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Note

Derivatization of amino acids to their N(O,S)-acyl alkyl esters for gas–liquid chromatographic determination

GERNOT GAMERITH
Institut für Medizinische Biologie und Humangenetik, Universität Graz, Harrachgasse 21/8, A-8010 Graz (Austria)

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Gas–liquid chromatography (GLC) is commonly used in the determination of amino acids in protein hydrolysates and in biological samples. It permits inexpensive and rapid analyses, with excellent resolution, and seems to be the most suitable technique for the quantitative screening of large numbers of samples[1,2]. Many different derivatives suitable for GLC analysis have been described[3]. Today, the method most often used is a two-step procedure involving esterification of carboxylic groups with acidified alcohols followed by acylation with acetic anhydride[4], trifluoroacetic anhydryde (TFAA) or heptafluorobutyric anhydride (HFBA) after prior evaporation of the first reaction medium. The N(O,S)-HFB isobutyl esters[5], the N(O,S)-HFB *n*-propyl esters[6] and the N(O,S)-TFA *n*-butylesters[7] have been used extensively, although other possible combinations are expected to behave similarly.

In our work on quantitative amino acid screening, it appeared that the procedures described for derivatization needed further investigation before application to large numbers of samples.

The procedure described in this paper allows the derivatization of up to 50 samples per day when preparing the N(O,S)-TFA *n*-propylesters. The number of samples will be lower if butanol or HFBA is used, because of longer evaporation times.

As described elsewhere[8], the time necessary for separation of N(O,S)-TFA *n*-propyl esters of more than 30 amino acids can be reduced to less than 20 min. Therefore, the overall advantage of GLC can be retained in spite of the requirement of the preparation of volatile derivatives prior to analysis.

EXPERIMENTAL

The reagents used were of analytical-reagent grade from Merck (Darmstadt, G.F.R.). TFAA was of reagent grade, purchased from Pierce (Rotterdam, The Netherlands). Dowex 50W-X8 (100–200 mesh) was obtained from Bio-Rad Labs. (Vienna, Austria), and amino acids from Fluka, Serva and Calbiochem.

Laboratory equipment included 100 × 16 mm glass tubes with PTFE-lined screw-caps (Sovirel), a block heater (Pierce) and a Büchi rotavapor. Heating to 150°C was performed using an aluminum block in which holes of depth 3 cm and diameter

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17 mm had been drilled. This allows good reflux during reaction. Addition of silicone oil to the holes to obtain a better heat transfer was tested, but offered no advantage and was subsequently omitted. For evaporation the rotavapor was equipped with a special attachment allowing the simultaneous evaporation of ten tubes. The tubes were connected to the rotavapor by screw-caps with a hole and PTFE-lined seals (Sovirel).

GLC was performed on a Hewlett-Packard 5880A gas chromatograph with one flame-ionization detector (FID), one nitrogen–phosphorus selective FID (NP-FID) and an HP 7672A automatic sampler. The other chromatographic equipment, including stock packings of 0.65% ethylene glycol adipate (EGA) on Chromosorb W AW (80–100 mesh) and 2% OV-17–1% OV-210 on Supelcoport (100–120 mesh), was obtained from Supelco.

The esterification reagent can be prepared either by bubbling anhydrous hydrogen chloride through the alcohol or by addition of acetyl chloride, the latter method being more convenient for routine applications. A 3.5 *M* solution is prepared by addition of 21.3 ml of acetyl chloride to 78.7 ml of 1-propanol at 0°C. The molarity may be ascertained by titration.

Derivatization

Pre-treatment of biological samples was performed according to Adams[4]. Samples containing up to about 10 μmole of amino acids were evaporated on the rotavapor at 40–50°C. To the dry residue were added 300 μl of the esterification reagent and the tubes were capped firmly. Esterification was performed at 110°C for 25 min. Then the tubes were cooled to room temperature with cold water and evaporated at 40°C. Meanwhile, the caps were dried in an incubator at *ca.* 90°C to ensure complete removal of condensed reagent. Trifluoracetylation was carried out by addition of 400 μl of methylene chloride or chloroform and 200 μl of TFAA. It is essential to cap the tubes firmly, as a fairly high pressure develops when heating the tubes to 150°C. Also, loss of reagent results in a reduced response for some amino acids.

When using HFBA instead, the samples were heated at 150°C for 10 min[9]. Again, the tubes were cooled with cold water to room temperature and evaporated at 30°C. Higher temperatures may result in losses of the more volatile derivatives. The dry residue was dissolved in an appropriate amount of ethyl acetate, depending on the amino acid concentration of the sample. Starting with 200 μl of a 2.5 m*M* standard solution, the derivatives were dissolved in 1 ml, resulting in a final concentration of 0.5 m*M*. When using the FID 3 μl were injected, but 1 μl was sufficient for the NP-FID.

RESULTS AND DISCUSSION

A method for amino acid determination retaining the advantages of GLC over classical ion-exchange chromatography must minimize the expense of time and money resulting from the derivatization necesary prior to the chromatography. This becomes even more important if the method is to be suitable for quantitative screening because of the large numbers of samples involved.

Although many different methods for the GLC analysis of amino acids have

been described[3], only a few were developed for clinical use[4,10–13]. However, none of them fulfilled the requirements we believed to be necessary for a screening method, as there were always some points inconvenient to the analysis of the large numbers of samples involved in our problem. Therefore, the procedure was improved by combining methods and suggestions made by different workers concerning the time and expense involved in the derivatization. Although the procedure was originally developed for the preparation of the N(O,S)-TFA *n*-propyl derivatives, it can also be applied to the preparation of other derivatives involving esterification and acylation, which are frequently used today.

The expenditure of time and money was considered with respect to (a) choice of the chemicals used for derivatization; inexpensiveness and simplicity of the preparation of the N(O,S)-TFA *n*-propyl esters led to investigations of their chromatographic behaviour[8]; and (b) the derivatization procedure, including evaporation, heating and laboratory equipment necessary or best suited to these steps.

It must be emphasized that large sample numbers require simultaneous sample preparation. This has already been noted previously[5,14], and the simultaneous preparation of six samples was mentioned by Desgrees *et al.*[11] without, however, the details of execution. For clinical investigations the previously described use of ordinary glass tubes for the derivatization[4,10,11] is sufficient.

Microvials[5,15], although effecting some savings of reagents, offer no real advantage, as they are expensive and not easy to wash by machine. Also, in many clinical investigations or an amino acid screening using urine samples, the sample volumes need not be very small; 100 × 16 mm glass tubes allow the derivatization of sample volumes over a wide range.

Heating is most often performed in aluminium block heaters with the advantage of good temperature control, and the occurrence of reflux, which also reduces pressure within the tubes at high temperatures. Samples processed together are exposed to identical conditions.

Although some workers employ a rotavapor for the evaporation steps[4,10], nitrogen is used for drying the samples and evaporation of excess of reagent in most instances[4,9,11,15]. This may be suitable for small volumes or a few samples, but becomes too expensive with large numbers of samples. Also, it proved difficult to treat all the samples of one batch in an identical manner. Using a six-fold outlet and syringe needles, the gas streams were found to differ from one another, leading to varying evaporation times of the samples of one batch. As the calibration mixture included in each batch should undergo exactly the same procedure as the other samples, such differences may lead to problems in quantitation. Therefore, the use of a rotavapor is advantageous. Here, conditions can be reproducibly controlled by adjusting the pressure and temperature, and can easily be maintained identical for all samples of one batch. Employing an attachment for ten tubes, up to five batches have been derivatized in one day.

The sample preparation steps are outlined in Fig. 1. Biological samples need a clean-up procedure, which was performed as described by Adams[4]. The eluate from the ion-exchange resin was allowed to drop into the reaction tubes. Evaporation, however, must be done using dry air or nitrogen, as the basic solution foams on the rotavapor. When the volume is reduced to approximately half, the remainder can easily be evaporated under vacuum. These problems may be reduced by using a

SAMPLE PREPARATION
CLEAN-UP
Sample → pH 2–2.5
Adsorption
Wash
Desorption
Evaporate with nitrogen
Evaporate with Rotavapor

ESTERIFICATION
Add 0.3 ml esterification reagent
Heat at 110°C for 25 min
Cool to room temperature
Evaporate with Rotavapor

ACYLATION
Add 0.4 ml solvent and 0.2 ml anhydride
Heat at 150°C for 5(10) min
Cool to room temperature
Evaporate with Rotavapor
Dissolve in ethyl acetate

Fig. 1. Flow chart of urine sample preparation.

recently published modified clean-up procedure[16].

No advantage was found in azeotropic removal of remaining traces of water before esterification, although this has sometimes been performed[15,17].

Addition of a water scavenger during esterification[3] did not influence the response values. This was also mentioned by MacKenzie and Tenaschuk[18] in the preparation of the N(O,S)-HFB isobutylderivatives, and the same conclusions were drawn for the N(O,S)-HFB *n*-propyl esters[6].

When using 1-propanol for esterification, excess of reagent was evaporated at 40°C after the tubes had been cooled to room temperature. During cooling, some alcohol condenses on the caps, so that they must be dried in an incubator while the tubes are on the rotavapor. 1-Propanol is evaporated after about 15 min, whereas when using butanol for this step, a higher temperature and a longer time are required. Azeotropic evaporation of the alcohol with benzene did not have any significant effect on responses. Acylation should be performed at 150°C for 5 min when using TFAA[7,8] or 10 min with HFBA[9].

Evaporation of the acylating reagent is performed in spite of the breakdown of a few TFA bonds (arginine and histidine lose one TFA group during this step) because this results in an excellent baseline, without the need for compensation columns and in reduction of the solvent peak. Using the N(O,S)-TFA *n*-propyl esters this step is performed at 30°C, whereas higher temperatures may be employed if less volatile derivatives are prepared.

Making use of 1-propanol for esterification and TFAA for acylation, together with the procedure described above, this method offers the advantage of inexpensive and rapid derivatization combined with a short separation time. However, this procedure may be applied to other acyl alkylesters, and was employed in a comparison of acylation times for the *n*-propyl-, isopropyl-, *n*-butyl- and isobytyl esters of ten amino acids with TFAA. Similar results were obtained for these esters[19]. Obviously, the

alcoholic group esterified to the carboxylic groups does not influence the acylation reaction significantly. It seems, therefore, that the derivatization procedure need not to be altered when preparing one of the above-mentioned esters. Only the acylation time has to be extended if HFBA is used instead of TFAA.

From these considerations and results, it can be concluded that when a rapid derivatization procedure together with simultaneous sample preparation are used, the requirement of derivatization is no longer a drawback in the GLC analysis of amino acids. The cost of an analysis of a biological sample is increased only 2- to 3-fold compared with two-dimensional thin-layer chromatography and remains far below that of ion-exchange chromatography. Excluding sample clean-up, which is necessary for most methods of sensitive quantitative analysis, as many as 50 samples can be derivatized in a day, each sample requiring about 20 min for separation and quantitation. Considering that about 120 min are needed for ion-exchange separation, the use of GLC for quantitative screening programmes, in spite of the derivatization procedure, remains advantageous.

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