Application Note 1006

Determination of Carbohydrates in Urine by HPAE-PAD

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High-Performance Anion-Exchange (HPAE) Chromatography, Pulsed Amperometric Detection (PAD), Mannitol, Rhamnose, Lactulose

Introduction

There are many methods to evaluate carbohydrates present in urine. Carbohydrates can be fluorescently labeled and then determined by high-performance liquid chromatography (HPLC) or derivatized and analyzed by gas chromatography-flame ionization detection (GC-FID). However, the derivatization reaction adds reagent costs and time to the sample analysis.

Another alternative is to determine the carbohydrates with direct detection by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD). Many HPAE-PAD methods have been published that evaluate concentrations of mannitol or rhamnose, xylose, 3-O-methylglucose, and lactulose in both urine and serum.[1-11]

In this work, two HPAE-PAD methods are compared for analysis of urine samples. The first method, using a Thermo Scientific Dionex CarboPac PA20 Analytical Column, minimizes eluent preparation with a Reagent-Free™ ion chromatography (RFIC™) eluent generator. Although previous methods have been published using the Dionex CarboPac™ PA1,[1-3] PA100,[4-5] and PA10[6-7] columns, the method described here takes advantage of the high resolution and short column format of the Dionex CarboPac PA20 column to separate within 16 min nine common carbohydrates that may be present in serum or urine samples.

This method illustrates resolution of the commonly measured carbohydrate probes as well as other potential carbohydrates of interest, including lactulose and lactose. If other carbohydrates are chosen, or resolution of endogenous carbohydrates is not necessary, shorter methods may be possible.

The second method discussed in this work takes advantage of the selectivity and high capacity of the Dionex CarboPac MA1 column. Use of this column allows mannitol to be strongly retained with minimal interferences. These advantages have led to many publications.[8-11]

Both methods are shown to successfully determine carbohydrates in urine with good precision and minimal matrix effects from the samples. However, the first method saves time, reagent costs, and adds the convenience and cost savings of eluent generation.

Goal

Demonstrate mono- and disaccharide determination in urine by HPAE-PAD methods.

Thermo SCIENTIFIC

Equipment

- Thermo Scientific Dionex ICS-5000 or ICS-3000 system, including:
  - SP Single Pump or DP Dual Pump module
  - EG Eluent Generator module
  - DC Detector/Chromatography compartment
  - AS or AS-AP Autosampler
  - ED Electrochemical Detector (P/N 061719)
  - Electrochemical Cell (P/N 061757)
  - Gold on PTFE Disposable Electrode (P/N 066480)
  - pH, Ag/AgCl Reference Electrode (P/N 061879)
  - EGC III KOH Potassium Hydroxide Eluent Generator Cartridge (P/N 074532)
  - CR-ATC Continuously Regenerated Anion Trap Column (P/N 060477)
  - EG Vacuum Degas Conversion Kit (P/N 063353)
  - 10 µL PEEK™ Sample Loop (P/N 042949)
- Vial Kit, 0.3 mL Polyprop with Caps and Septa (P/N 055428)
- Vial Kit, 1.5 mL Polyprop with Caps and Septa (P/N 061696)
- Polypropylene Microcentrifuge Screw Cap Tubes, 1.5 mL (Sarstedt® P/N 72.692.005)
- Thermo Scientific Nalgene 1000 mL, 0.2 µm Nylon Filter Units (P/N 09-740-46)
- Thermo Scientific Dionex Chromeleon Chromatography Data System software version 7.0 or higher

Reagents and Standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistivity or better
- Sodium Hydroxide Solution, 50% (w/w) (Fisher Scientific P/N SS254-500)
- Lactulose (Fisher Scientific P/N AC22593-1000)
- β-Lactose (Fisher Scientific P/N 50-701-7415)
- L-(+)-Rhamnose Monohydrate (Fisher Scientific P/N AC17408-0250)
- D-Mannitol (Fisher Scientific P/N BP686-500)
- Sucrose (Fisher Scientific P/N S6-500)
- D-Glucose (Fisher Scientific P/N D15-500)
- Synthetic Urine Solution (Fisher Scientific P/N 83611)
- 3-O-methyl-D-glucose (Fisher Scientific P/N AC22695-0010)
- D-(+)-Galactose (Fisher Scientific P/N AC15061-1000)
- D-(+)-Xylose (Fisher Scientific P/N BP708-250)
- D-(+)-Cellobiose (Fisher Scientific P/N AC10846-0250)
- D-(-)-Ribose (Fisher Scientific P/N AC13236-0250)

Samples

Synthetic Urine Solution spiked with carbohydrates to mimic mammalian urine samples

Conditions: Method 1

Columns:	Dionex CarboPac PA20 Analytical (3 × 150 mm)
Eluent Gradient:	10 mM KOH from -7 to 1 min, 10–30 mM KOH from 1–9 min, 30–35 mM KOH from 9–16 min
Eluent Source:	Dionex EGC III KOH with CR-ATC Or: 100 mM NaOH, manually prepared
Flow Rate:	0.5 mL/min
Inj. Volume:	10 µL (full loop)
Temperature:	30 °C (column and detector compartments)
Detection:	Pulsed amperometric, disposable Au on PTFE electrode
Background:	~45 nC (using the carbohydrate 4-potential waveform)
Noise:	~30 pC
System Backpressure:	~2400 psi

Carbohydrate 4-Potential Waveform for the ED

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

*Setting required in the Dionex ICS-3000/5000 system but not used in older Dionex systems; reference electrode in Ag mode (Ag/AgCl reference)[12]

Conditions: Method 2

Columns:	Dionex CarboPac MA1 Analytical (4 × 250 mm) and Guard (4 × 50 mm)
Eluent:	480 mM NaOH
Eluent Source:	1.0 M NaOH, manually prepared
Flow Rate:	0.4 mL/min
Inj. Volume:	25 µL (full loop)
Temperature:	30 °C (column and detector compartments)
Detection:	Pulsed amperometric, disposable Au on PTFE electrode
Background:	~37 nC (using the carbohydrate 4-potential waveform)
Noise:	~50 pC
System Backpressure:	~1345 psi

Preparation of Solutions and Reagents

Eluent Solutions: Method 1

Generate the potassium hydroxide (KOH) eluent on line by pumping high-quality degassed DI water through the Dionex EGC III KOH cartridge. The Chromeleon™ software will track the amount of KOH used and calculate the remaining lifetime.

If desired, the method can be executed with manually prepared eluents—NaOH is used in place of KOH. Add 5.2 mL of 50% w/w NaOH to 994.8 mL of degassed DI water to prepare 1 L of 100 mM NaOH. Proportion the 100 mM hydroxide solution with DI water to produce the described hydroxide gradient. See Dionex (now part of Thermo Scientific) Technical Note 71 for detailed information on manual eluent preparation.[13]

Eluent Solutions: Method 2

Add 52.0 mL of 50% w/w NaOH to 948.0 mL of degassed DI water to prepare 1 L of 1.0 M NaOH. Proportion the 1.0 M NaOH solution with degassed DI water to generate 480 mM NaOH.

Stock Standard Solutions

Prepare stock standards as shown in Table 1 to generate individual 100 mg/mL, nominal, stocks. Prepare mannitol at a concentration of 50 mg/mL.

Internal Standard Stock Solutions

Method 1: Add 10.71 mg of ribose to 100 µL of DI water.

Method 2: Add 145.31 mg of cellobiose to 2906 µL of DI water.

Working Standard Solutions

Method 1: Add 4.90 mL (4947 mg) of DI water to a 7 mL vial. Add 20.00 µL of 50 mg/mL mannitol stock to the vial. Add 10.00 µL each of rhamnose, 3-*O*-methylglucose, xylose, lactulose, lactose, galactose, sucrose, and glucose to the vial. This will be a 200 µg/mL intermediate stock of each carbohydrate. Add 50.0 µL of the combined stock to 950 µL of DI water to prepare a 10.0 µg/mL intermediate stock.

Method 2: Add 4.90 mL of DI water to a 7 mL vial. Add 20.00 µL of 50 mg/mL mannitol stock to the vial. Add 10.00 µL each of 3-*O*-methylglucose, xylose, lactose, and lactulose to the vial. Add 40 µL of DI water to the vial. This combined stock will contain 200 µg/mL of each of the added carbohydrates.

Dilute these two intermediate stocks as necessary to generate standard curves for Method 1 and Method 2 as detailed in Table 2.

Table 1. Calibration standard stock solution preparation amounts.

Analyte	Analyte Mass (mg)	DI Water Volume, Mass (µL, mg)	Stock Concentration (mg/mL)
Rhamnose	89.47	893.7	100
3-*O*-methylglucose	91.76	920.2	99.7
Lactulose	99.54	993.3	100
Xylose	100.8	1012	99.7
Mannitol	58.41	1167	50.0
Lactose	256.8	2568	100
Sucrose	268.4	2684	100
Glucose	160.0	1600	100
Galactose	134.9	1349	100

Table 2. Volumes used for preparing working calibration standards for two calibration ranges used for the two independent methods discussed in this study.

Calibrant Concentration (µg/mL)	Method 1			Method 2	
	10 µg/mL Stock (µL)	1.00 µg/mL Standard (µL)	DI Water (µL)	200 µg/mL Stock (µL)	DI Water (µL)
0.050		15.0	285		
0.10		30.0	270		
0.25		50.0	150		
0.50		100	100	0.50	199
1.00	100		900	1.00	199
1.67	25.0		125		
2.50	50.0		150		
5.00	100		100	5.0	195
10.0				10.0	190
25.0				25.0	175
50.0				50.0	150
100				100	100
150				150	50.0

Spiked Urine Preparation

Add 4.70 mL of synthetic urine to a 7 mL polypropylene vial. Add 100.0 µL of 50.0 mg/mL mannitol and 50.0 µL each of 100 mg/mL rhamnose, 3-*O*-methylglucose, xylose, and lactulose to the synthetic urine.

Sample Preparation: Method 1

Pipet 500 µL of sample into a 100 mL Class A polypropylene or polymethylpentene (PMP) volumetric flask containing ~80 mL of DI water. Fill the flask to the mark with DI water, cap the flask, and invert several times to mix the solution.

Further dilute the samples as necessary to remain within the linear response range of the carbohydrate standards. For example: Transfer 100 µL of sample into a 300 µL injection vial containing 200 µL of DI water. Cap the vial and vortex it to mix the solution. Inspect the solution to ensure that no air bubbles are trapped at the bottom of the vial after vortexing.

Sample Preparation: Method 2

Pipet 500 µL of sample into a 100 mL Class A polypropylene or PMP volumetric flask containing ~80 mL of DI water. Fill the flask to the mark with DI water, cap the flask, and invert several times to mix the solution. Transfer a 1 mL aliquot of sample into a 1.5 mL polypropylene injection vial.

Precautions

Carbohydrates have limited stability unless sterility is maintained. Store solutions and samples at -40 °C. Avoid multiple freeze/thaw cycles to preserve the carbohydrates. Mannitol solubility at low temperatures is limited. Do not prepare a 100 mg/mL stock of mannitol because it will precipitate during freezing. Ensure that all carbohydrate solutions are well mixed after thawing stock standards prior to preparing working standards and spiking solutions.

When using the Dionex ICS-5000 EG Eluent Generator module, install the vacuum degas conversion kit (P/N 063353). This degasser will remove gasses generated by the EG and help maintain a stable baseline. This kit is not necessary when preparing eluents manually. In addition, when using the EG module, a Dionex CarboPac PA20 guard column should not be installed because there is potential for the system pressure to exceed 3000 psi.

For Method 1, perform a column-wash every 40 injections during a sequence to maintain column performance. The column wash consists of 40 min of 100 mM hydroxide generated by the EG or proportioned from manual eluent. This wash will preserve both stable retention times and assist in maintaining a clean electrode.

The Dionex CarboPac MA1 column is a high-capacity column; when it is first installed, it will require adequate equilibration time at elution conditions before injecting samples. A minimum of 4 h of equilibration is recommended. For convenience, the column may be equilibrated overnight before samples are injected to ensure the most stable retention times.

Health Precaution

When working with human- or animal-derived fluids, procedures must be performed in accordance with the CDC's biosafety level protocol. See the CDC publication "Biosafety in Microbiological and Biomedical Laboratories" for guidance.[14]

Results and Discussion

Separation: Method 1

Figure 1 illustrates the separation of 10 carbohydrates on the Dionex CarboPac PA20 column. By using a hydroxide gradient, six of the 10 carbohydrates are fully resolved. Mannitol, 3-*O*-methylglucose, rhamnose, xylose, and lactulose—the most common probes of intestinal permeability—are generally well resolved from one another. Lactose is included in the standards for reference. This carbohydrate is unlikely to be present in urine samples.

The total analysis time is 16 min with an additional 7 min equilibration prior to sample injection. In Figure 1, ribose is included as a potential internal standard candidate for methods that require more complex sample preparation, as is common with analysis of serum samples.[5]

Glucose is well separated from all five of the indicative carbohydrates and although it is not commonly present in the urine, if it is present, it is unlikely to interfere with sample analysis. Mannitol is weakly retained, which can potentially lead to interferences from the samples. Rhamnose tests the same physiological processes as mannitol, and under these separation conditions, can be used as an additional probe of passive transcellular diffusion in intestinal permeability.

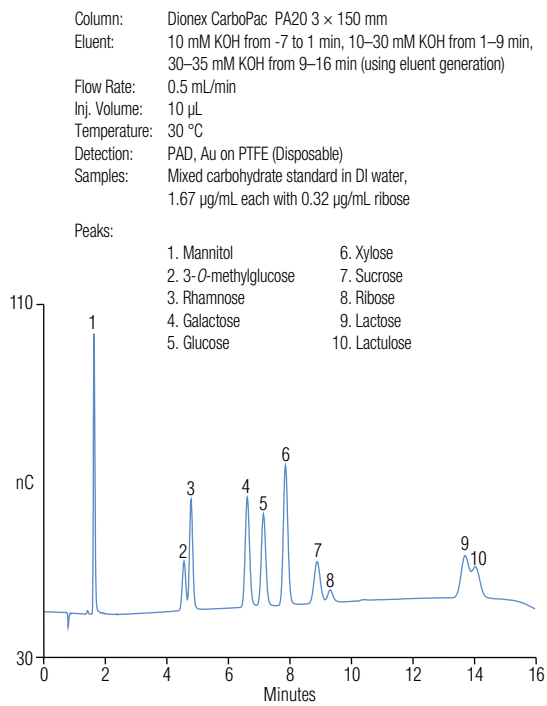

Figure 1. Separation of ten carbohydrates of interest using a gradient elution from the Dionex CarboPac PA20 column.

Table 3. Linearity (0.050–2.5 µg/mL for each carbohydrate), precision, LOQs (as 10 times SN), and recoveries from synthetic urine for nine chosen carbohydrates when samples are analyzed on the Dionex CarboPac PA20 column.

Analyte	Retention Time (min)	Retention Time Precision (RSD)	Peak Area (nC • min)	Peak Area Precision (RSD)	Coeff. of Determin. (r^2)	Recovery (%)	LOQ (µg/mL)
Mannitol	1.62	0.27	1.918	1.16	1.00	95.9	0.006
3-*O*-methylglucose	4.56	<0.01	0.7491	0.54	1.00	112	0.030
Rhamnose	4.79	0.08	1.449	1.14	1.00	103	0.020
Galactose	6.63	0.07	2.398	0.25	1.00	93.8	0.020
Glucose	7.16	<0.01	2.169	0.54	1.00	113	0.020
Xylose	7.88	0.05	3.280	0.45	1.00	97.3	0.010
Sucrose	8.93	0.05	1.208	0.61	1.00	95.5	0.050
Lactose	13.76	0.05	1.907	0.99	0.999	99.6	0.030
Lactulose	14.09	0.05	1.525	1.47	1.00	101	0.050

Quantification Linearity, Precision, and Limits of Quantification: Method 1

Table 3 shows the retention times for the nine carbohydrates used for calibration. Retention times were stable with RSDs generally <0.08. The primary exception was mannitol, which has a short retention time of 1.62 min. Due to the short retention time, small changes result in a larger RSD of 0.27.

Peak area precisions were evaluated by seven injections of a standard composed of 1.0 µg/mL of each carbohydrate. Peak area precisions were excellent with RSDs <1.5. Limits of quantification (LOQs) were evaluated for the nine chosen carbohydrates. For most of the targeted carbohydrates, the limits of detection will not be critical due to strong absorption by the small intestine with minimal metabolism. However, for lactulose, the amounts absorbed can be much smaller and will depend on the integrity of the small intestine. The lactulose LOQ was 0.050 µg/mL, or 500 pg for a 10 µL injection.

LOQs for the other carbohydrates are listed in Table 3. Linear ranges for the carbohydrates varied, but each was linear with coefficients of determination ≤0.999 within the range of 0.050–2.5 µg/mL as shown in Table 3. Example calibration curves are shown in Figure 2.

Sample Analysis: Method 1

Figure 3 shows the chromatogram overlay of two samples: a control of DI water spiked with five carbohydrates of interest and a sample of synthetic urine spiked in the same manner. This figure shows that retention times and response are not impacted by the presence of the urine matrix; the two chromatograms are nearly identical. The major differences observed are around mannitol, where there is a baseline disturbance from the salts and urea in the synthetic urine. The ability to quantify mannitol is not impacted by this disturbance.

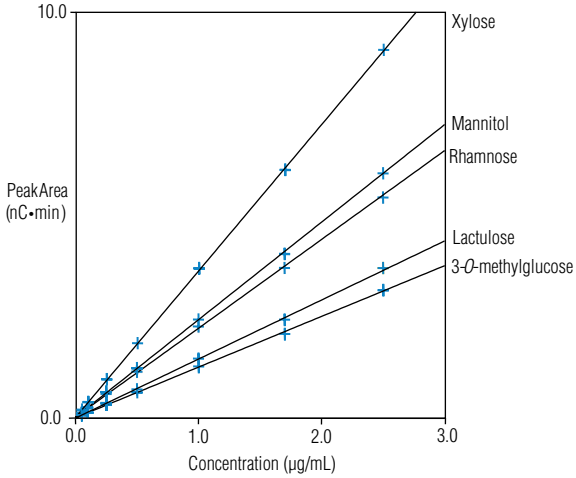

Figure 2. Calibration curves for the five carbohydrates most frequently used to evaluate intestinal permeability; all coefficients of determination are ≥0.999.

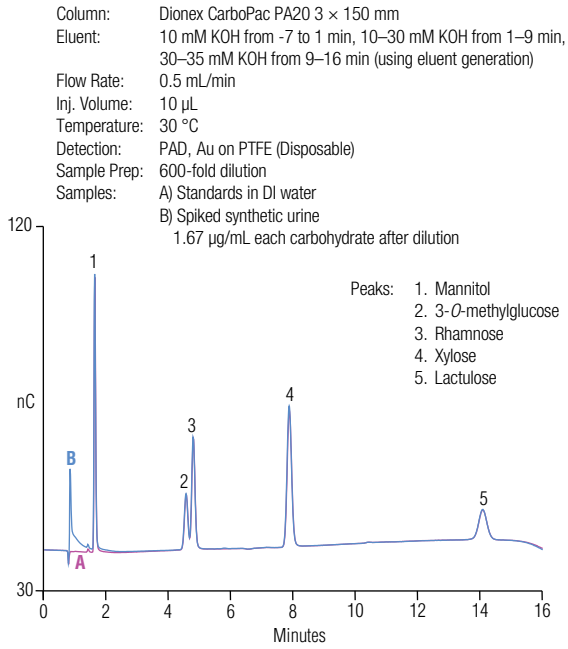

Figure 3. Comparison of standards in DI water and synthetic urine spiked with 1.67 µg/mL of each of the five commonly used carbohydrates for intestinal permeability evaluation.

Table 4. Method precision and accuracy evaluations: both intraday and between-day precisions as well as accuracy, as measured by recoveries, are reported.

	Analyte	Amount Spiked (mg/mL)	Amount Determined (mg/mL)	Peak Area Precision (RSD)	Recovery (%)	Intraday Precision (RSD)	Between-Day Precision (RSD)
Replicate #1	Mannitol	1.67	1.67	2.82	99.8	2.80	3.64
	3-*O*-methylglucose	1.67	1.75	2.07	105	2.39	3.70
	Rhamnose	1.67	1.84	1.81	110	2.52	2.66
	Xylose	1.67	1.69	3.36	102	3.21	4.47
	Lactulose	1.67	1.71	2.57	102	2.38	2.95
Replicate #2	Mannitol	1.67	1.68	0.30	100		
	3-*O*-methylglucose	1.67	1.83	0.31	109		
	Rhamnose	1.67	1.84	1.19	110		
	Xylose	1.67	1.78	1.66	107		
	Lactulose	1.67	1.73	0.53	104		
Replicate #3	Mannitol	1.67	1.64	0.26	98.0		
	3-*O*-methylglucose	1.67	1.81	0.12	108		
	Rhamnose	1.67	1.81	0.25	108		
	Xylose	1.67	1.73	0.57	104		
	Lactulose	1.67	1.70	0.23	102		
Low-level lactulose spike	Mannitol	1.67	1.68	0.23	100		
	3-*O*-methylglucose	1.67	1.79	0.68	107		
	Rhamnose	1.67	1.84	0.33	110		
	Xylose	1.67	1.75	0.24	105		
	Lactulose	0.33	0.35	2.48	105		

Sample Analysis Precision and Accuracy: Method 1

Synthetic urine samples were analyzed for three days using samples that were freshly spiked each day. Table 4 shows the results for one day of analysis as well as precision results for three days of triplicate sample analysis (nine total samples). Because lactulose will be present at reduced concentrations compared to the other intestinal permeability probes, a sample was also prepared with a low-level lactulose spike. Precision between triplicate sample preparations ranged between 2.4 and 3.2 as RSD. Recoveries of 85–115% were determined across three days of sample analysis (data not shown).

Separation: Method 2

Figure 4 shows the separation of synthetic urine, standards in DI water, and spiked synthetic urine when separated on the Dionex CarboPac MA1 column. This column is well suited for retaining sugar alcohols such as mannitol. As shown in Figures 4A and 4C, mannitol is significantly retained on this column with retention times longer than those of 3-O-methyl glucose. Xylose and lactulose are nearly baseline resolved with a resolution of 1.5. Under the conditions described here, 3-O-methylglucose and rhamnose will coelute; however, with the strong retention of mannitol, it is not necessary to incorporate rhamnose as a probe of intestinal permeability.

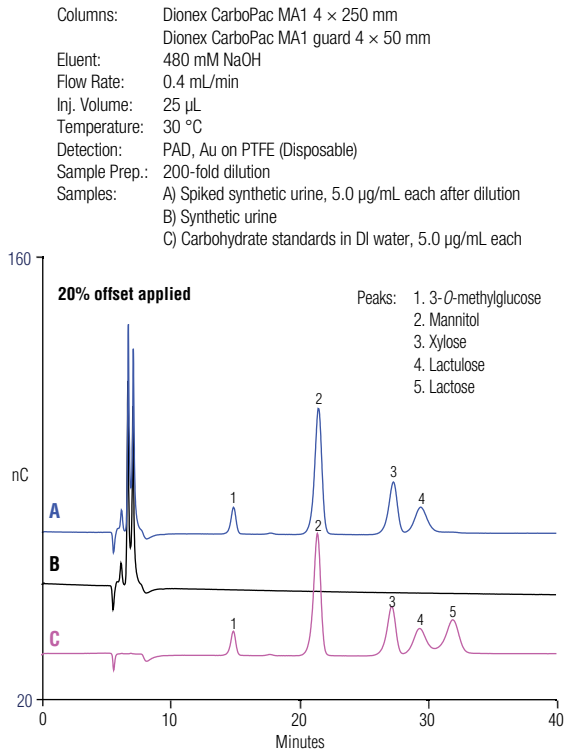

Figure 4. Comparison of the separation of carbohydrate standards in A) synthetic urine without added lactose and C) DI water with added lactose, on the Dionex CarboPac MA1 column: Chromatogram B) shows an unspiked synthetic urine sample for comparison. Note the excellent resolution between the matrix components and the first eluting carbohydrate probe, 3-*O*-methylglucose.

Quantification Linearity, Precision, and Limits of Quantification: Method 2

Due to the increased hydroxide eluent concentration for this isocratic method, the linear range of the carbohydrates of interest is expanded. As shown in Table 5, the calibration of four carbohydrates used as probes are each linear, with coefficients of determination >0.999 between 0.5 and 150 µg/mL. Retention times are stable, with precision, as RSD of <0.14. Peak area precision is <2 RSD for each carbohydrate studied.

Sample Analysis: Method 2

Spiked samples of synthetic urine were analyzed to evaluate recoveries for this method. Results for one day of analysis are presented in Table 6. Recoveries were excellent, ranging between 90.6 and 107%. Although this method is more time consuming than the previous method discussed, the high capacity of the Dionex CarboPac MA1 column reduces the need for dilution, and the method is isocratic with only one eluent to prepare. Furthermore, the resolution of lactulose and lactose is improved on this column in comparison to the separation on the Dionex CarboPac PA20 column. Cellobiose has been reported as an internal standard candidate and is an appropriate choice for these conditions, eluting after lactose at 47.5 min (data not shown).[11]

Method Comparisons

Both methods described in this work will separate the carbohydrates of interest in urine samples. However, each method has distinct advantages. Method 1 is much faster in terms of sample analysis with a total analysis time of 23 min between samples compared to 40 min for Method 2. Method 1 also takes advantage of the RFIC capability of the Dionex ICS-5000 system, allowing continuous operation without preparation of eluents. Due to the weak retention of mannitol, rhamnose—which probes the same biological pathway—is recommended as a mannitol substitute when analyzing samples using Method 1.

In contrast, Method 2 provides a simple isocratic elution method that has an extended calibration range compared to Method 1. Method 2 retains mannitol much longer, thereby reducing potential interferences; however, the high capacity of this column leads to longer retention times compared to Method 1. Both methods have good recoveries, suggesting accuracy, with excellent peak area and retention time precisions. No matrix effects were observed with samples injected at the dilutions described here.

Table 5. Linearity (0.500–150 µg/mL for each carbohydrate) and precision data for carbohydrates determined using the Dionex CarboPac MA1 column (25 µL injection volume): precision was evaluated with 1.00 µg/mL standards.

Analyte	Coeff. of Determination (r^2)	Retention Time (min)	Retention Time Precision (RSD)	Peak Area (nC • min)	Peak Area Precision (RSD)
3-*O*-methylglucose	0.999	15.18	0.04	0.7861	1.12
Mannitol	1.00	21.90	0.02	5.426	1.11
Xylose	1.00	28.11	0.04	2.746	1.30
Lactulose	1.00	30.51	0.14	2.068	1.48
Lactose	1.00	33.25	0.05	5.426	1.11

Table 6. Recoveries from 1/200 dilutions of synthetic urine spiked with four carbohydrates and analyzed using the Dionex CarboPac MA1 column.

Replicate Number	Analyte	Retention Time (min)	Amount Spiked (µg/mL)	Amount Determined (µg/mL)	Recovery (%)	Peak Area Precision (RSD)
1	Mannitol	21.48	4.96	5.01	101	0.90
	3-*O*-methylglucose	14.90	5.03	5.27	105	1.72
	Xylose	27.30	5.02	5.00	99.5	1.43
	Lactulose	29.44	5.03	4.56	90.6	0.68
2	Mannitol	21.48	4.95	5.12	103	0.23
	3-*O*-methylglucose	14.90	5.00	5.33	107	0.33
	Xylose	27.30	5.03	5.07	101	1.64
	Lactulose	29.44	5.03	4.86	98	1.54
3	Mannitol	21.48	4.96	4.79	96.6	1.31
	3-*O*-methylglucose	14.90	3.11	3.05	98.0	1.78
	Xylose	27.30	4.99	4.88	97.7	1.14
	Lactulose	29.44	1.02	1.03	101	1.69

Conclusion

This work discusses two methods for the determination of carbohydrates of interest in urine matrices. Both methods are capable of resolving mannitol, 3-*O*-methylglucose, xylose, and lactulose. The Dionex CarboPac PA20 column separates these carbohydrate probes as well as additional endogenous carbohydrates within 16 min—with a total method time of 23 min—with excellent precision and recoveries. This method takes advantage of the RFIC system capabilities, requiring only a source of degassed DI water for eluent generation. Neither method requires the derivatization steps needed for other chromatographic methods, which saves both time and costs during sample analysis.

For Research Use Only. Not for use in diagnostic procedures.

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