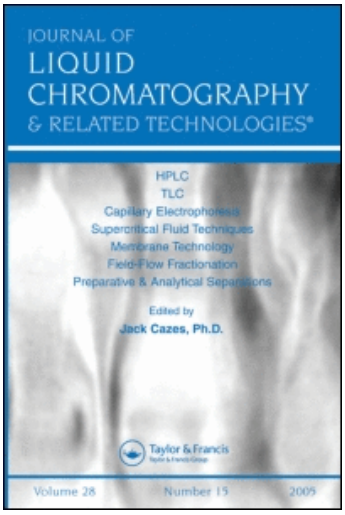


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# APPLICATION OF AQUEOUS GEL PERMEATION CHROMATOGRAPHY WITH IN-LINE MULTI-ANGLE LASER LIGHT SCATTERING AND DIFFERENTIAL VISCOMETRY DETECTORS FOR THE CHARACTERIZATION OF NATURAL PRODUCT CARBOHYDRATE PHARMACEUTICALS

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## ABSTRACT

Natural product complex carbohydrate polymers are currently being developed as prophylactic and/or therapeutic drugs. These water-soluble carbohydrate pharmaceuticals, which are primarily $\beta$-1,3-D-glucan polymers, belong to the class of drugs known as biological response modifiers (BRMs). A major obstacle to the development, understanding and clinical utilization of carbohydrate BRMs has been the difficulty involved in accurately characterizing high molecular weight (MW) carbohydrate polymers. Recent advances in aqueous gel permeation chromatography (GPC), differential viscometry (DV) and multi-

angle laser light scattering (MALLS) photometry make it possible to accurately characterize high MW carbohydrate BRMs. Herein, we report on the application and reduction-to-practice of aqueous GPC/DV/MALLS in the characterization of the water soluble carbohydrate BRM, Krestin. Molecular weight averages, weight average RMS radius ($r_{gw}$), polydispersity ($I$) and intrinsic viscosity ([$\eta$]) were established. Krestin, a complex ß-linked proteoglycan BRM, was observed to have three polymer peaks ($M_w$ = #1 - 1.35 x $10^7$, #2 - 8.77 x $10^5$, #3 - 2.40 x $10^5$ g/mol) with a $r_{gw}$ of 41.1 nm and [$\eta$] of 0.157 dL/g. These data demonstrate the utility of multi-detector GPC for the characterization of complex carbohydrate pharmaceuticals.

## INTRODUCTION

Water soluble complex carbohydrate polymers derived from natural sources have been shown to augment various facets of immune responsiveness in humans (1) and animals (2-5). These natural product carbohydrate polymers belong to the class of drugs known as Biological Response Modifiers (BRMs). The ability of complex carbohydrate BRMs to exert beneficial effects in a wide variety of experimental (2-5) and clinical disease states (1) has stimulated research into their potential biomedical applications. Among the more widely-known drugs belonging to this class are the BRMs Krestin (6), Lentinan (7,8), schizophyllan (9), and glucan phosphate (2,4,5). All of these drugs are water soluble to facilitate oral and/or parenteral administration.

A primary barrier to the development and understanding of complex carbohydrate pharmaceuticals has been the difficulty involved in accurately characterizing carbohydrate polymers with molecular weights ranging from $10^4$ to $10^7$. High performance gel permeation chromatography offers one important approach to the characterization of complex carbohydrate BRMs. While the basic principle of gel-filtration chromatography has been widely employed for decades, recent developments in aqueous high performance GPC separation technology, in concert with the development of new detectors and microcomputer based data acquisition systems make it possible to rapidly, simply and accurately determine a number of important physicochemical characteristics including molecular weight

moments (number-, weight-, and z-average molecular weights, respectively), rms radius moments, polydispersity (*I*) and intrinsic viscosity ([$\eta$]). Specifically, the development of in-line multi-angle laser light scattering (MALLS) photometry and differential viscometry (DV) detectors, coupled with sophisticated data acquisition packages, have greatly facilitated this effort (10-12).

Herein, we describe the application and reduction-to-practice of aqueous high performance GPC/MALLS/DV in the characterization of a complex carbohydrate BRM. Specifically, we demonstrate the applicability of this technique by examining the BRM, Krestin. Krestin, a protein-containing polysaccharide which is a mixture of $\beta$-1,4, $\beta$-1,3- and $\beta$-1,6 interchain linkages (6), was selected for this study because of its complex nature.



## MATERIALS AND METHODS

### Krestin, Pullulan and Dextran.

Krestin (PSK), a protein-containing (~25-38%) polysaccharide derived from *C. versicolor*, was obtained as a powder from Kureha Chemical Ind., Tokyo, Japan. Dextran standards (Pharmacia, Piscataway, NJ), and pullulan standards (Showdex P-82 series, J.R. Science, NY) were used for calibration of the GPC system.

### High-Performance Aqueous Gel Permeation Chromatography.

To evaluate polymer distribution Krestin was analyzed by aqueous GPC. The basic GPC system consisted of a Waters 600E solvent delivery system, a U6K manual injector and a column heating chamber (Waters Chromatography Division, Millipore Corp., Milford, MA). The mobile phase, 0.05 M sodium nitrite, was stored in a sterile reservoir (Kontes, Vineland NJ), and was thoroughly degassed by sparging and blanketing with helium prior to use. Mobile phase was delivered at a flow rate of 0.5 ml/min. Three Ultrahydrogel (Waters Chromatography Division, Milford, MA) aqueous GPC columns having exclusion limits of 2 x $10^6$ D, 5 x $10^5$ D and 1.2 x $10^5$ D were connected in series along with an Ultrahydrogel guard column. The columns were maintained at 30°C.

Flow rate, column temperature, and pump operating conditions were controlled by Maxima 820 GPC software (Waters Chromatography Division, Milford, MA). A schematic of the multi-detector GPC system is presented in Figure 1.

The system was calibrated using narrow-band pullulan standards. For analysis, Krestin was dissolved in mobile phase at a concentration of 2-3 mg/ml by gentle rocking until completely hydrated (~2-3 hrs). A 200 $\mu$l injection volume was used for all analyses.

Determination of Molecular Weight and RMS Radius by Multi-angle Laser Light Scattering.

Molecular weight was determined by in-line multi-angle laser light scattering (MALLS) photometry employing a Dawn-F MALLS photometer fitted with a K5 flow cell (Wyatt Technology Corp, Santa Barbara, CA). Molecular weight distribution, molecular weight moments (number-average MW, weight-average MW, Z-average MW, peak MW), polydispersity and rms radius moments were established with ASTRA software (v. 2.02). A differential index of refraction (*dn/dc*) of 0.146 $cm^3/g$ was assumed. Reported MWs of pullulan and dextran standards used to check column calibration showed good agreement with MALLS data.

Determination of Intrinsic Viscosity by Differential Viscometry.

Intrinsic viscosity ($[\eta]$) was determined by in-line differential viscometry (DV). For determination of $[\eta]$ the column eluent was passed through a Viscotek Model 200 differential refractometer/viscometer and data were analyzed with Unical software v. 3.11 (Viscotek, Porter, TX). Molecular weight determinations of standards using this technique showed good agreement with MALLS data. Intrinsic viscosity of pullulan standards was determined to be in close agreement with previous data (13).

## RESULTS

Molecular Weight Averages, Average RMS Radius, Polydispersity, Intrinsic Viscosity and Distribution of Krestin.

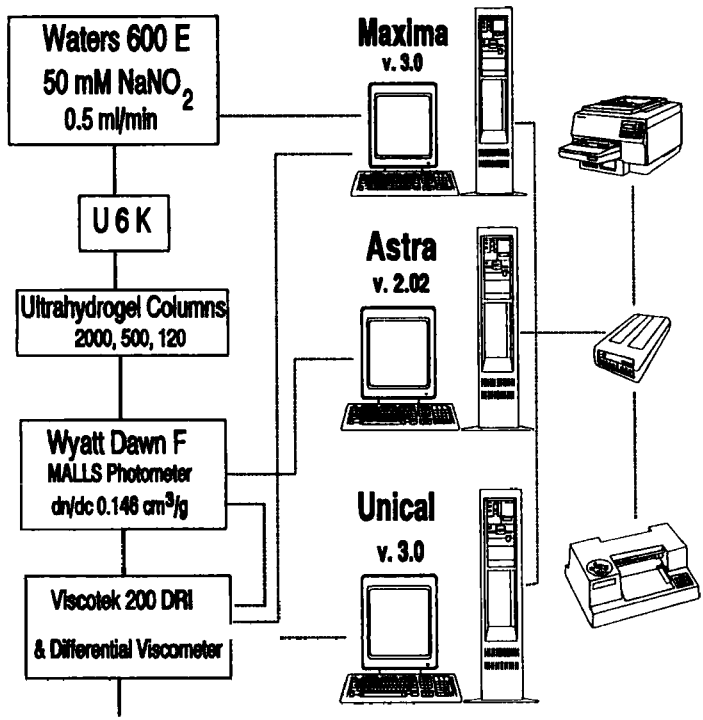


**Figure 1.** Schematic of the GPC system with MALLS, DV and DRI detectors connected in series. The mobile phase was 0.05 M $NaNO_2$ delivered at a flow rate of 0.5 ml/min. Three ultrahydrogel columns with exclusion limits of $2 \times 10^6$, $5 \times 10^5$ and $1.2 \times 10^5$ were connected in series along with a Ultrahydrogel guard column. Column temperature was maintained at 30°C. The mobile phase was sterile filtered and maintained in a Kontes sterile reservoir. The solid line represents the fluid path. The dashed line represents data paths.

TABLE 1

Characterization of Krestin by Aqueous Gel Permeation Chromatography with Multi-Angle Laser Light Scattering and Differential Viscometry Detectors.

| Peak | $M_n$ | $M_w$ | $M_z$ | $r_{gw}$ | $I$ | $[\eta]$ | % |
|---|---|---|---|---|---|---|---|
| peak 1 | $9.82 \times 10^6$ | $1.35 \times 10^7$ | $2.11 \times 10^7$ | 41.0 | 1.38 | | 2 |
| peak 2 | $4.05 \times 10^5$ | $8.77 \times 10^5$ | $1.78 \times 10^6$ | 41.1 | 2.17 | | 30 |
| peak 3 | $1.83 \times 10^5$ | $2.40 \times 10^5$ | $4.94 \times 10^5$ | 24.5 | 1.31 | 0.175 | 65 |

$M_n$ - number-average molecular weight in g/mol
$M_w$ - weight average molecular weight in g/mol
$M_z$ - z-average molecular weight in g/mol
$r_{gw}$ - weight average RMS radius in nm
$I$ - polydispersity (as $M_w/M_N$)
$[\eta]$ - intrinsic viscosity for entire polymer distribution in dl/g
% - percent of total polymers



Data for the number-average, weight-average and z-average MW moments of Krestin are presented in Table 1. Also presented are data concerning the viscosity average, polydispersity and polymer distribution. Krestin, a branched ß-1,4 protein bound polymer, was observed to have three polymer peaks ($M_w$ = #1 - $1.35 \times 10^7$, #2 - $8.77 \times 10^5$, #3 - $2.40 \times 10^5$ g/mol) and a $r_{gw}$ of 41.1 nm. Intrinsic viscosity $[\eta]$ across the entire polymer distribution was 0.157 dl/g. The low $M_w$ peak ($2.40 \times 10^5$ g/mol) represented 65.3% of the total polymers.

The gel permeation chromatograms for Krestin are presented in Figure 2. The top chromatogram represents analysis of the column eluent by MALLS. The data represents the 90° light scattering angle (detector 11). The middle chromatogram represents analysis of the column eluent by differential viscometry. The bottom chromatogram is the differential refractive index detector or concentration response (Fig. 2).

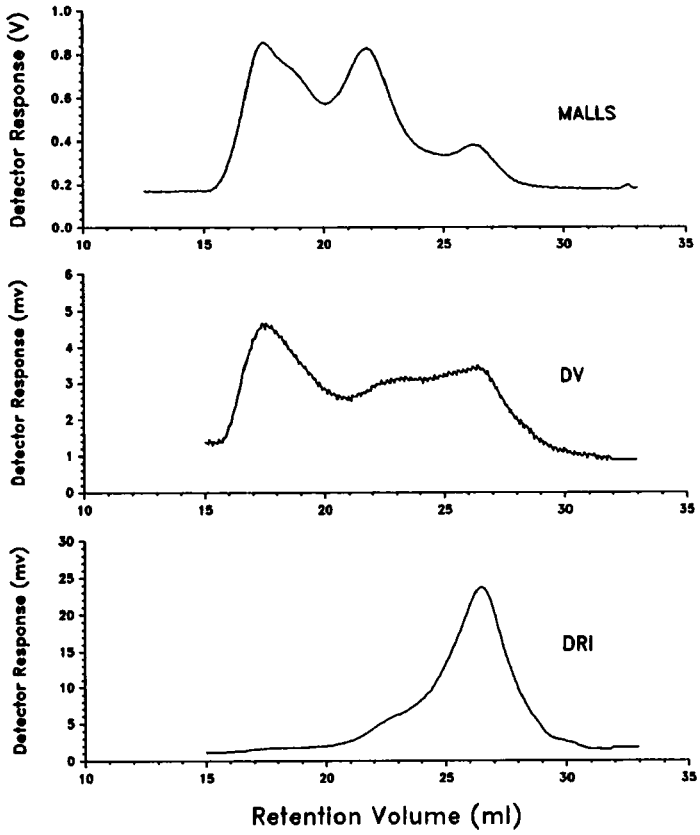


**Figure 2.** Gel permeation chromatograms of Krestin as determined by multi-angle laser light scattering (MALLS) photometry (top chromatogram), differential viscometry (DV) (middle chromatogram), and differential refractive index (DRI) (bottom chromatogram) detectors. The MALLS data represents the 90° light scattering angle (detector 11).

## DISCUSSION

Herein, we describe the application and reduction-to-practice of high performance gel permeation chromatography with multiple in-line detectors in the analysis of natural product complex carbohydrate pharmaceuticals. Up to this point-in-time, GPC with multiple in-line detectors has primarily been employed for the characterization of polystyrene and related synthetic polymers in organic mobile phases (10). Very little data exists concerning the application of aqueous high performance GPC with multiple detectors in a biomedical setting. This is particularly true with regard to development of natural product pharmaceutical. Recently, our laboratory group has employed aqueous GPC/MALLS/DV for the partial characterization of a $\beta$-1,3-linked glucopyranose BRMs isolated from *Sclerotium glucanicum* (12) and *Saccharomyces cerevisiae* (14,15).



Quality control of natural product carbohydrate BRMs has relied heavily on determination of *in vivo* immunobiological activity in a variety of experimental animal models (12). While this data is essential, it sheds virtually no light on the physicochemical characteristics of the BRM in question and, more importantly, these *in vivo* bioassays provide no structural and/or physicochemical information concerning batch-to-batch product quality. Previously, the molecular weight averages of natural product carbohydrate BRMs had been established by aqueous low pressure gel filtration chromatography or, in some cases, by aqueous high-performance GPC employing a traditional concentration detector, such as a differential refractive index (16). This type of analysis is subject to considerable error. Specifically, error is introduced by employing calibration curves which are generated with standards that are structurally dissimilar from the sample being analyzed. By way of example, in order to establish a GPC calibration curve for studies such as those described above, it is necessary to obtain standardized, water soluble, complex carbohydrate standards of varying molecular weight. At present, dextrans and pullulans are the only commercially available carbohydrate standards for establishing such aqueous calibration curves. Dextrans are water soluble $\alpha$-1,6-linked carbohydrate polymers with discontinuous branching (17,18).

Pullulans are water soluble $\alpha$-1,4-linked, random coil glucose polymers (13). While both of these carbohydrates are available in pure form over a wide range of molecular weights, as is indicated below, they are still not entirely suitable for establishing a calibration curve which will be valid for analysis of natural product carbohydrate BRMs.

Numerous studies have demonstrated that those carbohydrate BRMs with the greatest and most specific immunobiological activity are $\beta$-1,3-linked polymers of glucose with varying degrees of $\beta$-1,6 glucosyl side-chain branching (12). In addition, it is known that naturally occurring $\beta$-1,3-linked glucopyranoses exist in an ordered or triple-helical conformation (9,19). Maeda *et al.* (20) have demonstrated that the anti-tumor activity of $\beta$-1,3-linked triple-helical glucopryranose BRMs is intimately linked to their higher structure. At present, there are no $\beta$-1,3-linked triple-helical carbohydrate standards available. Therefore, analysis of complex carbohydrate BRMs by classical GPC employing a calibration curve based solely on structurally dissimilar standards, such as pullulan or dextran, may introduce substantial error.



In our laboratory, we have sought to overcome this problem by employing multiple in-line detectors to analyze carbohydrate polymers in the column eluent. Specifically, we have employed multi-angle laser light scattering (MALLS) and differential viscometry (DV) detectors along with a conventional differential refractive index (DRI) detector. The decision to employ a multiple detector system was carefully considered. As can be seen in Fig. 2, both MALLS and DV detectors increase the sensitivity of the analysis. More importantly, establishing molecular weight averages with the MALLS detector is not dependent on a calibration curve (10), thereby eliminating this source of error. The DV detector also provides data on the MW, intrinsic viscosity, polydispersity and radius of the polymer (11). In our hands, the MALLS detector fitted with a helium-neon laser (632.8 nm) is most efficient for examining polymers with molecular weights ranging from $\sim 1.5 \times 10^4$ to $1.25 \times 10^7$. However, it is has been our experience that establishing the radius of polymers with $M_w$ of $<20,000$

g/mol is exceedingly difficult with the helium-neon MALLS system. In contrast, the DV detector is most useful in the analysis of polymers with $M_w < 5.0 \times 10^5$. This is particularly true when attempting to establish the radius of low MW polymers. By employing in-line MALLS and DV it is possible to rapidly, simply and reproducibly establish a number of important physicochemical characteristics of the carbohydrate polymers in question. In addition, parameters such as $M_n$, $M_w$, $M_z$, radius and polydispersity can be independently established by MALLS and DV for a given polymer during a single run. Thus, the use of multiple in-line detectors allows comparison and confirmation of the results obtained as well as extends the analytical range of the technique. Based on these considerations we conclude that MALLS and DV are complimentary technologies which provide the most effective and desirable means of analysis of complex carbohydrate pharmaceuticals.



The advantages of employing in-line MALLS and DV detectors in the analysis and characterization of complex carbohydrate pharmaceuticals is illustrated in Figure 2. The $M_w$ of Krestin is reported to be $\sim 1.0 \times 10^5$ (6). Analysis of the column eluent employing conventional GPC with a DRI detector (Fig. 2, bottom panel) and Maxima v. 3.31 GPC software reveals a single broad peak with a $M_w$ of $9.6 \times 10^4$ using the narrow band pullulan calibration curve. This agrees well with previous reports in the literature employing similar technology (6). However, analysis of Krestin in the column eluent by MALLS and DV revealed three polymer peaks and a considerably higher $M_w$. MALLS data indicates that Krestin is composed of three polymer peaks, which can be analyzed separately. In addition, we established the MW average across the entire polymer distribution (*i.e.* across the three peaks). MALLS data indicates that Krestin has a $M_w$ of $5.4 \times 10^5$ g/mol. Viscometric analysis of Krestin also revealed three polymer peaks and a $M_w$ of $5.04 \times 10^5$ across the entire polymer distribution. Thus, both MALLS and DV analysis provide additional important insights concerning the physicochemical properties of Krestin that are not obtainable with conventional GPC (*i.e.* DRI detector) methodology. The practical

significance of these observations are readily apparent. As stated above, the characterization and quality control of natural product carbohydrate BRMs has, in the past, relied heavily on bioassays and conventional GPC. The data presented demonstrate the applicability of high performance GPC with multiple detectors in the analysis of complex carbohydrate BRMs. This technology when combined with other analytical techniques such as high-field $^{13}C$ and $^{1}H$-NMR, elemental analysis, helix coil transition analysis and exhaustive methylation/gas chromatography/mass spectrometry (12) provide a much more complete description of this novel class of pharmaceuticals.

The data presented demonstrate the utility of aqueous multi-detector GPC in the characterization of natural product, complex carbohydrate pharmaceuticals. This type of precise physicochemical data is required for: 1) a better understanding of the properties and higher structure of complex carbohydrate BRMs; 2) development of quality control methods for the isolation of natural product, complex carbohydrate BRMs and 3) the development of carbohydrate BRMs with greater and/or more specific immunobiological activity.



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