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Rapid analysis of membrane glycopeptides by gel permeation high-performance liquid chromatography

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SUMMARY

A rapid procedure for the analysis of glycopeptides has been developed using gel permeation high-performance liquid chromatography (HPLC). Glycopeptides derived by exhaustive pronase digestion of glycoproteins from radiolabeled human tumor and normal cell lines were chromatographed on DuPont GF-250 and GF-450 gel permeation columns in buffers containing non-ionic detergents. Effective separations of glycopeptides ranging in molecular mass from less than 600 daltons to more than 20 000 daltons, equivalent to the separation range of Sephadex G50 chromatography, were achieved in 7 min. The separations were dependent upon the use of an isocratic mobile phase, that contained a low-ionic-strength Tris buffer and Nonidet P-40 or Triton X-100. The mobilities of protein standards indicated the occurrence of a biphasic elution system, which favored the separation of species with molecular masses below 20 000 daltons. Glycopeptides isolated by this method could be applied directly to lectin or ion-exchange columns or could be digested with neuraminidase, endo H or other enzymes without further treatment. Removal of sialic acid from the glycopeptides caused a dramatic increase in retention time. Using this method, glycopeptides could be isolated rapidly and in high yield. The ease, speed and reproducibility of the separations and compatibility of the solvent systems with affinity or ion-exchange chromatography techniques make this gel permeation HPLC method an ideal initial step in the purification of glycopeptides.

INTRODUCTION

Glycopeptides derived by proteolytic cleavage of glycoproteins have been analyzed by various chromatographic methods. Separations of glycopeptides

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by gel permeation on Sephadex G50 have been used to demonstrate structural differences between the membrane sialoglycoproteins of transformed [1] and tumor cell lines [2,3] and human [4] and animal tumors [5] and those of normal cells. Standard chromatographic methods, however, require 4–6 h for each separation, limiting the number of analyses that can be accomplished per day to approximately three for a single column. High-performance liquid chromatographic (HPLC) techniques offer exceptional advantages in speed and reproducibility. Glycopeptides have been analyzed successfully by HPLC methods utilizing reversed-phase [6] and ion-exchange [7] techniques. Although these methods provide valuable information on the relative charge or hydrophobicity of glycopeptides, they do not provide information on the size relationship of glycopeptides. We have sought an HPLC system that would be rapid, simple and reproducible, could accommodate a large number of analyses in a single day and would permit separations of glycopeptides on the basis of molecular size.

We report here the development of an HPLC method that utilizes DuPont GF (gel filtration) columns for fast analysis of glycopeptides. Using this method, glycopeptides were eluted from the columns in 7 min with aqueous buffers containing non-ionic detergents. Separations of glycopeptides were achieved that were superior to separations requiring 4–6 h to develop using standard chromatographic methods. The HPLC method permits at least three analyses to be conducted per hour. Using this method, we have performed as many as twelve glycopeptide analyses in a single day.

EXPERIMENTAL

Materials

Chemicals were standard reagent grade. HPLC solvents were HPLC grade. Zorbax Bio Series GF-250 (150 Å pore size) and GF-450 (300 Å pore size) gel filtration columns were purchased from DuPont (Wilmington, DE, U.S.A.).

Cells and cell culture

Human colon (HT-29) and breast (MCF-7, BT-549) adenocarcinoma and normal breast (HBL-100) cell lines were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were grown in McCoy's 5A medium with 10% fetal bovine serum (FBS) in a 37°C humidified 5% CO_2-in-air atmosphere.

Radiolabeling

Glycoproteins were radiolabeled metabolically in their carbohydrate moieties. Cells were plated at $2 \cdot 10^6$ in 25-cm^2 culture dishes and were cultured until they reached logarithmic growth, usually within 24 h. At that time, culture

medium was exchanged with fresh medium containing 5 μCi/ml each of D-[2-^{3}H]mannose and D-[^{14}C(U)]glucosamine hydrochloride (New England Nuclear, Boston, MA, U.S.A.). Cells were cultured in the presence of isotope for 24 h.

Solubilization

Radiolabeled cells growing in monolayers were rinsed twice with cold Tris-buffered saline (TBS) and lysed by incubation in cold detergent buffer containing 0.15 *M* NaCl, 0.015 *M* $MgCl_2$, 0.025 *M* Tris–HCl pH 7.0, 0.02% phenylmethylsulfonyl fluoride (PMSF) and 0.5% Nonidet P-40 (NP-40) for 5 min on ice. Monolayers were scraped to remove all attached cellular material. Lysates were pipetted into centrifuge tubes, vortex-mixed ten times, centrifuged at 49 000 *g* for 20 min to remove nuclei and insoluble debris and used immediately or stored at −70°C. Protein concentrations were determined in detergent extracts using Markwell et al.'s modification of the Lowry protein determination assay [8].

Preparation of glycopeptides

Glycopeptides were prepared from NP-40-solubilized, radiolabeled glycoproteins by exhaustive digestion with pronase (Calbiochem) [1]. Cell extracts containing 5 mg protein and approximately 100 000 cpm of ^{3}H and 300 000 cpm of ^{14}C activity were incubated in the presence of 1 mg/ml pronase at 37°C for five days under toluene. Fresh pronase was added daily.

Neuraminidase digestion

Pronase-digested glycopeptides were treated with 20 U of Vibrio cholera neuraminidase (Calbiochem) (EC 3.2.1.18) in 50 μM sodium acetate, pH 5.2, and 10 μM $CaCl_2$ for 2 h at 37°C.

Analysis of glycopeptides on Sephadex G50

Pronase-digested radiolabeled glycopeptides from HT-29 cells were subjected to gel permeation analysis on a column (100 cm × 1.6 cm I.D.) of Sephadex G50 equilibrated with 0.2 *M* Na_2HPO_4, adjusted to pH 7.0 with HCl. Glycopeptide samples were eluted at a flow-rate of 14 ml/h. Fractions of 0.7 ml were collected. Aliquots of 20 μl were counted in a liquid scintillation counter.

High-performance liquid chromatography

Detergent-solubilized glycoproteins or pronase-digested glycopeptides were separated on GF-250 or GF-450 HPLC columns (DuPont) equilibrated and eluted with mobile phases consisting of either sodium phosphate or Tris buffers at neutral pH and containing various concentrations of NP-40 or Triton X-100. Radiolabeled samples of 10 μl volume containing 25–50 μg of protein

and 1000–3000 cpm of ^{3}H and 3000–9000 cpm of ^{14}C radioactivity were mixed with 90 μl of test mobile phase solvent, centrifuged at 130 000 *g* in a Beckman Airfuge and injected into a Hewlett-Packard 1090 HPLC system. Columns were eluted at a flow-rate of 1 ml/min. Fractions of 0.15 ml were collected directly into scintillation vials and were counted in a liquid scintillation counter. The columns were calibrated by the analysis of standard protein molecular mass markers and by comparison of equivalent samples on Sephadex G50. Molecular mass markers were thyroglobulin (669 000), apoferritin (443 000), β-amylase (200 000), bovine serum albumin (68 000), ovalbumin (43 000), soybean trypsin inhibitor (21 500), cytochrome C (12 500) and aprotinin (6000). Molecular mass markers were dissolved in each of the mobile phases tested.

RESULTS

Glycoprotein separations

To investigate the capabilities of the DuPont GF series columns for the separation of glycoproteins, [^{3}H]mannose- and [^{14}C]glucosamine-radiolabeled glycoproteins from HT-29 human colorectal carcinoma cells were solubilized in NP-40 and were subjected to isocratic elution on GF-250 or GF-450 gel filtration columns in sodium phosphate or Tris buffers containing various concentrations of NP-40 or Triton X-100 detergents. The results of these studies are presented in Fig. 1.

Glucosamine- and mannose-labeled HT-29 glycoproteins initially were subjected to chromatography in sodium phosphate buffers with low concentrations of NP-40. Separations of glycoproteins in a 0.2 *M* Na_2HPO_4 buffer containing 0.05% NP-40 are shown in Fig. 1A. Glycoproteins eluted in two broad, poorly resolved peaks. A two-fold increase in NP-40 concentration (Fig. 1B) produced only a slight improvement in the resolution of the early-eluting peak and a minimal separation of a third peak at fractions 82–83. Increasing the NP-40 concentration to 0.3% (Fig. 1C) decreased the tailing of the early-eluting peak and improved the separation of the late-eluting second and third peaks. Attempts to increase the NP-40 detergent concentration in the sodium phosphate buffer above 0.3% resulted in precipitation of detergent at room temperature. To accommodate increased concentrations of NP-40, solvents containing sodium phosphate were replaced with Tris-buffered solvents. Mobile phases containing Tris permitted testing of higher concentrations of NP-40. Buffers containing Tris at low-ionic-strength concentrations that would be compatible with subsequent ion-exchange or affinity procedures were tested. Buffers containing 10, 20 or 25 m*M* Tris and 1.0% NP-40 did not cause a major difference in the elution of proteins or glycopeptides (data not shown). In contrast, increasing the concentration of NP-40 by as much as twenty-fold had a dramatic effect on the mobility of both proteins and glycoproteins, causing an inverse change in mobilities. A mobile phase containing 20 m*M* Tris and 1.0% NP-40

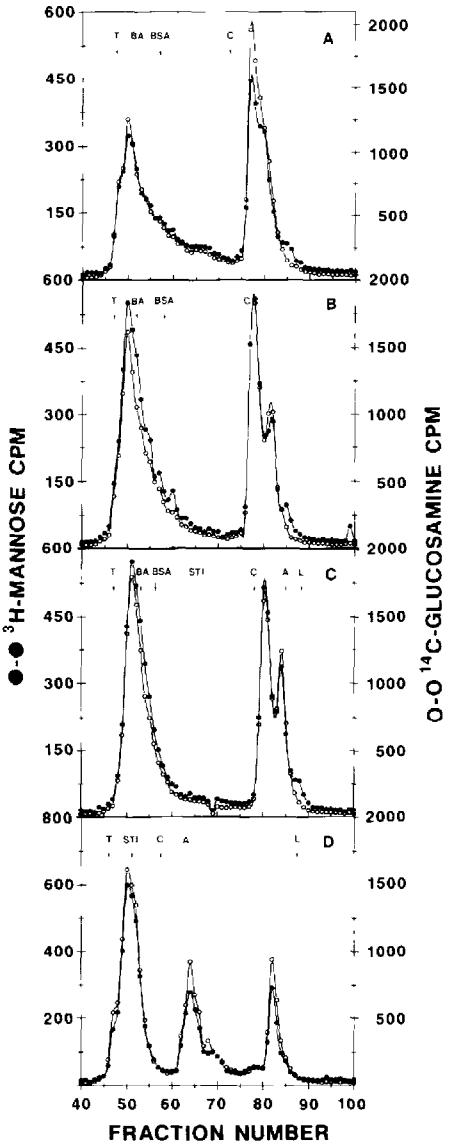

Fig. 1. HT-29 membrane glycoproteins analyzed by gel permeation HPLC. Radiolabeled membrane glycoproteins were solubilized in NP-40 detergent and subjected to gel permeation chromatography on a DuPont GF-250 column in 0.2 *M* Na_2HPO_4 containing 0.05% NP-40 (A), 0.1% NP-40 (B) or 0.3% NP-40 (C) or in 20 m*M* Tris and 1.0% NP-40 (D). Molecular mass markers were thyroglobulin (T), β-amylase (BA), bovine serum albumin (BSA), soybean trypsin inhibitor (STI), cytochrome C (C) and aprotinin (A). The elution position of sodium azide, the low-molecular-mass resolution limit, is indicated (L).

produced baseline separations of the HT-29 membrane glycoproteins into three major peaks which eluted at fractions 50, 64 and 82 (7.5, 9.5 and 12.75 min) (Fig. 1D).

To compare the resolving capabilities of the GF-250 and GF-450 columns for membrane glycoproteins, identical samples of the HT-29 glycoprotein preparation shown in Fig. 1D were analyzed separately on the GF-250 column (Fig. 2A) and on the GF-450 (Fig. 2B) column using the same 20 m*M* Tris–1.0% NP-40 mobile phase. These studies demonstrated that the early-eluting GF-250 peak could be separated into three or more components on the GF-450 column, while the mid-range GF-250 glycoprotein peak was shifted to a later-eluting position. The late-eluting glycoprotein peak did not show a change in position, although it became more narrow and exhibited a three-fold increase in peak height. Analysis of three glycoprotein peaks by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown) indicated that each of the peaks was a collection of glycosylated components. Samples of the three components of the early-eluting peak showed considerable overlap, while glycoproteins eluting in the mid- and late-eluting positions were separated according to molecular mass.

Protein molecular mass standards were used to calibrate the GF columns.

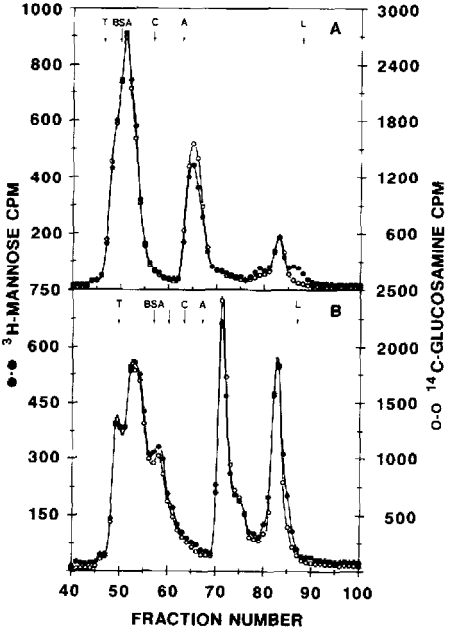

Fig. 2. Radiolabeled HT-29 tumor cell membrane glycoproteins separated on a GF-250 column (A) compared with the identical glycoprotein sample separated on a GF-450 column (B). Both columns were eluted with a mobile phase containing 20 m*M* Tris and 1.0% NP-40. Molecular mass markers were as in Fig. 1.

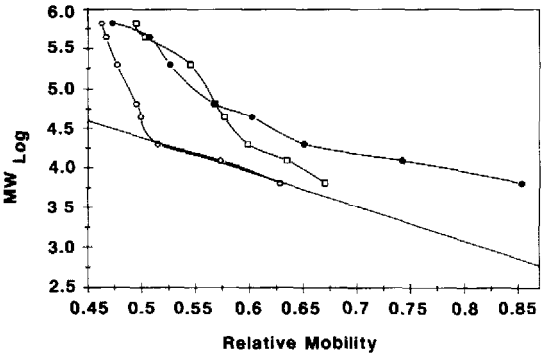

Fig. 3. Mobilities of protein standards on GF series columns in the presence of non-ionic detergents. Molecular mass markers were thyroglobulin (669 000), apoferritin (443 000), β-amylase (200 000), bovine serum albumin (68 000), ovalbumin (43 000), soybean trypsin inhibitor (21 500), cytochrome C (12 500) and aprotinin (6000). Column and buffer combinations were: GF-250, 0.2 *M* Na_2HPO_4 with 0.05% NP-40 (□); GF-250, 20 m*M* Tris with 1.0% NP-40 (○); GF-450, 20 m*M* Tris with 1.0% NP-40 (●)

Soluble protein standards were thyroglobulin, β-amylase, bovine serum albumin, soybean trypsin inhibitor, cytochrome C, and aprotinin. The elution positions of the protein standards and of sodium azide, the low molecular mass elution limit of the column, are indicated in Figs. 1 and 2. The addition of increasing amounts of detergent to the sodium phosphate mobile phase (Fig. 1A–C) did not affect the mobilities of thyroglobulin, β-amylase or bovine serum albumin and only slightly increased the mobilities of cytochrome C and aprotinin. Elution in the 20 m*M* Tris–1.0% NP-40 mobile phase (Fig. 1D) caused a marked decrease in mobility of all of the molecular mass markers. Plots of the mobilities of the protein standards eluted on the GF-250 column in the phosphate buffer and on the GF-250 and GF-450 columns in the Tris buffer are shown in Fig. 3. The mobilities of soybean trypsin inhibitor, cytochrome C and aprotinin eluted in mobile phases containing 20 m*M* Tris and various concentrations of NP-40 suggested that separations of low-molecular-mass molecules might be achieved on the GF-250 column in the presence of detergent. NP-40 at a concentration of 0.5% permitted the separation of species with molecular masses of 35 000 and below. The use of 1.0% NP-40 permitted the separation of 20 000 and below. NP-40 at a concentration of 1.5% did not afford improved separations of molecules below 20 000. Because glycopeptides typically occur with molecular masses below 20 000 and the analysis of glycopeptides by standard column chromatographic methods requires elution times of 4–6 h, we evaluated the separation of glycopeptides on the GF columns in the presence of detergent.

Analysis of glycopeptides

Glycopeptides were derived from D-[2-^{3}H]mannose- and D-[^{14}C]glucosamine-labeled HT-29 colon tumor cell glycoproteins by exhaustive

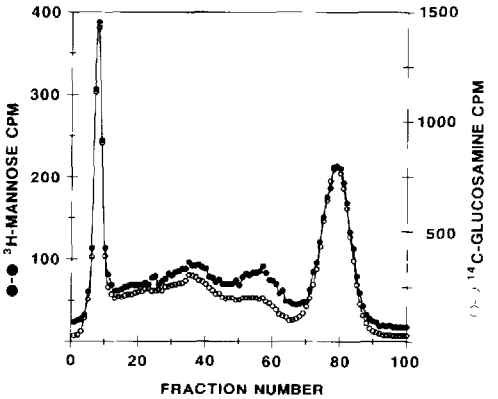

Fig. 4. Glycopeptides derived by exhaustive pronase digestion of [^{3}H]mannose- and [^{14}C]glucosamine-radiolabeled HT-29 glycoproteins fractionated on a Sephadex G50 column eluted with 0.2 *M* Na_2HPO_4, pH 7.0 at a flow-rate of 14 ml/h. Fractions of 0.7 ml were collected. Total elution time was 5 h.

digestion with pronase. The released glycopeptides were analyzed initially on a column of Sephadex G50 in sodium phosphate buffer in the absence of detergent (Fig. 4). The Sephadex G50 separations of radiolabeled HT-29 glycopeptides without detergent were typical, based on the distribution of molecular sizes of cell membrane glycopeptides determined in previous analyses on Sephadex G50 [1–4]. A pronase-resistant peak was eluted from the column initially, followed by two broad, poorly resolved mid-range peaks and, finally, a third low-molecular-mass peak (Fig. 4). When the same HT-29 glycopeptide sample was analyzed on the GF-250 column without detergent, the results shown in Fig. 5A were obtained. The elution positions of proteins of known molecular mass determined by prior calibration of the GF-250 column in the absence of detergent indicated that several high-molecular-mass pronase-resistant components may have been eluted first (Fig. 5A), followed by a single, large, complex peak containing a nearly continuous distribution of glycopeptides falling in the range of 20 000 to less than 4000. Changes in buffer ionic strength did not appreciably change the elution pattern (data not shown). The effects of neuraminidase digestion of the HT-29 glycopeptides could not be distinguished when neuraminidase-treated samples were analyzed without detergent (data not shown).

When the HT-29 glycopeptides were analyzed on the GF-250 HPLC column in the 20 m*M* Tris–1.0% NP-40 buffer (Fig. 5B), a marked difference in the elution of the glycopeptides was observed. In the presence of high detergent concentration, the glycopeptides eluted in separate, clearly resolved peaks that were more or less uniformly distributed across the separation range of the column. The occurrence of the individual peaks and the positions of their elution upon repeated analyses of the same sample were highly reproducible.

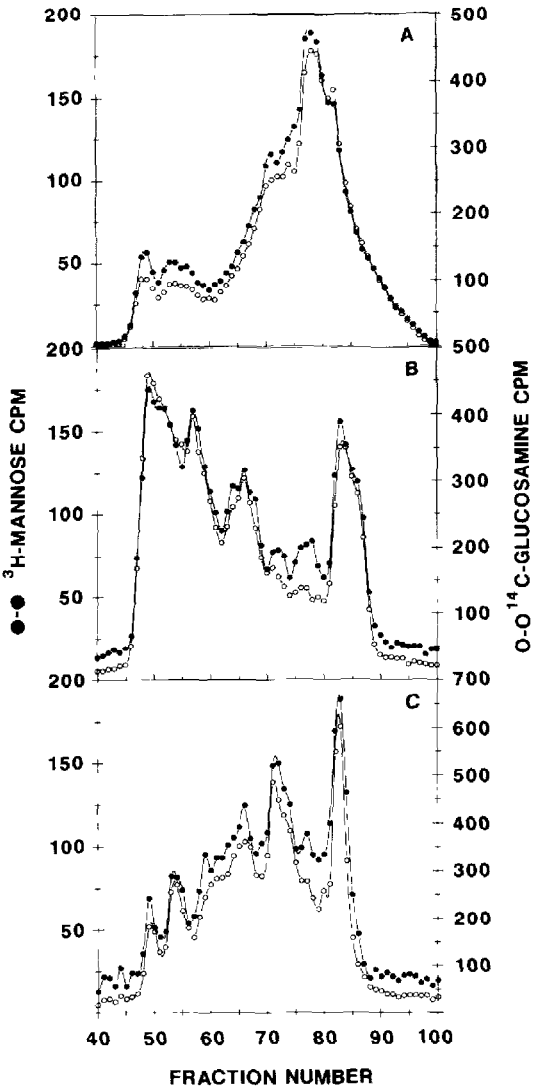

Fig 5. Radiolabeled HT-29 glycopeptides separated on DuPont GF columns. (A) Separation of HT-29 glycopeptides on the GF-250 column without detergent. The column was eluted with a mobile phase containing 0.2 *M* Na_2HPO_4, pH 7.0. (B) HT-29 glycopeptides on the GF-250 column, eluted with a mobile phase containing 20 m*M* Tris and 1.0% NP-40. (C) HT-29 glycopeptides separated on the GF-450 column, eluted with the 20 m*M* Tris and 1.0% NP-40 buffer.

When the HT-29 glycopeptide preparation was fractionated in the presence of detergent on the GF-450 column (Fig. 5C), there appeared to be a shift of glycopeptide peaks toward lower-molecular-mass positions. Because the GF-450 column has a larger pore size and is designed for the fractionation of higher-

molecular-mass macromolecules, the increased separation of larger glycopeptides was not unexpected.

To determine the effect of the presence of negatively charged sialic acid moieties that are known to occur on many membrane glycopeptides, we treated the HT-29 glycopeptides with neuraminidase and analyzed the desialylated glycopeptides on the GF-250 column in the Tris–NP-40 mobile phase. Neuraminidase digestion caused a dramatic change in the elution position of the glycopeptides (Fig. 6) when compared with the pattern before digestion. Nearly all of the glycopeptide peaks were effected by the neuraminidase treatment, which caused an increase in retention time of 2 min to more than 5 min. Neuraminidase-treated glycopeptides eluted in three major positions concentrating at fractions 68, 74 and 84 (10, 11 and 12.5 min).

To determine if characteristic patterns of glycopeptides would be obtained when samples derived from different cell lines were analyzed by the gel permeation system, we performed separations of glycopeptides from two breast tumor cell lines (MCF-7 and BT-549) and one normal breast cell line (HBL-100) on the GF-250 column in the Tris–NP-40 mobile phase. The results of these analyses are shown in Fig. 7. Glycopeptides from all three cell lines were distributed across the same elution times as those of HT-29 (Fig. 5B) but did not exhibit the concentration of components in the 7–9 min region (fractions 46–60) that was characteristic of the HT-29 glycopeptides. The glycopeptides from the three breast cell lines appeared to have been separated into similar size classes and were quantitatively nearly equal, but unlike those from the colon tumor cell line, a number of mannose-containing breast glycopeptides in lower molecular mass regions did not exhibit equivalent glucosamine labeling. Mannose-containing glycopeptides from the BT-549 breast tumor cell line showed a marked quantitative increase relative to MCF-7 or HBL-100 at frac-

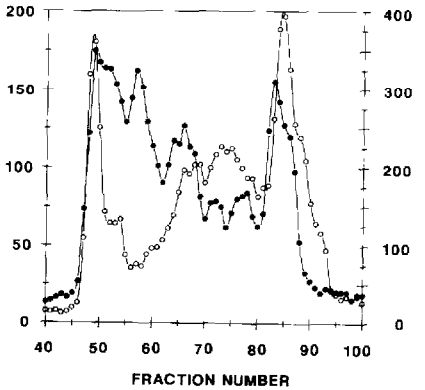

Fig. 6. HT-29 glycopeptides radiolabeled with [^{3}H]mannose before (●) and after (○) digestion with neuraminidase separated on the GF-250 column, eluted with 20 m*M* Tris and 1.0% NP-40.

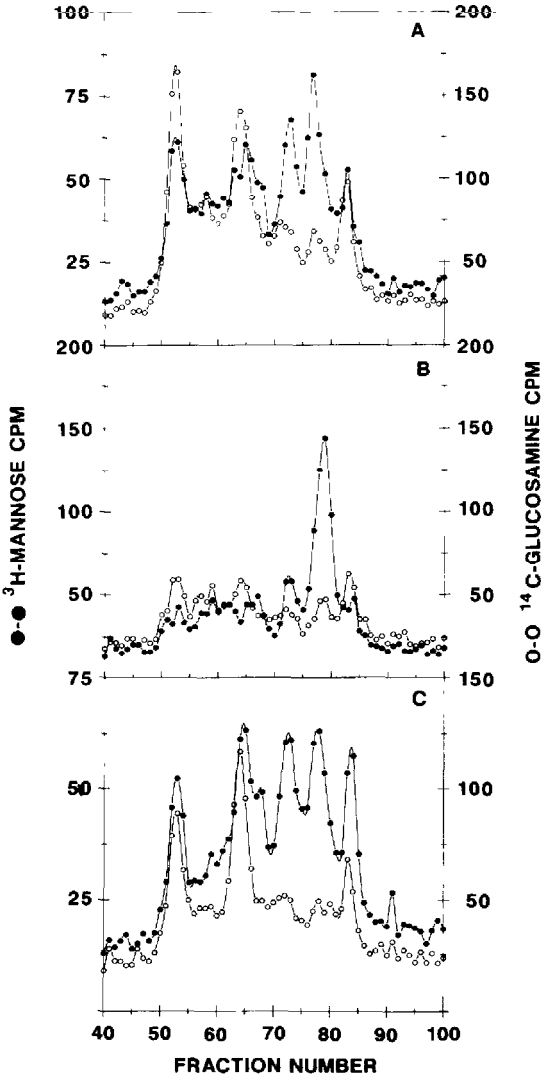

Fig. 7. [^{3}H]Mannose- and [^{14}C]glucosamine-radiolabeled glycopeptides from MCF-7 (A) and BT-549 (B) breast tumor cells and from HBL-100 (C) normal breast cells separated on the GF-250 column, eluted with 20 m*M* Tris and 1.0% NP-40.

tions 76–81. The glycopeptide pattern characteristic of each of the breast cell lines, as well as the characteristic but different glycopeptide pattern from the colon tumor cell line, each occurred consistently in subsequent preparations.

DISCUSSION

The results of these studies show that the DuPont GF-250 and GF-450 columns can provide an excellent chromatography medium for the rapid separa-

tion of detergent-solubilized glycopeptides in non-denaturing mobile phases. At a flow-rate of 1.0 ml/min, separations of glycopeptides into a number of size groups was achieved in 7 min. Using this method, glycopeptides from different cell lines were resolved into peaks that were characteristic of the specific cell line. The characteristic glycopeptide patterns we obtained for individual cell lines were reproducible. Each subsequent preparation of labeled cells produced a glycopeptide pattern that was nearly identical.

The use of low-ionic-strength buffers and non-ionic detergents permits this method to be used as a preliminary step in the purification of glycopeptides prior to lectin or antibody affinity or ion-exchange chromatography without the necessity of dialysis. We have used this method to purify tumor cell glycopeptides, which were pooled and applied directly to lectin affinity columns, or ion-exchange columns or were used as immunogens for the production of monoclonal antibodies without further treatment.

The addition of increasing amounts of detergent to the mobile phases caused decreased mobilities of the HT-29 glycoproteins on the GF-250 column (Fig. 1) resulting in effective separation conditions for glycoproteins in the 600–20 000 range. Glycoproteins in the 5000–200 000 range were effectively separated on the GF-450 column (Fig. 2).

Separations of glycopeptides without detergent were predictable according to the anticipated molecular mass range of the cell-derived glycopeptides from previous studies using Sephadex G50. HPLC separations of glycopeptides in the presence of non-ionic detergents were not predicted and may involve effects of charge and detergent binding. The shift in elution position when glycopeptides were analyzed on the GF-450 column, however, suggests that separations may occur on the basis of the three-dimensional size of the detergent-clad glycopeptide species. To determine the specific nature of the separation system, it will be necessary to perform detailed compositional and structural analyses of individual glycopeptide peaks to determine if structural characteristics of glycopeptides, such as branching, chain length, amino acid composition or other factors are identifiable determinators of elution position.

The results of GF-250 analyses of glycopeptides obtained from several breast tumor and normal cell lines shown in Fig. 7 demonstrate the uniqueness of the glycopeptide pattern that can be associated with a particular cell type when the analysis is performed by this method. A comparison of the uniform distribution of the breast tumor and normal cell glycopeptides with the more early-eluting pattern of the glycopeptides from the HT-29 colorectal carcinoma cell line (Fig. 5B) suggests that possible tissue-specific characteristics may be definable in analyses using this method.

The mild conditions required to obtain successful separations should make this method useful for the purification of low-molecular-mass biologically active peptides and glycopeptides. Because of the large shift in glycopeptide mobility following neuraminidase treatment, the method also should be extremely

useful to indicate the transfer of sialic acid to or from glycopeptides. It will be valuable to investigate the effects of other non-ionic detergents as well as the effects of controlled alteration of charge on the mobility of species. The results we present here suggest that below a certain molecular size, charge and detergent-binding character of a glycopeptide molecule play an increasingly important role in gel permeation HPLC separations.

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