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Correlation of structure and retention behaviour in reversed-phase high-performance liquid chromatography II. Methionine-enkephalin-related glycoconjugates

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Abstract

Reversed-phase high-performance liquid chromatographic elution data for methionine-enkephalin-related glycoconjugates were analysed as a function of the identity and position of the sugar–peptide linkage. It was shown that binding to the column could be correlated with the degree of sugar moiety protection. Replacement of either the phenylalanine or methionine residue in the peptide backbone of the glycoconjugates with its D-enantiomer leads to a considerably stronger retention on a reversed-phase column. The dependence of retention times on the methanol concentration in the mobile phase suggested that, under the conditions studied, there are different retention mechanisms for glycopeptides containing unprotected sugar moieties in the molecule.

1. Introduction

The results of a previous study [1] showing that glycation significantly influenced the retention characteristics of the endogenous opioid pentapeptide leucine-enkephalin [2] (Tyr–Gly–Gly–Phe–Leu) led us to investigate the chromatographic behaviour of the structurally similar opioid pentapeptide methionine-enkephalin [2] (Tyr–Gly–Gly–Phe–Met) and related glycopeptides under reversed-phase HPLC conditions. This seemed important as we observed that incorporation of the identical carbohydrate moieties into either the leucine- or methionine-enkephalin molecule produced different effects on the biological activity of the parent peptide compounds [3,4]. Therefore, the objectives of this work were to determine the retention parameters of some C-terminally glycated methionine-enkephalin derivatives under various conditions and to find correlations between the retention properties and physico-chemical parameters of the investigated glycopeptides.

2. Experimental

2.1. Instrumentation

The chromatographic system consisted of a Varian Model 9010 liquid chromatograph equipped with a Rheodyne Model 7125 injector, Varian Model 4400 integrator and Varian Model 9050 variable-wavelength UV–Vis detector. The

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detection wavelength was set at 280 nm and the flow-rate was 0.5 ml/min. A Serva *n*-octadecyl Si 100 column (250 × 4.6 mm I.D., 5 μm) was employed for both analytical and semi-preparative separations. The column dead volume (3.58 ml) was determined from the retention of uracil. The RP-HPLC conditions are given in the tables and figures. For analytical HPLC the samples were dissolved in 0.1% trifluoroacetic acid (TFA) in methanol–water (40:60) at a 0.5 mg/ml concentration and 100 μl of the solution were injected.

2.2. Chemicals

Methanol was of HPLC grade (Aldrich, Milwaukee, WI, USA) and trifluoroacetic acid was of spectroscopic grade (Uvasol; Merck, Darmstadt, Germany). Methionine-enkephalin ([Met^5]E), leucine-enkephalin ([Leu^5]E) and [D-Ala^2, Met^5]enkephalin were purchased from Sigma (St. Louis, MO, USA).

The methionine-enkephalin-related glycoconjugates **1–8** (Fig. 1) and leucine-enkephalin glycoconjugates **9–11** were synthesized as described previously [3–5]. HPLC analysis of each glycoconjugate indicated the presence of two peaks (found to be diastereomers) which were separated by repetitive injections (100 μl, concentration of 15 mg/ml in 40% methanol–0.1% TFA) under the conditions given in Section 3. The structures and homogeneity of the glycopeptides were confirmed by microanalysis, NMR spectroscopy using a Varian Gemini 300 instrument and RP-HPLC.

3. Results and discussion

A series of glycoconjugates (**1–8**, Fig. 1) in which opioid peptide, methionine-enkephalin or related analogue, [D-Ala^2, Met^5]-enkephalin (Tyr–D-Ala–Gly–Phe–Met), have been linked through an ester bond either to the 6-OH or 1-OH of various D-glycopyranose moieties were synthesized and analysed by RP-HPLC using trifluoroacetic acid as ion-pairing agent and methanol as modifier of the aqueous phase.

Depending on the synthetic conditions, two isomeric products were obtained in each instance due to the racemization at either the C-terminal amino acid residue (Met) or the penultimate residue (Phe). The diastereomers were separated by RP-HPLC (the only exceptions being glycoconjugates **6** and **8**, for which only a small amount of racemized product was produced

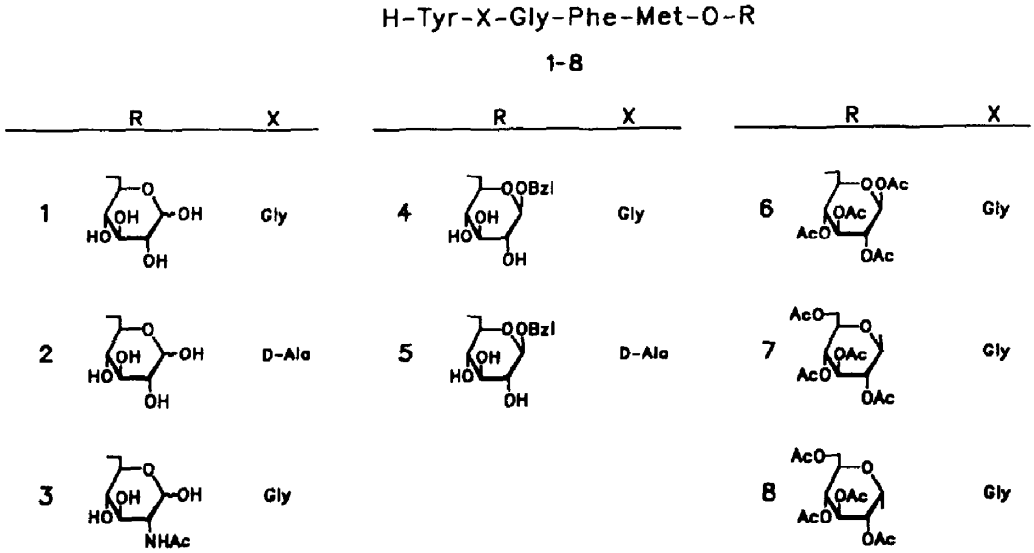

Fig. 1. Structures of methionine-enkephalin-related glycoconjugates **1–8**.

Table 1
Retention data for methionine-enkephalin (Tyr–Gly–Gly–Phe–Met) and related glycoconjugates

Compound	Sugar	Type of linkage	Position of linkage	Retention time (min)	
				L-Isomer	D-Isomer
1	Glc	Ester	6	9.45	13.39[a]
2	Glc	Ester	6	9.95	13.39[a]
3	GlcNAc	Ester	6	9.74	13.60[a]
4	GlcBzl	Ester	6	20.33	25.06[a]
5	GlcBzl	Ester	6	20.98	25.78[a]
6	$GlcAc_4$	Ester	6	20.55	
7	β-$GlcAc_4$	Ester	1	26.71	29.79[b]
8	α-$GlcAc_4$	Ester	1	27.57	
[Met^5]E				11.53	16.61[c]
[D-Ala^2, Met^5]E				11.74	

RP-HPLC conditions: linear gradient of methanol (40 to 57.5%) in 0.1% aqueous TFA over a 30-min time period; flow-rate, 0.5 ml/min; load, 50 μg per 100 μl of 0.1% TFA in methanol–water (40:60); temperature, 25°C; UV detection at 280 nm.
[a] Methionine-enkephalin-related glycoconjugate having D-Met at the fifth position of the peptide part of the molecule.
[b] Methionine-enkephalin-related glycoconjugate having D-Phe at the fourth position of the peptide part of the molecule.
[c] Tyr–Gly–Gly–Phe–D-Met.

during synthesis) and their chromatographic behaviour was examined using gradient (Table 1) and isocratic elution at different concentrations of methanol.

The results in Table 1 demonstrate that incorporation of a single sugar moiety caused either a decrease or an increase in the elution time of non-glycated parent peptides depending on the structure of the carbohydrate moiety introduced. The retention times of glycopeptides **1–3** containing unprotected sugar moieties, compared with methionine-enkephalin or its D-Ala^2-analogue, were shortened owing to the incorporation of the hydrophilic carbohydrate [1,6,7], whereas increased retention times were observed when partially (**4** and **5**) or fully protected monosaccharides (glycoconjugates **6–8**) were introduced into the enkephalin molecule. Fig. 2 illustrates the gradient elution pattern of methionine-enkephalin and of some related glycoconjugates.

Interestingly, although it might be expected that amino acid sequence would have an effect on opioid peptide retention, replacement of the Gly^2 residue in methionine-enkephalin with D-Ala did not lead to a significant increase in the hydrophobic interaction in RP-HPLC in either the peptide Tyr–D-Ala–Gly–Phe–Met or in the corresponding glycoconjugates **2** and **5** (Table 1). In contrast, inversion of the amino acid configuration at the Met^5 or Phe^4 position caused a dramatic increase in the retention times of all the compounds studied (Table 1). This is in accordance with the elution order of epimers of leucine-enkephalin-related glycoconjugates previously observed in RP-HPLC, with L–D isomers having longer retention times than the corresponding L–L isomers [1]. It could be concluded that replacement of the Met or Phe residue by the corresponding D-enantiomer results in a decreased molecular polarity and much larger hydrophobic contact area. According to the data in Table 1, under the RP-HPLC conditions employed, excellent column selectivity and resolution were obtained for all the diastereomeric pairs studied.

In order to find an explanation for the different biological activities of structurally related leucine- and methionine-enkephalin glycoconjugates [3,4], we compared changes in the retention time of the parent peptide, with gradient elution, caused by glycation with the same carbohydrate moiety. We observed (Table 2) that the introduction of the free D-glucose moiety into

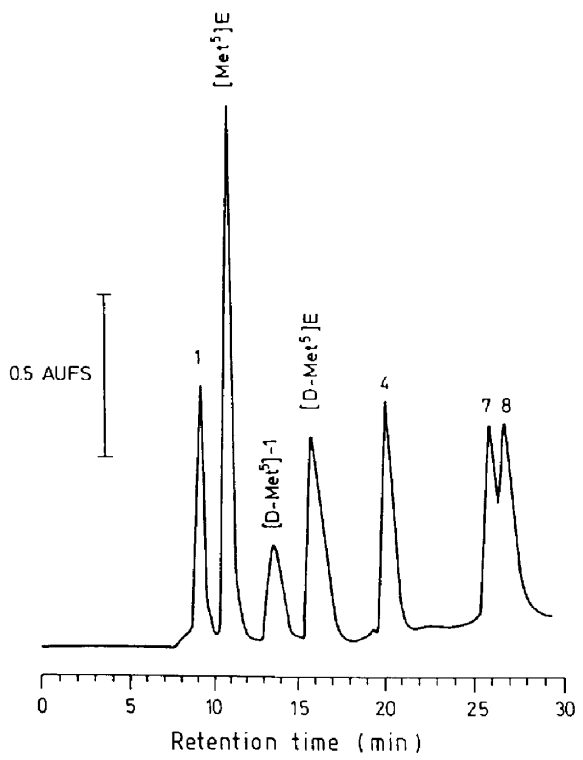

Fig. 2. Gradient elution profile of methionine-enkephalin and related glycoconjugates. Solutes: 6-O-(Tyr–Gly–Gly–Phe–Met)-D-glucopyranose (**1**); Tyr–Gly–Gly–Phe–Met ([Met^5]E); 6-O-(Tyr–Gly–Gly–Phe–D-Met)-D-glucopyranose ([D-Met^5]-**1**); Tyr–Gly–Gly–Phe–D-Met ([D-Met^5]E); benzyl 6-O-(Tyr–Gly–Gly–Phe–Met)-β-D-glucopyranoside (**4**); 2,3,4,6-tetra-O-acetyl-1-O-(Tyr–Gly–Gly–Phe–Met)-β-D-glucopyranose (**7**); 2,3,4,6-tetra-O-acetyl-1-O-(Tyr–Gly–Gly–Phe–Met)-α-D-glucopyranose (**8**). RP-HPLC conditions as in Table 1.

methionine-enkephalin (**1**) decreases the retention time less (compared with the parent opioid pentapeptide) than incorporation of the same sugar moiety into leucine-enkephalin (**9**). A similar trend was observed for their isomers in which Met or Leu residue in the peptide backbone was replaced by the corresponding D-enantiomer ([D-Met^5]-**1** and [D-Leu^5]-**9**, respectively). However, just the opposite trend was observed when methionine- and leucine-enkephalin were modified by incorporation of either partially protected (benzyl β-D-glucopyranoside) (**4** and **10**, respectively) or fully protected (1,2,3,4-tetra-O-acetyl-β-D-glucopyranose) (**6** and **11**, respectively) carbohydrates (Table 2). The sugar moieties mentioned increased the retention times of the parent unmodified enkephalins, but now the methionine-enkephalin molecule was influenced much more than leucine-enkephalin. Moreover, D-isomers of glycoconjugates **4** and **10**, having Tyr–Gly–Gly–Phe–D-Met and Tyr–Gly–Gly–Phe–D-Leu as the peptide moiety, showed the largest difference in retention times, Δt being +8.4 min for [D-Met^5]-**4** and −1.8 min for [D-Leu^5]-**10**. This confirmed the suggestion that the general hydrophobicity of unfolded peptides [8] and the contribution of the free or derivatized carbohydrates [9] on the retention time in RP-HPLC are not the only determining factors of the elution pattern of glycopeptides [10]. We assume that a chiral incorporated sugar moiety caused different conformational changes in the parent opioid peptides, influencing the overall hydrophobicity of the related glycopeptides and consequently produced also a different effect on the biological activity of the parent peptide molecule.

Fig. 3A and B illustrate the dependence of the chromatographic behaviour of **1–8** on the methanol concentration in the mobile phase obtained in a series of isocratic experiments. A remarkable change in the retention of glycopeptides **1–8** was observed when methanol concentration was changed from 40% to 50%, as can be seen from the log k'_L versus methanol concentration plots in Fig. 3A and B. Unlike partially or fully protected glycoconjugates **4–8**, for which the plots of log k'_L versus methanol concentration were almost linear (Fig. 3B), the plots for **1–3**, having an unprotected monosaccharide moiety in the molecule, were non-linear under the conditions studied (Fig. 3A). If we assume that the requirements for linearity of the log k'_L versus methanol concentration for the hyprophobic (solvophobic) retention mechanism on an ODS ligand were fulfilled [11–13] then the chromatographic behaviour of methionine-enkephalin-related glycoconjugates **1–3**, having the 1-OH position of the sugar moiety free is affected by processes other than solvophobic. It is important to mention that in these experiments the pH change was negligible (from pH 2.62 to 2.70),

Table 2
Comparison of the observed differences in the retention times of parent peptides after incorporation of the identical monosaccharide moieties into methionine- and leucine-enkephalin

Compound	Parent peptide	Carbohydrate moiety	Observed difference in retention time, Δt (min)
1	Tyr–Gly–Gly–Phe–Met	Glc	−2.08
9[a]	Tyr–Gly–Gly–Phe–Leu	Glc	−5.72
[D-Met^5]-**1**	Tyr–Gly–Gly–Phe–D-Met	Glc	−3.22
[D-Leu^5]-**9**	Tyr–Gly–Gly–Phe–D-Leu	Glc	−7.23
4	Tyr–Gly–Gly–Phe–Met	GlcBzl	+8.80
10[a]	Tyr–Gly–Gly–Phe–Leu	GlcBzl	+1.79
[D-Met^5]-**4**	Tyr–Gly–Gly–Phe–D-Met	GlcBzl	+8.45
[D-Leu^5]-**10**	Tyr–Gly–Gly–Phe–D-Leu	GlcBzl	−1.79
6	Tyr–Gly–Gly–Phe–Met	$GlcAc_4$	+9.02
11[a]	Tyr–Gly–Gly–Phe–Leu	$GlcAc_4$	+6.45

Based on the retention times data for methionine-enkephalin and related glycoconjugates presented in Table 1 and on the data for leucine-enkephalin and related glycoconjugates presented in Table 1 in Part I [1]. Difference in retention time calculated by comparison with the retention of the parent peptides.

[a] Compounds **9**–**11** are leucine-enkephalin-related glycoconjugates; **9** = 6-O-(Tyr–Gly–Gly–Phe–Leu)-D-glucopyranose [3]; **10** = benzyl 6-O-(Tyr–Gly–Gly–Phe–Leu)-β-D-glucopyranoside [3]; **11** = 1,2,3,4-tetra-O-acetyl-6-O-(Tyr–Gly–Gly–Phe–Leu)-β-D-glucopyranose [3]. For structures of compounds **9**–**11**, see Part I [1].

hence the effect of pH on the retention time of **1**–**8** can be neglected.

4. Conclusions

In this study we undertook a detailed analysis of the retention times of methionine-enkephalin-related glycoconjugates and their optical isomers as a function of the identity and position of the sugar–peptide linkage. We found that the major determinant of the elution time in RP-HPLC is the degree of the sugar moiety protection. Whereas unprotected sugars decreased the retention time, partially or fully protected monosaccharides dramatically increased the retention time of the parent peptide compound.

All pairs of diastereomers of the glycoconjugates studied can be well separated on the reversed-phase column, without using any chiral column. The retention times obtained for L-isomers were smaller than those for D-isomers, reflecting the increased hydrophobicity of the glycoconjugates in which either the phenylalanine or methionine residue was replaced by the corresponding D-enantiomer.

It has been demonstrated that glycation of the two closely related opioid pentapeptides methionine- and leucine-enkephalin, with identical monosaccharides, produced different effects on the retention times of the parent peptides.

The investigation of the dependence of the capacity factors on solvent composition suggested that the retention mechanisms of methionine-enkephalin-related glycoconjugates containing unprotected sugars in the molecule are influenced by the free hydroxyl group at C-1 of the carbohydrate moiety.

Abbreviations used for monosaccharides

Glc	D-glucopyranose
$GlcAc_4$	1,2,3,4-tetra-O-acetyl-β-D-glucopyranose
α-$GlcAc_4$	2,3,4,6-tetra-O-acetyl-α-D-glucopyranose
β-$GlcAc_4$	2,3,4,6-tetra-O-acetyl-β-D-glucopyranose
GlcBzl	benzyl β-D-glucopyranoside
GlcNAc	2-acetamido-2-deoxy-D-glucopyranose

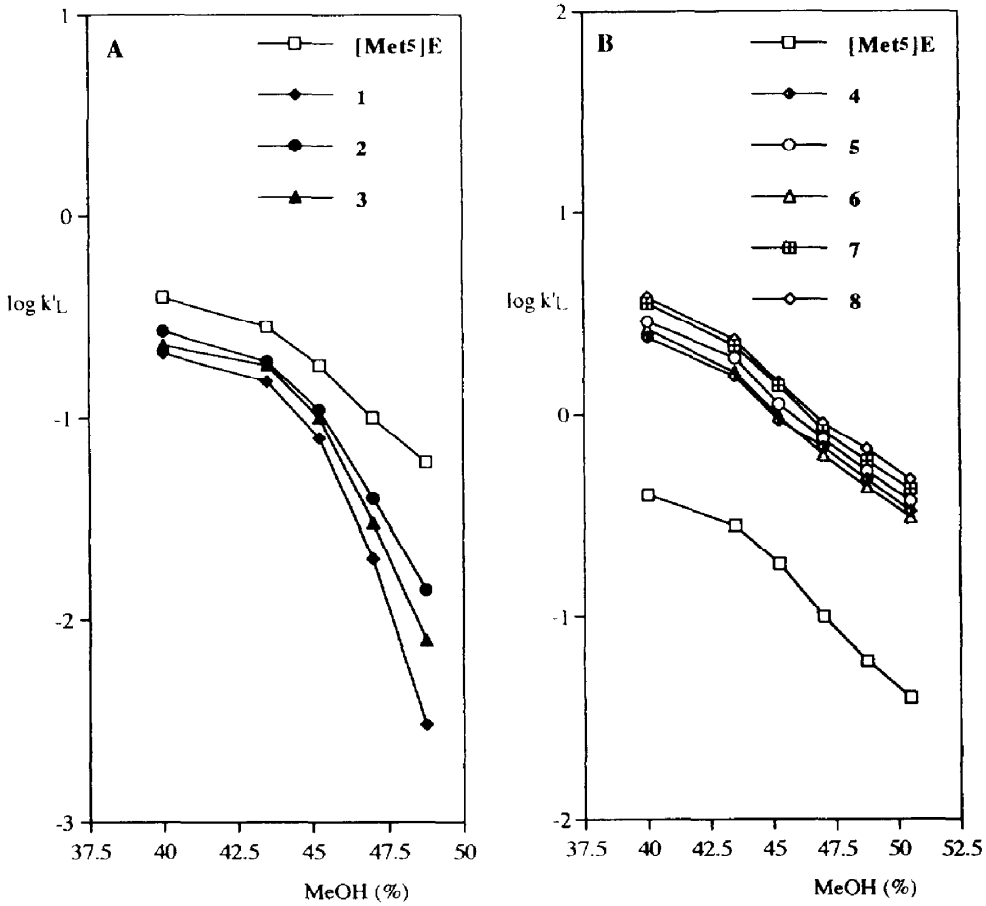

Fig. 3. Log k'_L values resulting from the chromatography of (A) glycoconjugates **1–3** and (B) glycoconjugates **4–8** with isocratic elution with concentrations of methanol in 0.1% aqueous TFA in the mobile phase of 40.00, 43.50, 45.25, 47.00, 48.75 and 50.50%. Other RP-HPLC conditions as in Table 1.

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