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Note

Possibilities and limitations in the analysis of amino acid oxazolidinones in the femtomole range by gas chromatography with electron-capture detection

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Both of the automated chromatographic techniques — high-performance liquid chromatography (HPLC) and gas chromatography (GC) — are nowadays routine tools in the analysis of amino acids because there is the possibility of separating these compounds several times more quickly than with the classical amino acid analysers. Simultaneously, there is a determined effort to increase the sensitivity of detection by use of selective detectors, such as the fluorometer in HPLC or the electron-capture detector in GC. Fluorescent derivatives for HPLC amino acid analysis are most frequently prepared by treatment of the compounds with *o*-phthaldialdehyde (OPA) [1—3], dansyl chloride [4—6] or, more recently, with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) [7], which in contrast to OPA reacts also with the secondary amino group of proline and hydroxyproline. Analysis at the picomole level is usual, the detection limit being in the range of a few femtomoles.

In GC analysis the necessary pre-column derivatization of amino acids leads in most cases to formation of fluorine-rich compounds, so that electron-capture detection (ECD), generally three orders of magnitude more sensitive than flame-ionization detection (FID), is the obvious choice. However, problems arise in conjunction with temperature-programmed operation as the increase in baseline with temperature programming of packed columns causes deterioration in the analysis of the higher amino acid members, especially when derivatives of lower volatility are used [8]. Under isothermal operation femtomole amounts of N(O,S)-TFA (trifluoroacetyl), or HFB (heptafluorobutyryl) amino acid alkyl esters could be analysed successfully [9—12]. Thus, the use of selective detectors with either of the chromatographic techniques affords comparable results concerning the sensitivity.

As the recently introduced procedure of amino acid treatment by action of

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a perhalogenated acetone [13, 14] can compete well with the commonly used esterification approaches [13], and a higher abundance of chemically introduced halogen atoms predestines the derivatives for ECD, we have studied the chromatographic behaviour of the compounds in mass ranges down to femtomoles. The GC—ECD temperature-programmed analysis of the acylated amino acid oxazolidinones succeeded well with both packed and open-tubular columns; however, variable losses of the compound amino acids were observed in packed columns at lower injected mass. Possibilities for analysis in the sub-picomole range together with limitations, caused by the presence of chromatographic support and/or reagent impurities, are discussed.

EXPERIMENTAL

Five nanomoles down to 100 pmol of each amino acid in an equimolar mixture of twenty protein amino acids, including hydroxyproline, ornithine and S-methylcysteine (not included in the sample for the capillary column), were subjected to derivatization with 1,3-dichlorotetrafluoroacetone (DCTFA) and reactive anhydrides, the heptafluorobutyric anhydride (HFBA) or the pentafluoropropionic anhydride (PFPA), as described previously [13, 14]. Following the procedure the derivatives were dissolved and injected in heptane only, so arginine and the two amides asparagine and glutamine could not be determined [13].

Gas chromatography was carried out with a Hewlett-Packard 5736A gas chromatograph equipped with a linear ^{63}Ni electron-capture detector and two flame-ionization detectors. A capillary inlet system 18740B with splitless injection mode was used for analysis with a 25 m × 0.31 mm capillary column made from fused silica and containing OV-1 cross-linked methylsilicone gum. Like previously [14] the column was run in a temperature range of 60 to 230°C (16°C/min) under a hydrogen flow-rate of 4.6 ml/min (100 cm/sec) and make-up nitrogen for ECD of 20 ml/min. A dual set of glass-packed columns (2 mm I.D.), a 2-m column with 3% OV-17 on Chromosorb W HP and a 0.5-m column with 1.5% SE-30 on Chromosorb G HP (or Chromosorb 750), operating at 8°C/min linear increase in the temperature ranges 80—230°C and 150—200°C under nitrogen flow-rates of 20 ml/min and 30 ml/min, respectively, was used [13] for the comparison studies. The injector and electron-capture detector temperatures were 200°C and 250°C for both types of column used.

RESULTS AND DISCUSSION

Already in our previous studies [13, 14] on the GC behaviour of the amino acid oxazolidinones it was concluded that these perhalogenated compounds are more prone to adsorption to the column fillings than the acylated amino acid alkyl esters. Especially the derivatives of histidine, cystine and tryptophan could not be eluted from packed columns with common fillings based on diatomaceous supports of the Chromosorb W type. As even the Chromosorb 750 support, being considered the best deactivated one, did not allow the elution of the three troublesome derivatives completely [14], the exclusion of

any support at all would be the best way how to succeed with quantification.

Using selective and halogen-sensitive ECD it was possible to evaluate the possible adsorption losses of the amino acid oxazolidinones near or below the picomole level. Success with GC—ECD temperature-programmed analysis of N(O,S)-HFB amino acid isobutyl esters in the picomole range using support-coated open-tubular (SCOT) columns was reported recently [15, 16]. The relative molar responses showed that some derivatives were detectable in much smaller amounts than others. Corkill et al. [17] studied responses of strong electrophores in the GC—ECD system and found that the most sensitive compounds were derivatized iodothyronines (the N,O-perfluoroacylated

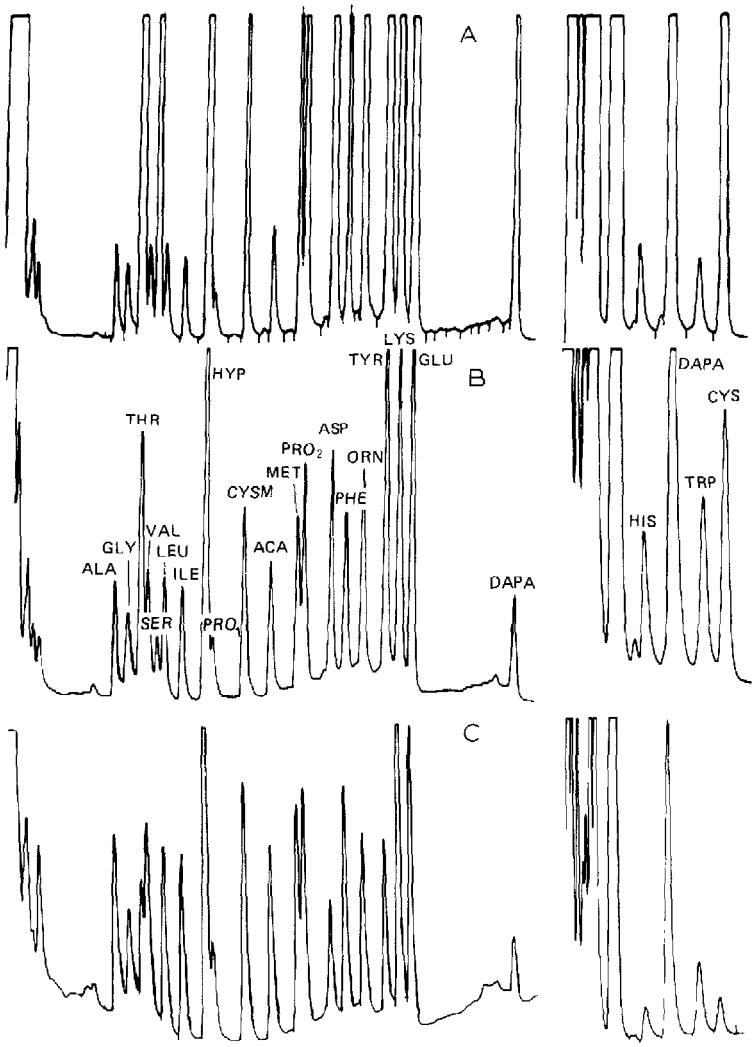

Fig. 1. GC—ECD analysis of (N,O)-HFB amino acid oxazolidinones in a dual set of packed columns [13] after derivatization of an equimolar mixture containing 5 nmol of each amino acid. The derivatives were submitted to analysis after dilution with heptane to the following final amount of each derivative per injected sample: (A) 100 pmol (attenuation × 512); (B) 10 pmol (× 64); (C) 1 pmol (× 4). The analytical conditions in all three cases were the same so that a constant elution order was maintained.

methyl esters) which were essentially twenty times more sensitive than lindane.

Fig. 1 shows GC—ECD-programmed analysis of N(O)-HFB amino acid oxazolidinones in packed columns in a range of 100 to 1 pmol per injected compound. In accordance with our previous study [13] it can be accepted that no or minimal adsorption losses of amino acid oxazolidinones occur in the packed columns down to 100 pmol per amino acid injected. This means that the responses in Fig. 1A represent real differences in ECD caused by the presence of an unequal number of electron-capturing groups in the molecule. Among the "low sensitive" electron-capturing derivatives we find the aliphatic amino acids histidine and tryptophan, i.e. amino acids containing the bis(chlorodifluoromethyl)oxazolidinone ring only. A higher response is given by the sulphur-containing amino acids (S-metylcysteine and methionine) and also phenylalanine because of the additional effect of the aromatic ring. "Highly sensitive" are the compound amino acids in which the side-chain reactive groups are converted to HFB-acylated forms, and also the diamino-dicarboxylic acids with two oxazolidinone rings in the molecule. Also the high response for the second derivative of proline (Pro_2, eluted after methionine in Fig. 1.) confirms our assumption that the HFB group participates in its formation. The presence of the additional electrophore in the molecule of the compound amino acids causes unexpectedly high enhancement of the ECD response, which is on average one order higher than that of the "low sensitive" oxazolidinones of the simple amino acids. Very similar results were obtained with the N(O,S)-HFB amino acid isobutyl esters [16], where the relative molar response (RMR) difference between the least sensitive valine (RMR 0.90) and the most sensitive tyrosine (RMR 32.7) was about 36-fold. Two to five times higher responses were shown by the sulphur-containing amino acids in comparison with the aliphatic ones, again in close agreement with our findings.

The picture, however, alters when the injected amount is lowered by one or two orders down to 1 pmol per amino acid derivative (Fig. 1B and C). Adsorption losses in the column fillings occur with the compound amino acids, where the presence of additional fluorine atoms in the HFB moiety makes the derivatives more prone to adsorption than those with an oxazolidinone ring only. The most drastic decline in RMR was observed with the hydroxyl-containing amino acids (except hydroxyproline, the large peak of which in Fig. 1C does not represent the pure compound but is a co-elution with an unknown impurity), especially serine, the adsorption of which is complete in the picomole range (disappearance of Ser from Fig. 1C). Also the response of cystine declines rapidly with the mass reduction. As the oxazolidinones of the simple amino acids do not seem to be adsorbed with the mass lowering, the relative responses of the protein members are close to equality in the picomole range. From Fig. 1C it is also apparent that the N,N-diHFB oxazolidinone of lysine is less prone to adsorption than any other compound amino acid and its analysis down to 10 fmol was possible with the packed column used.

Approximately ten times higher sensitivity can be achieved using a capillary column (Fig. 2). As the fused-silica column with bonded OV-1 methylsilicone does not separate the HFBA-treated oxazolidinones completely, the PFPA-treated oxazolidinones were used instead [14]. The lower amount of fluorine atoms in the PFP moiety results in a partial lowering of the ECD responses for

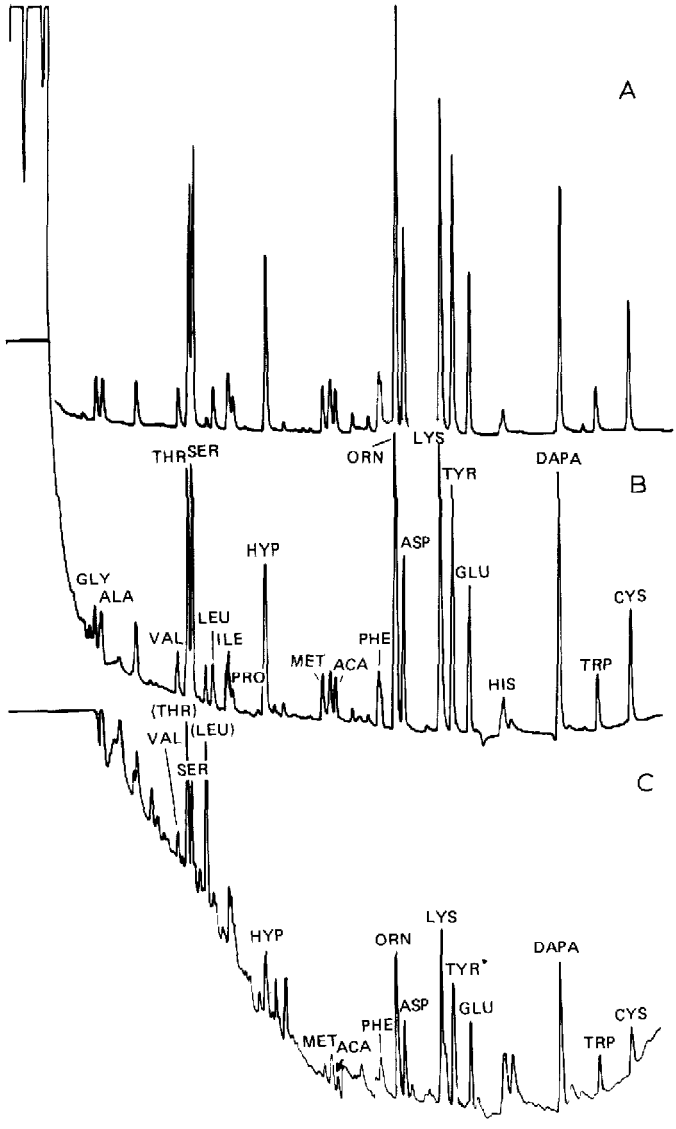

Fig. 2. GC—ECD analysis of (N,O)-PFP amino acid oxazolodinones in a fused-silica capillary column with OV-1 bonded phase [14]. The same initial amount as in Fig. 1 was derivatized and the sample was diluted to the following final amounts injected: (A) 1 pmol (attenuation × 32); (B) 0.1 pmol (× 4); (C) 0.01 pmol (× 1). Amino acids in brackets are co-eluted with an unknown impurity having identical retention time.

the compound amino acids. From Fig. 2A it can be seen that the highest response is given by the dibasic amino acids (Orn and Lys), being approximately ten times higher than that of the aliphatic amino acids. However, the most important finding is that the responses of the protein members are independent of the mass injected. From Fig. 2A—C it follows visually that the RMR values are comparable for all concentration ranges down to 10 fmol and that they were essentially the same in the range of 1 to 0.01 pmol of injected mass. This means, therefore, that employment of open-tubular columns without a chro-

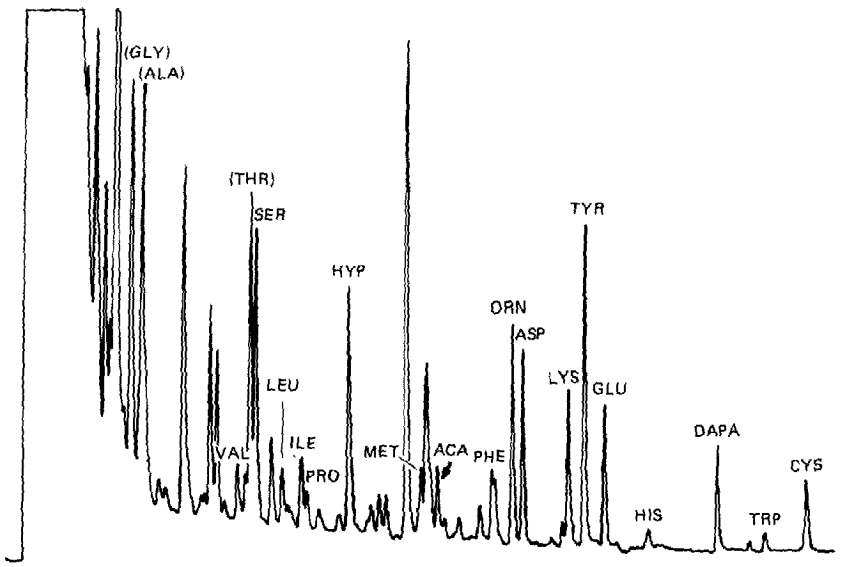

Fig. 3. GC—ECD analysis of a sample containing 100 pmol of each amino acid initially. After derivatization, 1 pmol of each amino acid derivative was injected into the capillary column as in Fig. 2. The brackets around some amino acids follow the same meaning as in Fig. 2.

matographic support is the only means of obviating losses of the perfluoroacylated amino acid oxazolidinones at concentration ranges below 100 pmol per injected amino acid derivative. Next to this, it is worth noting that the presence of hydrogen, being the carrier gas for the capillary column, did not seem to influence the process of electron-capture in the detector provided that nitrogen as the make-up gas was added. Both the sensitivity and the linearity did not change significantly as far as we noticed.

At higher instrument sensitivities, i.e. lower mass injections, another problem arises with the presence of impurities, as can be seen from Figs. 2C and 3. Even when the samples with the lowest derivative concentrations are prepared by dilution of the initial concentrated sample (nanomole range) by heptane of nanograde purity, extraneous peaks appeared on the chromatogram (Fig. 2C) and the quantification of the first protein members was impossible. Impurities with identical retention times to those of some amino acid derivatives present a serious problem when samples with a low amino acid content are derivatized (Fig. 3). We found later that many of the impurities originated from the analytical grade hexane (Fluka, Buchs, Switzerland) used for extraction [13] and that its repeated distillation brought a great improvement, even though some extraneous peaks survived. The results show that the GC—ECD analysis of perfluoroacylated amino acid oxazolidinones near and below the picomole range is well possible; however, one has to face impurities of various origins when the initial derivatized amount is low.

APPLICATION

The capillary column with OV-1 cross-linked phase connected to an electron-
[illegible] (attenuation × 512) or even a flame-ionization detector

(attenuation × 4) enabled us to estimate the protein amino acids in 5 μl of human serum routinely. The amino acids were isolated by means of cation-exchange resin (Dowex 50W-X2, H^+, 100—200 mesh), which was placed with a plug of synthetic wool directly in the lower part of a polypropylene conical tip (used for the push-button pipettes and cut off below at O.D. of about 2 mm) thus forming a bed of 6—8 mm of wet resin. The 5-μl serum sample was diluted ten times with 25% aqueous acetic acid containing 0.5 nmol of the internal standards (α-aminocaprylic acid, diaminopimelic acid) [13], dropped on the resin bed and left to penetrate it. The bed was then washed with two times 30 μl of water and the amino acids were eluted subsequently with 150 μl of 2 *M* aqueous ammonia. The amino acids in dry residue were then converted to the N(O)-PFP oxazolidinones and after extraction and evaporation of the extraction solvent the amino acid derivatives were dissolved in 40 μl of heptane and subjected to GC analysis. Minimal presence of disturbing peaks was observed.

CONCLUSIONS

The GC–ECD temperature-programmed analysis of PFP- or HFB-acylated amino acid oxazolidinones brought results comparable to those obtained with N(O,S)-HFB amino acid isobutyl esters [15, 16]. Amino acids with the perfluoroacylated side-chain reactive groups afford responses of about one order higher than the others; however, they tend to be adsorbed in the column filling more readily than those with the oxazolidinone ring only. Significant adsorption losses appear in packed columns at levels below 100 pmol per amino acid injected. Elimination of chromatographic support in the capillary columns resulted in undisturbed analysis down to femtomole amounts without apparent losses of the troublesome amino acid derivatives; however, problems with impurities appear to be serious at that level. Hydrogen can be used as carrier gas for the capillary without deterioration in the performance of the electron-capture detector if nitrogen as the make-up gas is added in excess.

The capillary column was used for estimation of the protein amino acids in 5 μl of human serum either by employment of ECD or FID routinely.

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