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FOCUS: BIO-ION CHEMISTRY: INTERACTIONS OF BIOLOGICAL IONS WITH IONS, MOLECULES, SURFACES, ELECTRONS, AND LIGHT: RESEARCH ARTICLE

Identifying the D-Pentoses Using Water Adduction to Lithium Cationized Molecule

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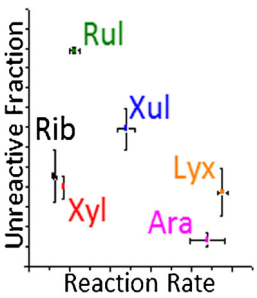

Abstract. A method has been developed that is capable of distinguishing an exhaustive list of underivatized D-pentoses with only a mass spectrometer. Electrospray ionization (ESI) of a solution containing a pentose and a lithium salt yields [Pentose + Li]$^+$. These lithiated pentoses adduct water in a quadrupole ion trap. The reaction rate of water adduction is unique for several of the pentose isomers. Additionally, there are multiple potential gas-phase lithiation sites to form [Pentose + Li]$^+$. A mixture of ions with at least one reactive (water adducting) and at least one unreactive (non-adducting) lithiation site is formed for each pentose. The water adduction reaction rate along with the unreactive fraction of lithiated pentose can be used to completely discriminate all D-pentoses.

Keywords: Pentose, Monosaccharide, Ion molecule reaction, MS/MS

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Introduction

Pentoses are involved a diverse range of biological and physiological processes in living systems such as building larger carbohydrates, modifying proteins and lipids, supporting the structure of RNA, and can also be found as free molecules [1–4]. Being able to discriminate between different pentose isomers is essential for developing a complete understanding of their role in biological systems. Like several other classes of biological molecules, pentoses have several relevant epimers; isomers that differ only in stereochemistry at one or more sites. Discriminating between the epimers within any group of monosaccharides is a challenging task [1, 5]. Pentoses are especially difficult to discriminate compared with larger monosaccharides because their structures differ only by the equatorial or axial placement of two hydroxyl groups [6].

Several analytical techniques have been employed for structural identification of monosaccharides. Techniques such as NMR and X-ray crystallography require very pure samples and millimolar concentrations [7–9]. Obtaining suitable samples from biological matrices for these techniques requires extensive purification to both increase the concentration and eliminate chemical background, greatly increasing analysis time. Using mass spectrometry (MS) as the detector allows lower concentrations (micromolar and lower) to be used, and the high specificity of MS does not require extensive sample purification [1, 10]; these factors combined with short analysis times make MS a much more favorable method for analysis.

Separation methods such as chromatography and ion mobility are often used prior to mass analysis to increase the selectivity of the analysis, and these techniques are often coupled to mass spectrometry to distinguish between regioisomers, diastereomers, and enantiomers. Chromatographic separations require long separation times and often need derivatization and/or specialized columns [11, 12]. Derivatization improves the limits of detection for both liquid chromatography (300 nM) [13] and gas chromatography (7 μM) [14]. However, when analyzing monosaccharides with these techniques, multiple peaks often result from a single monosaccharide because of separation of the α and β anomers as well as pyranose and furanose forms of the monosaccharides [15]. Because monosaccharides are freely able to interchange between these various forms in solution, the extra peaks reduce sensitivity as the ion signal is spread across multiple peaks and convolute data analysis when the ultimate objective is only identification and quantification of the monosaccharide [16, 17]. Ion mobility techniques provide much faster separations, usually on millisecond timescales. However, to date ion

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mobility techniques lack the resolving power needed to distinguish underivatized pentose isomers.

Several methods have been developed for distinguishing isomeric and diastereomeric compounds using tandem mass spectrometry (MS/MS) with no prior separation technique. Dissociative techniques have been used to distinguish various types of monosaccharides with MS/MS, especially after first derivatizing the molecules with a transition metal complex. This has been used to distinguish diastereomeric hexoses [18], hexosamines [19], and *N*-acetylhexosamines [20]. However, distinguishing epimers with dissociative techniques without prior derivatization is very challenging. There have been no reports in the literature on distinguishing pentose isomers using only dissociative techniques without first adding to the analyte solution a chiral reagent, which then coordinates to the pentose.

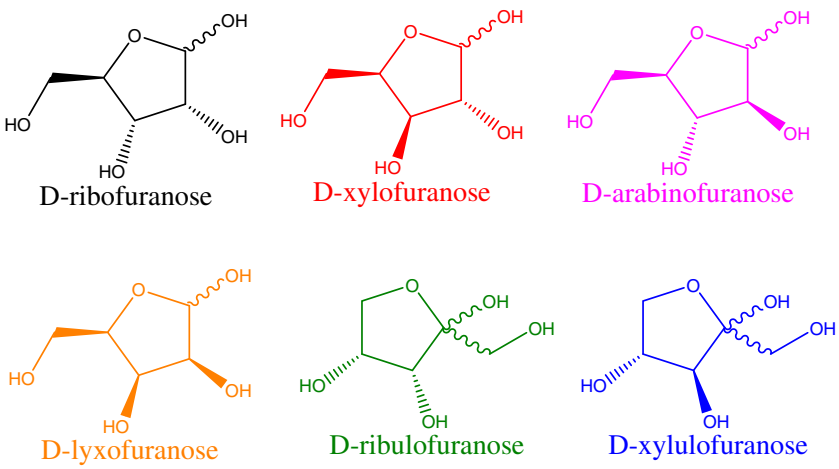
Forming intermolecular complexes between the analyte and added reagents is the first step in the analytical techniques that use only MS/MS for distinguishing pentose isomers. These techniques are based on the kinetic method. The simplest approach requires addition of a divalent metal (typically a transition metal) and a chiral ligand to the electrospray solvent. Electrospray ionization of this solution produces a noncovalent tetrameric complex of analyte, divalent metal, and two chiral ligands. Collision induced dissociation of this complex results in competitive dissociation with loss of either one of the chiral ligands or the analyte of interest while the other remains bound to the divalent metal. The relative intensity of the product ions is a function of the binding energies of the different species in the complex [21–23]. This method has been used to distinguish both diastereomers and enantiomers. A derivative of this method, the fixed ligand kinetic method, where one chiral ligand is replaced with a different chiral ligand that will not dissociate during CID (a "fixed" ligand), was used to distinguish all pentoses. This requires forming two fixed ligand tetrameric complexes for each isomer: one pentose electrosprayed from solution with Ni(II), L-Asp, and 5′-guanosine monophosphate, and a second sample of the same pentose electrosprayed from solution with Cu(II), L-Ser, and 5′-guanosine monophosphate. This method was reported using high concentrations of analyte, divalent metal, chiral ligand, and fixed ligand (all 100 μM or greater) as well as high ESI flow rates to provide sufficient signal for reproducible results [17].

Herein an MS/MS method is presented capable of distinguishing all D-pentoses using a simple ion-molecule reaction without any exotic reagents. Electrospray ionization of pentoses in a solution containing a lithium salt generates $[Pentose + Li]^+$. Once inside the quadrupole ion trap, some fraction of these lithiated ions adduct water, resulting in new peak 18 mass-to-charge units higher. This ion/molecule reaction has been previously observed with lithium-cationized saccharides [24, 25]. Here, the kinetics of the water adduction reaction and unreactive fraction of lithiated pentose can be used to distinguish all six D-pentoses.

Methods

The six pentoses are shown in Scheme 1 in the furanose form. D-arabinose (Ara), D-lyxose (Lyx), D-xylose (Xyl), D-ribulose (Rul), and D-xylulose (Xul) were purchased from Carbosynth (Berkshire, UK) and used without derivatization or further purification. D-ribose (Rib), L-arabinose, lithium acetate, deuterium oxide (99%), and methanol-d_4 (CD_3OD, 99%) were purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC grade methanol (Fischer Optima Brand) and HPLC grade water (Fischer Optima Brand) were purchased from Fischer Scientific (Fairlawn, NJ, USA).

Each pentose was analyzed individually in a solution of 10 μM pentose, 100 μM lithium acetate in 50/50 water/methanol unless otherwise noted. Data reported herein were obtained with a Bruker Esquire 3000 Ion Trap mass spectrometer. Similar results were obtained with two Bruker HCTUltra Ion Traps (Billerica, MA, USA) and in the linear ion trap of a Thermo LTQFT. Samples were ionized by electrospray ionization (ESI) with a flow rate of 2 μL/min. The inlet capillary voltage was set to –5000 V in the instrument control software. The remaining ion optics were



Scheme 1. All pentoses shown in their furanose forms

adjusted in the instrument control software to achieve the maximum signal intensity for $[M + Li]^+$ (*m/z* 157). Helium (ultra-high purity) was purchased from AirGas (Durham, NC, USA) and leaked into the ion trap to achieve a pressure of 1 mTorr.

The reaction rate for $[M + Li]^+$ with H_2O was determined for each isomer. The parent ion, $[M + Li]^+$ (*m/z* 157), formed by ESI was isolated in the quadrupole ion trap. $[M + Li]^+$ was then held in the ion trap and allowed to react for a set amount of time with background water before the parent and product ions (*m/z* 175) were ejected for mass analysis. Reaction times were controlled by adding a delay time between isolation of the lithiated pentose and the subsequent mass analysis ejection of ions out the ion trap to the detector. Delay times of 0, 10, 20, 30, 40, 50, and 1000 ms were used. The total reaction time is the sum of the delay time and the time until *m/z* 157 was scanned out of the trap during the mass analysis step. The instrument was set to start scanning at *m/z* 50 with a scan rate of 13,000 mass-to-charge units per second. Using these instrumental parameters, it takes 8.2 ms from the start of the scan to eject *m/z* 157, marking the end of the reaction time. Therefore, with the end of the isolation portion of the scan function serving as t = 0, the final reaction times used were 8.2, 18.2, 28.2, 38.2, 48.2, 58.2, and 1008.2 ms. A total of 10 mass spectra were averaged for each of the individual data points. All data were analyzed using the Bruker DataAnalysis software package. Reported intensities are peak areas summed ± 0.5 *m/z* from the peak center.

Results

ESI of the pentose and lithium acetate solution produces mostly $[M + Li]^+$, though some $[M + Na]^+$ is observed at less than 10% the intensity of the $[M + Li]^+$ peak. The $[M + Na]^+$ for all pentoses was found to be completely unreactive with water; however, the lithiated pentoses were found to adduct water in the quadrupole ion trap, and the mass spectrum shows two ions: $[M + Li]^+$ (I_{157}) and $[M + Li + H_2O]^+$ (I_{175}). Plotting $\left(\frac{I_{157}}{I_{157}+I_{175}}\right)$ versus reaction time yields an exponential decay curve (Figure 1). The exponential decay curve for each pentose

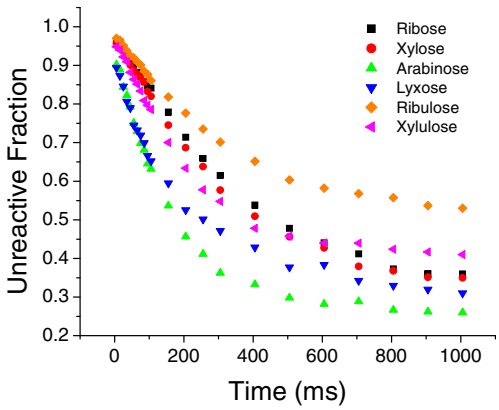


Figure 1. For the reaction of $[M + Li]^+ + H_2O$ in a quadrupole ion trap exponential decay curves

asymptotes without all $[M + Li]^+$ ions adducting water. The asymptote is due to some fraction of each lithiated pentose being unreactive. Each pentose contains five oxygen atoms to which the lithium cation can potentially coordinate, allowing for multiple sites of lithiation to form $[M + Li]^+$. Some sites may be able to adduct water while other sites are unreactive. For all pentoses, over 99% of the molecule will be cyclized in either a furanose or pyranose form in solution, restricting the flexibility of the pentose, and limiting the number of oxygens that are capable of coordinating to the lithium at one time relative to the linear structure.

Previous experiments and density functional theory calculations show that as the first hydration shell forms around a lithium cation, the bond dissociation enthalpy decreases for each sequential water binding [26]. Therefore it is believed that sites where the lithium can coordinate to one or two oxygens may remain reactive, and a water molecule would be able to adduct. Lithiation sites where the lithium is bound to three or more oxygens are less likely to be able to also form a coordination bond to the water, causing these sites to be unreactive. This unreactive fraction of lithiated pentose $\left(\frac{I_{157}}{I_{157}+I_{175}}\right)$ produced during ESI is very reproducible (with RSDs ≤ 8.0%). This fraction can be used to distinguish ribulose, xylulose, and arabinose from all other pentoses (with $p \leq 0.027$ using Student's *t*-test). However, ribose, lyxose, and xylose are not able to be distinguished from each other using only the unreactive fraction.

Complete discrimination between all pentoses requires the kinetics of the water adduction reaction to be measured. Because the concentration of water in the trap is expected to be much greater than the number of ions, pseudo-first order kinetics can be assumed. The generic first order kinetics equation for a reactant, A, $\frac{A_t}{A_0} = -kt$, was therefore used to measure the water adduction reaction kinetics. In this system A_t, the concentration of reactant at any time, t, can be represented by I_{157}, and A_0, the initial concentration of reactant, can be represented by $I_{157} + I_{175}$, assuming negligible losses during trapping and ejection.

For kinetic studies I_{157} and I_{175} were measured after reaction times of 8.2, 18.2, 28.2, 38.2, 48.2, and 58.2 ms. As previously discussed, ESI of each pentose has reactive and unreactive lithiation sites. Therefore, the unreactive fraction of ions must be subtracted from the measured I_{157} before the reaction rate can be calculated. The fraction of I_{157} that is unreactive (R_U) can be measured as $\left(\frac{I_{157}}{I_{157}+I_{175}}\right)$ after a reaction time sufficient for all reactive species to adduct water (greater than 800 ms). The reactive fraction of lithiated pentose remaining, R_R, defined by Equation 1, is then used in the determination of the reaction rate.

$$R_R = \frac{(1-R_U)(I_{157}+I_{175})-I_{175}}{(1-R_U)(I_{157}+I_{175})} \quad (1)$$

In this equation, the denominator is the total reactive species, including species that have adducted water and species

that has not yet adducted water. The numerator is simply the reactive species that has not yet adducted water. Plotting $\ln(R_R)$ versus time yields a linear plot as expected for psuedo-first order reaction kinetics (Figure 2). Using R_R is preferred to simply using the remaining reactive species (the numerator in Equation 1 more conventionally used in first order kinetics) because R_R is unaffected by variations in signal intensity in successive mass spectra. These variations are mostly a result of fluctuations in the number of ions that are successfully transmitted from the ion source into the ion trap in successive mass analyses.

Linear regression of the resulting plots of $\ln(R_R)$ versus time gives R^2 values that are all greater than 0.997. The slopes of these lines are the reaction rates (i.e., the product of the reaction rate constant and the concentration (unknown) of water in the quadrupole ion trap). Although the concentration of water is unknown, it is not expected to vary significantly with time. This is confirmed by the small relative standard deviations (<8%) of the reaction rate measured for any lithiated monosaccharide across several months.

The reaction rate measured was found to be unique for all of the pentoses studied (with $p \leq 0.025$ using Student's *t*-test), with the exception of Ara and Lyx (Supplementary Table 1). However, Ara and Lyx can be confidently distinguished from one another using the unreactive fraction as discussed previously. Therefore, when both unreactive fraction and reaction rate are used, all six pentoses can be readily distinguished. This is shown in Figure 3 where the unreactive fraction is plotted versus the reaction rate, separating all the isomers in a two-dimensional space.

This method was also used to compare distinguishing the absolute configuration of two pentoses, D-arabinose and L-arabinose. The reaction rates and unreactive fractions were measured for each as previously explained. The enantiomers were unable to be distinguished within experimental error. The reaction rates for D-Ara and L-Ara were 64.1 ± 4.2 and 63.9 ± 3.9, respectively, and the unreactive fraction for D-Ara and L-Ara were 0.333 ± 0.009 and 0.336 ± 0.005, respectively. This result is expected as the two enantiomers would have identical binding sites for Li^+ (i.e., the distances and angles between all oxygen atoms in the pentose are same in each enantiomer).

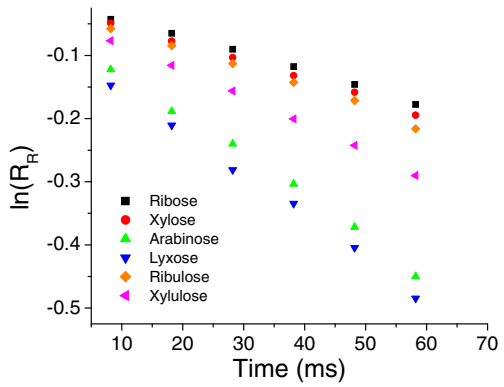


Figure 2. Natural log of the remaining reactive fraction versus reaction time

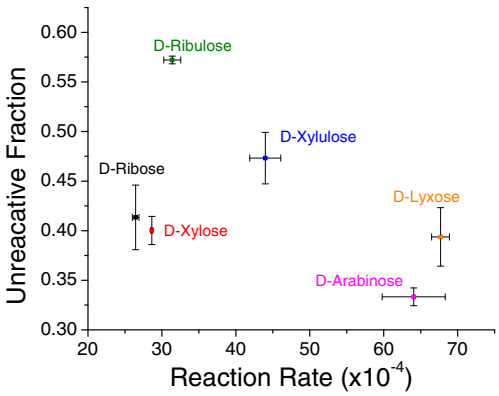


Figure 3. Using both the unreactive fraction and the uncorrected reaction rate allows for all six isomers to be distinguished

The effect of pentose concentration on the unreactive fraction and the reaction rate was tested with ribose. Experiments were performed with 100 μM lithium acetate and different concentrations of ribose ranging from 500 μM to 250 nM. The reaction rate remained unchanged (within one standard deviation) throughout the entire range of concentrations tested. However, at 250 nM the relative standard deviation was greater than 10% (compared with less than 5% for all other concentrations). The unreactive fraction remains constant (within one standard deviation) at all concentrations tested, and the relative standard deviation of all measurements remains below 10%.

Experiments were performed to determine whether the origin of the trace water concentration came from the electrospray solvent. To do this, lithiated monosaccharide was produced via ESI from deuterated methanol and deuterated water. The observed mass-to-charge ratio of [M + Li] shifted as a result of all hydroxyl hydrogens exchanging with deuterons. After allowing time for the water adduction reaction only $[M + Li + H_2O]^+$ and not $[M + Li + D_2O]^+$, was observed. This indicated that the water adducting in the ion trap did not come from the electrospray solvent. It is therefore believed that the most likely source of the water is that it is just the ambient background level. The reaction has been observed on four different quadrupole ion trap systems. The reaction rate varies from system to system, but is very reproducible (RSD <8%) over several months for a given system. Each system has a bit different base pressure, which explains the different in reaction rates from system to system.

Conclusions

A method was developed that is capable of distinguishing between all D-pentoses using only mass spectrometry. ESI of a solution containing pentose and lithium acetate produces $[M + Li]^+$, which adducts water in a quadrupole ion trap. The reaction rate of the water adduction was observed to be unique for all pentoses except Ara and Lyx. Additionally, ESI produces a mixture of ions that includes at least one reactive (water adducting) lithiation site and at least one non-reactive (non-water adducting) lithiation site for each pentose. The unreactive

fraction can be used to distinguish several of the pentoses. The reaction rate and the unreactive fraction are very reproducible (with RSDs ≤8%), and when used together all lithiated pentoses can be discriminated with $p \leq 0.025$ using Student's *t*-test. This method could be used with pentoses in a complex matrix and does not require ion mobility or chromatography to distinguish the isomers. Additionally, it requires only the addition of a common lithium salt to the analyte solution followed by direct infusion ESI, making it simpler and faster than chromatographic methods previously reported for identifying pentoses. It uses order of magnitudes lower concentrations than similar methods that use only mass spectrometry or other analytical techniques.

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