# Influence of different N- and O-linked carbohydrates on the retention times of synthetic peptides in reversed-phase high-performance liquid chromatography

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## ABSTRACT

Glycopeptides consisting of 6–19 amino acid residues and different mono- and disaccharides attached to single asparagine and serine residues were synthesized on solid-phase and were characterized by reversed-phase high-performance liquid chromatography and circular dichroism. It was shown that the decreased retention times due to glycosylation could be correlated with the increasing length of the sugar moiety. Phosphorylation of the same sequences reduced the retention times 1.6 times more than glycosylation with monosaccharides did. The binding to the column was dependent on the structure of the disaccharide when derivatized and glycosylated asparagine, the building block of N-glycopeptide syntheses was studied. However, this structural dependence of the elution times disappeared in the final glycopeptides. Although both glycosylation and phosphorylation resulted in altered secondary structure of the peptide backbone, it appears that the retention times reflect the increased hydrophilicity more strongly than induced conformational orientation on the surface of the bonded phase.

## INTRODUCTION

Recently the chemical synthesis of post-translationally modified peptides has become the center of interest of the biotechnology sector. Although non-modified protein fragments are readily available through procedures of gene technology, the biological function of the proteins can only be fully understood if the characteristic peptide fragments are properly glycosylated and phosphorylated. Most viral proteins are N-glycosylated, for example gp 120 of HIV-1 is extremely heavily glycosylated [1], and the full pathogenic potential of HIV-1 *in vitro* is manifested only if its viral envelope proteins are N-glycosylated [2]. Serine- and threonine-bound carbohydrate systems are found in mucins and in related proteins, and are considered to be tumor-associated antigens [3].

We and others have developed several novel solid-phase synthetic strategies for glycopeptides and phosphopeptides [4–9]. The purification of these post-translationally modified peptides, similarly to the parent, non-modified analogues, is accomplished almost exclusively by reversed-phase high-performance liquid chromatography (RP-HPLC). We regularly use RP-HPLC to quantify the coupling efficiencies, the crucial step for solid-phase glycopeptide synthesis [10]. RP-HPLC separation of glycosylated and non-glycosylated forms of isolated N- and O-glycopeptides has been reported [11–13], and the influence of N-glycosylation on the retention times of peptides has been estimated based on isolated rat neurointermediary lobe peptides [14], but systematic studies on the contribution of different sugars to the RP-HPLC retention times of synthetic peptides have not been reported yet. Furthermore, in this paper we compare the influence of glycosylation and phosphorylation of the same serine residue, and serine-phosphorylation and asparagine-glycosylation of the same peptide backbone to refine the predictive algorithms. The observed differences in retention times are examined in context with the detected conformational changes upon glycosylation to gain insights into a

possible conformational orientation on the surface of the bonded phase. Finally, we show why caution should be exercised when conclusions from retention time differences, based on RP-HPLC of derivatized sugars [15], are drawn on the contribution of the same sugars when incorporated into final peptides.

EXPERIMENTAL

*Peptide synthesis*

$N^{\alpha}$-9-Fluorenylmethoxycarbonyl-asparagine $N^{\beta}$-glycosides [Fmoc-Asn(sugar)derivatives] were prepared as described earlier [16]. Asparagine-linked glycopeptides were synthesized on solid phase using a BioSearch SAM2 automated synthesizer. Carbohydrates were incorporated by the building block strategy with different Fmoc-Asn(sugar)-O-pentafluorophenyl derivatives [5]. Unprotected serine residues were introduced for O-glycopeptide synthesis and glycosylated with peracetylated glucose oxazoline after the peptide chain assembly was completed [6]. For phosphopeptide synthesis, the same resin was phosphorylated with dibenzyl phosphochloridate [8]. Peptides were cleaved off the resin with trifluoroacetic acid–anisole/thioanisole (95:5, v/v). The O-glycopeptides were finally deacetylated with 0.1 $M$ NaOH. Phosphorylation of VF13 and the varicella peptide (for the sequences see Table II) was made with polyphosphoric acid after the peptides were cleaved from the solid support. Glycopeptides and phosphopeptides were analyzed by fast-atom bombardment mass spectrometry, two-dimensional NMR and phosphate analysis. Amino acid analyses were performed in The Wistar Institute Protein Microchemistry Facility.

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[a] Abbreviations for the amino acid residues are: A (Ala) = L-alanine; R (Arg) = L-arginine; N (Asn) = L-asparagine; D (Asp) = L-aspartic acid; C (Cys) = L-cysteine; Q (Gln) = L-glutamine; E (Glu) = L-glutamic acid; G (Gly) = L-glycine; I (Ile) = L-isoleucine; L (Leu) = L-leucine; K (Lys) = L-lysine; M (Met) = L-methionine; F (Phe) = L-phenylalanine; P (Pro) = L-proline; S (Ser) = L-serine; T (Thr) = L-threonine; Y (Tyr) = L-tyrosine; V (Val) = L-valine. Abbreviations for the monosaccharides are: Gal = D-galactopyranose; Glc = D-glucopyranose; GalNAc = 2-acetamido-2-deoxy-D-galactopyranose; GlcNAc = 2-acetamido-2-deoxy-D-glucopyranose; Man = D-mannopyranose.

*Chromatography*

Chromatographic system A consisted of two Beckman 110A pumps, regulated by a 421A controller, an Altex Ultrasphere ODS 250 × 10 mm I.D. column, a Beckman 160 fixed-wavelength detector operating at 214 nm, 0.1 a.u.f.s., and a Shimadzu C-R6A integrator. Solvent A was 0.1% aqueous trifluoroacetic acid; solvent B was 0.1% trifluoroacetic acid in acetonitrile. The samples were loaded in 5% solvent B. The flow-rate was 3 ml/min. A linear gradient of 1.33%/min of solvent B was used for screening the N-glycopeptides (gradient a), and 0.67%/min for O-glycopeptide and detailed N-glycopeptide analysis (gradient b). Gradient b with Beckman 114 M pumps was used to analyze the Fmoc-Asn(sugar) derivatives (chromatographic system B). The loads were 10–100 $\mu$g.

*Circular dichroism (CD)*

CD spectra were taken on a Jasco J720 spectrograph. Spectograde trifuoroethanol and water were used as solvents. All measurements were made in 0.02 cm cells. The peptide concentrations were 0.4 mg/ml throughout.

RESULTS

*Separation of Fmoc-Asn(sugar)-O-tert.-butyl derivatives*

Glycosylated N-fluorenylmethoxycarbonyl-asparagine *tert.*-butyl esters are the starting materials for solid-phase N-glycopeptide synthesis. Since the reaction route to these derivatives proceeds through unpurified intermediate products, their final purification is necessary before cleavage and activation occurs. Although the major factors in binding of these derivatives to RP-HPLC columns are the hydrophobic fluorenylmethyl and *tert.*-butyl groups, structural information of the exposed regions of the sugars can still be obtained through their binding. Table I lists the retention times of a series of Fmoc-Asn(sugar)-O-*tert.*-butyl derivatives. Disaccharide derivatives bind to the column less than monosaccharide derivatives do, as expected. Asparagine with sugars containing 2-acetamido groups bind to the column stronger than sugars without acetamido groups, because of the methyl group. Nevertheless, the acetamido group is still hydrophilic, as the strongly increased retention time of the 2-deoxy de-

TABLE I

REVERSED-PHASE SEPARATION OF Fmoc-Asn (SUGAR)-O-*tert.*-BUTYL DERIVATIVES

| Carbohydrate | Retention time (min) |
|---|---|
| Galβ(1→3)GlcNAc | 62.59 |
| Glcβ(1→4)Glc (cellobiose) | 62.59 |
| Glcα(1→4)Glc (maltose) | 62.59 |
| GlcNAcβ(1→4)GlcNAc (chitobiose) | 63.14 |
| Glc | 65.08 |
| Gal | 65.08 |
| GlcNAc | 65.72 |
| GalNAc | 65.72 |
| Man | 65.72 |
| 2-Deoxy-Glc | 67.83 |

rivative suggests. While the configuration of the substitutents at C-4 does not affect the observed retention times, the substitutents at C-2 do. The most interesting feature of Table I is the separation of disaccharides conjugated to the asparagine residue. While Galβ(1→3)GlcNAc, Glcβ(1→4)Glc (cellobiose) and Glcβ(1→4)Glc (maltose) derivatives can-
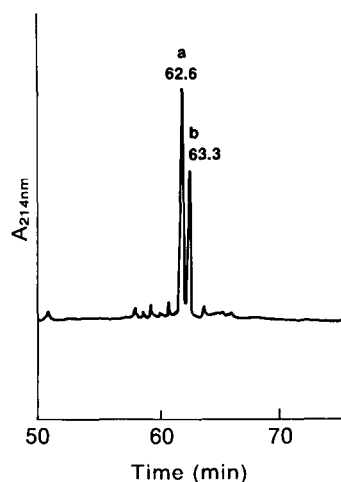


Fig. 1. Co-injection of Fmoc-Asn(maltose)-O-*tert.*-butyl, Fmoc-Asn[Galβ(1→3)GlcNAc]-O-*tert.*-butyl and Fmoc-Asn(chitobiose)-O-*tert.*-butyl. Chromatographic conditions as described under Experimental. Peaks: a = the disaccharide derivatives with glucose or galactose, as the outer sugar moiety; b = the chitobiose derivative with the outer sugar containing 2-acetamido group (GlcNAc). The inner sugar does not seem to play a role in the binding to the column.

not be separated, the GlcNAcα(1→4)GlcNAc (chitobiose) derivative exhibits increased retention time, suggesting that the "outer" sugar plays a role only in binding to the surface of the column. Since monosaccharides still bind, it appears that (regardless of the sterochemistry of the glycosidic bond) the outer sugar prevents contact of the inner sugar with the bonded phase, most probably through folding. The maltose, Galβ(1→3)GlcNAc and chitobiose derivatives were injected together in Fig. 1. Peak a represents the two disaccharide derivatives with Glc or Gal as the outer sugar, peak b represents the disaccharide derivative with GlcNAc as the outer sugar. It should be noted that the Fmoc-Asn(Man) and Fmoc-Asn(Glc) derivatives (differing in the position of the hydroxyl group at C-2) could not be separated once the C-terminal *tert.*-butyl group was removed.

*The influence of N-glycosylation on the retention times of synthetic peptides*

One set of our model peptides is excised from glycoprotein sequences of rabies, heptatis B, and varicella viruses, and contains the natural Asn-Xxx-Ser/Thr consensus glycosylation pattern (Table II). The presence or absence of carbohydrate on the asparagine residues was suggested to be involved in antigenic activity [17,18]. We employed mainly GlcNAc as the sugar moiety, since this carbohydrate is attached to the asparagine in all natural N-glycosylated systems, and the conformational transition due to glycosylation was detected immediately after incorporation of the first moiety [19]. Unnatural glycosylated analogues of the principal neutralizing determinant (PND) of HIV gp 120 were made to break tolerance in the hypervariable region of the V3 loop through slight changes in the conformation caused by the incorporation of the various sugar moieties [20]. As Table II shows, incorporation of monosaccharides reduced the retention times of the parent, non-modified peptides. Phosphorylation of VF13 reduced the retention 1.6 times more than glycosylation with GlcNAc did. The extent of the reduction is heavily sequence dependent and apparently less effective and selective at the termini than in mid-chain positions. As the data of peptides GM12 show, incoporation of disaccharides repeatedly reduced the retention times further than incorporation of monosaccharides did.

TABLE II

THE DECREASE OF THE RETENTION TIME OF SYNTHETIC PEPTIDES ON RP-HPLC DUE TO N-GLYCOSYLATION AND COMPARISON WITH O-PHOSPHORYLATION OF THE SAME SEQUENCES

Conformation of parent and modified peptides as determined by circular dichrosim. Chromatographic system A, gradient a was used in all these experiments. Glycosylated asparagine residues are marked with an asterisk; phosphorylated serines are italicized.

| Peptide | Sequence | Side-chain modification | Decrease of retention time compared to unmodified analogue (min) | Proposed conformation |
|---|---|---|---|---|
| GM12$^a$N | GKAYTIFN*KTLM | — | — | α-Helical |
| GM12G | | GlcNAc | 1.0 | Type Iβ-turn |
| GM12C | | Chitobiose | 1.4 | Stronger type Iβ-turn |
| GM12B | | Cellobiose | 1.4 | Type I β-turn |
| VF13$^a$N | VVEDEGCTN*LSGF | — | — | α-Helical |
| VF12G | | GlcNAc on Asn | 0.8 | Type I β-turn |
| VF13P | | Phosphate on Ser | 1.3 | Distorted |
| Hep71$^b$N | TKPSDGN*CTCIPIPS | — | — | Type II β-turn |
| Hep71G | | GlcNAc | 0.5 | Stronger turn |
| VgpIV$^c$N | VFIGQELPTGTN*YS | — | — | Type II β-turn |
| VgpIVG | | GlcNAc on Asn | 0.2 | No change |
| VgpIVP | | Phosphate on Ser | 0.2 | No change |
| PND$^d$N | N*GPGRAFY | — | — | Type II β-turn |
| PNDGlc | | Glc | 0.7 | Stronger turn |
| PNDGlcNAc | | GlcNAc | 0.7 | Stronger turn |
| PNDGal | | Gal | 0.7 | Stronger turn |
| PNDGalNAc | | GalNAc | 0.7 | Stronger turn |
| PNDC | | Chitobiose | 0.8 | Stronger turn |
| PNDB | | Cellobiose | 0.8 | Stronger turn |

$^a$ Corresponding to rabies virus G (glyco-)protein.
$^b$ Corresponding to hepatitis B virus surface antigen protein.
$^c$ Excised from varicella virus gp IV (glyco-)protein.
$^d$ GPGRAFY corresponds to the principal neutralizing determinant of the V3 loop of gp 120 of human immunodeficiency virus (HIV) 1.

In contrast to the glycosylated asparagine residues, however, the selectivity based on the identity of the outer sugar disappeared. Fig. 2 represents a co-injection of equal amounts of GM12C and GM12B where only a single peak could be detected, verifying the identical retention times for these two disaccharide-containing glycopeptides. Co-injection of PND glycopeptides that contained only monosaccharides (GlcNAc and Glc) resulted in a single peak, but co-injection of a PND with both monosaccharide and disaccharide (GlcNAc and chitobiose) resulted in two closely eluting peaks (data not shown).

*Reversed-phase characterization of O-glycopeptide sequences*

Abnormal phosphorylation of peptide tau7 (corresponds to amino acid residues 189–207 of human τ protein) may play a role in the deposition process of Alzheimer's disease [21]. To test this possibility
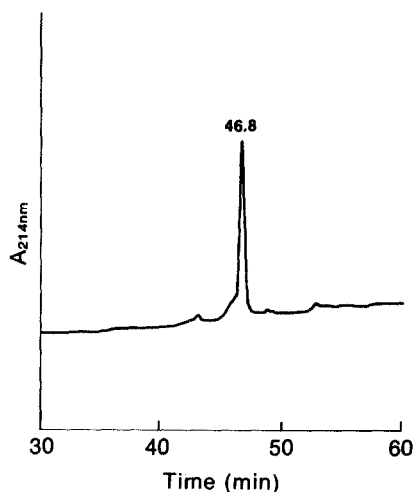


Fig. 2. Co-injection of rabies peptide GM12 glycosylated on its asparagine residue with two disaccharides, chitobiose and cellobiose. Chromatographic system A with gradient b was used. The conditions are found in Experimental. No separation of the two glycopeptides is observed.

TABLE III

COMPARATIVE CHROMATOGRAPHIC AND CONFORMATIONAL DATA ON O-GLYCOSYLATED AND PHOSPHO-RYLATED PEPTIDES

Chromatographic system A gradient b was used in all these experiments. Glycosylated and phosphorylated serine residues are marked with an asterisk. Ac = Acetyl.

| Peptide | Sequence | Side-chain modification | Decrease of retention time compared to unmodified analogue (min) | Proposed conformation |
|---|---|---|---|---|
| Tau7[a] | PKSGDRSGYSSPGS*PGTPG | – | – | Type II $\beta$-turns |
| Tau7P | | Phosphate | 1.44 | Mostly random |
| Actau7 | Ac-PKSGDRSGYSSPGS*PGTPG | – | –[b] | |
| Actau7G | | GlcNAc | 0.97 | Mixture of $\beta$-turns |
| Hexa[c] | Ac-GS*PVEK | – | – | Mostly random |
| HexaP | | Phosphate | 4.48 | Distorted |
| HexaG | | GlcNAc | 2.71 | Turn-like |

[a] Peptide sequence is excised from human $\tau$ protein.
[b] Acetylation was necessary for the successful synthesis of O-glycopeptides but not for phosphopeptides. Phosphorylated and acetylat-ed peptide has not been prepared. In fact, acetylation increased the retention time of the non-acetylated, unmodified peptide by 1.9 min; the retention time of the acetylated and side-chain unmodified peptide served as the starting point for the HPLC study of the glycosylated and acetylated peptide.
[c] Synthetic and chromatographic test sequence.

and to test the specificity of the post-translational modification on antibody recognition, we synthe-sized tau7, as well as tau7 containing either a phos-phate or GlcNAc, on serine 14 (for the sequence see
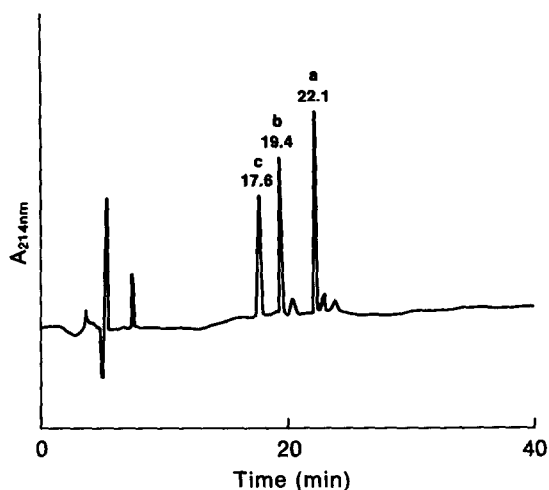


Fig. 3. Co-injection of the unmodified test hexapeptide (peak a), and its glycosylated (GlcNAc, peak b) and phosphorylated (peak c) analogues. Chromatographic conditions as described under Experimental. Phosphorylation reduced the retention time fur-ther than glycosylation of the same serine residue did.

Table III). We also prepared a test hexapeptide which was either O-glycosylated or phosphorylated to further verify the synthetic and chromatographic results [6]. Because of the hydrophilic nature of the peptide sequences, a slower gradient was used in Table III than in the study of N-glycopeptides. Nevertheless, O-glycosylation and N-glycosylation reduced the retention times similarly. It is striking that the ratio of the decrease of the retention times due to phosphorylation compared to glycosylation is consistently 1.6 for both peptide series. Fig. 3 rep-resents a coinjection of the unmodified test hexa-peptide and its glycosylated and phosphorylated analogues. All these components are well separated, as expected from the retention time changes deter-mined in Table III.

DISCUSSION

Prediction of retention times of peptides on RP-HPLC is of great value in the process of isolating and identifying protein fragments [14,22]. Although the use of synthetic peptides offers a much wider database, especially when positional environmental domains alter the retention coefficients [23], the in-fluence of post-translational modifications was

thought to be based on only a few isolated N-glyco-peptides and O-phosphopeptides [14]. The quantitative effect of O-glycosylation had not been examined earlier.

In the present study we undertook a detailed analysis of the RP-HPLC retention times of glycopeptides as a function of the identity, number, and location of the sugar moieties. We found that the major determinant in the elution time of N-glycopeptides (corresponding to mainly natural glycosylation sites) on RP-HPLC is the number of sugar moieties incorporated, which is in agreement with earlier reports. Increasing levels of artificial glycosylation of lysine residues reduces the retention times on RP-HPLC and the number of sugars is the main determinant in the elution time [24]. On asparagine, however, the reduction of retention time is not a linear function of the number of carbohydrate moieties. Incorporation of the first sugar reduced the retention time much more than a subsequently added sugar moiety. Glycosylation of the asparagine residue of a pentapeptide IMMNG (our test sequence for studying the coupling efficiency [10]) with a trisaccharide (chitotriose) almost did not decrease the elution time further than glycosylation with a disaccharide (chitobiose) did (data not shown).

Separation of sugars alone on RP-HPLC has been accomplished [25], and the contribution of derivatized carbohydrates on the retention time in RP-HPLC has been estimated [15]. However, as our findings with the separation of sugar-conjugated asparagine derivatives and final glycopeptides suggest, conclusions drawn merely on data concerning the carbohydrate moieties should be handled with caution. Asparagine coupled to disaccharides differing in the outer sugar could be separated, but this difference in the retention times disappeared when the same glycosylated asparagine residues were incorporated into a T cell epitopic peptide of rabies virus. This fact suggests that the general hydrophobicity of unfolded peptides [26] is not the only determining factor of the elution pattern of glycopeptides. Divergencies occurred in the prediction of peptide retention times due to conformation dependent changes in hydrophobic contact area occupancy at the stationary phase surface [27,28]. Peptides with a tendency to assume both $\beta$-pleated sheets [29] and $\alpha$-helices [30] were reported to

change their conformation during reversed-phase separation. Later, these conformational change were investigasted by CD studies in polar non-polar solvent systems. In an earlier report, we discussed the possibility that the delay in the elution of a synthetic phosphopeptide isomer was due to a distinctive $\beta$-turn formation [31]. A type III $\beta$-turn, generally having a class C CD, can be regarded as a single turn of a $3_{10}$-helix [32], and a series of these turns can be considered periodic structures. Conformational changes are invaluable tools for RP-HPLC screening of potential T cell epitopes [30]. Our T cell epitopes, GM12 and VF13, exhibit unordered CD spectra in water, but in trifuoroethanol assume typical $\alpha$-helical structure. Glycosylation results in a two-step conformational change. Incorporation of monosaccharides as a first step breaks the $\alpha$-helix [19], and the decreased retention times of the glycopeptides is in agreement with this process. The incorporation of the second sugar into GM12 (besides increasing the overall hydrophilicity), however, as a second step stabilizes the newly formed $\beta$-turn only slightly [19], and the retention time should increase instead of decreasing further, as has been recorded. Apparently, the increase of the hydrophilicity and the increase of the ordered (turn) structure affect the retention times in opposite directions, and the final result favors the hydrophilicity. This idea is further supported by the data on the hepatitis and PND sequences, where although stronger $\beta$-turns were formed after glycosylation, the retention times of the glycopeptides are still decreased.

Glycosylation of serine residues decreases the retention times less than phosphorylation of the same residues does, and this can be explained by both mechanisms. Phosphorylation generally results in a distorted structure [19,33], which can be regarded as a long-range intramolecular turn [33], but not as a periodic structure [19]. On the other hand, the phosphate group is very hydrophilic; it is in anionic form even at pH 2, where the chromatographic runs are made [33]. The definite reduction of retention times due to phosphorylation compared to both types of glycosylation, however, contradicts an earlier algorithm [14] that predicts a much greater effect of glycosylation. Based on our results, it appears that such an algorithm needs major revision or refinement.

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