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Gas chromatography of permethylated peptides

Gas chromatography (GLC) of peptides has excited less attention than GLC of amino acids (*e.g.* refs. 1–3), possibly because of the constraints imposed by volatility and possibly because the applications are less obvious. The potential of this technique has, however, been indicated by the determination of structure of antamanide[4] and an oxytocin analogue[5], as well as its use to monitor both the presence of error peptides in synthetic products[6] and the extent of racemization during synthesis[7].

As part of our investigations into separation of peptide mixtures by GLC, we have examined derivatives obtained by the permethylation technique[8], widely employed for mass spectrometry of peptides, with the aim of extending analysis by combined gas chromatography and mass spectrometry (GC–MS) to peptide amides and to peptides with blocked N- or C-termini which are difficult to study by standard methods of peptide chemistry (for discussion see *e.g.* refs. 9 and 10). Soon after this work began, investigation by McDONALD *et al.*[11] of the specificity of dipeptidyl aminopeptidase I showed that this enzyme provided the necessary control over degradation of polypeptides to yield peptides amenable to GLC. Its use would permit systematic exploitation of the technique developed by PROX *et al.*[4] and BAYER *et al.*[5,6], which relied on partial acid hydrolysis to give random mixtures of small peptides. The value of such a method, based on controlled enzymatic hydrolysis followed by GC–MS analysis, has been indicated previously[12]. This paper is confined to some results of general applicability regarding GLC of permethylated peptides. Other aspects of this sequencing technique will be described elsewhere.

Methods and materials

Peptides and derivatives. Peptides were obtained from the following sources: BDH Chemicals, Poole; Koch-Light, Colnbrook; Fluka, Buchs; Schuchardt, Munich; Cyclo Chemicals, Los Angeles; and Sigma, London. Derivatives were prepared at room temperature. Methyl esters were prepared with 1–2 *N* HCl in methanol overnight. Acylation was carried out with trifluoroacetic anhydride[13] for 1 h or with acetic anhydride–methanol (4:1)[14] for 1–3 h. Permethylation was performed by a number of methods already described[8,10,15,16].

Gas chromatography. This was carried out with a Perkin-Elmer F 11 equipped with a flame ionization detector. Carrier gas: nitrogen at 10–60 ml/min (2.2 mm I.D. stainless-steel columns) or 60–100 ml/min (3 mm I.D. glass columns). Columns were 1 or 2 m long, filled with 2½% OV-1 or 2½% OV-17 on AW-DMCS Chromosorb G 80–100 mesh.

Results

Table I gives the retention indices for three derivatives of a number of peptides on two silicone stationary phases. N-Trifluoroacetyl (TFA) methyl esters are most volatile followed by N-acetyl (Ac) permethylated derivatives. Two other types of derivatives were examined but considered unsuitable. Neither N-ethoxycarbonyl methyl esters[17] nor N-TFA permethylated peptides gave satisfactory GLC traces. Yields of the latter were poor, probably because cleavage of the TFA group occurs

TABLE I

RETENTION INDICES[a] OF SELECTED PEPTIDE DERIVATIVES ON SILICONE PHASES

Peptide	*Derivative*[b]	*Phase*[c]	
		2½% *OV*-1	2½% *OV*-17
Pro–Gly–Phe	TFA/Me	2760	3600
	Ac/perMe	2980	3750
Phe–Tyr	TFA/Me	2700	3300
	Ac/perMe	2970	3650
Ala–Phe	TFA/Me	2020	2270
	Ac/perMe	2360	2670
	Ac/Me	2410	2860
Leu–Ala	TFA/Me	1720	1920
	Ac/perMe	1835	2110
	Ac/Me	1940	2200
Ala–Leu	Ac/perMe	1865	—
	Ac/Me	1940	2150

[a] Most values are the mean of several runs.
[b] TFA/Me = N-TFA methyl ester; Ac/perMe = N-Ac permethyl; Ac/Me = N-Ac methyl ester.
[c] Glass columns 3 mm I.D. × 1 m. Isothermal in range 184–281°.

under the strongly basic conditions required for permethylation, which appears to preclude their use for quantitative analysis although they are satisfactory for mass spectrometry[18].

Table II provides details of the retention behaviour of a number of N-Ac permethylated peptides. It shows that peptide amides can be analysed successfully as N-Ac permethyl derivatives, whereas previous observations showed N-TFA derivatives to be unsuitable[19]. The retention indices indicate that practical limits have not been reached with packed columns and it seems likely that many other tri- and some tetra- and higher peptides will be amenable to analysis by this method. Although the results given in Tables I and II were obtained with glass columns, stainless-steel columns are also satisfactory. An all-glass system has been preferred to reduce the risk of decomposition.

Products obtained by a number of permethylation techniques were examined by GLC. Extensive C-methylation (confirmed by mass spectrometry) occurred when the COGGINS–BENOITON method[20] was applied to Ala–Ser and at least four major peaks were observed (OV-1 column). The sodium hydride–dimethyl sulphoxide–methyl iodide method[8] has proved generally satisfactory. The scheme proposed by LECLERCQ AND DESIDERIO[10] has provided the most consistent results while the large excess of reagents employed previously appears to lead to more extensive side reactions, as judged by the complexity of the GLC traces. This observation accords with those of POLAN *et al.*[21], who showed that mass spectra of products obtained in the presence of excess methyl iodide gave no typical sequence peaks.

GLC of derivatives of peptides known to be mixtures of enantiomers often yielded multiple peaks indicating resolution of the isomers. Thus N-TFA DL-Ala–DL-Phe methyl ester gave two major peaks with an rDL/LL of 1.09 on OV-17, compared with $r = 1.13$ for the same separation on polyphenyl ether[22]. R values of

TABLE II

RETENTION INDICES[a] OF SOME N-Ac, PERMETHYLATED PEPTIDES ON TWO SILICONE PHASES

Peptide	*Phase*[b]	
	2½% *OV*-1	2½% *OV*-17
Pro–Gly–Phe	2980	3750
Gly–Phe–Ala	2840	—
Phe–Phe–NH_2	2960	3635
Gly–Phe–NH_2	2565	3060
Phe–Tyr	2970	3650
Ala–Phe	2360	2670
Leu–Ala	1835	2110
Trp–Phe	3220	—
Ala–Ser	1740	—
Ala–Leu	1865	—
Gly–Leu	1920	—
Trp–Gly	—	3400
Tyr–Leu	—	3230
Thr–Phe	—	2850
Ser–Tyr	—	3160
Asp–Ser	—	2540

[a] Most values mean of several runs; peptides have all L-configuration.
[b] Glass columns 3 mm I.D. × 1 m. Isothermal runs in range 171–281°.

1.00–1.18 are observed for amino acids as N-TFA isopropyl esters on optically active phases[3] but analysis times up to 6 h are required.

Discussion

The successful GLC of N-Ac permethylated peptides offers a number of advantages over previous methods for GLC of peptides. The permethylated derivatives are not substantially less volatile than the N-TFA methyl esters which were employed earlier[4–7], and their use extends GLC analysis to amidated peptides which are not amenable otherwise to this technique[10]. The method may also be applicable to other peptides with blocked N- or C-termini. The method of acetylation and permethylation described by LECLERCQ AND DESIDERIO[10] appears to offer most promise for quantitative analysis although HALSTROM *et al.*[23] have cautioned against acidolytic cleavage of peptides during acetylation under more drastic conditions. On the other hand, some other derivatization procedures[3] lead to varying degrees of racemization.

Although peptides with at least three residues can be successfully analysed, a major application of this GLC technique is likely to be mixtures containing mostly dipeptides derived from polypeptides by degradation with dipeptidyl aminopeptidase-I. Many, if not most, of the 400 possible dipeptides should be separable on suitable GLC columns but in practice it is unlikely that separation of such complex mixtures would be required and the demands on the GLC system would be correspondingly less severe. For instance, we and others[10] have found that peptides with reversed sequences as well as α- and γ-Glu peptides are separable on silicone columns. Overlapping or unresolved doublets would be distinguished by low resolution mass spectrometry (*e.g.* Phe–Tyr and Phe–Phe–NH_2). An additional advantage is the possibility of examining racemization in synthetic peptides or optical configuration of

natural products at the same time, since there is evidence[11] that the enzyme hydrolyzes peptides containing residues of the D-configuration and our results indicate separation of enantiomeric dipeptides on silicone columns in less than 60 min. The total analysis time employing temperature programming could probably be kept within 2 h (*e.g.* ref. 4).

Recent investigations (refs. 16 and 18 and refs. cited) have shown that permethylated derivatives of peptides containing any of the protein amino acids can be prepared successfully on the 10–100 nanomolar scale and sequenced by mass spectrometry. We have found GLC analysis of N-Ac permethylated peptides to be reproducible within the same range. GC–MS has the inherent advantages over conventional mass spectrometry of cleaning up the sample, separating byproducts and mixtures and providing a higher instantaneous concentration of a given compound in the mass spectrometer (because of the short elution time of a GLC peak) than is possible using a direct inlet system — thus improving sensitivity, while the major disadvantage, the volatility required for GLC, is largely overcome when the peptides involved are small, as is the case with dipeptidyl aminopeptidase I digests. In addition, mass spectra of small peptides are not subject to the severe loss of sensitivity at high mass observed in those of large peptides.

We believe that a procedure based on degradation with dipeptidyl aminopeptidase I followed by permethylation and analysis by GLC or GC–MS will prove a useful addition to existing peptide sequencing methods and that it has advantages over analysis of unfractionated peptide mixtures by mass spectrometry alone[21,25]. Quantitative aspects of derivatization and GLC, together with applications of the complete sequencing technique, are now being investigated. Since the results described in this paper were obtained, OVCHINNIKOV AND KIRYUSHKIN[26] have described the separation of dipeptides from dipeptidyl aminopeptidase I digests, as N-TFA methyl esters on 1 % OV-17.

National Institute for Medical Research, D. H. CALAM
Mill Hill, London, N.W. 7 1AA (Great Britain)

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