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High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD) Analysis of Mannose-6-Phosphate

INTRODUCTION

D-Mannose-6-phosphate (M-6-P) is a terminal monosaccharide of asparagine-linked oligosaccharides that is an important intermediate in glycoprotein production and, when incorporated in the glycoprotein's final oligosaccharide, is needed for targeting and recognition on some lysosomal proteins. Genetic glycosylation defects or malfunctions in the synthesis or processing of protein-bound oligosaccharides are known collectively as congenital disorders of glycosylation (CDG).[1] The most common disorders, CDG type 1a, are deficiencies in phosphomannomutase.[1] Human phosphomannomutase, a single substrate, single product isomerase, converts M-6-P to α-D-mannose-1-phosphate (M-1-P). Deficiencies or defects in human phosphomannomutase can cause numerous disorders, including mental retardation, hypoplasia (underdevelopment of organs), ataxia (loss of motor control), and seizures.

Inclusion-cell (I-cell) disease, or mucolipidosis II, is caused when M-6-P is not formed on lysosomal enzymes by GlcNAc-phosphotransferase and phosphodiester α-GlcNAcase. Without M-6-P, the enzyme is not targeted by the trans-Golgi network for transfer to or from the endosomes and lysomes. Improper transport of these enzymes and build-up of these proteins in the cell can cause over 40 diseases.[2] Studies of M-6-P–containing glycoproteins and M 6-P receptors are active areas for medical research with the goals of understanding and hopefully treating or controlling these and other diseases.[1–3]

When characterizing a lysosomal glycoprotein, it is important to determine how much M-6-P, if any, is present. When producing recombinant lysosomal glycoproteins known to have M-6-P terminal oligosaccharides, it is important that the glycoprotein be purified without loss of M-6-P. Recombinant proteins containing M-6-P enter the cell and subsequently the lysosome so that they can take action. A recombinant protein without M-6-P would be ineffective because it will be eliminated before reaching its site of action. Thus, sensitive, accurate, and selective determinations of M-6-P are important for these therapeutic recombinant glycoproteins.

HPAE-PAD is widely used to determine a large variety of carbohydrates, including monosaccharides, oligosaccharides, alditols, sugar phosphates, and sugar acids including sialic acids. In a 2001 publication, M-6-P was quantified in recombinant human α-galactosidase A by HPAE-PAD using a CarboPac® PA10 with an AminoTrap™ pre-column.[3] The separation used a sodium hydroxide and sodium acetate mobile phase, which is typical of HPAE-PAD separations of charged carbohydrates. The PAD used a three-potential

waveform (Waveform B, TN 21).[4] While this method is effective, a four-potential waveform introduced in 1997 (Waveform A, TN 21[4] and Table 1) delivers greater long term peak area reproducibility and would be used today. The AminoTrap pre-column may not be necessary for this method. Eluent consumption and thus eluent preparation can be reduced using a comparable column, the CarboPac PA200, with a lower flow rate of 0.5 mL/min.

This application note describes a method for determining M-6-P in glycoproteins using HPAE-PAD. Acid-hydrolyzed BSA is used as a generic protein matrix because a commercially available M-6-P–containing protein could not be found. M-6-P added to hydrolyzed BSA was separated on a CarboPac PA200 analytical column using 100 mM sodium hydroxide and 100 mM sodium acetate at 0.5 mL/min for 30 min, and detected using Waveform A. M-1-P (3.3 min) and M-6-P (10.7 min) peaks were fully resolved. Peak identification was confirmed by the absence of both peaks after dephosphorylation with alkaline phosphatase, and by determining mannose with a second HPAE-PAD assay.[5] This application note and reference 3 show that HPAE-PAD is a fast, direct (no sample derivatization) method for determining the M-6-P content of a glycoprotein.

EQUIPMENT

Dionex ICS-3000 Ion Chromatography system[a] consisting of:

- SP Single Gradient Pump, with degas option
- DC Detector/Chromatography module equipped with single or dual temperature zones and a 6-port injection valve
- ED Electrochemical Detector (P/N 061718),
- AS Autosampler with Sample Tray Temperature Controlling option, and 1.5 mL sample tray
- An electrochemical cell containing a combination pH/Ag/AgCl Reference Electrode (cell and reference electrode P/N 061756, reference electrode P/N 061879) and a conventional (P/N 061749) or disposable (P/N 060139, package of six; P/N 060216, package of 24) gold (Au) working electrode

[a]*The ICS-3000 IC system can be configured to sequentially determine M-1-P and M-6-P on System 1 and mannose on System 2. The ICS-3000 will need a Dual Gradient Pump (DP) instead of an SP, a diverter valve in the AS Autosampler for two injection lines, two EDs, and two electrochemical cells.*

Chromeleon® Chromatography Management Software

Sample Vial Kit, 0.3 mL polypropylene with caps and septa (P/N 055428)

Centrifuge (Eppendorf® 5400 series)

SpeedVac® evaporator

Sterile assembled micro-centrifuge tubes with screw cap, 1.5 mL (Sarstedt 72.692.005)

Filter unit, 0.2 µm nylon (Nalgene® Media-Plus with 90 mm filter, Nalge Nunc International, P/N 164-0020) or equivalent nylon filter

Vacuum pump

Thermolyne® Dri-Bath heater and heating block

UV/Vis spectrophotometer

REAGENTS AND STANDARDS

Deionized water, Type 1 reagent grade, 18 MΩ-cm resistivity, freshly degassed by ultrasonic agitation and applied vacuum

Use only ACS reagent grade chemicals for all reagents and standards.

Alkaline phosphatase, bovine intestinal mucosa, lyophilized (Aldrich, P/N P6772-2KU)

Bovine serum albumin, essentially fatty acid free, ≥96% (agarose gel electrophoresis), lyophilized powder (Aldrich, P/N A6003)

Mannose ($C_6H_{12}O_6$, Aldrich, P/N M6020) (FW 180.16)

Mannose-1-phosphate, sodium salt hydrate ($C_6H_{12}O_9P$-xNa^+-yH_2O, Aldrich, P/N M1755) (FW 300.15)

Mannose-6-phosphate, sodium salt ($C_6H_{12}O_9PNa$, Aldrich, P/N M3655) (FW 282.12)

pH 7 (yellow) and pH 10 (blue) buffer solutions (VWR International, P/N 34170-130, 34170-133)

Micro BCA™ Protein Assay kit (Pierce Biotechnology, P/N 23250)

Sodium acetate, HPLC grade (CH_3COONa, Aldrich, P/N 71185; Dionex, P/N 059326) (FW 82.03)

Sodium hydroxide, 50% (w/w) (NaOH, Fisher Chemicals, P/N SS254-500)

Trifluoroacetic acid, 1 mL ampoules (CF_3COOH, Aldrich, P/N 91701) (FW 114.02)

Tris (tris-hydroxymethylaminomethane, [$NH_2C(CH_2OH)_3$, Aldrich, P/N 252859] (FW 121.14)

CONDITIONS

M-1-P and M-6-P Determinations

Column:	CarboPac PA200 Analytical, 3 x 250 mm (P/N 062896)
Eluent:	A: 100 mM Sodium hydroxide B: 100 mM Sodium hydroxide, 1 M sodium acetate
Method:	90% A, 10% B
Flow rate:	0.5 mL/min
Column temperature:	30 °C
Tray temperature:	10 °C
Inj. volume:	20 µL
Inj. loop:	100 µL (P/N 030391)
Detection:	Pulsed amperometric detection (PAD), conventional or disposable Au working electrode
Waveform:	Table 1
Reference electrode mode:	AgCl
Typical background:	30 nC
Typical pH:	12.5–12.7
Noise:	30–50 pC
Typical system backpressure:	~2500 psi
Run Time:	30 min

Mannose Determinations[5]

Column:	CarboPac PA20 Analytical, 3 x 250 mm (P/N 060142)
Eluent:	A: 100 mM Sodium hydroxide B: Degassed Type 1 deionized water
Method:	10% A, 90% B from 0–12 min, 100% A, 0% B from 12–20 min, 10% A, 90% B from 20–30 min
Flow rate:	0.5 mL/min
Column temperature:	30 °C
Tray temperature:	10 °C
Inj. volume:	10 µL
Inj. loop:	100 µL
Detection:	Pulsed amperometric detection (PAD), conventional or disposable Au working electrode
Waveform:	Table 1.
Reference electrode mode:	AgCl
Typical background:	20 nC
Typical pH:	11.9–12.1
Noise:	30–50 pC
Typical system backpressure:	~2500 psi
Run time:	30 min

Table 1. Waveform A, Four-Potential Carbohydrate Waveform[4]

Time (s)	Potential (V)	Gain Region*	Ramp*	Integration
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	-2.0	Off	On	Off
0.42	-2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	-0.1	Off	On	Off
0.50	-0.1	Off	On	Off

*Settings required when using the ICS-3000, but not used for older Dionex systems.

PREPARATION OF SOLUTIONS AND STANDARDS

When manually preparing eluents, it is essential to use high quality, Type 1 water, 18.2 MΩ-cm resistivity or better, that contains as little dissolved gas as possible. Degas the deionized water before eluent preparation using a Nalgene filter flask with 0.2 µm nylon filter and applied vacuum. Use degassed Type 1 deionized water for the AS Autosampler flush solution and change weekly.

Preparation of Eluents for M-1-P and M-6-P Determinations

Preparation of Eluent A, 100 mM Sodium Hydroxide Eluent

It is essential to use a high quality, low carbonate sodium hydroxide solution, such as Fisher 50% (w/w) solution, for sodium hydroxide eluent preparation. Sodium hydroxide pellets should never be used for eluent preparation because the pellets are coated with sodium carbonate, which causes poor chromatography. Because sodium carbonate tends to settle on the bottom, do not shake or stir the 50% sodium hydroxide bottle. Remove aliquots from the center of the solution volume, a minimum of 1 in. from the bottom of the bottle. For more information on preparing hydroxide eluents, see Technical Note 71 (Eluent Preparation for High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection).[6] Pipette 10.4 mL of 50% sodium hydroxide

solution into a 2 L plastic eluent bottle containing 1989.6 g of freshly degassed Type 1 deionized water. Immediately cap the bottle, connect it to the Eluent A line, and place the eluent under 4–5 psi of helium or inert gas. Swirl the eluent bottle gently to thoroughly mix the eluent. Prime the pump with the new eluent.

Preparation of Eluent B, 100 mM Sodium Hydroxide, 1 M Sodium Acetate Eluent

To prepare 2 L of 100 mM sodium hydroxide with 1 M sodium acetate eluent, add ~100 mL Type 1 deionized water into a 250 mL HDPE bottle containing 82.04 g of sodium acetate (FW 82.04, Dionex), and shake the bottle until the sodium acetate is fully dissolved. Transfer to a 1 L container and dilute to 1 L with deionized water. Filter and degas the sodium acetate solution using the Nalgene filter flask with 0.2 µm nylon filter and applied vacuum. Transfer all of the filtered sodium acetate solution into a 2 L plastic eluent bottle. Cap the 2 L eluent bottle. Prepare a second liter of sodium acetate solution in the same way. Transfer the second degassed filtered sodium acetate solution into the same 2 L eluent bottle. Pipette 10.4 mL of 50% sodium hydroxide solution into the 2 L eluent bottle containing the filtered sodium acetate solution. Immediately cap the bottle, connect it to the Eluent B line, and place the eluent under 4–5 psi of helium or other inert gas. Swirl the eluent bottle gently to thoroughly mix the eluent. Prime the pump with the new eluent.

Preparation of Eluents for Mannose Determinations

Prepare 100 mM sodium hydroxide (eluent A), degassed Type 1 deionized water (eluent B), and 2 M sodium hydroxide (wash solution) as described above.

Preparation of Standards for M-1-P and M-6-P

Preparation of Stock Standards

To prepare separate solutions of 1.0 mM M-1-P and M-6-P, dissolve 6.0 mg of sodium mannose-1-phosphate hydrate (FW 300.15) or 5.8 mg of sodium mannose-6-phosphate (FW 282.12) solids in 20.0 g of Type 1 deionized water. Store the stock standard solutions at -20 ºC or below.

Preparation of Working Standards

To prepare 0.5, 1, 2, 5, 10, 20, and 50 µM M-1-P and M-6-P combined working standards from 1.0 mM M-1-P and 1.0 mM M-6-P stock standards, pipette 5, 10, 20, 50, 100, 200, and 500 µL of each sugar phosphate into separate 20 mL scintillation vials. Dilute with Type 1 deionized water to 10.0 g total weight. Prepare a 100 µM M-6-P spiking standard solution in a similar way by diluting 500 µL of the 1.0 mM M-6-P stock standard to 5.0 g total weight with Type 1 deionized water. The working standards should be stored at 4 ºC.

Preparation of Standards for Mannose

Preparation of Stock and Working Standards

To prepare 10 mM mannose (FW 180.16) stock solution, dissolve 36.0 mg of mannose in 20.0 g of Type 1 deionized water. To prepare 1, 5, 10, 20, and 50 µM mannose working standards from 10.0 mM mannose, pipette 1, 5, 10, 20, and 50 µL into separate 20 mL scintillation vials. Dilute with Type 1 deionized water to 10.0 g total weight.

Preparation of 50 mM Tris Buffer (pH 9) and Alkaline Phosphatase Working Solutions

To dephosphorylate M-1-P and M-6-P containing samples with alkaline phosphatase, prepare a 50 mM Tris buffer (pH 9) and 2 units/µL alkaline phosphatase mixture according to the instructions in reference 3. To prepare 50 mM Tris Buffer (pH 9), dissolve 606 mg of tris-hydroxymethylaminomethane (Tris, FW 121.14) in 100 mL of Type 1 deionized water. Adjust to pH 9 with dilute HCl. Store the Tris buffer solution at 4 ºC. To prepare 2116 units/mL (2 units/µL) working solution of alkaline phosphatase, pipette 1 mL of Type 1 deionized water into the bottle containing 1.76 mg (entire bottle) of 35% alkaline phosphatase, bovine intestinal mucosa (3436 units/mg protein). Shake gently to dissolve. Store at -20 ºC or below.

Sample Preparation

Bovine Serum Albumin (BSA) Samples

To prepare 5 mg/mL BSA (69 kD), dissolve 25 mg of BSA solid with 5 mL of deionized Type 1 water in a 20 mL HDPE scintillation vial. Swirl the solution until thoroughly mixed. Store stock BSA solutions at -20 ºC or below.

Acid Hydrolysis Samples

Prepare the BSA samples by pipetting 20 µL of 5 mg/mL (µg/µL) BSA (100 µg) into 1.5 mL Sarstedt screw top centrifuge tubes containing 125 µL of concentrated TFA (13.5 M, ampoules) and 125 µL of deionized water. Prepare the blanks similarly with 125 µL of concentrated TFA and 145 µL deionized Type 1 water.

Hydrolyze BSA samples with 6.75 M trifluoroacetic acid (TFA) in the Thermolyne Dri-Bath at 100 °C for 1.5 h.[3] After hydrolysis, quench the vials in ice water, centrifuge at 14,000 rpm for 2 min, and dry using a SpeedVac evaporator at room temperature and vacuum (0.5–1 Torr). Store the dried samples and blanks at -40 °C. Prior to use, thaw samples at room temperature, reconstitute with 200 µL of Type 1 deionized water, and centrifuge at 14,000 rpm for 5 min. Reconstituted TFA-hydrolyzed BSA samples were spiked with 2, 3, 5, or 10 µM M-6-P by pipetting 4, 6, 10 or 20 µL of 100 µM M-6-P, respectively.

Dephosphorylation Samples

To prepare samples for mannose and M-6-P determinations after dephosphorylation, pipette 50 µL of 50 mM Tris buffer (pH 9) and 3 µL of 2 units/µL alkaline phosphatase into a 1.5 mL screw top vial containing reconstituted method blank, TFA-hydrolyzed BSA, or TFA-hydrolyzed BSA with M-6-P samples described in the previous section. Prepare separate 10 µM M-6-P and M-1-P control samples in the same way by using 197 µL of Type 1 deionized water, 50 µL of 50 mM Tris buffer (pH 9), 25 µL of 100 µM M-6-P or 100 µM M-1-P, and 3 µL of 2 units/µL alkaline phosphatase. Prepare 3 µM M-6-P or M-1-P control samples with 7.5 µL of 100 µM M-6-P or 100 µM M-1-P and the same amounts of Tris buffer, water, and alkaline phosphatase as previously described. Digest all samples at 37 °C for 5 h. Quench the vials in ice, centrifuge at 14,000 rpm for 5 min, and dry by SpeedVac evaporator at room temperature and vacuum (0.5–1 Torr) for 2–3 h. Store the dried solutions at -20 °C or below. Reconstitute the samples with 200 µL of Type 1 deionized water prior to analysis.

Heat-Quenched Dephosphorylation Samples

Spike dephosphorylated samples with M-6-P as a control. To prepare these samples, heat-quench the reconstituted dephosphorylated samples and controls in boiling water for 5 min. Then add 5 µL of 100 µM M-6-P to 100 µL of the sample.

Determination of Protein Concentrations

Use the Micro-BCA test kit to measure protein concentrations. Prepare samples and standards for determination of protein concentrations according to the manufacturer's instructions.

SYSTEM PREPARATION AND SETUP

The setup for the individual modules, components, and system is thoroughly described in the ICS-3000 Operator's Manual,[7] ICS-3000 Installation Manual,[8] AS Autosampler Operator's Manual,[9] and the column manuals.[10,11] In this application note, phosphorylated mannose (M-1-P, M-6-P) and mannose are determined separately on one system using different conditions and columns. If desired, the ICS-3000 system can be configured to determine M-1-P and M-6-P on System 1 and mannose on System 2 using sequential mode and partial loop injections. To configure the AS Autosampler in sequential mode, refer to Technical Note 64 (TN 64): Using the AS Automated Sampler in the Simultaneous, Sequential, and Concentrate Modes.[12]

Plumbing the Chromatography System

Use red PEEK (0.127 mm or 0.005 in. i.d.) tubing for all eluent lines from the pump to the cell inlet. Install the CarboPac PA200 column on System 1 for M-6-P and M-1-P determinations, according to the CarboPac PA200 Product Manual[10] and Figure 1. Install a 100 µL loop in DC Inj. Valve 1. Install CarboPac PA20 for mannose determinations in a similar way as CarboPac PA200 according to the CarboPac PA20 Product Manual.[11]

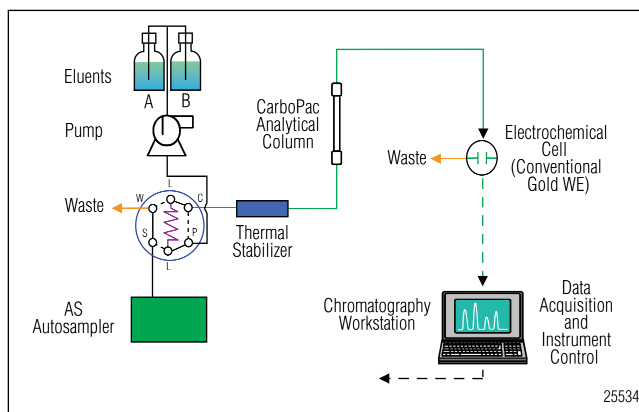

Figure 1. HPAE-PAD system for carbohydrate determinations.

Configuring the AS Autosampler

Configure the AS Autosampler and connect the sample syringe according to the AS Autosampler Operator's Manual.[9] Select the syringe size of the sample syringe from the pull down menus, under the Module Setup Menu/System Parameters. Also select Normal for Sample Mode under System Parameters in the Module Setup Menu. To configure the system in partial loop mode, enter the loop size (100 µL) in Loop Size V1, on the AS front panel, under Module Setup Menu/Plumbing Configuration. Install the injection port tubing into Port S of Inj. Valve 1. Partial loop mode will withdraw a total volume of 30 µL from the sample vial (the sample loop volume of 20 µL plus 2x cut segment volume of 5 µL). Partial loop limited sample mode will withdraw only the sample loop volume (20 µL) from the sample vial. Partial loop, partial loop limited sample injection, and sequential injection modes are thoroughly discussed in the Chromeleon Help program and TN 64. Recent software changes in Chromeleon (>6.8) and the AS Autosampler (>2.1), contrary to TN 64, allow different volumes for the injection lines when in the sequential injection mode. To calibrate the injection lines, follow the instructions under the Calibrate IPTV button located on the Autosampler tab of the panel.

Configuring the System

Install the ED module in the upper DC chamber, above Inj. Valve 1, before turning on the DC and configuring the system. Do not remove or install the ED module while the DC is turned on. To configure the system and create a timebase (named "Mannose6phosphate") refer to the instructions in Application Note 188: *Determination of Glycols and Alcohols in Fermentation Broths Using Ion-Exclusion Chromatography and Pulsed Amperometric Detection* (AN 188).[13] To determine both mannose and M-6-P sequentially, follow the instructions in TN 64.

Configuring Virtual Channel to Monitor pH

It is useful to monitor and record the pH during sample analyses. To continuously record the pH during sample determinations, create a Virtual Channel in Server Configuration according to the instructions in AN 188. The pH virtual channel becomes one of the available signal channels. More information on Virtual Channels can be found in Chromeleon Help program.

Amperometry Cell

Calibration, handling, and installation tips for the reference electrode and conventional gold working electrodes are thoroughly described in the ED Service Procedures of the ICS-3000 Operator's Manual.[7] Handling and installation tips for disposable gold working electrodes are described in the Disposable Electrode Product Manual.[14] To calibrate the combination pH-Ag/AgCl reference electrodes, use pH 7 and pH 10 buffers to determine the pH offset and pH slope, respectively. Follow the instructions in AN 188[13] and under the Calibration button on the ED tab of the Panel.

Assembling the Electrochemical Cell

Assemble the electrochemical cell according the instructions in AN 188. In this application the working electrode is a conventional gold working electrode, but a disposable gold working electrode is also suitable. When used with a recommended waveform and integration, the conventional and disposable gold working electrodes have a background specification of 20 to 50 nC against the reference electrode in AgCl mode. Typically, the background will stabilize within 10 min. However, the pump may cause minute fluctuations in the background for up to an hour after electrode installation. For trace analysis it is advisable to allow for an hour of equilibration before running samples.

Program

To make a new program, use the Program Wizard to enter the parameters from Table 2 and the Conditions section. Enter the title of the program and select Review Program. The new program will open in command mode into a new window. Review and check the program (using the Control, Check commands) for any errors and save it when completed.

Table 2. Entries in Program Wizard to Create a New Program		
Parameter	**Value**	**Section**
Cut (Segment) Volume	5	Sampler Options
Integrated Amperometry	Select	EDet 1 Mode Options
Detector Channels	pH, Pressure, EDet1	Acquisition Options
Amperometry Cell Channels Data Collection Rate pH Lower Limit pH Higher Limit Autozero Waveform Selector	On Select all 2.00 (Hz) 10 13 No Carbohydrate (standard quad)[a] (Reference Electrode Mode: AgCl)	EDet1 Options
Formula Type Step	pH.value Analog Auto, Step	Virtual Channel Options

[a]Waveform from Table 1

RESULTS AND DISCUSSION

To determine the M-6-P content of a glycoprotein with a limited amount of protein requires a sensitive analysis method. HPAE-PAD is a well-established analytical method for selectively determining low concentrations of carbohydrates without derivatization. Native carbohydrates are separated by high performance anion-exchange chromatography and detected by pulsed amperometry with a gold working electrode. HPAE-PAD has been used to assay the M-6-P content of a recombinant therapeutic glycoprotein.[3] Lacking a commercially available M-6-P containing protein, we used acid-hydrolyzed BSA as a generic protein matrix and added M-6-P to show the feasibility of determining the M-6-P content of a glycoprotein.

M-1-P and M-6-P Method Optimization and Evaluation

The CarboPac PA200 column was selected for determination of M-6-P in proteins because it delivers the highest resolution separations of neutral and charged oligosaccharides, making it ideal for sugar phosphate determinations. The eluent conditions were optimized at the standard flow rate of the column (0.5 mL/min) to obtain the shortest possible retention time for M-6-P after most of the hydrolyzed protein had eluted from the column. Changing the sodium acetate concentration changes the M-6-P retention time on the PA200 (i.e., increased sodium acetate concentration results in less M-6-P retention). Experiments showed that 100 mM sodium hydroxide and 100 mM sodium acetate eluent conditions were optimal, and produced short elution times and good resolution from most of the protein. M-1-P was included in this evaluation because a direct simultaneous assay of M-1-P and M-6-P is ideal for determining the activity of phosphomannomutase.[1] Figure 2 shows a separation of 200 pmol of M-1-P and M-6-P. Both M-1-P and M-6-P peaks show good chromatography as indicated by peak asymmetry (1.1–1.3 EP).

To evaluate this method, we determined the relationship between peak area response and concentration, the peak-to-peak noise, and method detection limits (MDLs). To determine the relationship between peak area response and concentration, we calibrated with triplicate injections of seven combined M-1-P and M-6-P standards prepared at 0.5, 1, 2, 5, 10, 20, and 50 µM. The corresponding amounts of 20 µL injections were 10, 20, 40, 100, 200, 400, 1000 pmol, respectively. The peak area responses were linear for M-1-P and M-6-P concentrations, with the r^2 values of 0.9996 and 0.9998, respectively. Peak-to-peak noise was determined from 40 to 60 min in triplicate 60 min sample runs without a sample injection. The average noise was 37.6 pC. The calculated MDLs for M-1-P and M-6-P were 0.11 ± 0.01 µM (2.2 ± 0.2 pmol, n = 7), S/N = 3.

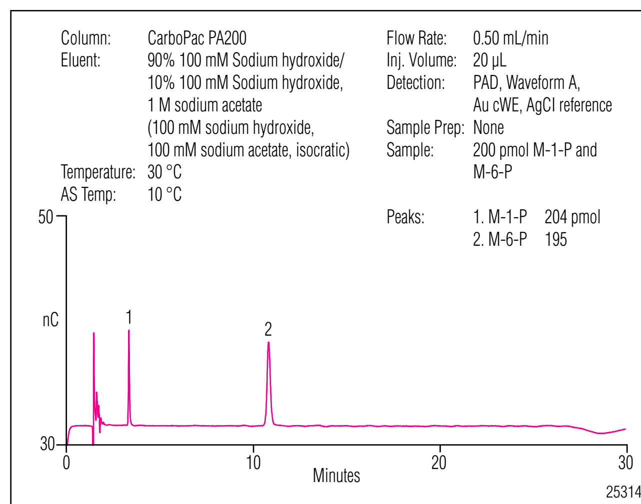

Figure 2. Separation of M-1-P and M-6-P.

Mannose Method Optimization and Evaluation

This method follows the conditions described in TN 40 with the exception that, instead of using an electrolytically generated gradient, manually prepared 100 mM sodium hydroxide eluent was proportioned with water to achieve the separation conditions. Manually prepared eluent was used to facilitate switching between the M-6-P and mannose methods. Mannose was separated on the CarboPac PA20 at 0.5 mL/min and detected by PAD. Manually prepared eluent will have a higher carbonate concentration than an electrolytically generated eluent. Therefore the wash and equilibration times were extended as compared to the method described in TN 40. To evaluate this method, we determined the relationship between peak area response and concentration, the peak-to-peak noise, and MDL. To determine the relationship between peak area response and concentration, we calibrated with duplicate injections of seven standards prepared at 0.5, 1, 2, 5, 10, 20, and 50 µM (5, 10, 20, 50, 100, 200, and 500 pmol respectively, using a 10 µL injection). The peak area responses were linear with $r^2 = 0.9990$. The MDL was determined in the same way as previously described. The results were similar to those previously reported in TN 40, 0.3 µM (3 pmol)

Table 3. Recoveries of Mannose-6-Phosphate and Mannose in TFA-Hydrolyzed BSA Samples

	Mannose-6-Phosphate[a]				Mannose[b]			
Sample	**Amount Added µM (pmol)**	**Amount Measured µM (pmol)**	**RSD[c]**	**Recovery (%)**	**Amount Added µM (pmol)**	**Amount Measured µM (pmol)**	**RSD[c]**	**Recovery (%)**
TFA Method Blank	11.3 (226)	11.3 ± 0.4 (226 ± 8)	3.3	99.6	—	—	—	—
BSA + TFA	10.2 (204)	9.64 ± 0.1 (193 ± 2)	0.1	94.9	—	—	—	—
	20.3 (406)	20.2 ± 0.8 (404 ± 1)	3.9	99.4	—	—	—	—
BSA + TFA + Alkaline Phosphatase (AP) Digestion	—	ND[d]	—	—	1.1[e] (11)	1.0 ± 0.09 (10.5 ± 0.8)	8.3	95.2
BSA + TFA + AP Digestion	—	ND	—	—	2.2[e] (22)	2.07 ± 0.02 (20.7 ± 1.7)	8.5	94.3
2.8 µM M-6-P + AP Digestion	—	ND	—	—	2.8[e] (28)	2.74 ± 0.08 (27.4 ± 0.8)	2.9	91.3
32.6 pmol M-6-P + AP Digestion + Heat Quenching	5.08 (102)	4.89 ± 0.13 (97.9 ± 0.5)	2.6	95.9	—	ND	—	—
BSA + TFA + AP Digestion + Heat Quenching	5.08 (102)	4.65 ± 0.11 (92.9 ± 0.05)	2.4	91.0	—	ND	—	—

[a]20 µL injection
[b]10 µL injection
[c]n = 3
[d]ND = not detected
[e]M-6-P was added prior to dephosphorylation

Determinations of M-6-P in Acid-Hydrolyzed BSA

To determine M-6-P in proteins, 10 and 20 µM (200 and 400 pmol using a 20 µL injection) of M-6-P were spiked into 90 ng (130 pmol) samples of previously dried and reconstituted acid-hydrolyzed BSA (Figure 3B). The amount of M-6-P added would represent 1.54 and 3.08 mol of M-6-P per mol of BSA, if BSA was a M-6-P containing glycoprotein. The TFA method blanks were spiked in the same way with 10 µM (200 pmol) of M-6-P (Figure 3A). The chromatograms show good peak shape and good resolution from neighboring peaks. The measured concentrations of M-6-P were 193 ± 2 and 404 ± 1 pmol, respectively in the TFA-hydrolyzed BSA and 226 ± 8 pmol in the method blank (Table 3). The M-6-P spiked BSA samples had good accuracy, 94.9 and 99.4% recoveries for 10 and 20 µM additions, respectively. There was also good M-6-P recovery of 99.6% for 10 µM added to the TFA method blank. In this example, M-6-P elutes after most of the hydrolyzed BSA. When developing an assay for a M-6-P containing protein, eluent strength may need to be modified to prevent interference with the M-6-P peak.

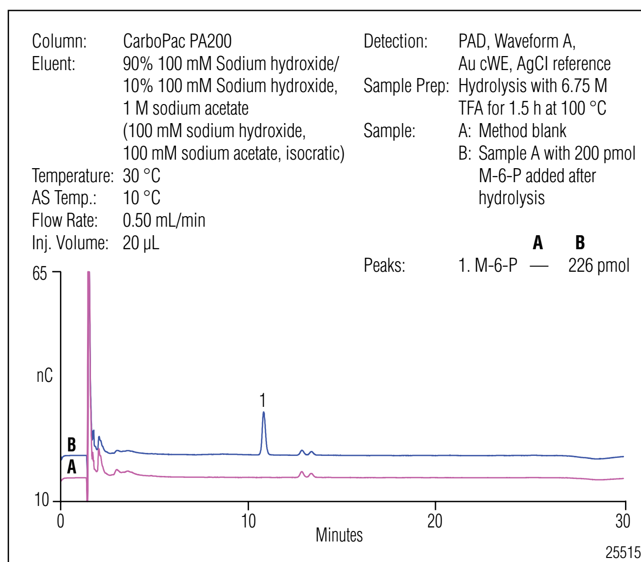

Figure 3A. Comparison of A) A TFA-hydrolysis blank, and B) blank spiked with 200 pmol of M-6-P after hydrolysis.

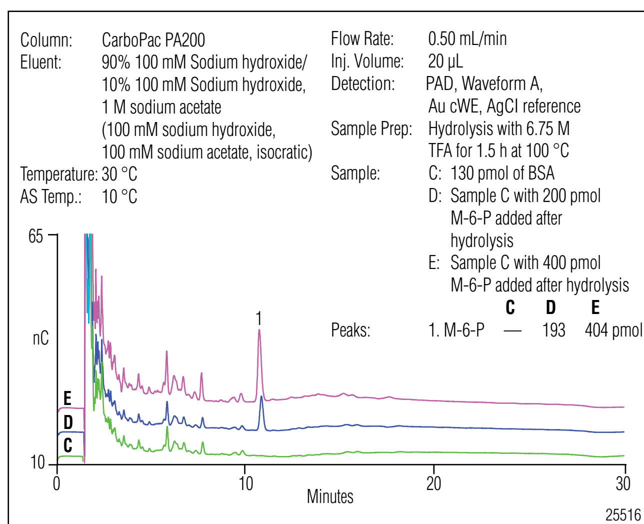

Figure 3B. Comparison of C) BSA after TFA hydrolysis, and D) and E) two TFA-hydrolyzed BSA samples spiked with M-6-P.

Determinations of M-6-P and Mannose in Dephosphorylated Acid-Hydrolyzed BSA

To confirm that the M-6-P peaks in the acid-hydrolyzed BSA samples are M-6-P, acid-hydrolyzed BSA samples spiked with 1.7 and 2.8 µM M-6-P (33 and 56 pmol for a 20 µL injection) were dephosphorylated with 6 units of alkaline phosphatase in Tris buffer. An assay of these solutions revealed the absence of the M-6-P peak, as expected (Figure 4A). Mannose concentrations determined in 10 µL injections of the same solutions (17 and 28 pmol mannose from the M-6-P) were 19.3 ± 1.1 and 27.4 ± 0.8 pmol (Figure 4B). The chromatograms confirmed the presence of mannose. The calculated recoveries of mannose from the dephosphorylation of M-6-P for the standard and BSA samples were 113.5 and 91.3%, respectively (Table 3).

To demonstrate that column overload by the alkaline phosphatase buffer is not the reason why M-6-P was not detected in the alkaline phosphatase-treated samples, the samples were heat-quenched to denature the alkaline phosphatase and spiked with 5 µM (100 pmol) M-6-P (Figure 5). The recovery of M-6-P in this experiment was 91.0%.

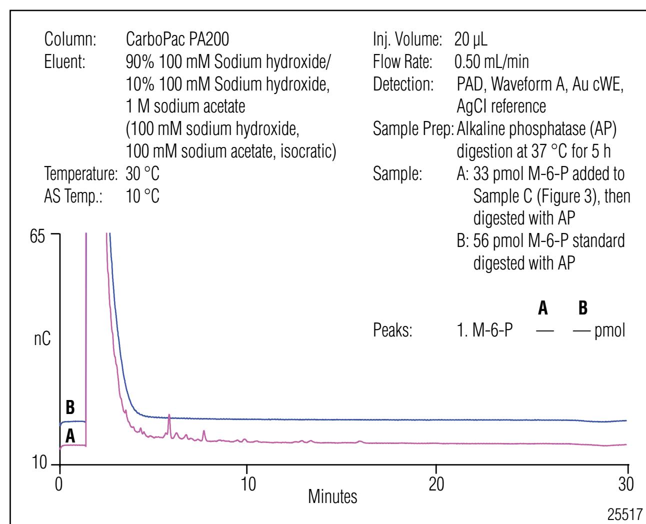

Figure 4A. The absence of M-6-P after alkaline phosphatase digestion.

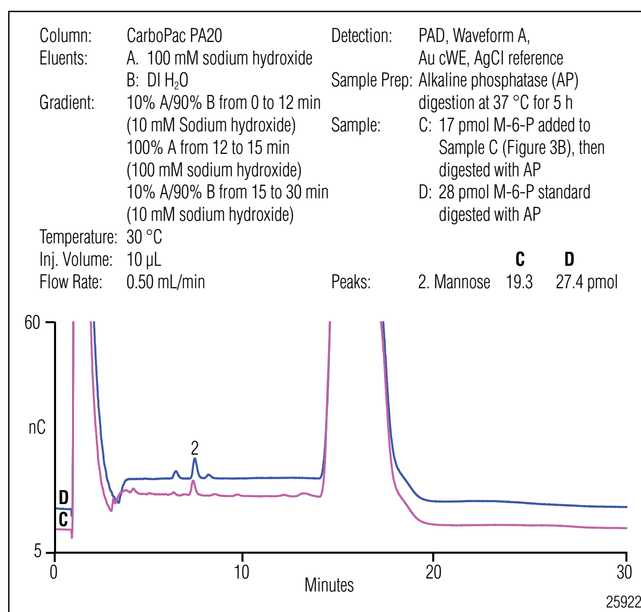

Figure 4B. Assay of mannose in alkaline phosphatase-treated samples.

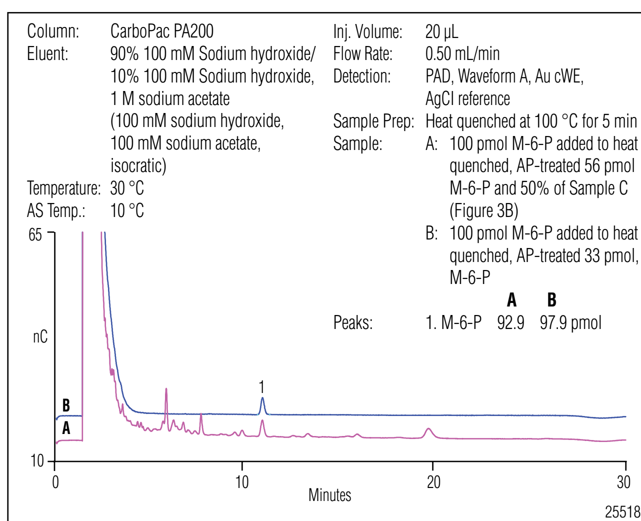

Figure 5. 100 pmoles of M-6-P spiked into heat quenched alkaline phosphatase-treated samples.

CONCLUSION

This application note demonstrates sensitive and accurate determinations of M-6-P spiked into acid hydrolyzed BSA using HPAE-PAD. The presence of M-6-P can be easily confirmed by treating the hydrolyzate with alkaline phosphatase and assaying for mannose product and for the disappearance of M-6-P. This assay, or minor modifications thereof, can be used to determine the M-6-P content of a glycoprotein. All three HPAE-PAD assays that included M-6-P, loss of M-6-P, and appearance of mannose require no sample derivatization.

PRECAUTIONS

It is important that the reference electrode not be allowed to dry out, especially when the cell is on. If the system will not be used for several days, reduce the eluent flow to 0.2 or 0.3 mL/min. If the system will be inactive for more than a week, disassemble the cell and remove and store the reference electrode in saturated KCl.

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