Journal of Chromatography, 350 (1985) 279–284
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 171

Note

Separation of some peptides and related isopeptides by high-performance liquid chromatography: structure–retention time relationships

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(First received August 6th, 1985; revised manuscript received September 9th, 1985)

During the past few years, high-performance liquid chromatography (HPLC) has been widely used in the separation of small peptides resulting from enzymatic digestion of large polypeptides and proteins[1–3]. Reversed-phase HPLC has also proved useful in accurately determining the extent of hydrolysis of a number of model isopeptides containing either a single methionyl residue or methionine oligomers covalently linked to the ε-amino group of lysine by both pancreatic and intestinal enzymes[4]. The released methionine and methionine oligomers up to pentamethionine, as well as their parent isopeptides, could easily be separated on alkylsilane-bonded silica using gradient elution with acetonitrile in an orthophosphoric acid solution. It has been shown that the nutritional quality of dietary proteins could be improved through the covalent attachment of essential amino acids to their lysyl residues, resulting in the formation of a so-called isopeptide bond[5–8].

The experimental determination of the retention behaviour of as many as 100 peptides in reversed-phase HPLC has shown that the retention time of any peptide could be directly derived from the sum of the contributions from the constituent amino acids[9–11]. However, if the amino acid composition of a given peptide is known to be of prime importance for retention, other factors such as conformation, sequence and charge distribution may also be involved. This is illustrated by the fact that some diastereoisomers as well as positional isomers with identical chemical compositions were found to be separated by HPLC[12].

It is the purpose of this paper to show that a number of peptides and related isopeptides can readily be separated by reversed-phase HPLC under different experimental conditions, and to correlate the retention time for each individual peptide or isopeptide to that predicted from their amino acid composition.

EXPERIMENTAL

Materials

All peptides and isopeptides contained exclusively L-amino acids. The two N^{α}-acetyloligopeptides, Ac^{α}-Met-Lys and Ac^{α}-Met-Lys-Ala-NH_2, and the corresponding isopeptides, $(Ac^{\alpha}\text{-Met})^{\varepsilon}$-Lys and $(Ac^{\alpha}\text{-Met})^{\varepsilon}$-Lys-Ala-$NH_2$, the isopeptides containing oligo(methionine) chains varying in length from two to five residues,

0021-9673/85/$03.30

$(Met)^{\varepsilon}_{2/3/4/5}$-Lys, the N^{α}-acetylisopeptides containing methionine, dimethionine and trimethionine, Ac^{α}-$(Met)^{\varepsilon}_{1/2/3}$-Lys-$NH_2$, as well as Met_4 and Met_5 were synthesized by solid-phase procedures as already described[4], whereas $(Met)^{\varepsilon}$-Lys was obtained by liquid-phase synthesis[6]. Other peptides were purchased from Bachem Fine Chemicals (Bubendorf, Switzerland). HPLC grade acetonitrile and orthoposphoric acid were obtained from Merck (Darmstadt, F.R.G.). Ammonium acetate, sodium perchlorate and trifluoroacetic acid were supplied by Fluka (Buchs, Switzerland) while sodium phosphate was obtained from Prolabo (Paris, France).

Methods

Retention times were measured on a Merck LiChrosorb C_{18} reversed-phase column (7 μm, 250 × 4 mm) using a Waters Associates HPLC system equipped with a M 6000 A and a M 45 solvent-delivery unit, a M 720 solvent programmer, an U6K injector, a M 441 fixed-wavelength detector (214 nm) and a M 730 two-channel chart recorder. The mobile phase was 0.1% orthophosphoric acid (pH 2.2), 0.1 *M* sodium perchlorate–0.1% orthophosphoric acid (pH 2.2), 0.05% trifluoroacetic acid (pH 2.3), 0.01 *M* ammonium acetate (pH 5.7) or 0.005 *M* sodium phosphate (pH 7.4). The mobile phase modifier was acetonitrile, the concentration of which was linearly increased from 0 to 60% over a 40-min period (1.5%/min). The flow-rate was 1 ml/min.

RESULTS AND DISCUSSION

Chromatography of peptides and isopeptides

Fig. 1 shows that the dipeptide Met-Lys and corresponding isopeptide $(Met)^{\varepsilon}$-

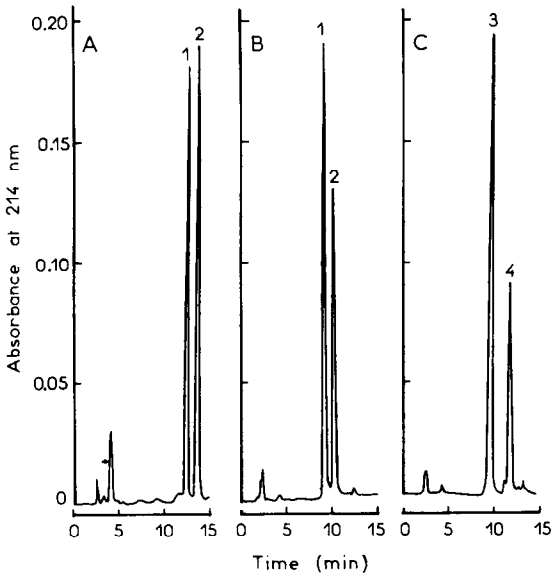

Fig. 1. Separation of two dipeptides and the corresponding isopeptides by HPLC on a 7-μm LiChrosorb RP-18 column. Elution was carried out with 0.1% phosphoric acid–0.1 *M* sodium perchlorate, pH 2.2 (A) and 0.005 *M* sodium dihydrogen phosphate, pH 7.4 (B, C) and an acetonitrile gradient (1.5%/min); flow-rate, 1.0 ml/min. Peaks: 1 = Met-Lys; 2 = $(Met)^{\varepsilon}$-Lys; 3 = Ac^{α}-Met-Lys; 4 = $(Ac^{\alpha}$-$Met)^{\varepsilon}$-Lys.

Lys were readily separated by reversed-phase HPLC with acetonitrile linear gradient elution under either acidic (pH 2.2) or neutral (pH 7.4) conditions. As expected, the retention times at the lower pH were found to be significantly higher since the carboxylic group was partly undissociated. Under such conditions it is likely that the separation of both the dipeptide and related isopeptide resulted from the position of the charged amino group in the chain. However, a possible small effect of some conformational difference between the peptide and isopeptide on their retention behaviour could not be ruled out. In contrast to the unprotected structure, the N-acetylated dipeptide Ac^{α}-Met-Lys and related isopeptide $(Ac^{\alpha}\text{-Met})^{\varepsilon}$-Lys had significantly different retention times only at neutral pH.

As indicated in Fig. 2, methionine oligomers (Met_3, Met_4 and Met_5), on the one hand, and isopeptides containing oligo(methionine) chains of varying lengths (two to five residues) covalently linked to the ε-amino group of lysine, on the other hand, were eluted from the 7-μm LiChrosorb column in the order of increasing hydrophobicity. However, the lower retention time for $Ac^{\alpha}(Met)^{\varepsilon}_3$-Lys-$NH_2$ as compared to $(Met)^{\varepsilon}_3$-Lys was rather unexpected, suggesting that differences in the ionization and position of charged groups might explain the retention behaviour. Lysine was found to have a marked negative effect on retention since all the isopeptides were eluted before the corresponding methionine oligomers. Finally, the presence of perchlorate in the mobile phase was observed to sharpen the peaks as compared to the situation in salt-free buffer, especially for isopeptides with unprotected amino groups (data not shown).

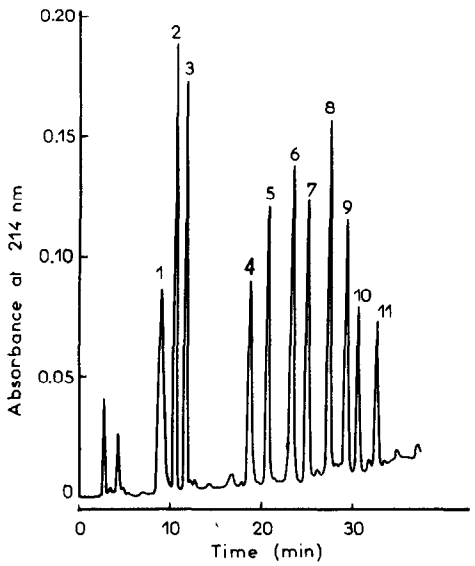

Fig. 2. Reversed-phase HPLC of some methionine oligomers and isopeptides. Chromatographic conditions as in Fig. 1a. Peaks: 1 = Met; 2 = Met-Lys; 3 = $(Met)^{\varepsilon}$-Lys; 4 = $Ac^{\alpha}(Met)^{\varepsilon}_2$-Lys-$NH_2$; 5 = $Ac^{\alpha}(Met)^{\varepsilon}_3$-Lys-$NH_2$; 6 = $(Met)^{\varepsilon}_3$-Lys; 7 = Met_3; 8 = $(Met)^{\varepsilon}_4$-Lys; 9 = Met_4; 10 = $(Met)^{\varepsilon}_5$-Lys; 11 = Met_5.

Retention behaviour of peptides and isopeptides

The dependence of peptide behaviour on the amino acid composition was first investigated using paper chromatography more than three decades ago[13–15]. Moreover, the retention time of small peptides during reversed-phase HPLC in a linear gradient elution system is known to be linearly related to the sum of the individual contributions from the constituent amino acids[9,10,16]. More recently, the prediction of retention times for peptides has been reinvestigated in the case of a volatile eluent system with acetonitrile as the mobile phase modifier[11]. Although some apparent discrepancies were found to exist between the experimental data obtained in volatile and non-volatile eluent systems, it is reasonable to consider that both conditions led to comparable relationships between the actual and predicted retention times, at least for small peptides (< 29 residues). It was therefore of interest to see whether or not a high degree of correlation between theoretically predicted and experimentally observed retention times exists for methionine oligomers as well as for methionine peptides and isopeptides containing oligo(methionine) chains covalently attached to the α- and ε-amino group of lysine, respectively (Table I).

Fig. 3 shows that the rather high correlation between actual and predicted retention times for seventeen methionine-containing peptides and isopeptides under both non-volatile and volatile eluent conditions at acidic pH. Correlation coefficients of 0.983 in 0.1% phosphoric acid, pH 2.2 and 0.992 in 0.05% trifluoroacetic acid, pH 2.3 (see Fig. 3a and b) were obtained by using Meek's retention coefficient val-

TABLE I

OBSERVED RETENTION TIMES OF PEPTIDES AND ISOPEPTIDES ON A LICHROSORB RP-18 COLUMN UNDER DIFFERENT EXPERIMENTAL CONDITIONS WITH ACETONITRILE AS THE MOBILE PHASE MODIFIER

Chromatographic conditions, as well as letters A–D, indicated in Fig. 3.

Peptide	*No.*	*Retention time (min)*			
		0.1% Phosphoric acid (A)	*0.05% Trifluoroacetic acid (B)*	*0.01 M Ammonium bicarbonate (C)*	*0.005 M Sodium dihydrogen phosphate (D)*
Met-Lys	1	4.5	9.0	4.6	9.0
$(Met)^{\varepsilon}$-Lys	2	9.6	10.1	9.5	10.2
Ac^{α}-Met-Lys-Ala-NH_2	3	11.3	12.4	12.6	14.6
$(Ac^{\alpha}$-Met$)^{\varepsilon}$-Lys-Ala-NH_2	4	11.3	12.4	12.6	16.1
Ac^{α}-$(Met)^{\varepsilon}$Lys-NH_2	5	11.6	11.7	12.2	17.6
Ac^{α}-Met-Lys	6	12.6	12.0	9.7	9.1
$(Ac^{\alpha}$-Met$)^{\varepsilon}$-Lys	7	12.6	12.0	10.7	10.9
$(Met)^{\varepsilon}_2$-Lys	8	13.2	15.6	11.8	–
Ac^{α}-$(Met)^{\varepsilon}_2$-Lys-NH_2	9	15.7	15.8	17.9	20.1
Met_2	10	16.7	15.5	10.1	11.1
$(Met)^{\varepsilon}_3$-Lys	11	17.7	19.6	19.0	22.0
Ac^{α}-$(Met)^{\varepsilon}_3$-Lys-NH_2	12	20.9	21.3	22.6	24.6
Met_3	13	21.1	22.5	15.3	14.2
$(Met)^{\varepsilon}_4$-Lys	14	22.7	23.7	23.9	26.4
Met_4	15	26.7	26.9	21.6	20.4
$(Met)^{\varepsilon}_5$-Lys	16	27.1	27.2	27.5	30.0
Met_5	17	30.8	30.0	24.9	23.8

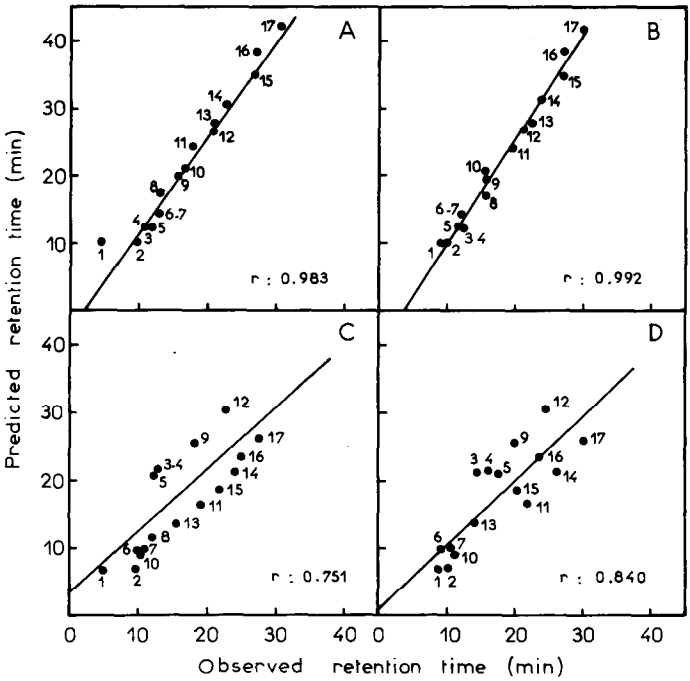

Fig. 3. Relationship between measured retention times and times obtained by summing retention coefficients for the amino acids and end groups at pH 2.1 (a, b) and pH 7.4 (c, d). Chromatography was done on a 7-μm LiChrosorb RP-18 column under four experimental conditions: A, 0.1% orthophosphoric acid, pH 2.2; B, 0.05% trifluoroacetic acid, pH 2.3; C, 0.01 *M* ammonium acetate, pH 5.7; D, 0.005 *M* sodium phosphate, pH 7.4. The mobile phase modifier was acetonitrile (1.5%/min). Numbers represent the peptides and isopeptides listed in Table I; *r* is the correlation coefficient.

ues[10,17]. By contrast, at pH 5.7 (0.01% ammonium acetate, Fig. 3c) and pH 7.4 (0.005 *M* sodium phosphate, Fig. 3d) a poor correlation was observed. In the former case, this might be due to the fact that the predicted retention times were derived from the retention coefficients at pH 7.4 instead of pH 5.7. Another reason would be the strong positive deviation observed for the peptides containing either an acetyl or an amide protecting group, *i.e.*, the predicted retention time was much higher than that observed experimentally. The contribution to retention of such groups was partially corrected by using the new retention coefficient values reported by Meek and Rossetti[17]. The correlation coefficients were improved to 0.94 and 0.88 in ammonium acetate, pH 5.7 and sodium phosphate, pH 7.4, respectively. However, some discrepancies were then observed under acidic conditions since the correlation coefficients decreased to 0.90 and 0.92 in orthophosphoric acid and trifluoroacetic acid, respectively.

CONCLUSIONS

This study clearly shows that reversed-phase HPLC on a C_{18} column using a volatile or non-volatile mobile phase under both acidic and neutral conditions, and

acetonitrile as the mobile phase modifier in a linear gradient elution system, is an effective means of separating a number of methionine oligomers as well as peptides and isopeptides containing up to five methionyl residues linked to the α- or ε-amino group of lysine. It is therefore suggested that differences in the ionization and charge distribution of some functional groups in peptides and isopeptides, in addition to the main contribution to retention from the constituent amino acids, are responsible for their separation. The linear relationship observed between the actual and predicted retention times was not affected by the isopeptide bond.

ACKNOWLEDGEMENTS

This work was supported in part by grant 820085 from A.E.C./Rhône-Poulenc. Our thanks are also due to Brigitte Videau for typing the manuscript and Paule Cassa for the illustrations.

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