Ion-Exchange Chromatography Followed by ESI-MS for Quantitative Analysis of Sugar Monophosphates from Glucose Catabolism

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The aim of this work is to establish a quantitative method to determine the ratio of [$U\text{-}^{13}\text{C}$] labeled to unlabeled hexose monophosphates isolated from yeast extracts. This is accomplished by anion exchange chromatography and mobile phase desalting followed by electrospray (ESI) mass spectrometry. We test the method with the analysis of a sample of biological origin. Previously developed analytical techniques are not adequate to accomplish mass spectrometric analysis of these and other small monosaccharide systems because of interference from salt clusters. By lowering the ionic strength of the mobile phase and using a simplified injection system to the mass spectrometer, we were able to obtain data on the relative abundance of the hexose monophosphates. (J Am Soc Mass Spectrom 2006, 17, 104–107) © 2005 American Society for Mass Spectrometry

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Our interest in the analysis of small monosaccharide monophosphates and related materials arose from the need to support investigations of biological channeling using yeast as a model [1]. The analytical goal is to measure the ratio of [$U\text{-}^{13}\text{C}$] labeled to unlabeled hexose monophosphate pathway intermediates produced in a yeast system by simultaneous incubation in [$U\text{-}^{13}\text{C}$] glucose and unlabeled galactose. The difficulty in analysis of small sugar phosphates [e.g., *D*-glucopyranose 6-phosphate (Glc6P) and 6-phospho-*D*-gluconic acid (6PGA)] in biological matrices belies their relative chemical simplicity. Prior work used derivatization of hexose monophosphates to permit separation by gas chromatography [2, 3]. Others have used ion-pair reagents and reverse phase chromatography followed by multiple reaction monitoring with a triple-quadrupole mass spectrometer to profile these sugar monophosphates [4]. In our work, the need to separate isomeric forms of the hexose monophosphates precludes this approach. We chose to avoid derivatization and ion-pairing since separation of the isomers can be achieved by anion exchange chromatography followed by pulsed amperometric detection using a sodium acetate

(NaOAc) gradient [5]. Isotopomers, however, are indistinguishable by chromatographic methods alone; thus, mass spectrometry (MS) is required.

Negative ion electrospray (ESI) of hexose monophosphates works well, but ESI cannot tolerate the high salt levels in the eluant from anion exchange chromatography. To prepare eluants for subsequent mass spectral analysis, Dionex Corporation developed an on-line desalting apparatus, carbohydrate membrane desalter (CMD) [6], and showed its suitability as a sample preparation method for samples submitted to MALDI analysis. While MALDI is a good choice for larger carbohydrates and their phospho-derivatives, it is not a good choice in the present context owing to matrix interference in the low-mass range where the analytes of interest will appear. We found, in fact, that none of the existing methodologies for desalting a chromatographic eluant containing $\mu\text{mol/L}$ hexose monophosphates and their isomers in the presence of nearly molar concentrations of salt to be effective for ESI analysis.

The purpose of this Application Note is to fill the need for analyses in this area. We describe a method to separate hexose monophosphates in complex mixtures, desalt the eluants, and analyze them by ESI-MS to determine the ratios of [$U\text{-}^{13}\text{C}$]/ ^{12}C -Glc6P and [$U\text{-}^{13}\text{C}$]/ ^{12}C -6PGA. This method would also be applicable to the general problem of analysis of small sugar phosphate metabolic intermediates of any type arising from the operation of various pathways.

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Experimental

After incubation, yeast cells were harvested by centrifugation and extracted with hot ethanol [7]. Separations were performed with a DX-500 chromatograph (Dionex Corporation, Sunnyvale, CA) equipped with an ED40 electrochemical detector using pulsed amperometric detection. Extracts were separated using strong anion-exchange liquid chromatography on a Dionex Carbpac PA1 column with Solvent A (50 mmol/L NaOH) and Solvent B (50 mmol/L NaOH and 600 mmol/L NaOAc) at a flow of 1 mL/min. Initial conditions were 85% A and 15% B with a linear gradient from 15 to 65% B in 40 min. After detection, the chromatographic eluant was desalted online with the CMD, and one mL fractions collected followed by vacuum drying for later mass spectral analysis. The dried LC fractions were reconstituted in HPLC-grade methanol containing 0.5% (v) acetic acid, providing the best sensitivity and the highest signal/noise for the analysis of the hexose monophosphates. Electrospray ionization in negative ion mode was performed on a Waters Q-TOF Global mass spectrometer by infusion of the methanol acetic acid solution using a syringe pump operating at 10 μ L/min. The limit of detection was determined by successive 2-fold dilutions of a stock 40 μ mol/L solution of the hexose monophosphates. Both the Glc6P and 6PGA compounds had a detection limit of 300 nmol/L at a signal to noise ratio of five. [U - ^{13}C] G6P was synthesized enzymatically by modifying the synthesis reported by Huck et al. [4]. A mixture of 25 mmol/L adenosine triphosphate, 4.9 mmol/L magnesium chloride, 1 mmol/L [U - ^{13}C] glucose, and 17 units hexokinase was incubated for 1 h at 30 $^{\circ}C$ in a total volume of 10 mL at pH 7.4. The mixture was subsequently spin filtered in two 0.5 mL aliquots through a 10,000 molecular weight cutoff membrane to remove proteins.

Results and Discussion

Optimization of the Desalting Protocol

The CMD exchanges Na^{+} for H^{+} in the anion exchange eluant. This exchange is facilitated by the counter flow of a regenerant solution [trifluoroacetic acid (TFA) or sulfuric acid] across an electrically charged membrane. However, when we used 150-mmol/L TFA as the regenerant to desalt LC-separated hexose monophosphates as recommended by Thayer et al. [6], the collected eluants still had sufficient sodium salts to prevent analysis of the reconstituted LC fractions by negative ion electrospray. At low sodium acetate concentrations (for early-eluting analytes), sodium trifluoroacetate clusters dominated the mass spectra. At high sodium acetate concentrations (for later eluting analytes), the mass spectra were dominated by sodium trifluoroacetate and sodium acetate clusters. In either case, the hexose monophosphates were not detected. Apparently, the regenerant was moving across the membrane,

resulting in a solution that gave the sodiated clusters we observed in our negative ion ESI spectra.

We addressed this issue by switching the regenerant to sulfuric acid, which was reported to have less desalting ability than TFA but should be less likely to form clusters. Upon analysis, sodium sulfate clusters were observed for all fractions, but at much lower abundance than sodium trifluoroacetate clusters, and more importantly, the analytes were readily detected. At lower levels of sulfuric acid (50 mmol/L), the ability to detect hexose monophosphates was not affected. However, the fraction that contained later-eluting 6PGA showed sodium acetate clusters upon ESI. These clusters were eliminated by modifying the gradient 15% B to 40% B for 1 min, subsequently maintained for 20 min, and then increased to 65% B for 15 min. Consequently, elution of 6PGA occurred at ~ 340 mmol/L Na_{+} ; a salt concentration which the CMD was able to reduce enough to allow MS detection.

Modified Direct Infusion Apparatus to ESI Source

For initial analyses, we used the switching valve and injection loop supplied with the mass spectrometer. Mass spectra of both hexose monophosphates showed significant carry-over upon consecutive injections, regardless of the amount of washing between injections. This tendency can be seen in the replicate injections shown in Figure 1a, where the peak width is considerably wider than the expected 0.4 min for a 4 μ L injection infused at 10 μ L/min. These compounds appear to be “sticky”, and the best way to avoid carryover and greater peak tailing was to minimize the length of plumbing from the injection port to the ion source. We constructed a simple tee injection inlet obviating the use of PEEK transfer lines and valves from the sample injection to the source, decreasing the distance from the syringe needle to the source to ~ 10 cm with a dead volume of ~ 5 μ L (Figure 1b). This injection scheme provided fast, reliable, and reproducible injections with minimal carry-over (Figure 1a).

Measuring Ratios of $^{13}C/^{12}C$ Containing Glc6P by Mass Spectrometry Alone

To test the effectiveness of the methodology, we measured the ratio of [U - ^{13}C] Glc6P to unlabeled Glc6P using prepared mixtures of the two hexose monophosphates. These mixtures had a constant concentration of unlabeled Glc6P (20 μ mol/L) and serial dilutions of [U - ^{13}C] Glc6P, from 24 μ mol/L to 0.76 μ mol/L (note: the starting concentration of unlabeled [U - ^{13}C] Glc6P was later determined to be ~ 1.2 times greater than the presumed 24 μ mol/L increasing the slope of the linear fit). These were analyzed by ESI-MS with triplicate manual injection of 4 μ L of each solution by direct infusion. The integrated areas under the reconstructed ion current profiles for the $[M - H]^{-}$ molecular ion of

Figure 1. (a) Reconstructed ion current profile of m/z 259, showing multiple injections of Glc6P using the low-pressure injector. (b) Diagram of low-pressure direct infusion injector.

both the completely labeled and unlabeled hexose monophosphates were measured, and the average area for each dilution was determined. The ratio of the average integrated area versus the ratio of the actual concentrations had a linear fit with a slope of 1.3 and an intercept of -0.02 . The R^2 value for the fit was 0.99 and the relative standard deviation for the 21 measurements was 7.7%.

Ion-Exchange LC-MS for $^{13}\text{C}/^{12}\text{C}$ -Glc6P

We initially checked the chromatographic separation using a test mixture of seven sugar monophosphates, all of which were baseline resolved. Glc6P and 6PGA were separated by nearly 14 min with Glc6P eluting in 240 mmol/L NaOAc and 6PGA eluting $\sim$ 350 mmol/L NaOAc. Subsequently, we analyzed four test mixtures of $[\text{U-}^{13}\text{C}]$ Glc6P and unlabeled Glc6P (1:1, 2:1, 4:1, 8:1). The desalted fractions (along with those of a second round including the $^{13}\text{C}/^{12}\text{C}$ ratios, 5:1, 1:1, 1:5) were analyzed in triplicate by ESI MS using the low dead-volume tee injector. The areas of the reconstructed ion current profiles of the ions of interest (m/z 265.1 for completely ^{13}C labeled Glc6P and m/z 259.1 for monoisotopic ^{12}C G6P) were integrated, and their ratios were plotted versus the ratio of concentrations $^{13}\text{C}/^{12}\text{C}$ Glc6P. The plot had a linear fit with a slope of 1.2 and an intercept of -0.02 . The R^2 value for the fit was 0.98 and the relative standard deviation for the 15 measurements was 12%.

Preliminary Analysis from a Yeast Extract

Yeast extracts were prepared as described above. The preparations were not provided with any source of ^{13}C labeled nutrient, therefore no ^{13}C labeled compounds would be expected. Chromatography of the yeast extract was performed twice; once spiked with 800 pmol of unlabeled Glc6P and 6PGA and once unspiked. The analysis of the spiked sample confirmed that the retention times of the analytes of interest were not affected by the complex mixture of compounds in the yeast extract. The elution profile of the compounds of interest was identical to that of the test standards. ESI-MS analysis of the fractions collected from the unspiked yeast extract revealed the $\text{M} - \text{H}$ ions of both Glc6P (259.1) and 6PGA (275.1) in their respective fractions, with very little Na^+ interference (Figure 2a and c). The product ion spectra for each $\text{M} - \text{H}$ ion, shown in Figure 2b and d, confirm the presence of both hexose monophosphates. Furthermore, accurate mass determinations (data not shown) were within 3 ppm of the theoretical mass for both compounds.

Conclusions

By modifying existing methods for the desalting of anion exchange eluants, we were able to determine quantitatively the ratios of $[\text{U-}^{13}\text{C}]$ labeled and unlabeled hexose monophosphates by negative ion electrospray mass spectrometry despite mmol/L salt concen-

Figure 2. (a) and (c) ESI mass spectrum of desalted and collected fractions from a yeast extract at the retention time for Gly6P (m/z 259.1) and 6PGA (m/z 275.1), respectively. (b) and (d) MS/MS product-ion spectrum of hexose monophosphate precursor ions for Gly6P (m/z 259.1) and 6PGA (m/z 275.1), respectively.

trations in the eluants. The method is now suitable for analyzing small sugar phosphates and for investigating enzyme channeling in yeast. In addition, this method could be applied to other areas of carbohydrate research when resolved separation of isomers is required in combination with isotope labeling for quantitation by electrospray mass spectrometry or for the identification of unknown components requiring MS.

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