Journal of Chromatography, 218 (1981) 519–533
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,234

ANALYSIS OF N-ACETYL-N,O,S-PERMETHYLATED PEPTIDES BY COMBINED LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Initial results are presented on the liquid chromatography–mass spectrometry (LC–MS) of N-acetyl-N,O,S-permethylated oligopeptides using a moving belt interface for the sequence analysis of the peptides. Using a quadrupole MS, it has been found that isobutane chemical ionization provides good intensity $[M + 1]^+$ ions, as well as well-defined N- and C-terminal fragment ions. It is shown that C-methylated peptides can be separated by LC and identified by MS. Examples are given on the LC–MS of N-acetyl-permethylated Leu-enkephalin as well as a mixture of derivatized peptides. In all cases sequence information is available by the LC–MS approach. Finally, initial and promising attempts at predicting chromatographic retention in a linear gradient condition in reversed-phase LC for the derivatized peptides are presented. These results establish that sequence analysis of permethylated peptides by LC–MS can be achieved.

INTRODUCTION

High-performance liquid chromatography (HPLC) using ultraviolet (UV) and fluorescence detection has emerged as a significant method for the separation of peptides[1–3] and more recently proteins[4]. The value of a powerful separation method is readily recognized considering the fact that there are 20 common amino acids which can yield 20^n possible *n*-amino acid peptides, *e.g.*, 400 for dipeptides, 8000 for tripeptides and 160,000 for tetrapeptides. Along with separation, the problem of identification of individual peptides can also be very great.

Since the early days of paper chromatography[5], attempts have been made to identify individual peptides from retention behavior. In this case, a Martin-type group additivity approach is tested, in which individual amino acids contribute a specific portion of the total-free-energy change in the distribution process. For example, the amino acid group contributions have been related to Rekker hydrophobic fragment constants in reversed-phase liquid chromatography (RPLC)[6]. In addition, empirically determined structural increments of retention have been used to obtain a rough estimate of total peptide retention in RPLC[7]. As these approaches are only approximations, the accurate identification of peptides based on retention often is not possible without some extra chromatographic information. Thus, there is need

0021-9673/81/0000–0000/$02.50

for a more definitive structural identification of the species eluting from an HPLC column.

One approach is to collect individual peptide bands and to perform standard Edman degradation procedures[8]. This method works well in many cases, is conventionally employed and can indeed lead to low picomolar sequencing possibilities of intact oligopeptides[9]. Nevertheless, the procedure is relatively slow, cannot be used for peptides whose N-terminal sides do not contain a free amino group (*e.g.*, pyroglutamate) and has difficulty with uncommon amino acids. Thus, other approaches are necessary to complement the Edman procedure.

The value of mass spectrometry (MS) for elucidating the amino acid sequence of oligopeptides–proteins is now widely recognized[10–12]. MS can provide valuable information on amino acid sequences from both the N- and C-terminals in one run, is relatively rapid, can handle unusual amino acids and end groups and is potentially highly sensitive. Because of the very large number of peptide possibilities, a desirable feature of MS analysis, in order to provide readily interpretable spectra, is the cleavage of the peptide at the amide bonds with little or no rearrangement. This has generally meant the need to employ derivatives for fragmentation purposes which at the same time enhance the volatility of the peptide. Generally, two types of MS analysis of peptides have commonly been employed: gas chromatography (GC)–MS and the direct insertion probe.

Analysis by GC–MS requires the formation of volatile derivatives of which the O-trimethylsilyl-polyamino alcohols, formed via a multi-step procedure, have proved to be the most developed for peptide sequencing thus far[13]. This approach has recently been combined with DNA sequencing procedures for the rapid sequencing of very-long-chain proteins[14]. Nevertheless, besides the complex and time consuming chemistry, a limitation of the GC–MS approach for oligopeptide sequencing is imposed by the low volatility of longer peptides. In general, the GC–MS analysis of peptides using O-trimethylsilyl ethers of polyamino alcohols is limited to chains containing not more than five or at best perhaps six amino acid residues[15]. It may also be noted that N-acetyl peptide esters generally work best for dipeptides in GC–MS[16].

For peptide analysis using the direct insertion probe, the most commonly employed procedure involves the formation of N-acetylated-N,O,S-permethylated derivatives. Following the pioneering work of Das *et al.*[17], the permethylation procedure, as modified by Morris[18] and Leclerq and Desiderio[19], involves N-acetylation and is followed by N,O,S-permethylation with methyl sulfinyl carbanion and methyl iodide. It has been reported that N,O,S-permethylation and sample introduction into the MS via the direct insertion probe can be satisfactory for the sequencing of peptides containing as many as fifteen amino acid residues using conventional ionization techniques [*i.e.*, electron impact (EI) or chemical ionization (CI)][20]. However, unlike GC–MS, analysis via the direct insertion probe is basically an off-line approach. While simple mixtures (two to five components) can be analyzed by fractional volatilization[21], complex mixtures from peptide–protein hydrolysates could become a rather involved procedure. It is interesting to note in passing that the N-trifluoroacetyl-N,O,S-permethylated peptide derivative has also been employed in GC–MS for sequence determinations[22].

Even though the most informative mass spectra of oligopeptides are obtained by formation of polyaminoalcohols or permethylated derivatives, as indicated above,

analysis of hydrolysates by GC–MS is limited to peptide mixtures containing relatively small peptides of, at the most, four to six amino acid residues. This limitation makes it more difficult to recognize overlapping sequences of peptides and ambiguities are likely. Since sample volatility is not an issue in HPLC, this procedure can, in principle, handle larger peptides than GC. Therefore, we sought to determine the applicability of combined HPLC–MS to peptide analysis. To this date, the technique of combined HPLC–MS for the analysis of peptide mixtures has been successfully utilized only in preliminary studies using N-acetyl peptide methyl ester derivatives[23]. However, these derivatives are relatively non-volatile and their mass spectra not fully characterized, especially for peptides containing four or more amino acid residues. In order to ensure compatibility with MS, it was important to consider derivatives which could yield structurally informative mass spectra. In view of the analogy between the moving belt interface and the direct insertion probe as far as sample introduction is concerned, we decided to examine the utility of the permethylated derivatives for this purpose. The results of our preliminary studies towards meeting this goal are described in this manuscript.

EXPERIMENTAL

Materials

The peptides were purchased from Sigma (St. Louis, MO, U.S.A.) and Vega Biochemicals (Tuscon, AZ, U.S.A.) and were used as received. The purities of these peptides were tested by HPLC when this seemed necessary. Pure peptides were collected by preparative HPLC where appropriate. Methyl sulfoxide (Mallinckrodt, St. Louis, MO, U.S.A.) and methyl iodide (Aldrich, Milwaukee, WI, U.S.A.) were freshly distilled before use. The sodium hydride was a 50% dispersion in oil and was obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Other reagent grade chemicals and HPLC grade or spectrograde solvents were purchased commercially and were not purified further.

Acetylation and permethylation

The acetylation and permethylation procedures described by Leclerq and Desiderio[19] were employed. Peptides containing arginine were treated with hydrazine to convert arginine to ornithine before acetylation, as described by Thomas *et al.*[24]. The samples were refrigerated when not in use.

Mass spectrometry

Both CI and EI mass spectra were recorded on a Model 4000 quadrupole mass spectrometer (Finnigan, Palo Alto, CA, U.S.A.) with a Finnigan LC–MS interface and Incos data system. Approximately 100 ng of material dissolved in acetonitrile were used for each mass spectrum run. The conditions were set as follows: electron multiplier voltage, −1670 V; electron energy, 70 eV; ionizer temperature, 270°C; manifold temperature, 110°C; vaporizer temperature, 230°C; and ionizer pressure, 0.05 Torr for isobutane, 0.11 Torr for ammonia, 0.12 Torr for methane.

High-performance liquid chromatography

Two HPLC instruments were used: a Model 6000A pump (Waters Assoc.,

Milford, MA, U.S.A.) with a Model 770 variable-wavelength UV detector (Schoeffel, Westwood, NJ, U.S.A.) and an HP 3385A automation system (Hewlett-Packard, Avondale, PA, U.S.A.); and a Model 5000 high-performance liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.) with a Varian UV-50 variable-wavelength detector. Detection was performed by UV absorption at 214 nm. A Supelcosil LC-8 column was used 150 × 4.6 mm I.D.) (Supelco, State College, PA, U.S.A.). Samples were introduced by using a microsyringe through a Model AH60 sample injector valve (Valco, Houston, TX, U.S.A.) actuated by air.

A standard linear gradient from 5 to 70% acetonitrile in water in 20 min with a flow-rate of 1 ml/min was employed. These conditions were used to obtain the preliminary retention data for assessment of retention prediction of the permethylated peptides. Generally, the amount of sample injected was about 1 μg in 5 μl acetonitrile.

High-performance liquid chromatography–mass spectrometry

The HPLC–MS system consisted of a Varian 5000 high-performance liquid chromatograph and a Finnigan 4000 mass spectrometer equipped with a Finnigan LC–MS moving-belt interface. In order to accomodate reversed-phase eluents, the interface was modified according to the suggestions of Smith and Johnson[25]. As shown in Fig. 1, a stream of hot nitrogen gas was passed through the HPLC effluent to create an aerosol spray for deposition of the effluent onto the belt. In addition, a block heater was placed under the belt just beneath the point of sample deposition to facilitate further the rate of solvent evaporation. The nitrogen gas heater was set from 140°–170°C and the block heater was maintained around 80°C. The combination of heated nitrogen gas and the block heater allowed operation of the HPLC–MS interface at flow-rates as high as 0.5 ml/min for *ca.* 30% acetonitrile in water. The conditions for CI mass spectra were the same as described above. A home-made 5-μm octyl-silica bonded-phase column (150 × 2.8 mm I.D.) was used. The linear gradient system was the same as mentioned above with a flow-rate of 0.5 ml/min.

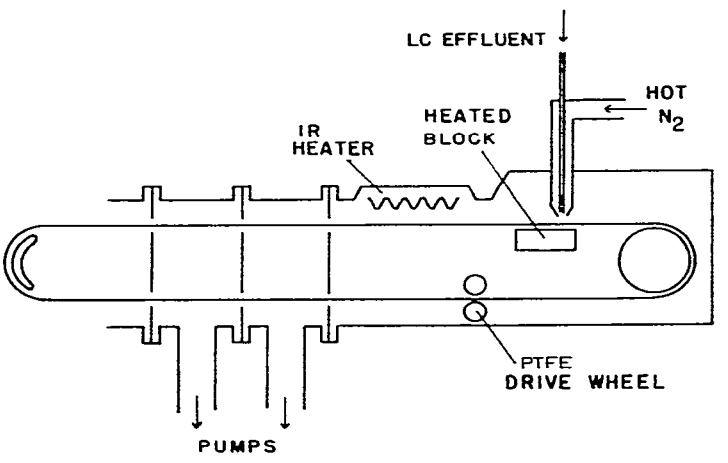

Fig. 1. Diagram of modification of moving belt interface to operate with aqueous–organic mobile phases. See text for description.

RESULTS AND DISCUSSION

Our investigation of the utility of N,O,S-permethylated derivatives of peptides for amino acid sequencing by HPLC–MS involved the following considerations: off-line evaluation of the mass spectral (EI and CI) properties of permethylated oligopeptides using the moving belt as the mode of sample introduction; and, examination of peptides and peptide mixtures by HPLC and on-line HPLC–MS.

Mass spectrometry

This portion of the work was necessary to establish the optimum conditions of our system, and in particular the moving belt interface (*e.g.*, interface vacuum and temperature for solvent removal, flash vaporizer temperature, etc.), for effective sample transfer and introduction into the mass spectrometer. Moreover, it was necessary to assess the relative merits of EI- and CI-MS for the sequencing of permethylated peptides in our specific system. This was particularly important because of the relative paucity of published spectral data in this area and previous observations to the effect that the overall signal-to-noise ratio was improved when the Finnigan 4000 HPLC–MS system was operated in the CI mode[26].

For this purpose, pure samples of permethylated peptides were dissolved in acetonitrile and aliquots containing *ca.* 100 ng of the solute were dripped onto the moving belt for transfer into the ion source. Typical mass spectra obtained are shown in Fig. 2a and b which compares the CI-isobutane spectrum and the EI spectrum of the permethylated derivative of the tripeptide Gly-Phe-Ala. Fig. 3 summarizes the observed fragmentation pattern under CI conditions and the "ideal" fragmentation expected from simple cleavages of the peptide bond under EI ionization conditions. Under CI conditions, two types of protonated ions are observed: the typical protonated molecular ion $[M + H]^+$ and the C-terminal ammonium ion [C-terminal fragment + $2H]^+$.

It is apparent that the CI-isobutane spectrum (Fig. 2a) gives a well-defined "molecular-ion" peak, characterized by the ion peaks at *m/z* 392, $[M + 1]^+$ and *m/z* 432, $[M + 41]^+$. "Molecular-ion" peaks, $[M + 41]^+$ and occasionally $[M + 57]^+$, are the adduct ions formed by combination of a solute molecule and the fragment ions of $C_3H_5^+$ and $C_4H_9^+$ of isobutane, respectively. In addition, sequence determining ions associated with both the N- (*m/z* 114, 247, 275, 332 and 360) and the C-terminals (*m/z* 118, 279 and 350) are for the most part easily discernible. These features should permit identification of the structure of the peptide. On the other hand, the EI mass spectrum of this peptide (Fig. 2b) yields a barely visible molecular-ion peak (*m/z* 391) and, aside from the C-terminal ion at *m/z* 116, it is dominated by N-terminal ions (*m/z* 86, 114, 247, 275, 332 and 360).

A second example of the advantages offered by CI-MS is given in Fig. 4, which shows the CI-isobutane mass spectrum of the permethylated derivative of the tetrapeptide Val-Ala-Ala-Phe. Sequence determining ions from both the N- and the C-terminals are again noted and, moreover, the molecular weight of the peptide is readily defined by the peaks at *m/z* 519, $[M + 1]^+$; *m/z* 559, $[M + 41]^+$; and *m/z* 575, $[M + 57]^+$. The peaks at *m/z* 86 and 134 are probably due to the formation of protonated imine ions of valine $[CH_3–\overset{+}{N}H=CH–CH(CH_3)_2]$ and phenyl alanine $[CH_3–\overset{+}{N}H=CH–CH_2–C_6H_5]$, respectively.

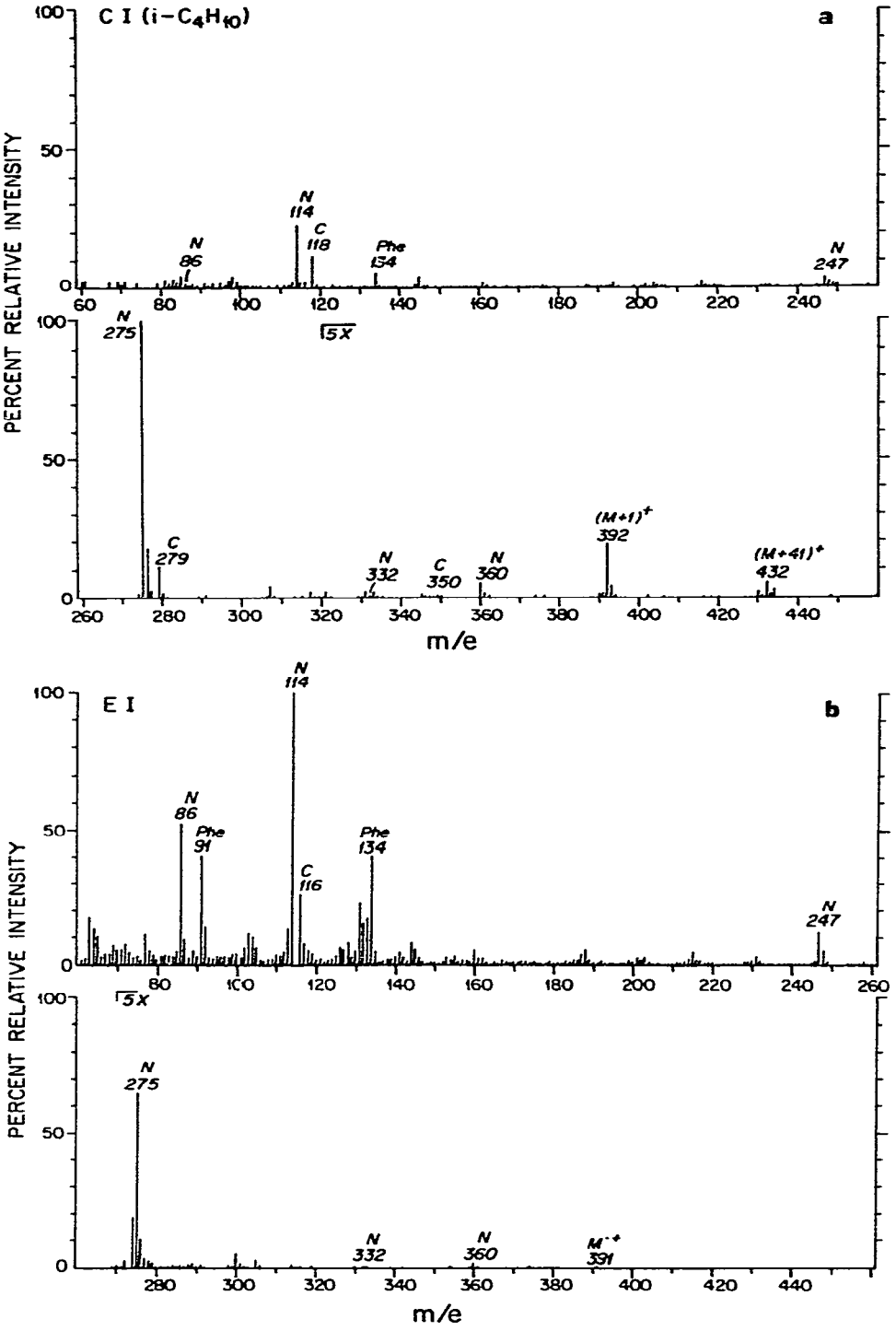

Fig. 2. a. Isobutane CI mass spectrum of Ac-Me · Gly-Me · Phe-Me · Ala-OMe. b, EI mass spectrum of Ac-Me · Gly-Me · Phe-Me · Ala-OMe. Ac = Acetyl; Me = methyl.

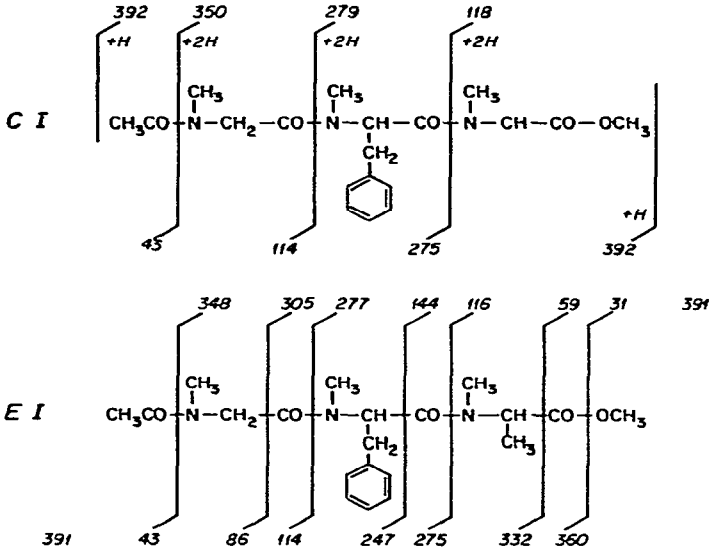

Fig. 3. Structure and fragmentation pattern of Ac-Me · Gly-Me · Phe-Me · Ala-OMe.

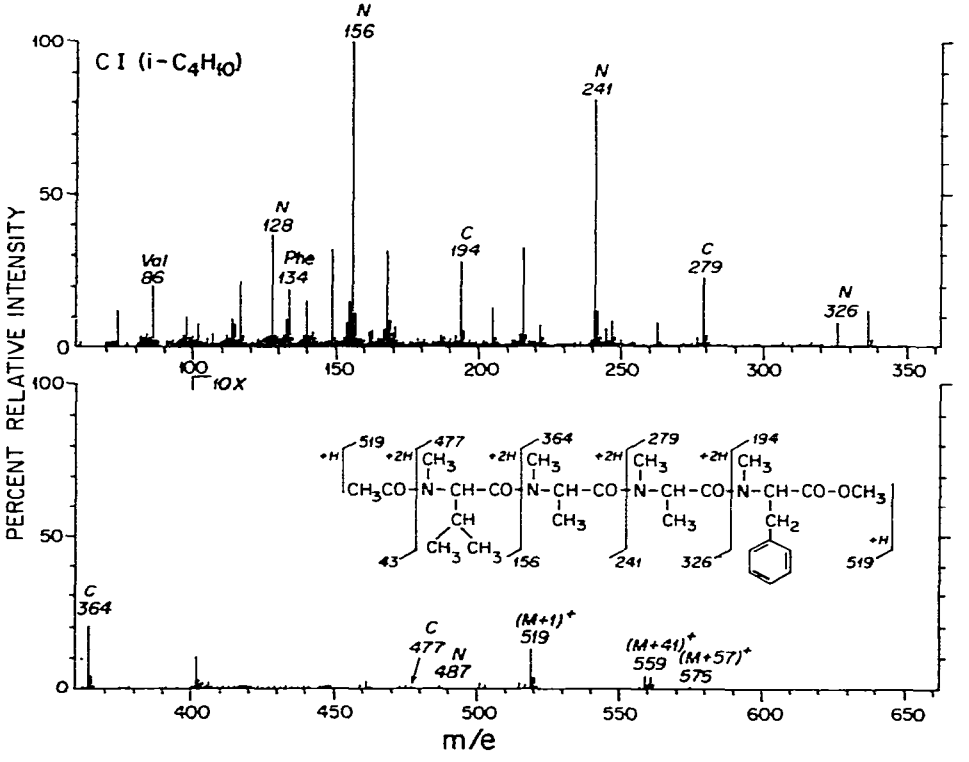

Fig. 4. Isobutane CI mass spectrum and fragmentation pattern of Ac-Me · Val-Me · Ala-Me · Ala-Me · Phe-OMe.

In general, we found that with our system the CI mass spectra of N-acetyl-N,O,S-permethylated peptides were more useful than the corresponding EI spectra. This is in agreement with the conclusions reached by Mudgett *et al.*[27] in their comparative study of the EI and CI spectra of a series of permethylated peptides. In our particular case, the advantages of CI over EI are realized not only in terms of the occurrence of both N- and C-terminal ions, but also because of the lower overall ion background level contributed by the polyimide belt under CI conditions. This increased signal-to-noise ratio permits a better definition of the "molecular-ion" peaks under CI rather than under EI conditions and, consequently, provides for more definitive structural identification of the peptides. It should be further noted that the CI-ammonia mass spectra of permethylated peptides were generally similar to those obtained with isobutane reagent gas. On the other hand, in agreement with the results of Mudgett *et al.*[27], use of methane instead of isobutane as the CI reagent gas resulted

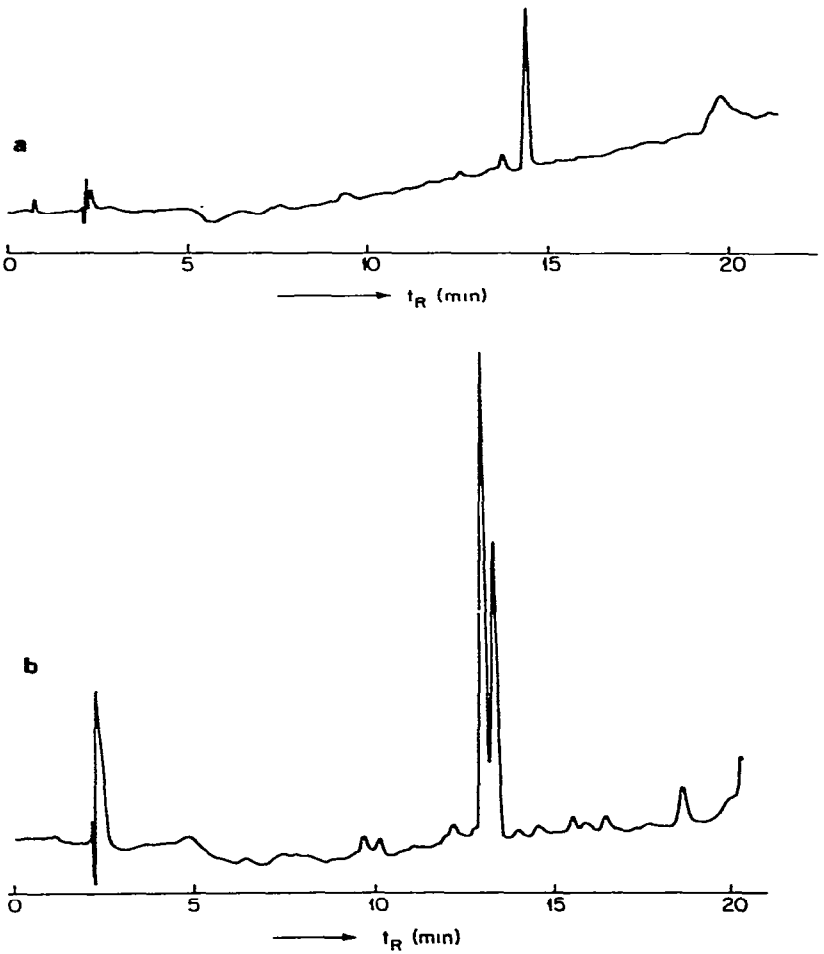

Fig. 5. a, HPLC chromatogram of Ac-Me-Phe-Pro-OMe. b, HPLC chromatogram of Ac-Me-Ala-Me-Val-OMe and the corresponding derivative C-methylated on the α-carbon of Val. Solvent A: water; solvent B: acetonitrile; gradient: from 5 to 70% acetonitrile in 20 min; flow-rate: 1 ml/min.

in spectra containing weaker molecular-ion peaks and relatively lower ion-peak intensities at the high mass region of the spectra.

High-performance liquid chromatography and high-performance liquid chromatography–mass spectrometry

The HPLC analyses of two N-acetyl-permethylated peptides, Phe-Pro and Ala-Val, are shown in Fig. 5 in order to illustrate the type of chromatograms observed. The gradient conditions and approximate sample sizes have been given in the Experimental section. In the case of Phe-Pro, a single chromatographic peak is observed, and the N- and C-terminal fragment ions as well as the protonated molecular-ion peak are readily recognized in the CI mass spectrum.

Of significance is the occurrence of a doublet in the chromatogram of the N-acetyl-permethylated derivative of Ala-Val (Fig. 5b). The mass spectra from the two peaks are shown in Fig. 6a and b. The CI spectrum from the first peak shown in Fig. 6a is easily identified as the expected permethylated derivative of the dipeptide. The CI spectrum from the second peak of this doublet is shown in Fig. 6b. The significant ion at 287, 14 mass units above the $[M + 1]^+$ ion of 273, strongly suggests an additional methyl group added to the molecule. The significant ions at 160 and 255 point to the methyl group being added to the α-carbon of valine. The chromatographic peak is thus identified as the C-methylated-N-acetyl-N,O-permethyl-Ala-Val, and its structure is shown in the figure. The major ions such as m/z at 146, 273 and 313 arise from the normal permethylated derivative which overlaps into the second peak and is in much higher quantity (see Fig. 5b).

C-Methylation is a well-known artifact in permethylation of peptides and has been studied specifically with peptides containing a glycine residue[28]. It is significant that gradient elution HPLC can separate the C-methylated analogues from the principal chromatographic peaks of interest, namely those of the N-acetyl-N,O,S-permethylated derivatives. The exact identity of the C-methylated analogues, including the position(s) of C-methylation, can readily be identified from the mass spectra during HPLC–MS. The incidence of C-methylation and a discussion of the reaction conditions required to suppress the extent of its occurrence will be covered in more detail in a later paper[29].

It was noted in the Introduction that the ultimate advantage of HPLC–MS over GC–MS will be realized by its ability to handle longer chain peptides of low volatility which are difficult or impossible to analyze by GC–MS. Fig. 7a shows an HPLC chromatogram of the N-acetyl-permethylated leucine-enkephalin derivative. The small second peak in this chromatogram arises from C-methylation. This peptide contains five amino acid residues, *i.e.*, Tyr-Gly-Gly-Phe-Leu. In the analysis by GC–MS to form an O-trimethylsilyl-polyamino alcohol, the GC retention index, as calculated from retention index increments[30], is 3970. This value is at the upper retention range where elution through the GC is relatively difficult. As shown in Fig. 7b, a total ion chromatogram was readily obtained in which two distinct peaks are observed, indicative of reasonable band broadening characteristics in this region of the gradient (*ca.* 70% acetonitrile). (Studies of band broadening with this LC–MS interface are in progress.) The corresponding mass spectrum of the derivatized peptide is shown in Fig. 7c with a $[M + 1]^+$ ion at 696. It is interesting to note that MS was originally used to aid in the identification of this peptide in brain samples[31].

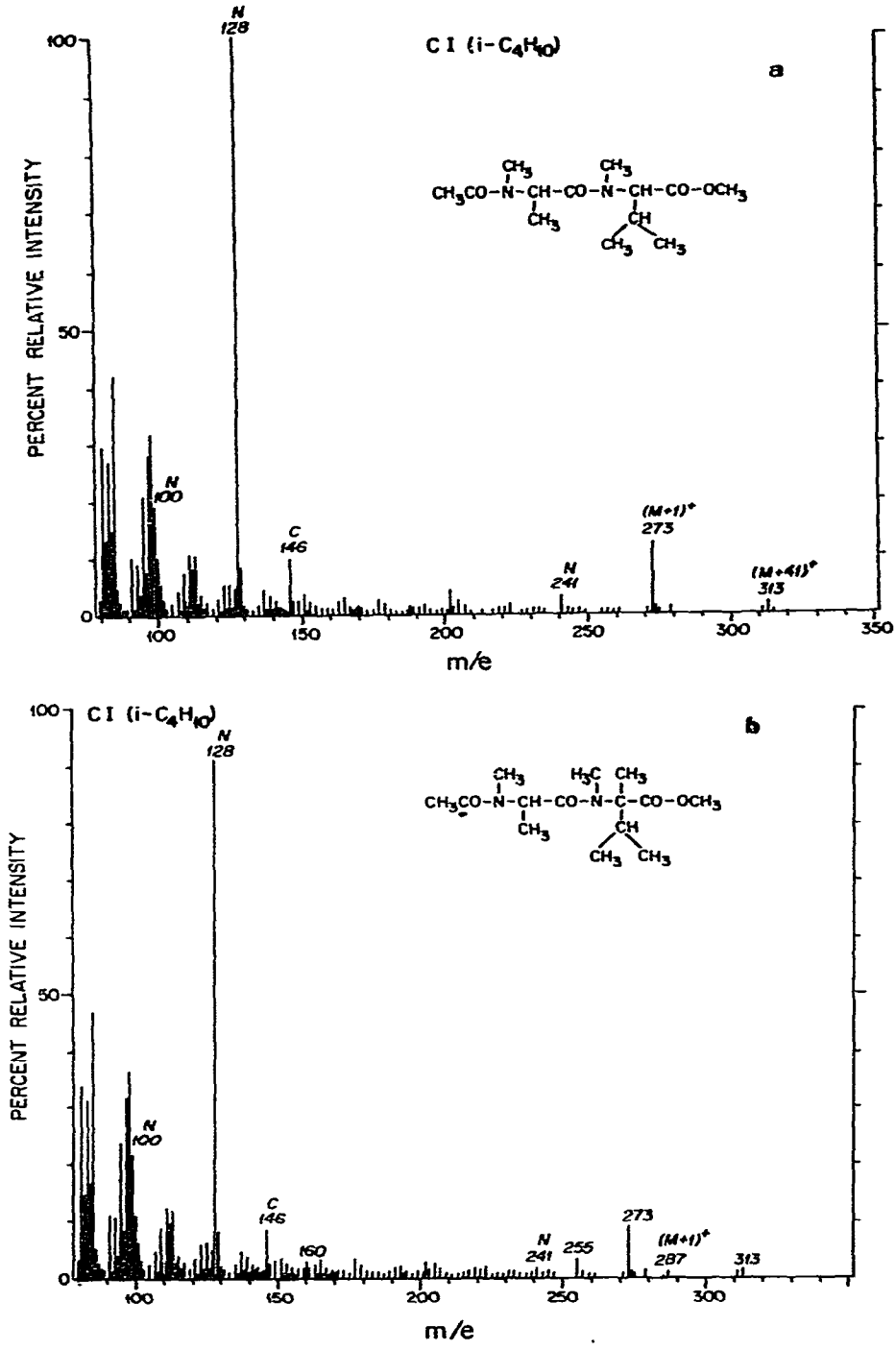

Fig. 6. a, Isobutane CI mass spectrum of the first peak of the Ala-Val chromatogram in Fig. 5b. The spectrum is readily interpreted as the expected permethylated derivative. b, Isobutane CI mass spectrum of the second peak of the Ala-Val chromatogram in Fig. 5b. The spectrum corresponds to the C-methylated derivative shown in the figure.

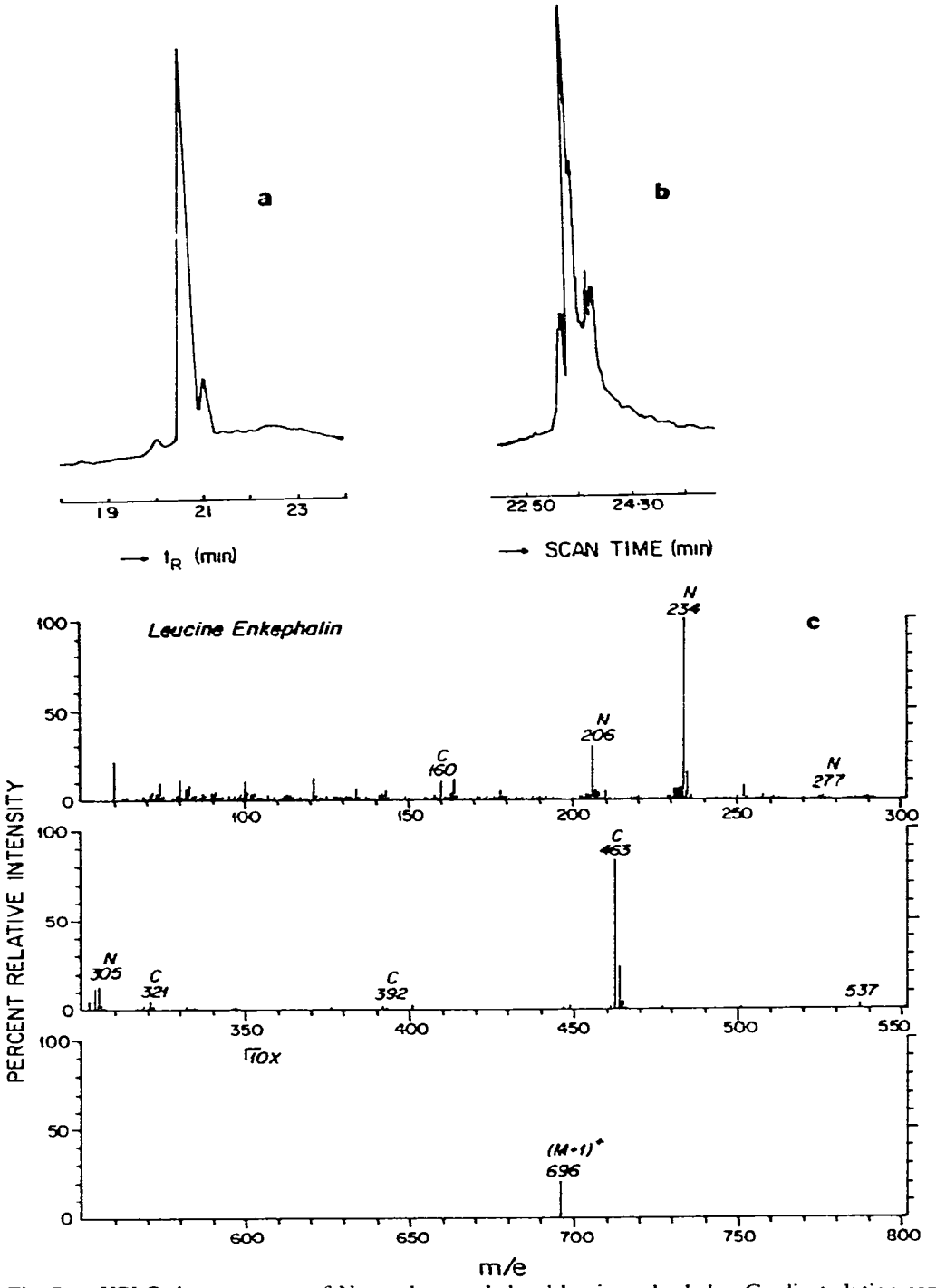

Fig. 7. a, HPLC chromatogram of N-acetyl-permethylated leucine-enkephalin. Gradient elution conditions given in the text. The second peak is a C-methylated derivative of the peptide. b, Total-ion chromatogram of N-acetyl-permethylated leucine-enkephalin obtained from moving belt system. c, Isobutane CI mass spectrum of N-acetyl-permethylated leucine-enkephalin obtained from the LC–MS system.

To assess whether or not HPLC–MS of permethylated peptides would be applicable to the analysis of peptide hydrolysates, we examined the HPLC–MS characteristics of a mixture of eight N-acetyl-permethylated peptides: tetra-Ala, Ala-Val, Gly-Phe-Ala, Phe-Pro, Trp-Gly, Gly-Phe-Phe, Leu-Phe and Val-Tyr-Val. The HPLC chromatogram of the mixture, using a UV detector, is shown in Fig. 8. The corresponding total ion chromatogram recorded during HPLC–MS and using the same HPLC conditions is shown in Fig. 9i. These chromatograms were obtained by injecting a mixture containing 5–10 nmol of each component into the HPLC. Mass chromatograms, utilizing the protonated molecular ions of the peptides are shown in Fig. 9a–h. All the peptides in the mixture could be further identified from their compleete mass spectra. It can be seen that, as noted above, C-Methylation products are noticeable in Fig. 8 as well as in Fig. 9.

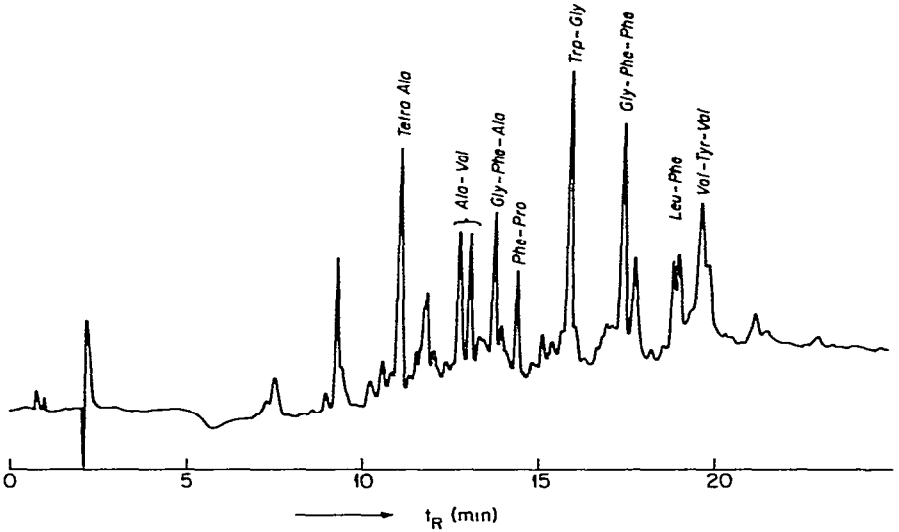

Fig. 8. HPLC separation of a mixture of eight N-acetyl N,O,5-permethylated peptides as indicated on the chromatogram. Gradient elution conditions given in the text.

It is apparent from the results of this preliminary study (Figs. 8 and 9) that detection of permethylated oligopeptides by HPLC–MS is greatly facilitated via the use of mass chromatograms or, if one is looking for a specific peptide(s), by selective ion monitoring. Obviously, this would be impractical when dealing with an unknown mixture. To overcome such difficulties in GC–MS, retention indices are calculated based on the summation of retention increments of individual amino acids[30]. This greatly aids in the mass spectral search for specific peptides and in the cross-checking of peptides identified by MS. A retention increment scale for LC of the permethylated peptides is in principle possible. Using linear solvent strength (LSS) gradient conditions, it can be shown that the apparent solute capacity factor of the gradient, k'_{app}, is roughly proportional to log k'_0 where k'_0 is the capacity factor of the solute under the starting mobile phase conditions[32]. Since the latter is a free energy term, it is possible to apply a Martin-type relationship to k'_{app} under LSS conditions.

As in the case of Nau and Biemann in GC[30] and Meek in LC[7], our preliminary

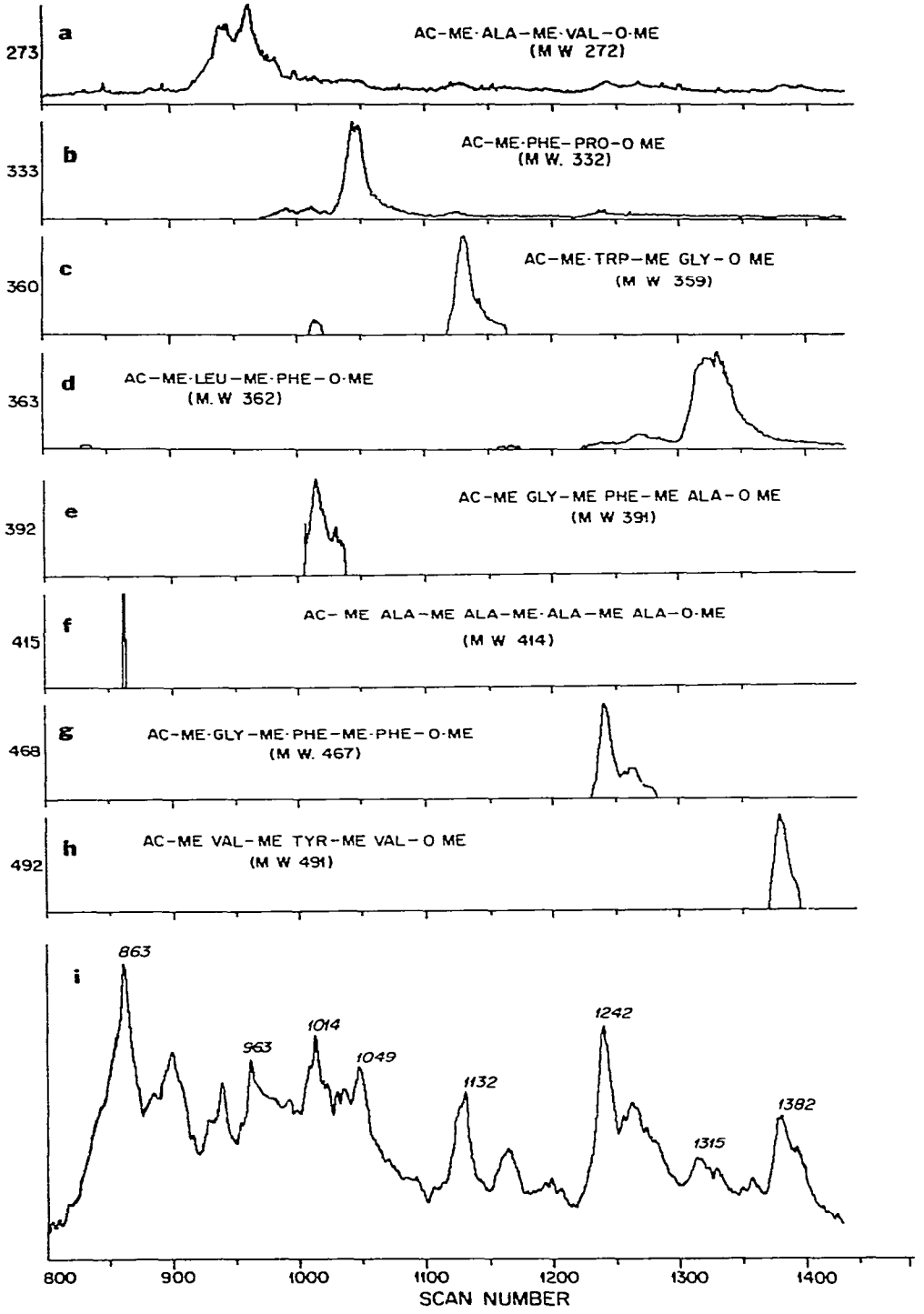

Fig. 9. a–h, Single ion monitoring $[M + 1]^+$ of the eight permethylated peptides eluting from the HPLC column (see Fig. 8). i, Total ion chromatogram of the coupled LC–MS analysis of the eight permethylated peptides. Sample size: *ca.* 5 nmol per peptide. AC = Acetyl; ME = methyl; M.W. = molecular weight.

approach has been to determine individual amino acid increments. We have obtained k'_{app} values by summing these increments for a specific peptide along with an end group contribution for the N-acetyl and $-OCH_3$ groups. Using a series of poly-alanines from $(Ala)_2$ to $(Ala)_5$ we have estimated the Ala contribution as 0.54 ± 0.12 and the end group contribution as 2.60 ± 0.50. Other amino acid contributions have generally been calculated from dipeptides containing Ala, except where other homo-peptides were available. We have examined predicted k'_{app} values *vs.* experimentally determined k'_{app} values for 40 di-, tri- and tetrapeptides, and some representative results are shown in Table I.

TABLE I

RETENTION PREDICTION OF PERMETHYLATED PEPTIDES USING A LINEAR SOLVENT STRENGTH GRADIENT

Permethylated peptide	k'_{app}, *exp.*	k'_{app}, *calc.*
Gly-Ser-Ala	3.72	3.61
Leu-Gly-Gly	5.05	4.99
Gly-Ala-Phe	6.47	6.40
Phe-Pro	6.82	6.98
Ser-Leu	6.99	6.60
Trp-Gly	7.79	7.61
Val-Ala-Ala-Phe	8.64	9.21
Leu-Phe	9.72	9.39
Val-Tyr-Val	10.22	9.71
Trp-Phe	10.48	10.65
Phe-Phe-Phe	11.13	13.52

In general, we find that k'_{app} calculated agrees with k'_{app} measured within 5–8%. This good agreement for a limited sample of peptides means that this approach is quite promising. Undoubtedly, the removal of a high degree of polarity in the peptide molecule upon derivatization aids in the additivity of the increments. We should also note that in a few cases, the difference in k'_{app} calculated *vs.* k'_{app} experimental can be as high as *ca.* 20%, as illustrated by Phe-Phe-Phe in Table I. This result may mean that steric correctional factors may at times be necessary to account for adjacent amino acids. Work is continuing to measure k'_{app} values for a wide variety of derivatized peptides in order to permit improved amino acid increment retention values for predictive purposes.

CONCLUSIONS

The data presented here demonstrate the feasibility of conducting on-line HPLC–MS of peptide mixtures using N-acetyl-permethylated derivatives. It is apparent that the moving belt interface can be used for the introduction of permethylated derivatives of peptides into the MS, in a manner analogous to the direct insertion probe. As a consequence, MS analysis of relatively long chain oligopeptides should in principle be possible. The combined use of MS data and chromatographic retention using HPLC–MS may thus augment the current state-of-the-art in the field of peptide

sequencing. Studies to optimize many of the parameters discussed in this paper *e.g.*, reaction conditions, chromatographic conditions, etc.) are currently in progress. In addition to these factors, our studies also include a consideration of other types of chemical derivatives and different interfacial designs, such as the direct liquid interface.

ACKNOWLEDGEMENTS

The authors wish to acknowledge NIH under grant numbers GM22787 (P.V.) and GM15847 (B.L.K.) for support of this work. Contribution number 101 from the Institute of Chemical Analysis.

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