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SINGLE-COLUMN GAS CHROMATOGRAPHIC ANALYSIS OF AMINO ACID OXAZOLIDINONES

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SUMMARY

A study was made of single-column analysis of cyclic oxazolidinone derivatives of protein amino acids. Employment of Chromosorb 750 as the most inert chromatographic support and hydrogen as the carrier gas in a packed column improved only slightly the elution of the most polar (histidine, arginine) and the heaviest (tryptophan, cystine) amino acid derivatives. Similar results were obtained with a support-coated (OV-17) open tubular column. The best analytical performance was afforded by wide-bore open tubular columns either with methylphenylsilicone liquid coated on the glass walls or with methylsilicone gum chemically bonded to fused silica. The total analysis time for all protein members was approximately 10 min.

INTRODUCTION

We recently reported a simple chemical approach to the ready conversion of all protein amino acids into cyclic derivatives at room temperature[1]. The amino acids are condensed with 1,3-dichlorotetrafluoroacetone (DCTFA) and the resulting oxazolidinones are further treated with reactive anhydrides in the same reaction medium. A stable derivative of histidine is formed instantaneously during the subsequent extraction and both the amides, asparagine and glutamine, are preserved and amenable to gas chromatographic (GC) determination. The total time for chemical treatment and analysis was about 1 h.

One drawback of this approach is the requirement for a second column, as the derivatives of histidine, tryptophan and cystine cannot be eluted from the first analytical column. The reason for this is to be found in the adsorptive properties of even the best deactivated chromatographic supports toward perhalogenated derivatives, which are more prone to adsorption than any other derivatives used. This problem was alleviated by reduction of the support surface area by increasing the particle size to 45–60 mesh[2,3] or by employment of a more inert chromatographic support[1], with a

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concomitant reduction of column length[1–3]. The interaction of the oxazolidinones with the chromatographic support proved to be largely independent of the stationary phase polarity.

A search for a chromatographic system with a higher performance in respect of single-column analysis of amino acid oxazolidinones led us to investigate open tubular columns. Our primary aim was not to increase resolution power or to accelerate the analysis, but explicitly to exclude the chromatographic support as the predictable source of adsorption. Simultaneously, we wished to retain the possibility for direct sample injection of larger volumes using conventional injection systems and from this reason we focused on wide-bore capillary columns. Attention was centred on capillaries with medium polar methylphenylsilicone phases, because of experience gained in our previous studies[1,3]. Nowadays, reliable and reproducible procedures are available for the preparation of long-lasting open tubular columns with a uniform layer of silicones of high polarity[4–6]. Moreover, recent advances in column technology have yielded capillaries drawn from fused silica, which seem to be superior to glass in terms of flexibility, overall inertness and efficiency[7]. Future developments may lie in the immobilization of gum phases by cross-linking or chemical bonding to the inner capillary wall[8–10]. The advantage of such capillaries is particularly apparent in the case of splitless injection, when a substantially higher resistance to solvents prolongs column life.

EXPERIMENTAL

Materials

The reagents, glassware and derivatization procedure were as reported previously[1]. Ten nanomoles of each amino acid in an equimolar mixture of twenty protein amino acids, including hydroxyproline, S-methylcysteine (CYSM), ornithine and two internal standards, α-aminocaprylic acid (ACA) and diaminopimelic acid (DAPA), were subjected to the derivatization procedure. (Amino acid abbreviations used follow IUPAC-IUB recommendations[11].)

In addition to the (N,O)-heptafluorobutyryl (HFB) oxazolidinones, also the (N,O)-PFP forms were prepared by treatment of the sample with pentafluoropropionic anhydride (PFPA; Pierce Eurochemie, Rotterdam, The Netherlands). PFPA was added in amount of 12 + 12 μl after the performed condensation. After the acylation step with 3–5 vol% of the corresponding anhydride in heptane, the samples were analysed not later than 30 min after the heat treatment; otherwise losses of tryptophan, caused by its progressive acylation on the free indolyl group, occur.

Analyses

A dual-column dual-FID Hewlett-Packard 5730A gas chromatograph, equipped with an Hewlett-Packard 18740 B capillary inlet system and connected to a computing integrator 3380 A, was employed for the linear temperature-programmed analysis. A rearrangement of the inlet part of the chromatograph was necessary in order to maintain the original dual-column capability. The heater block (No. 18709-20020) of the original dual-column heated injection ports (Model 18709 A) was divided into two and half of it was reinstalled in the inlet space. The new capillary inlet block with gas lines directed to FID-cover assembly was placed behind it and the

heater-sensor cable was connected to the auxiliary temperature source of the basic instrument.

The following chromatographic columns were employed in the analysis of the derivatized amino acids:

2 m × 2 mm I.D. glass column filled with 2% OV-17–OV-22 (1:1) on Chromosorb 750 (80–100 mesh); the packing was supplied by Supelco (Crans, Switzerland) and the column was conditioned under nitrogen (20 ml/min) at 270°C (1°/min linear increase from 60°C) overweekend at an injector port temperature of 250°C.

15 m × 0.5 mm I.D. glass support-coated open tubular (SCOT) column containing OV-17 [half of an originally 30 m long SCOT column (Scientific Glass Engineering, Milton Keynes, Great Britain)]; it was conditioned under nitrogen (10 ml/min) at 270°C overnight

12 m × 0.5 mm I.D. glass wall-coated open tubular (WCOT) column containing OV-17 [half of an originally 25 m long column (Packard-Becker, Delft, The Netherlands), with the following specifications: layer thickness 0.45 μm; coating efficiency 98%; number of plates per metre 2700 at $k = 5$]; it was conditioned under hydrogen (5 ml/min) at 240°C for 5 h

25 m × 0.25 mm I.D. glass WCOT column containing OV-101 (supplied by Packard-Becker, with the following characteristics: layer thickness 0.23 μm; coating efficiency 82%; number of plates per metre = 3800 at $k = 7.7$); it was conditioned under hydrogen (2 ml/min) at 250°C for 5 h

10 m × 0.25 mm I.D. fused-silica open tubular (FSOT) column containing OV-17 [Alltech Europe, Eke, Belgium; Cat. No. FSOT-7, without further specifications]; it was conditioned under hydrogen (2 ml/min) at 230°C for 5 h

25 m × 0.31 mm I.D. FSOT column containing OV-1 cross-linked silicone gum [Helwett-Packard, Avondale, PA, U.S.A. Specifications: coating thickness 0.17 μm; theoretical plates per metre $\geqslant$ 2600 at $k = 7$–9; max. operating temperature 350°C]; it was conditioned under hydrogen (3 ml/min) at 250°C for 2 h. Both the FSOT columns and the small-bore WCOT column containing OV-101 were operated in connection with the capillary inlet system having a glass insert of 1.5 mm I.D. under splitless mode with a 35-sec delay. The wide-bore WCOT and SCOT columns containing OV-17 were operated under flash vaporization conditions. The unstraightened column ends were connected by means of shrinkable PTFE tubing (0.8 mm I.D.) to a piece of glass stem (5 cm × 1.5 mm O.D. × 0.5 mm I.D.), the oven end of which was narrowed to approximately 1 mm O.D. to fit the PTFE connection. The glass stem was inserted 3.5 cm into the 9 cm × 2 mm I.D. glass insert of the 1/8 in. O.D. inlet metal liner and sealed with a polyimide (Vespel) reducing ferrule (1/8 to 1/16 in.). The same connection was used at the detector end. On-column injection was performed with the packed column.

The particular analytical conditions for each column are given in the figure legends. The following parameters applied in each case: total flow of nitrogen used as make-up gas and hydrogen through the FID jet, 60 ml/min at a mixing ratio of 1:1 (except for the packed column where the hydrogen flow was higher, see Fig. 1); injector and detector temperatures, 200 and 250°C; rate of linear temperature increase, 16°C/min. The given flow-rates were measured always at the initial temperature of the stated range.

Each chromatogram represents the response of an equimolar mixture of amino

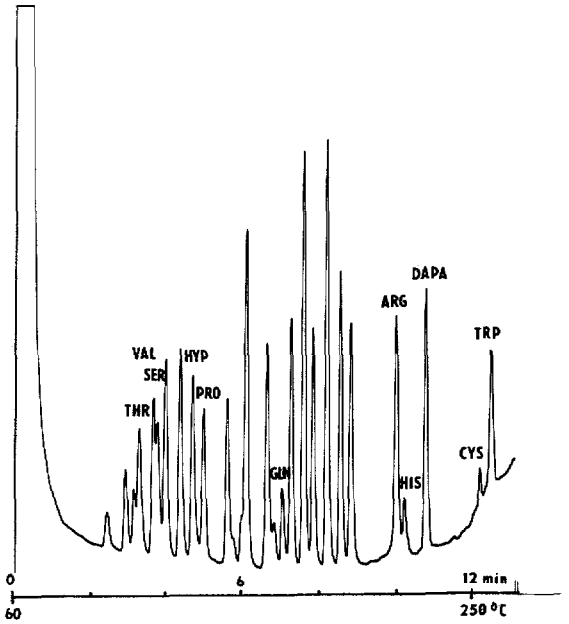

Fig. 1. PFPA-treated oxazolidinones analysed on a 2 m × 2 mm glass column packed with 2% OV-17–OV-22 (1:1) on Chromosorb 750 (80–100 mesh) operated in the temperature range 60–250°C under an hydrogen carrier gas flow of 60 ml/min. For assignment of amino acids to corresponding peaks see Fig. 3.

acid oxazolidinones treated either with HFBA or PFPA. The concentration of the solutes in the injected volume (usually 1–2 μl) did not exceed 0.1 nmol per amino acid derivative for open tubular and 1 nmol for support-coated and packed columns.

RESULTS AND DISCUSSION

The appearance of histidine, tryptophan and cystine on the chromatograms in Figs. 1–5 confirms our assumption that the chromatographic support itself and not the kind of stationary phase used is responsible for their losses in the column. It is apparent that an elimination of the support in open tubular columns with a concomitant suppression of the glass wall adsorptivity by an effective deactivation improves elution of these compounds substantially if higher flow-rates of carrier hydrogen are used. A glass capillary coated with OV-17, used for gas chromatographic–mass spectrometric (GC–MS) analysis of amino acid (N,O)-HFB oxazolidinones in our earlier study[12] and run under low flow-rate of carrier helium (0.8 ml/min for 0.3 mm I.D. capillary), did not enable the elution of the three troublesome amino acids at all. The dependence of the responses of the mentioned amino acids on the carrier gas flow-rate was particularly apparent with columns containing support and even with the glass WCOT column (Fig. 4). The weakest dependence was found with the FSOT column (Fig. 5). Hydrogen was the only carrier gas recommended, as with nitrogen the three amino acids were practically not eluted, the best results again being obtained with the bonded phase.

Even when a partial elution of histidine, tryptophan and cystine was possible

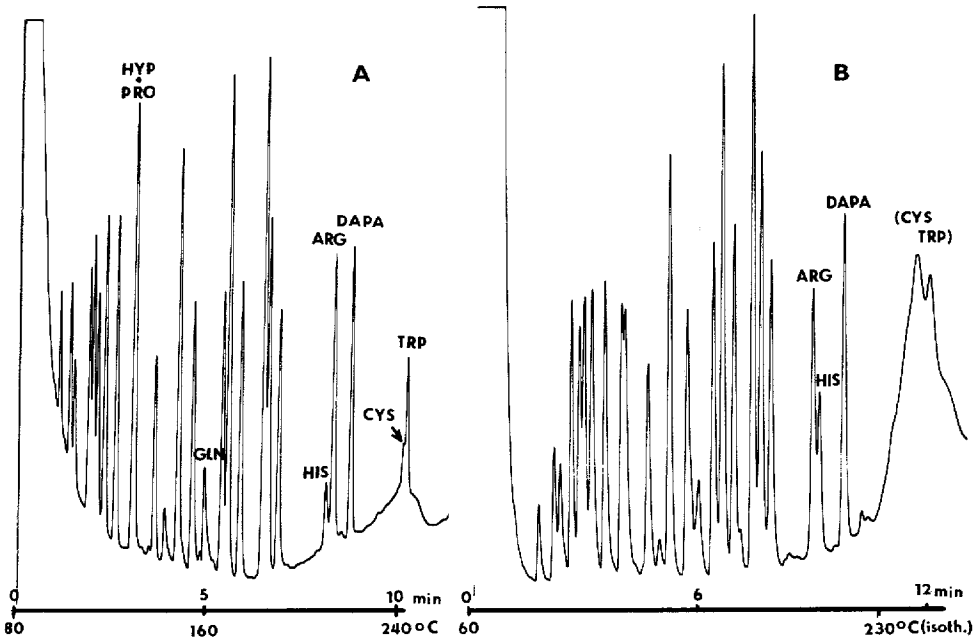

Fig. 2. HFBA-treated oxazolidinones analysed on: A, 15 m × 0.5 mm SCOT (OV-17) column in the temperature range 80–250°C under an hydrogen flow of 24 ml/min (200 cm/sec); B, 10 m × 0.25 mm FSOT (OV-17) column in the temperature range 60–230°C under an hydrogen flow of 3 ml/min (100 cm/sec). For amino acid elution order compare with Fig. 3.

from the packed column (Fig. 1) at a high flow-rate of carrier hydrogen, the separation efficiency of the better deactivated Chromosorb 750 support is less than that of the W type[1]. Moreover, the OV-17 phase does not allow one to separate CYS and TRP in a packed column, so that co-mixing of the more polar OV-22 methylphenylsilicone was necessary. The unsatisfactory separation of the CYS–TRP pair on the OV-17 phase at higher flow-rates is apparent from Fig. 2A. Analysis of amino acid oxazolidinones in the 0.5 mm I.D. SCOT column revealed a linear dependence between the gas flow and the magnitude of the HIS, CYS and TRP responses. In the hydrogen flow-rate range of 10–50 ml/min their responses varied from nearly zero to nearly maximum. However, in the latter case (*i.e.*, at a linear velocity of 400 cm/sec!) the resolution power of the column was lost and many derivatives were coeluted. The PFPA treatment of amino acid oxazolidinones was chosen in those cases when the HFB forms could not be separated under the selected conditions. The (N,O)-HFB oxazolidinones of TYR and LYS on the phenylmethylsilicones and those of TYR and GLU on the methylsilicones are practically unresolved at higher flow-rates, while the (N,O)-PFP oxazolidinones with slightly lower retention times are well separated.

The best results were obtained as shown in Fig. 3. Since the OV-17 stationary phase enables a good separation of all protein amino acid oxazolidinones even in 2 m long packed column[1], a capillary of high resolution power is unnecessary. Bearing this in mind, we investigated a wide-bore open tubular column, enabling a direct sample injection without a special sampling device. A 0.5 mm I.D. glass WCOT

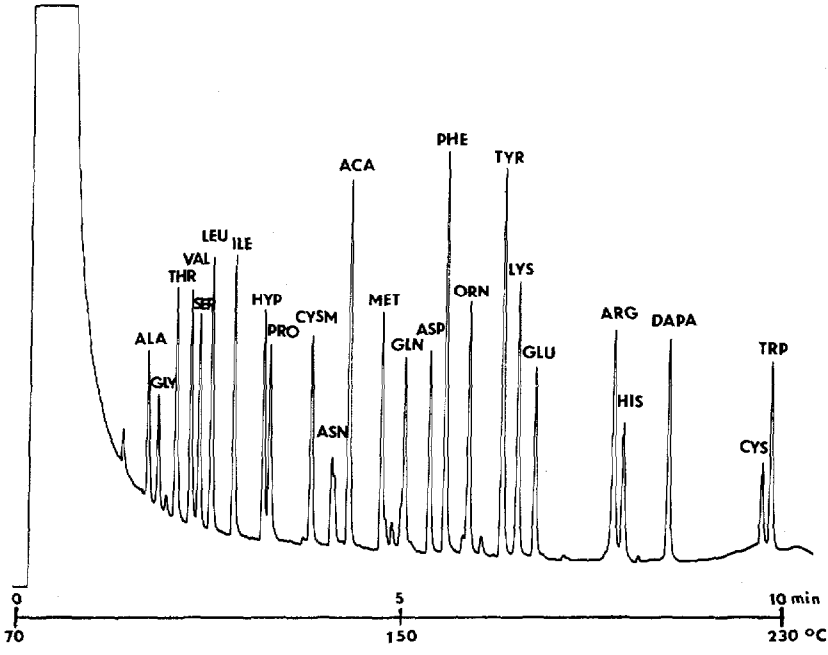

Fig. 3. HFBA-treated oxazolidinones analysed on a 12 m × 0.5 mm glass WCOT (OV-17) column in the temperature range 70–230°C under an hydrogen flow of 5.8 ml/min (50 cm/sec).

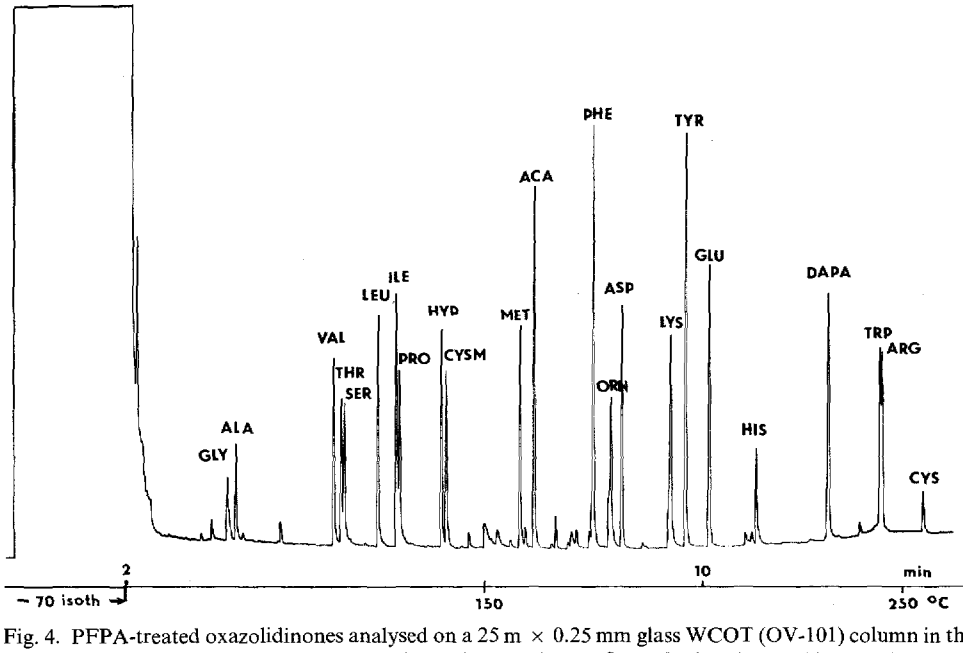

Fig. 4. PFPA-treated oxazolidinones analysed on a 25 m × 0.25 mm glass WCOT (OV-101) column in the temperature range 70 (hold for 2 min)–250°C under a hydrogen flow of 2.9 ml/min (100 cm/sec).

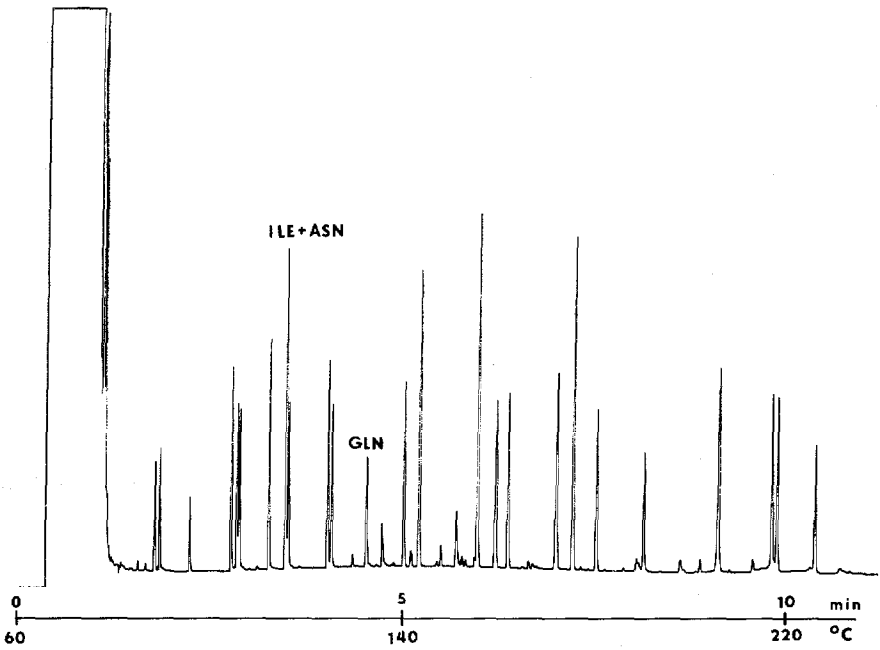

Fig. 5. PFPA-treated oxazolidinones analysed on a 25 m × 0.31 mm FSOT (OV-1 cross-linked) column in the temperature range 60–230°C under an hydrogen flow of 4.6 ml/min (100 cm/sec). For amino acid elution order see Fig. 4.

capillary of only 12 m in length fulfilled our requirements, as this enabled not only elution of all derivatives but also gave a short time of analysis. Unfortunately the method of wall deactivation was not specified by the manufacturer. Similar results were obtained regardless of whether the column was connected to the inlet as described or by means of 0.2 mm I.D. platinum–iridium tubing, being part of a rigid cage for column housing supplied by Packard. Connection of the column by means of glass-lined metal tubing and special unions (SGE patent) resulted in worse results, probably due to contact of sample vapours with the tubing metal surface in the evaporation zone.

Because of the known difficulties in coating FSOT columns with polar silicone phases[6], it was rather surprising that Alltech was the only known distributor offering a FSOT column containing OV-17. The analysis in Fig. 2B demonstrates clearly that the analytical performance of this column is very unsatisfactory, worsening with time. The high degree of phase bleed and low resolution power suggest stripping of the phase with time and rearrangement of the phase film inside the column so that uncoated areas of the glass surface occur. New developments in this area are the coating of FSOT columns with the recently introduced phase OV-1701 (Ohio Valley, U.S.A.), a silicone gum similar in polarity to OV-17 liquid. Such columns are now offered from SGE and we will test them in due course.

Comparing Figs. 4 and 5 it is obvious that the analytical performance of the FSOT column with bonded methylsilicone gum is superior to that of the WCOT column, even when the former capillary has a wider bore (*i.e.*, lower resolution cap-

ability, theoretically). The FSOT column offers an improved separation of the TRP–ARG pair, better elution of the terminal members and also the basic amino acids. Both the amides, GLN and ASN, which are converted into nitriles during the chemical treatment[1,12], are not present on the chromatogram of Fig. 4, confirming the presence of residual adsorption in this column. Unfortunately, ASN is the only amino acid which cannot be separated from another one, ILE, on the FSOT OV-1 cross-linked column. This column of high quality should thus be reserved for analysis of amino acids in hydrolysates.

As mentioned in Experimental, the sample should be analysed as soon as possible after the final acylation if tryptophan is present. Otherwise a progressive acylation on the indolyl hydrogen takes place and the acylated form of TRP elutes between ARG and HIS in the OV-17 coated columns; this is undesirable because the derivatives of ARG and HIS are eluted close together.

Another problem is connected with the analysis of histidine. The presence of anhydride in the heated inlet zone is a possible source of HIS degradation loss, because of its attack on the methoxycarbonyl moiety of the imidazole. This was observed in those cases where glass wool was present in the heated evaporation zone. Thus, the use of glass wool in the injection port should be avoided or reduced to a minimum (as it was with the packed column).

To conclude, a wide-bore well deactivated glass capillary column containing OV-17 stationary phase and with a relatively short length of 10–12 m, connected simply to a flash vaporizing tube and operated under direct sample injection, is recommended for single-column analysis of protein amino acids as their cyclic derivatives. Alternatively, a more expensive 25 m long FSOT wide-bore column of chemically bonded methylsilicone gum is able to separate all the derivatives with the exception of asparagine; it is suitable for estimation of amino acids in acidic protein hydrolysates. In both cases the time of analysis is approximately 10 min.

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