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Note

Simple method for the determination of histidine, tryptophan, cystine and homocystine by gas chromatography

PETR HUŠEK
Research Institute of Endocrinology, Národni třída 8, CS-116 94 Prague 1 (Czechoslovakia)
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Numerous previous studies on the gas chromatography of amino acids in packed columns have indicated that the use of silicone phases and silanized supports is essential for the determination of the most polar (histidine, arginine) and the heaviest (tryptophan, cystine) protein amino acids[1]. Further, it was shown that tryptophan can be eluted from a chromatographic column even as a base, *i.e.*, with a free indolyl group, whereas free imidazolyl causes adsorption of histidine in the column filling[2,3]. As trifluoroacetyl (TFA)- and to lesser extent also heptafluorobutyryl (HFB)-acylated imidazole (N^{im}-acyl derivative) is prone to hydrolysis, the co-injection of the corresponding anhydride together with the sample is necessary in order to maintain the N^{α},N^{im}-diacylated histidine derivative in the column[4,5]. In spite of this, partial cleavage at the imidazolyl nitrogen occurs and co-injection of acetic anhydride to create a more stable N^{im}-acetyl-N^{α}-HFB compound via on-column acetylation is therefore recommended[6–9]. Another method is heat treatment (110–150°) of the derivatized amino acids with ethoxyformic anhydride[3,10], but the additional reaction step required renders it impractical. The imidazolyl hydrogen can, however, be removed preferentially by treatment with chloroformates; this reaction proceeds smoothly at room temperature and the derivatives are resistant to hydrolysis and are stable in the column[11,12].

Nearly 1 h is usually required for derivatization of the amino acids mentioned according to existing techniques. Condensation of amino acids with 1,3-dichlorotetrafluoroacetone makes it possible to reduce this time to about 15 min. Tryptophan, cystine and homocystine can be determined immediately after the condensation, and histidine after subsequent extraction with an organic solvent containing a small amount of isobutyl chloroformate. A short packed column permits the determination of the derivatives of the four amino acids together with diaminopimelic acid as an internal standard within a few minutes.

EXPERIMENTAL

Materials and equipment

L-Histidine hydrochloride hydrate (HIS), L-tryptophan (TRP), L-cystine (CYS), L-homocystine (HCYS) (all grade A quality) and D,L-α,ω-diaminopimelic acid (DAPA) (grade B) were obtained from Calbiochem (Lucerne, Switzerland). The reagents, 1,3-dichlorotetrafluoroacetone (DCTFA) and isobutyl chloroformate (IBCF), were purchased from Fluka (Buchs, Switzerland) and E. Merck (Darmstadt, G.F.R.), respectively.

The glassware and apparatus were the same as described earlier[13], but the reaction tubes were silanized by treatment overnight with dichlorodimethylsilane in toluene (1:10). The chromatographic conditions were as follows. Glass column (1 m × 2 mm I.D.) filled with 3% SE-30 on 45–60-mesh Chromosorb W AW DMCS, programmed from 160° to 220° at 8°/min, with a carrier gas (nitrogen) flow-rate of 30 ml/min. The injector and detector temperatures were 200° and 250°, respectively, and the attenuation was 4×10^{-10} A. Average molar responses with respect to the internal standard (DAPA) were HIS 0.93, TRP 1.40, CYS 0.76 and HCYS 0.96.

Procedure

Amino acids in the dry residue (not more than 50 nmole of each amino acid in the mixture) were condensed with 15–20 μl of DCTFA in the presence of acetonitrile (50–60 μl), dichloromethane (15–20 μl) and pyridine (8 μl). After keeping the sample at 40° for 10 min, extraction was started by shaking the mixture for 10–15 sec with 500 μl of organic solvent (light petroleum (b.p. 40–60°)–dichloromethane, 3:1) and 400 μl of 1 *M* sodium carbonate solution in tightly closed reaction vial. Then 1 μl of IBCF was added and shaking was continued until the organic phase cleared (usually after 20–30 sec). The aqueous phase was discarded and pyridine in the organic solvent removed by shaking for 10–15 sec with 400 μl 1 *M* hydrochloric acid and then 400 μl of water. After removing the aqueous layer, the organic extract was transferred into another vial and evaporated just to dryness at room temperature under a gentle stream of nitrogen. The derivatized forms were dissolved in 50–100 μl of *n*-heptane (or *n*-hexane) and subjected to gas chromatographic analysis.

RESULTS AND DISCUSSION

With the exception of TRP, the diaminodicarboxylic acids and HIS are very difficult to dissolve in the condensation medium and the presence of polar acetonitrile is necessary for rapid disssolution and a satisfactory reaction course[14]. On the other hand, the presence of acetonitrile in the medium hinders extraction because of its miscibility in both the aqueous and organic phases. Partial replacement of acetonitrile with dichloromethane does not lower the dissolution rate substantially and provides a good compromise with respect to subsequent acylation of the polyfunctional protein amino acids[15] and the following extraction. DAPA, the internal standard, usually requires 15–30 min to be fully cyclized on both ends of the molecule. However, the condensation process was completed after about 10 min when DAPA was mixed with the other amino acids.

All of the amino acids except HIS can be analysed directly after condensation merely by injection of the reaction medium into the column. HIS with a free imidazolyl group is fully absorbed in the column filling. Moreover, if the extraction medium is not treated with IBCF, a cation is formed on the imidazole end during shaking with hydrochloric acid and HIS passes into the aqueous phase. The employment of IBCF proved to be the best means of blocking the imidazolyl hydrogen effectively. The reaction proceeds instantaneously in the presence of small amounts of pyridine and carbonate in the organic phase and the bulky isobutyloxycarbonyl (IBOC) group contributes to decreasing the imidazole polarity to such an extent that the elution of the N^{im}-IBOC-oxazolidinone of HIS from the column is possible. The size of the attached group is critical in view of the interaction of histidine with the chromatographic support[15].

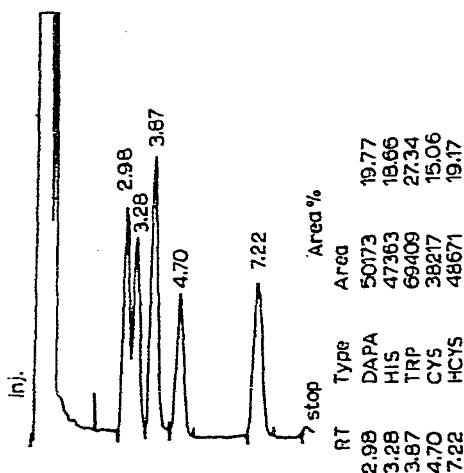

Fig. 1. Chromatogram of equimolar amounts (1 nmole each) of amino acids derivatized as described.

The oxazolidinones of amino acids treated in this work are more prone to column absorption than any other amino acid derivatives, *e.g.*, the N,O-acylated alkyl esters. Partial or even full absorption of these cyclic forms in the column occurs when a silicone phase is coated on a silanized support of the usual particle size (80–100 mesh). The interaction of the oxazolidinones with the chromatographic support, regardless of the phase used for the support coating, appeared to be responsible for the losses. Thus, only a reduction of the surface area of the support by increasing the particle size from 80–100 to 45–60 mesh and the employment of a short column with a small internal diameter (see Fig. 1) enabled the adsorption to be suppressed to a minimum (the influence of particle size on the molar response values will be given in a subsequent paper[15]). The use of another deactivation procedure, *e.g.*, formation of a thin PEG 20M layer by thermal heating of acid-washed supports[16], was ineffective in this respect. These findings also show that efficient deactivation procedures for diatomaceous supports are still lacking.

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