabolized and excreted amino acids is considered to be a helpful tool in the taxonomy of proteolytic bacteria. The determination of amino acid excretion by two-dimensional TLC has been used for

bacterial taxonomy, however the lack of quantitation results in decreased accuracy of the conclusions[23].

Standardizations of the media, necessary for identifying bacteria by means of their organic acid excretion[24], are also important in the determination of amino acids for taxonomy. The metabolic pathways synthesizing aminobutyric acids, and particularly their regulation, are as yet only poorly understood and need to be clarified in further investigations.

ACKNOWLEDGEMENTS

We thank Professor W. Rosenkranz for the facilities for carrying out the analytical work in his institute. This work was supported by the Fonds zur Förderung der Wissenschaftlichen Forschung, Project No. 5559.

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