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

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Abstract

The chromatographic behaviour of leucine-enkephalin-related glycoconjugates with an ester-, ether- and amide-type of linkage was investigated by reversed-phase high-performance liquid chromatography using trifluoroacetic acid as ion-pairing agent and methanol as modifier of the aqueous phase. The results show that the position and the type of sugar–peptide linkage, the type of sugar moiety introduced and the degree of carbohydrate protection contribute to the overall retention of the glycopeptides studied.

1. Introduction

In recent years, reversed-phase high-performance liquid chromatography (RP-HPLC) has been widely used for the separation of naturally occurring glycoproteins [1,2] and in the final purification of synthetic glycopeptides [3–7]. The carbohydrate chain of the glycoproteins often carries a highly specific biological recognition structure and extensive studies have been performed on the chromatographic analysis of these oligosaccharide systems [8–10]. However, only a few reports have appeared [11–13] dealing with the relationships between the structure and RP-HPLC retention characteristics of natural or synthetic glycopeptides.

Since the discovery of the enkephalins [14] (Tyr–Gly–Gly–Phe–Leu/Met), many endogenous opioid peptides have been detected in mammals. This important group of peptides produce a wide range of central and peripheral effects, which, in addition to spinal and supraspinal analgesia, include tolerance and physical dependence, respiratory depression, euphoria and other behavioural effects, effects on gastrointestinal motility and cardiovascular and immune functions [15].

We have shown that the introduction of the sugar moiety at the fifth position of the enkephalins significantly influenced the biological activity profile of the parent peptide. Interestingly, both the type of linkage and the kind of sugar moiety introduced were of major consequence with regard to opioid receptor selectivity [16–20]. In addition, leucine-enkephalin (Tyr–Gly–Gly–Phe–Leu) glycoconjugates exhibited antiviral activity against HIV-1 which was significantly

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higher than the activity of the parent peptide itself [21].

In continuation of our investigations on the influence of incorporated sugar moieties on different aspects of the behaviour and activity of leucine-enkephalin, in this paper we give the correlation between the incorporated sugar moieties and the chromatographic behaviour, using RP-HPLC of leucine-enkephalin-related glycoconjugates **1–12** (Fig. 1). The study was carried out with both gradient and isocratic elution with methanol, using trifluoroacetic acid as ion-pair-

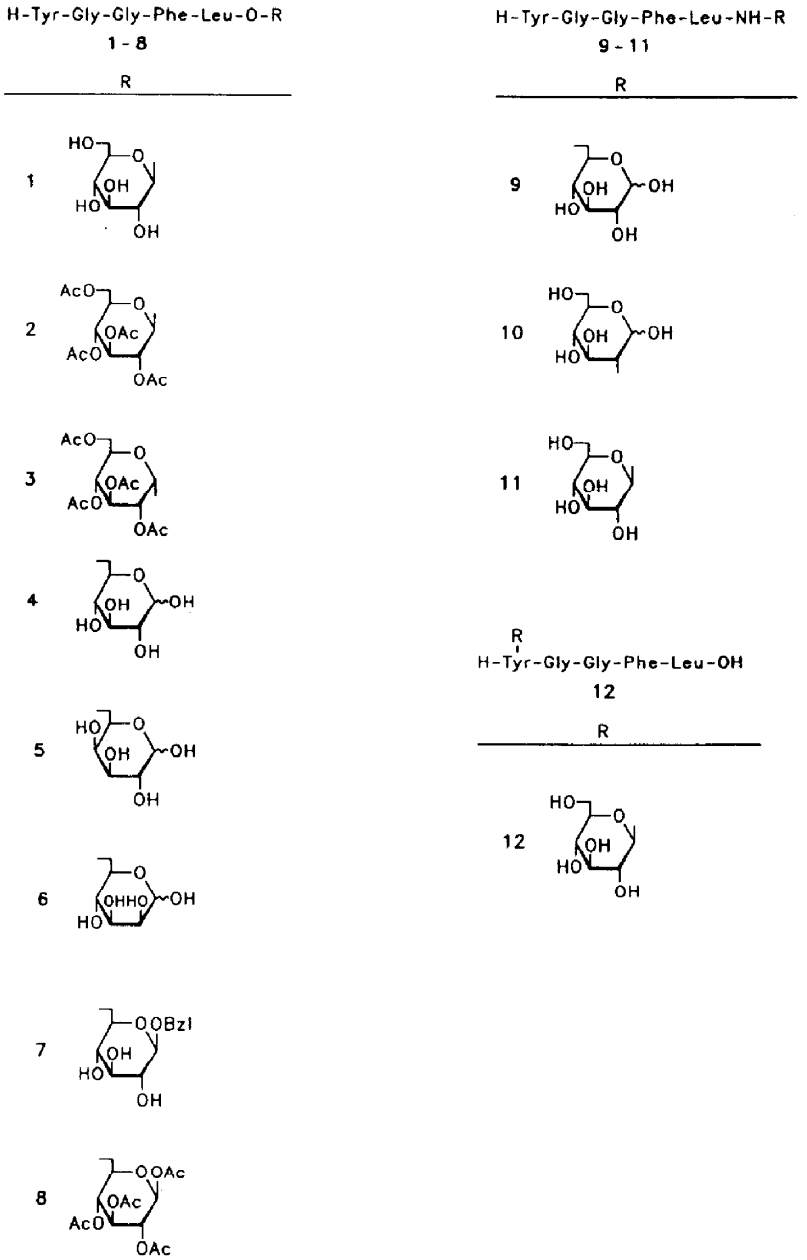

Fig. 1. Structures of leucine-enkephalin-related glycoconjugates with ester (**1–8**), amide (**9–11**) and ether (**12**) types of linkage.

ing agent. The effects of carbohydrates on the retention of the parent peptide were determined as a function of the type of sugar moiety, the degree of sugar moiety protection and the type and position of the sugar–peptide linkage.

2. Experimental

2.1. Column

An *n*-octadecyl Si 100 analytical column (250 × 4.6 mm I.D., 5 μm) (Serva) was used. The dead volume was determined to be 3.58 ml by injection of 5 μg of uracil. The column was operated at a flow-rate of 0.5 ml/min. All measurements were made at 25°C.

2.2. Instrumentation

The HPLC equipment consisted of Varian Model 9010 liquid chromatograph equipped with a Rheodyne Model 7125 injector, a Varian Model 4400 integrator and a Varian Model 9050 variable-wavelength UV–Vis detector. UV detection was performed at 280 nm.

2.3. Mobile phase

Solvent A was 0.1% trifluoroacetic acid (TFA) in methanol–water (40:60) (pH 2.50) and solvent B was 0.1% TFA in methanol–water (75:25) (pH 2.82). Samples were eluted in the linear gradient mode with methanol (100% A → 50% A + 50% B; 40.0% → 57.5% methanol and 100% A → 25% A + 75% B; 40.0% → 66.25% methanol) in 0.1% TFA over a 30-min time period, and also in the isocratic mode with different concentrations of methanol.

For analytical HPLC the samples were dissolved in solvent A at 0.5 mg/ml and 100 μl of the solution were injected.

2.4. Chemicals

Methanol was of HPLC grade (Aldrich, Milwaukee, WI, USA) and trifluoroacetic acid was of spectroscopic grade (Uvasol; Merck, Darmstadt, Germany). Leucine-enkephalin ([Leu^5]E) and leucine-enkephalinamide ([Leu^5]E-NH_2) were purchased from Sigma (St. Louis, MO, USA). Leucine-enkephalin methyl ester ([Leu^5]E-OMe) was prepared as described previously [22]. Compounds **1–12** were synthesized as described [16–20]. HPLC analysis of some glycoconjugates indicated the presence of two peaks (found to be diastereomers) which were separated by repetitive injections (100 μl, concentration of 15 mg/ml in solvent A) under the conditions given in Section 3. The structure and homogeneity of glycopeptides were confirmed by microanalysis (C, H, N), NMR spectroscopy using a Varian Gemini 300 instrument and RP-HPLC.

3. Results and discussion

The results obtained with linear gradient elution for leucine-enkephalin-related glycoconjugates with ester (**1–8**), amide (**9–11**) and ether (**12**) types of linkage, and also for some of their D-Leu^5 isomers [two isomeric products were obtained during the synthesis of **4–7**, while in others only small amounts (**1–3** and **8**) or no (**9–12**) of racemized products were detected] are shown in Table 1.

In general, incorporation of an unprotected carbohydrate, owing to the increased hydrophilicity of the molecule, decreased the retention time of the parent unmodified leucine-enkephalin. Differences in the retention times indicate that the type of unprotected sugar moiety and the type and position of the linkage affect the HPLC retention behaviour of the glycoconjugates studied. Compounds **1** and **4** in which the peptide is linked through the ester bond to either C-1 or C-6 of the identical carbohydrate moiety (D-glucose) indicate a smaller hydrophilicity of the 1-O-glycoconjugate **1**, reflected in a stronger retention, than that of the 6-O-derivative **4**. The investigation of the chromatographic behaviour of **4–6** having identical type and position of the sugar–peptide linkage but different monosaccharide moieties (D-

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

Compound	Sugar	Type of linkage	Position of linkage	Retention time (min)	
				L-Isomer	D-Isomer
1	Glc	Ester	1	11.60	
2	β-$GlcAc_4$	Ester	1	24.49	
3	α-$GlcAc_4$	Ester	1	24.49	
4	Glc	Ester	6	10.60	16.61[a]
5	Gal	Ester	6	11.53	18.19[a]
6	Man	Ester	6	10.60	16.47[a]
7	GlcBzl	Ester	6	18.11	22.05[a]
8	$GlcAc_4$	Ester	6	22.77	
9	6-NH_2-Glc	Amide	6	10.81	
10	2-NH_2-Glc	Amide	2	11.03	
11	1-NH_2-Glc	Amide	1	11.67	
12	Glc	Ether	1	11.10	
[Leu^5]E				16.32	23.84[b]
[Leu^5]E-OMe				19.12	22.70[c]
[Leu^5]E-NH_2				12.82	

RP-HPLC conditions: linear gradient of methanol in 0.1% TFA (compounds **2** and **3**, 40.0 to 66.25% methanol over a 30-min time period; all aother compounds, 40.0 to 57.5% methanol over a 30-min time period); flow-rate, 0.5 ml/min; load, 50 μg per 100 μl of solvent A; temperature, 25°C; UV detection at 280 nm.
[a] Leucine-enkephalin-related glycoconjugates containing D-Leu at the fifth position of the peptide backbone.
[b] [D-Leu^5]E.
[c] [D-Phe^4,Leu^5]E-OMe.

glucose, D-galactose, D-mannose) revealed that with gradient elution the retention remained unaffected with configurational change at C-2 (**4** and **6**). In contrast, introduction of D-galactose into the leucine-enkephalin molecule (**5**) decreased the hydrophilicity of the overall molecule, resulting in increased retention on the column. Concerning the effect of the type of C-1 sugar–peptide linkage, we found no significant difference in the retention behaviours of ester **1** and amide **11**. The ether derivative **12** showed increased hydrophilicity, probably owing to the presence of a free carboxyl group in the molecule. Comparison of the chromatographic behaviours of the C-6 glycoconjugates **4** and **9** revealed that an amide bond contributes to a slightly stronger retention on a reversed-phase column. Different chromatographic behaviour of glycoconjugates with the amide type of linkage at C-6, C-2 and C-1 positions of the sugar molecule (**9**, **10** and **11**, respectively) was also observed. Thus, the 1-NH-glycopeptide **11** binds to the column more strongly than **9** and **10**, and this is reflected in the decreasing order **11** > **10** > **9**. Amidation of the C-terminal carboxyl group of leucine-enkephalin also decreases the retention of the parent peptide compound but to a lesser extent than in **9**–**11**.

Inversion of the amino acid configuration at the Phe^4 or Leu^5 position in leucine-enkephalin in addition to the glycoconjugates studied leads to a considerably stronger retention on a reversed-phase column and the retention times of negative (L-D) isomers have consistently been found to be greater than those for positive (L-L) isomers (Table 1).

As expected, the incorporation of partially or fully protected monosaccharides into leucine-enkephalin resulted in increased hydrophobicity of the overall molecule and thus in stronger retention on the column (Table 1). Among the fully acetylated glycopeptides studied (**2**, **3** and **8**) we observed the same elution order as with the corresponding unprotected glycopeptides **1**

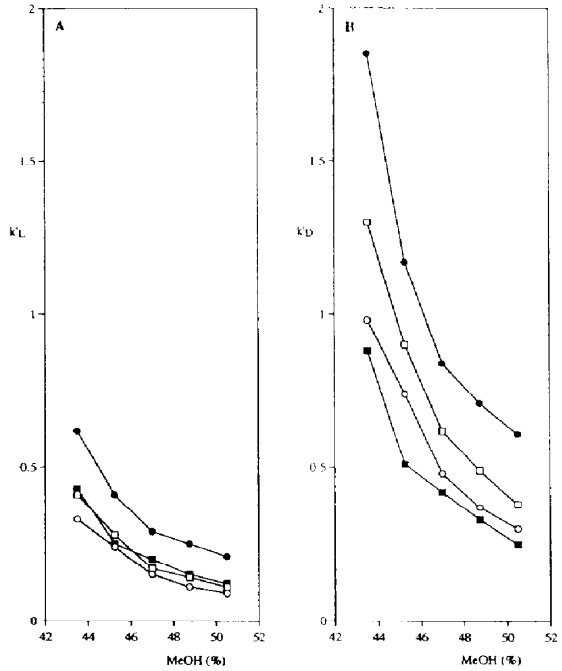

Fig. 2. Effect of methanol concentration on (A) capacity factors (k'_L) of glycoconjugates **4–6** and (B) capacity factors (k'_D) of D-Leu^5-isomers of compounds **4–6**. RP-HPLC conditions: isocratic elution at different concentrations of methanol in 0.1% trifluoroacetic acid; flow-rate, 0.5 ml/min; load, 50 μg per 100 μl of solvent A; temperature, 25°C; UV detection at 280 nm. ○ = **4**; □ = **5**; ■ = **6**; ● = [Leu^5]E.

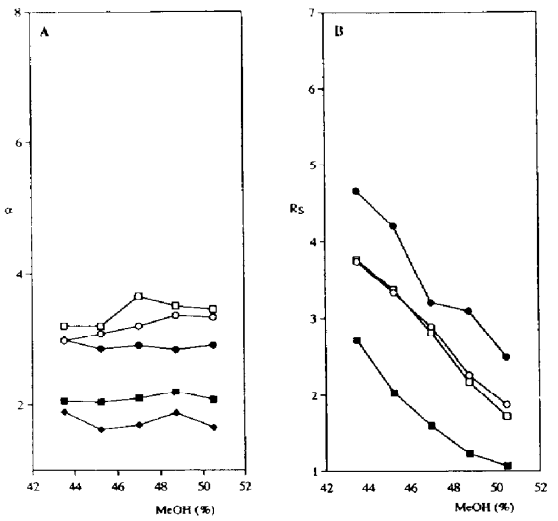

Fig. 3. Effect of methanol concentration on (A) separation factor (α) and (B) resolution (R_s) of some epimeric leucine-enkephalin-glycoconjugates. RP-HPLC conditions as in Fig. 2. ○ = **4**; □ = **5**; ■ = **6**; ◆ = **7**; ● = [Leu^5]E.

and **4**. Accordingly, 1-O-conjugates **2** and **3** were retained considerably more strongly on the column than the 6-O-derivative **8**. Concerning the influence of the anomeric form on retention, under the gradient conditions employed there was no difference in the chromatographic behaviour of **2** (β-anomer) and **3** (α-anomer). The same observation was made with isocratic elution (50.5% methanol in 0.1% trifluoroacetic acid). However, under the same chromatographic conditions, the epimer of **2**, containing at the C-terminal peptide backbone D-Leu residue, was retained more strongly than the corresponding epimer of **3**.

We investigated the chromatographic behaviour of **1–12** at different methanol concentrations. A remarkable change in the retention was observed when the methanol concentration was changed from 40% to 50%. With increasing percentage of methanol in the mobile phase, the retention of all the compounds studied decreased, following the same elution pattern as under gradient conditions, except for **4–6**. As can be seen from the k'_L versus methanol concentration plot in Fig. 2A, the retention decreased in the order (except at 45.25% methanol) *manno* (**6**) >*galacto* (**5**) > *gluco* (**4**). Interestingly, the elution orders were the same under gradient and isocratic conditions for the corresponding isomers of conjugates **4–6** having D-leucine in the peptide part of the molecule (Fig. 2B).

The effect of different methanol concentrations in the mobile phase on the separation factor α of epimeric glycoconjugates and also the parent epimeric peptides is presented in Fig. 3A. As shown, for all the compounds examined the composition of the mobile phase has no or little influence on the α values.

Resolutions (R_s) of the studied diastereomers of leucine-enkephalin-related glycoconjugates were inferior to those of epimeric leucine-enkephalin (Fig. 3B).

In conclusion, the investigation of the chromatographic behaviour of leucine-enkephalin-related glycopeptides **1–12** demonstrated that the

position and type of sugar–peptide linkage, the structure of the sugar moiety and the degree of the sugar moiety protection, influence their retention on a reversed-phase column. Further, the feasibility of RP-HPLC for separating epimeric glycopeptides has been demonstrated.

Abbreviations used for monosaccharides

Gal	D-galactopyranose
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
Man	D-mannopyranose
1-NH_2-Glc	β-D-glucopyranosylamine
2-NH_2-Glc	2-amino-2-deoxy-D-glucopyranose
6-NH_2-Glc	6-amino-6-deoxy-D-glucopyranose

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