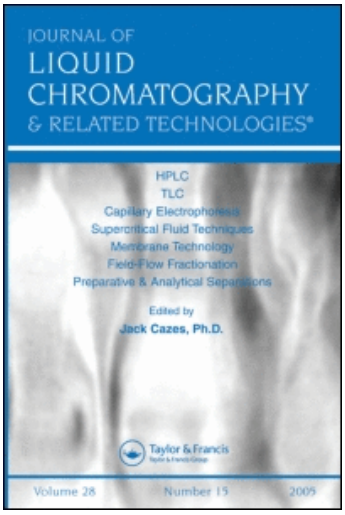


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# REVERSE PHASE ION PAIR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF VIRAL TRYPTIC GLYCOPEPTIDES

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## ABSTRACT

Tryptic glycopeptides from influenza A/WSN ($H_0N_1$) and mink cell focus (MCF)-inducing (MCF-247) murine leukemia virus were subjected to high performance liquid chromatography (hplc) for mapping purposes. Hydrophilic ion-pairing was accomplished using 0.1% phosphoric acid on $C_{18}$ μBondapack and LiChrosorb RP-18 ODS commercially available columns. The samples were eluted from the column with acetonitrile with sample recovery in the range of 70 to 80%. The advantages of hplc over the conventional techniques previously used are detailed.

## INTRODUCTION

Recent studies on the importance of glycoproteins for cellular interactions has necessitated the development of analytical techniques for the molecular characterization of peptides and glycopeptides available in minute quantities. When high performance liquid chromatography was introduced as an analytical tool it was expected that this technique would allow rapid, discrete

separation of peptides and proteins. Unfortunately early analyses of underivatized peptides were not completely successful due to poor resolution, lack of reproducibility, peak broadening, long retention times and poor yields. However, the introduction of small hydrophilic ion pairing agents into the mobile phase offered a solution to these problems and made it possible to analyze and purify a wide variety of peptides.

Ion pair partition chromatography has been used effectively with a variety of compounds (1-5). Hydrophobic ion pairing agents such as heptane sulfonic acid and tetrabutyl ammonium phosphate have been used to increase the retention times of molecules. Hydrophilic ion pairing agents such as phosphoric acid (1-3), acetic acid (1), formic acid (1), picrates (6), trialkyl-ammonium phosphate (7), and ammonium acetate (10) have been used with good success for complex peptide separations. The use of hydrophilic pairing agents has greatly enhanced the scope of ion-pair reverse phase liquid chromatography. On reverse phase supports the hydrophilic modifiers increase polarity, thereby reducing the retention time of the sample components (1,11). Considerable flexibility is afforded to the chromatographer in that retention times of sample components may be manipulated allowing the resolution of complex mixtures with increased yields in the case of peptides. In addition combinations of hydrophobic and hydrophilic ion pairing agents may be used simultaneously to further resolve difficult sample mixtures.

Over the past few years this laboratory (1,3,10,12,15,21) and others (2,4,5,11,13,14) have developed techniques utilizing hplc for the rapid, discrete separation of peptide mixtures that are available only in minute quantities. Resolution was sufficient to use reverse phase hydrophilic ion pairing for peptide mapping of several biologically active proteins. Concurrent with

these efforts, this laboratory was carrying out peptide and glycopeptide mapping studies of viral proteins and glycoproteins using classical gel permeation and ion exchange techniques. These studies were time consuming and resolution of complex tryptic glycopeptide mixtures was often incomplete. We have now found that tryptic glycopeptides may be chromatographed using reverse phase ion pair partition chromatography with a hydrophilic pairing agent, phosphoric acid. The usefulness of this method for the separation of tryptic glycopeptides is demonstrated in this report.



## MATERIALS AND METHODS

### Chemicals and Apparatus

All work was carried out on a Waters high performance liquid chromatographic system that included two M6000 solvent delivery units, a M660 solvent programmer and a U6K injector or on a Laboratory Data Control System including Constametric IIG and III solvent delivery units and a gradient master. All samples were monitored by radioactivity with a LKB 1210 Ultrobeta Liquid Scintillation counter. All solvents were filtered using either a Millipore FA or HA 40μ solvent filters. Phosphoric acid was purchased from Fisher Chemicals and acetonitrile was purchased from Burdick and Jackson. Water was passed through a Millipore reverse osmosis Super-Q deionization System using a 0.22μ filter to approximately 13 megaohm and passed over activated charcoal cartridges prior to use on the high pressure system. The $C_{18}$ μBondapak (Waters Associates) and LiChrosorb RP-18 (E. M. Laboratories) columns that were used in this procedure were purchased commercially. TPCK-trypsin was purchased from Millipore Corporation.

## Chromatographic Procedures

All chromatograms were developed at room temperature. Peptides and glycopeptides were dissolved in the eluting solvent before injection and centrifuged in a Beckman Eppendorff centrifuge to remove any insoluble sample components. The columns were allowed to equilibrate in the appropriate mobile phase for about 120 mls prior to chromatography. The A solvent in each study was 0.1% $H_3PO_4$, pH 2.85, and the organic solvent was 60% acetonitrile ($CH_3CN$) in the A buffer.

## Sample Preparation



Influenza A/WSN ($H_0N_1$) virions were grown in MDBK cells, labeled with $^3$H-glucosamine, and purified as previously described (16). Virion polypeptides were solublized under reducing conditions and separated by SDS-polyacrylamide gel electrophoresis on a 10% slab gel. Following electrophoresis the slab gel was fixed and the fluorographic procedure of Bonner and Laskey (17) was applied to each gel. Fluorography on dried gels was carried out with Kodak XR-2 X-ray film at -70°. Gel segments containing viral glycoproteins were excised and rehydrated in a solution of 0.1 M ammonium bicarbonate containing 50 µg/ml of TPCK-trypsin (Millipore Corp.). The gel slices were incubated for 24 hr at 37° and the treatment was repeated at least three times. The enzymatic digests were pooled and lyophilized. The lyophilized material was rehydrated in water and three to five drops of concentrated formic acid was added to each digest to liberate residual ammonium bicarbonate. The $^3$H-glucosamine labeled tryptic glycopeptides were then filtered through a 0.2µm Millex filter (Millipore Corp.) and relyophilized. The dried samples were then stored until analyzed.

The envelope precursors of mink cell focus (MCF) - inducing (MCF-247) murine leukemia virus (MuLV) were kindly provided by Dr. Nancy Famulari (Sloan-Kettering Cancer Center, New York).

These glycoproteins were labeled with $^{3}$H-mannose in SC-1 or mink lung cells as previously described (18,19). The glycoproteins were immunoprecipitated from cell lysates and separated by SDS-polyacrylamide gel electrophoresis on 7.5% slab gels using methods previously described (18). The slab gel, following electrophoresis, was treated as decribed above and the tryptic glycopeptides of SC-1/MCF-247 and mink/MCF-247 $Pr^{env}$ were prepared in the same manner described for the influenza viral glycoproteins.

## RESULTS AND DISCUSSION



Influenza A hemagglutinin (HA) glycoproteins are located on the surface of the virions. HA glycoproteins may be present in virions as uncleaved HA or as two cleavage products, $HA_1$ and $HA_2$, which are linked by disulfide bonds. Previous studies showed that two major types of oligosaccharides are present in HA: complex (type I) oligosaccharides containing glucosamine, mannose, galactose and fucose, as well as high mannose (type II) oligosaccharides which lack galactose and fucose (16). For the A/WSN strain, $HA_1$ was shown to have both type I and type II oligosaccharides whereas $HA_2$ possessed only type I oligosaccharides (16,20).

Our laboratory is interested in determining the number and type of glycosylation sites in HA and developing a method to compare glycoproteins when only small amounts of material can be obtained. To fulfil these objectives we have analyzed the tryptic glycopeptides of A/WSN influenza HA virus after extensive digestion with TPCK-trypsin, which produces more peptides containing a larger number of amino acid residues than pronase treatment (21,22). The tryptic glycopeptides were fractionated on DE52 cellulose (Whatman Inc.) followed by further fractionation on Bio-gel P-6. These procedures are laborious and application of this method to characterization of other viral

glycoproteins is often impossible because of the limited amount of material that can be obtained. Therefore, to demonstrate the applicability of reverse phase hplc to characterization of the glycosylation sites of glycoproteins, $^{3}$H-glucosamine labeled HA was digested with TPCK-trypsin and prepared for chromatography as described in Materials and Methods. The HA tryptic glycopeptides were dissolved in 0.1% $H_3PO_4$ and applied to a $C_{18}$ μBondapack column. The tryptic glycopeptides were eluted from the column by a gradient containing increasing amounts of acetonitrile from 0 to 36%. Under these conditions a total of about eight glycopeptide peaks were resolved as previously described (15,21). We felt resolution would be improved by altering the hplc techniques. Previously we had noted that LiChrosorb RP-18 columns had a longer retention time and it was felt that this property might aid in the resolution of tryptic glycopeptides with low hydrophobic properties. HA glycoprotein labeled with $^{3}$H-glucosamine was prepared as described in Materials and Methods. The $^{3}$H-glucosamine labeled tryptic glycopeptides from HA were dissolved in 0.1% $H_3PO_4$ and applied to a RP-18 column. The glycopeptides were eluted by a 0 to 40% acetonitrile gradient (Fig. 1A). As can be seen from this profile a major glycopeptide eluting between fraction 38 and 50 was clearly resolved from the complex of tryptic glycopeptides that eluted at higher acetonitrile concentration. This major tryptic glycopeptide appears to be highly ionic as demonstrated by its selective binding in different ion pairing agents. When HA tryptic glycopeptides were dissolved in formic acid and applied to a $C_{18}$ μBondapack column and eluted with 0 to 40% acetonitrile gradient the major glycopeptide eluted in the void volume of the column.

As mentioned above, HA may exist as two cleavage products, $HA_1$ and $HA_2$. $HA_2$ has a greater electrophoretic mobility than $HA_1$ and it has been shown to possess a single type I glycosylation site (16). Therefore, we prepared $^{3}$H-glucosamine labeled $HA_2$,

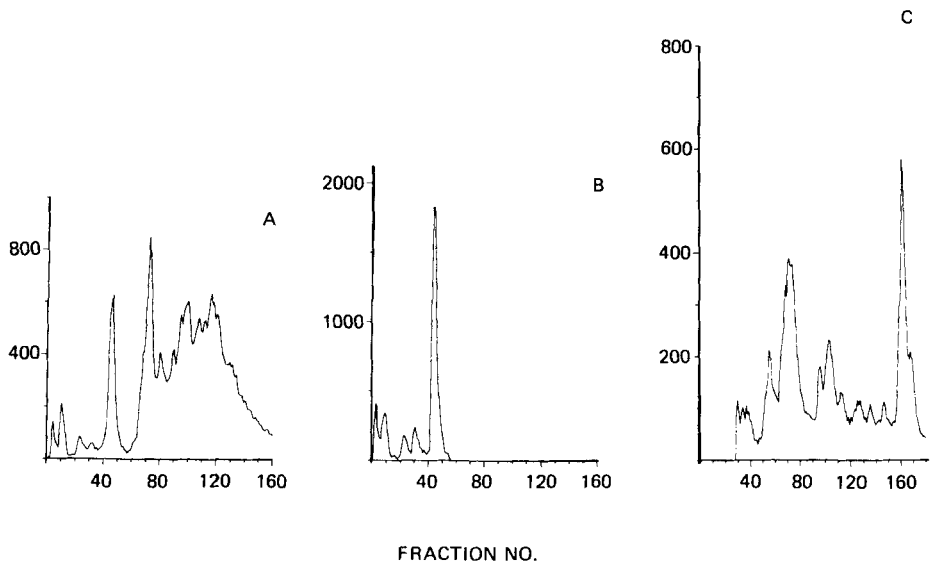


Figure 1. Chromatograms of (a) HA tryptic glycopeptides, (b) $HA_2$ tryptic glycopeptides and (c) $HA_1$ tryptic glycopeptides. Chromatographic conditions for (a) and (b): The labeled tryptic glycopeptides were applied to a RP-18 ODS column in 0.1% $H_3PO_4$, pH 2.85, and were eluted from the column by a linear gradient increasing in acetonitrile concentration to 40%. The sample was applied at 0% acetonotrile and allowed to run for 10 minutes before beginning the gradient. The flow rate was 2.0 ml/min. One minute fractions were collected and the radioactivity was determined by liquid scintillation counting. Chromatographic conditions for (c) are the same except the flow rate was 1.0 ml/min and 20 minutes after injection the gradient began at 12.5% acetonitrile concentration to final concentration of 35%.

digested it with TPCK-trypsin, and chromatographed it on an RP-18 column under the same conditions used for the elution of HA tryptic glycopeptides (Fig. 1B). As can be seen from Fig. 1B, the elution profile of the $HA_2$ tryptic glycopeptides is nearly identical to that of the first part of the HA elution profile. The major difference is an increased proportion of the material which elutes at an acetonitrile concentration of ~8%. The minor

tryptic glycopeptides in this profile may reflect slight charge differences in the amino acid portion of the tryptic glycopeptides or altered charge in the oligosaccharide part since complex type I oligosaccharides are often sulfated (16,23).

As shown in Fig. 1A, only tryptic glycopeptides associated with $HA_2$ were resolved under the gradient conditions used. To increase the resolution of the $HA_1$ tryptic glycopeptides the gradient conditions were altered. The glycopeptides were dissolved in phosphoric acid and applied to an RP-18 column, and eluted by a 12.5 to 35% acetonitrile gradient, 1 ml fractions were collected. The elution profile of the $HA_1$ tryptic glycopeptides is shown in Fig. 1C. As can be seen from this profile, the complex of tryptic glycopeptides that were unresolved by the conditions used for chromatography of HA appear to be resolved into five or six major tryptic glycopeptide constituents. Taken together, the results of these analyses suggest that the HA of the A/WSN influenza virus strain has six or seven glycosylation sites. These results are similar to an analysis carried out using ion exchange and open column methods that required weeks of labor. The analysis of the three glycoproteins can be accomplished in less than 12 hours with approximately a ten fold reduction in starting material. In addition, this method can be used for preparative separation of the tryptic glycopeptides for further biochemical and biological analyses.

To demonstrate further the general applicability of this method to the comparison of minute amounts of viral glycoproteins the envelope precursor proteins of MCF-247 MuLV were analyzed. Precursor proteins ($Pr75^{env}$) grown in two different cells were labeled with $^3$H-mannose and prepared as described in Materials and Methods. Mink/MCF-247 and SC-1/MCF-247 $Pr75^{env}$ tryptic glycopeptides were dissolved in phosphoric acid and chromatographed on a $C_{18}$ μBondapack column. The tryptic glycopeptides were eluted by a 0 to 36% acetonitrile gradient. A total of 160

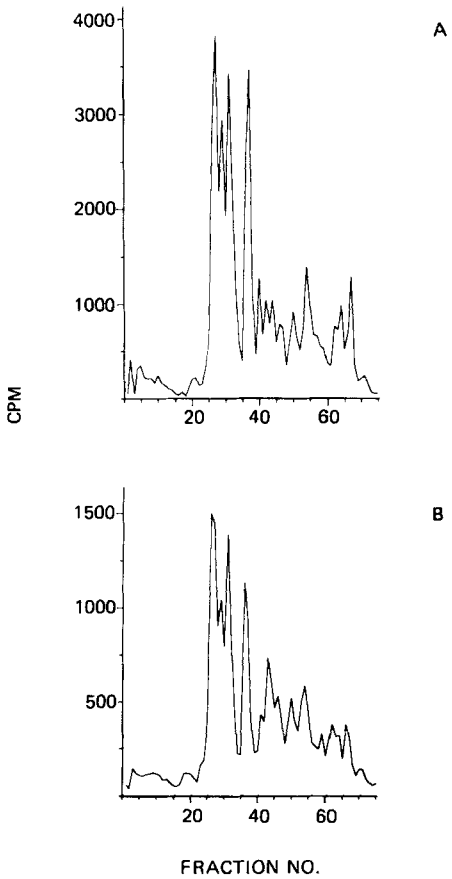




Figure 2. Chromatograms of (a) Mink/MCF-247 and (b) SC-1/MCF-247 envelope precursor tryptic glycopeptides. Chromatographic conditions were the same as described for Figure 1a and 1b except the column was a $C_{18}$ μBondapak.

fractions were collected but all of the tryptic glycopeptides eluted at an acetonitrile concentration of less than 25% (Fraction 75). As can be seen from Fig. 2A (mink/MCF-247) and Fig. 2B (SC-1/MCF-247) the elution profiles are very similar which allows us to conclude that the precursors are identical (18). These analyses were performed on material immunoprecipitated from cell

lysates and separated by polyacrylamide gel electrophoresis. Such analyses would be nearly impossible using conventional ion exchange and open column methods due to the small amounts of material available.

In conclusion, the results described in this procedure for tryptic glycopeptides are comparable to previous work done by this laboratory (22) and other (23,24) for tryptic peptide mapping, and, as with the peptide mapping procedures, offers distinct advantages over open column conventional chromatographic techniques.



## ACKNOWLEDGEMENTS

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