Rapid on-column analysis of glucosamine and its mutarotation by microchip capillary electrophoresis

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Abstract

A novel electrophoretic microchip method for analyzing α- and β-α-glucosamine and their interconversion in solution is presented. α- Glucosamine is labeled with fluorescamine and analyzed by capillary electrophoresis in under 2 min revealing its pH-dependent mutarotation between the α- and β-anomers. The forward interconversion rates for the labeled sugars, based on an iterative analysis of the plateau heights between the peaks, are 0.72 ± 0.09, 1.3 ± 0.1, and 2.2 ± 0.3 × 10⁻³ s⁻¹ at pH 8.99, 9.51 and 10.01, respectively. In a separate experiment, the mutarotation of the unlabeled α-α-anomer was followed; the relative intensities of the α- and β-peaks as a function of reaction time at pH 9.51 give a forward rate constant of 0.6 ± 0.1 × 10⁻³ s⁻¹. These results demonstrate that fast microchip separations, previously exploited for amine, amino acid, and nucleobase analysis, can also be used to analyze amino sugars and their mutarotation.

Keywords: Lab-on-a-chip; Fluorescamine; Glucosamine; Mutarotation; Astrobiology; Biomarkers

1. Introduction

In addition to amino acids, nucleobases, and mono- and diamines, amino sugars are important biomarkers for both past and present biological activity. Amino sugars such as glucosamine are found in shells of crustaceans and arthropods among other natural products. Recently amino sugars were found to be a major source of organic carbon in particulate and dissolved organic matter in marine environments [1]. The amino sugar glucosamine has tumor-inhibiting activity [2], is in wide therapeutic use to treat symptoms of osteoarthritis [3,4], has been reported to have immunosuppressive effects [5], and has recently been utilized in the synthesis of dendrimers for biomedical applications [6]. The widespread appearance and use of glucosamine thus makes the development of fast and accurate analysis methods for purity and anomeric composition of general interest.

Two general approaches have been used for glucosamine analysis. First, polarimetric measurements and proton NMR have been used to determine the anomeric composition [7]. A single measurement of the average specific rotation or the chemical shifts is used to determine the composition of the mixture. Observation of that same solution over time yields kinetic information about the mutarotation between the α- and β-anomers. Second, chromatographic methods, such as ion-exchange chromatography, gas chromatography, and capillary electrochromatography have successfully separated the two anomers [8–12]. In a single trace, the α- and β-anomers are resolved, allowing determination of the mixture composition. In addition, on-column mutarotation between the two anomers has been observed [8,10]. Analysis of the peak and plateau heights between the interconverting species can yield reaction rate constants from a single trace [13,14], but those calculations were not applied to the chromatographic separations discussed above. Time scales of 10–20 min are commonly reported which can yield poor resolution separations because of on-column mutarotation. A technique is needed that delivers fast, high resolution separations of both anomers in solution as well as mutarotation rates from a single trace.

Capillary zone electrophoresis (CE) is a widely used technique for the rapid analysis of biomolecules, such as carbohydrates [15–18], amines, and amino acids [19–22], that can deliver both high resolution and high efficiency separations with short (~3 min) separation times. We previously developed a portable microchip capillary electrophoresis instrument called the Mars...
Organic Analyzer (MOA) and demonstrated its utility for composition and chirality analysis of amino acid biomarkers. Amino acids were labeled with fluorescamine and analyzed with ppb to part-per-trillion sensitivities, and the method has been extended to other primary amines and nucleobases [21,23]. Here we apply our fluorescamine labeling technique to the analysis of glucosamine and exploit the speed of the microchip CE separations to observe the α- and β-anomers and their interconversion in real time. In addition, by applying the iterative unified equation developed by Trapp [14], the mutarotation rates are determined. Finally, the utility of this method for the determination of α- and β-glucosamine in complex mixtures of biologically relevant analytes is demonstrated.

2. Materials and Methods

2.1. Identification of glucosamine anomers

d-glucosamine hydrochloride (0.6 mM, mixed α- and β-anomers, 10 mM Na₂CO₃ buffer, pH as specified) was labeled with fluorescamine as described previously [20,21]. Briefly, 2 μL aliquots of the sample mixture were combined with 3 μL of 20 mM fluorescamine in dimethylsulfoxide (DMSO) for labeling. After 10 s of reaction at room temperature, the solutions were diluted 1/10 for analysis. To determine the dependence of mobility on pH, amino acid standard “AS” (1 mM total amino acid concentration: 133 μM each valine, alanine, serine, glutamic acid and aspartic acid, 66 μM glycine, and 266 μM aminoisobutyric acid (AIB). [20,21]) was added to provide a stationary set of peaks for alignment (final injected concentrations 8 μM sugar, 80 μM AS). To identify the glucosamine peaks, α-d-glucosamine hydrochloride (98+%, Alfa Aesar, Pelham, NH, USA) was dissolved in 10 mM Na₂CO₃ pH 9.51 buffer to 0.6 mM. After incubation for the desired time, aliquots were immediately labeled (<10 s reaction time) and diluted as above with pH 9.51 buffer for analysis. Aliquots were analyzed at time points ranging from 140 to 2609 s after the initial preparation of the unlabeled glucosamine solution. The indicated reaction times include ~120 s for labeling, diluting, and loading the sample and chip in the instrument.

2.2. Labeling of amino sugars, amines and amino acids

Aqueous solutions of bioamines were prepared in 10 mM Na₂CO₃ pH 9.78 and individually labeled with fluorescamine (20 μL of the solution plus 30 μL of 20 mM fluorescamine in acetonitrile). After 10 min of reaction at room temperature, the acetonitrile was removed under house vacuum (3 min) and the solutions were diluted (1/20 to 1/500, final concentrations as indicated) and then combined in 10 mM Na₂CO₃ pH 9.78 for analysis.

2.3. CE analysis of fluorescamine-labeled bioamines

All samples were analyzed at room temperature using all-glass microfabricated devices made in-house as described previously in detail [20,21]. The 150-μm-wide by 21-μm-deep separation channels are 21.4 cm-long (effective separation length 19 cm) and contain 0.6 cm-long cross injection channels. The uncoated channels are conditioned between runs with 5 min flushes of 0.1 M NaOH followed by dH₂O. The four CE reservoirs (sample, waste, cathode and anode) are surrounded by polydimethylsiloxane (PDMS) moats to increase the reservoir volume. CE separations were performed at 700 V/cm on a portable CE instrument described previously [21]. The instrument contains the electronics and power supplies for CE separations and pneumatics to control the PDMS valves and pumps. The instrument uses a 15 mW, 400-nm diode laser for excitation, 430-nm long-pass and 522-nm band pass emission filters, and a 100-μm-diameter fiber optic-coupled photomultiplier tube for fluorescence detection. The temperature for all kinetic and analytical analyses was 22 ± 1 °C.

2.4. Analysis of CE traces to determine mutarotation rates

All traces were analyzed using Grams 32 (Thermo, San Jose, CA, USA) to determine the chromatographic parameters. To calculate interconversion rate constants for the glucosamine anomers based on single traces, migration time, full-width at half height, peak height, and relative plateau height were determined. These values were entered into the expressions for interconversion used previously [13,14,20,24]. The unified equation was used with iterative calculations because the anomers were not initially present in a 1:1 ratio [14]. Eq. (1) is used to determine $k_f$:

$$k_f = a\ln(\beta e + d) - e = R$$

where $a$, $b$, $c$, $d$, and $e$ are constants determined by the chromatographic parameters described above (see [14]) and $R$ is the residual. The equation is solved by performing iterative calculations with different input values of $k_f$ to minimize $R$. The iterations were performed in Excel™ using the goal seek tool, allowing for a maximum of 5000 iterations and no minimum value change before the iteration stopped.

The mutarotation rates were also determined by analysis of an initially pure α-d-glucosamine solution as a function of time. In this study, the normalized areas (true peak area divided by total area of α- and β-anomers) were determined at each time point. Measurements were taken until the equilibrium concentrations were maintained with repeat analyses. A first-order kinetic plot was then used to determine the forward rate constant for the interconversion of α- to β-d-glucosamine.

3. Results

The separation of fluorescamine-labeled glucosamine over a range of buffer pHs is shown in Fig. 1. In all separations, there are two distinct peaks due to glucosamine. The first glucosamine peak does not shift with increasing pH, while the second peak shifts towards the −2 charge region (~80 s) indicating an increase in net charge as pH increases. At pH ≥ 8.99, the area between the peaks is filled in (inset), indicating that we are observing an interconversion between these two species during the separation [13,25,26]. The height of the plateau does not obviously increase with increasing pH; however, the area
between the peaks (a function of plateau height and difference in migration time between the two peaks) does increase, indicating qualitatively that the interconversion between the two species proceeds at a faster rate at higher pH.

Ring sugars in solution interconvert between different anomers (here α- and β-glucosamine) via an open structure in a process called mutarotation (Fig. 1 top). In addition, amino sugars can also convert to a different epimer; glucosamine epimerizes to mannosamine. An additional complication is that the fluorescamine dye undergoes a pH-dependent interconversion between different dye enantiomers [20]. In order to determine which process was causing the observed exchange, the rate constants were determined for each of the traces by calculating peak migration times, heights, full-widths at half height and plateau heights. The rate constants for the forward interconversion (peak 1 to peak 2) were determined to be 0.72 ± 0.09, 1.3 ± 0.1, and 2.2 ± 0.3 × 10^{-3} s^{-1} at pH 8.99, 9.51 and 10.01, respectively.1 These interconversion rates are ∼10-fold lower than the enantiomerization rates determined for fluorescamine over a similar pH range [20] suggesting this process is due to the amino sugar interconversion rather than fluorescamine racemization.

To determine which sugar interconversion process (epimerization versus mutarotation) was being observed, a solution containing both glucosamine and mannosamine was analyzed (not shown). A single broad peak for mannosamine was observed that eluted between the two glucosamine peaks at all buffer pHs, demonstrating that neither of the peaks observed is due to the epimerization of glucosamine to mannosamine. The breadth of this peak is presumably due to fast mutarotation of the fluorescamine-labeled mannosamine.

A solution of pure α-D-glucosamine was next prepared, and aliquots were removed at different time points and labeled quickly (<10 s) with fluorescamine before analysis (Fig. 2). The initial separation at \( t = 140 \) s (time from preparation of initial unlabeled glucosamine solution to injection) produced a strong peak at \( \sim 57 \) s and a much weaker peak at longer migration times. As this sample incubated in pH 9.51 buffer, \( T = 21^\circ C \), the relative height of the second peak increased. The area of the second peak relative to area of the first peak as a function of time is plotted in Fig. 3 A. At long times (\( \geq 2600 \) s), the area ratio reaches a plateau. Repeat analysis of the solution after \( t = 2600 \) s yielded the same β/α ratio, so the point at \( t = 2609 \) s was determined to be at equilibrium. Based on the exponential fit of the plot, the equilibrium concentration was determined to be \( K_{eq} = 0.39 \pm 0.06 \) (72.2 ± 0.5% α-anomer and 27.8 ± 0.5% β-anomer). The equilibrium ratios in our traces at pH 9.51 are shifted slightly toward the α-form compared to those reported for glucosamine hydrochloride by Horton et al. (∼63% α-glucosamine and ∼37% β-glucosamine [7]) but otherwise support our assignment.

1 At pH 8.99: peak A: \( t = 55.5 \) s, FWHH = 0.28 s, \( h(A) = 0.78 \); peak B: \( t = 56.5 \) s, FWHH = 0.31 s, \( h(B) = 0.22 \); \( h(p) = 2.0\% \). At pH 9.51: peak A: \( t = 56.5 \) s, FWHH = 0.31 s, \( h(A) = 0.77 \); peak B: \( t = 59.3 \) s, FWHH = 0.36 s, \( h(B) = 0.23 \); \( h(p) = 1.5\% \). At pH 10.01: peak A: \( t = 53.5 \) s, FWHH = 0.30 s, \( h(A) = 0.70 \); peak B: \( t = 59.1 \) s, FWHH = 0.35 s, \( h(B) = 0.30 \); \( h(p) = 1.2\% \). These values are then used to determine \( a, b, c, d, e \) following the equations in [14].
Fig. 2. Analysis of α-D-glucosamine mutarotation as a function of time at pH 9.51. Pure α-D-glucosamine was allowed to react and then the resulting solution was labeled and injected. The initial run at 140 s (time from the preparation of the un-labeled glucosamine to injection of the sample) exhibited a weak second peak at ∼60 s due to β-D-glucosamine that increased in intensity in later aliquots. All traces are scaled to identical α-D-glucosamine height and shifted in time to display the increasing height of the β-D-glucosamine peak. Temperature = 22 ± 1 °C.

A pseudo-first order reversible reaction was assumed, and Eq. (2) was used to determine the rate constants for the forward (kf) and reverse (kr) reactions based on the normalized area of the α-anomer as a function of time:

\[ \ln (\alpha_t - \alpha_{eq}) = -(k_f + k_r)t + \ln (\alpha_o - \alpha_{eq}) \]  

The plot of \( \ln (\alpha_t - \alpha_{eq}) \) versus \( t \) yielded a slope of \( -2.1 \pm 0.4 \times 10^{-3} \text{ s}^{-1} \), which was equal to \( -(k_f + k_r) \). Using \( K_{eq} = k_f/k_r = 0.39 \), the rate constant for the forward interconversion was determined to be \( 0.6 \pm 0.1 \times 10^{-3} \text{ s}^{-1} \).

Finally, a mixture of 5 μM glucosamine, 25 μM AS, 30 μM methylamine, 30 μM ethylamine, and 51 μM cadaverine was prepared and analyzed on the portable system at pH 9.78 with the goal of determining whether amino sugars can be detected in complex mixtures. The low mutarotation plateau did not interfere with the separation, and all species in addition to both
glucosamine anomers were successfully resolved (Fig. 4). The two product peaks for cadaverine (a diamine) were due to both the singly (cadaverine-1, 45 s) and doubly (cadaverine-2, 64 s) fluorescamine-labeled species which have a −1 and −2 charge, respectively.

### 4. Discussion

Amino sugars were successfully labeled with fluorescamine and analyzed on the Mars Organic Analyzer microchip CE system. The two glucosamine peaks were identified as α- and β-glucosamine and their mutarotation rates were determined. The resolution and mutarotation of glucosamine anomers has previously been observed by ion-exchange chromatography [8,10], and peak profiles similar to those seen here were also noted; however, the time scales of the earlier separations were ~20 min. Capillary electrophoresis with mass spectrometer detection has been shown to give anomic resolution of maltose, fructose, N-acetylgalactosamine and glucose, but separation times were ~10 min, and they did not observe on-column interconversion [11]. Here, we were uniquely able to observe the mutarotation in real time, determine the kinetics of forward and reverse interconversion, and conclusively identify the two species with high sensitivity in a single trace.

The forward mutarotation rates determined here by analyzing the interconversion on-column and by observing the peak areas as a function of time agree within a factor of 2, with the peak/plateau method consistently yielding higher values. It is likely that the values determined from the on-column interconversion are influenced by the presence of the fluorescamine dye-label: the mutarotation observed in the on-column study is for the fluorescamine-labeled anomers, while the time course method allows the mutarotation to proceed before labeling takes place. Although the time course method is likely the more accurate method for determining the mutarotation constants of glucosamine, the single trace approach offers a good approximation and reduces the overall analysis time from ~2 h to ~2 min.

The direct observation of the mutarotation of glucosamine is facilitated because the mobility of the β-isomer depends on pH. Previous chromatographic methods that resolved the two anomers did so by preferentially binding one anomer to a metal ion, increasing the retention time, or by utilizing a packed column and an organic buffer. In our case, differences in migration time are indicative of differences in charge, therefore the β-anomer must be going through an acid-base equilibrium. This equilibrium is fast relative to the time scale of the separation so only one peak is observed that represents the average charge on the molecule. It has been reported that the α-anomer of glucose (pKₐ = 12.49) is less acidic than the β-anomer (pKₐ = 12.20) due to a larger entropy of ionization [27]. Analogously Blasko et al. determined pKₐ values of 12.44 and 12.20 for α- and β-glucosamine, respectively, in D₂O, and estimated pKₐ values of 11.8 and 11.56 in H₂O [28]. Based on our identification of the second peak as β-D-glucosamine, we can confirm that the β-anomer of this fluorescamine-labeled pair is also more acidic. The response of mobility to the buffer pHs suggests that the pKₐ of labeled α-D-glucosamine is well above pH 10 because its mobility was not altered at this buffer pH while the pKₐ of labeled-β-D-glucosamine is significantly lower. Clearly the differences in pKₐ observed here are greater than those reported in the literature for unlabeled glucose or glucosamine. The fluorescamine at the equatorial position must be further stabilizing the axial alkoxy ion relative to the un-derivatized amine, resulting in an appreciably lower pKₐ for β-glucosamine.

Analysis methods for amines, amino acids, and amino sugars are relevant for many fields, including astrobiology, neurobiology, and food science. Amino sugars form polymers that are structurally important in the shells of crustaceans and arthropods and are therefore important biomarkers for the analysis of fossilized remains. The development of a portable system capable of sensitive analysis of these compounds has set the stage for astrobiology research on Mars [21,29,30] and should also enable the use of this instrument for a broad range of terrestrial applications.

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### References


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2 At pH 10, the β-glucosamine peak has moved approximately 1/3 the distance between the α-glucosamine peak and the valine peak. A fully-charged (net −2) glucosamine peak would appear in front of the valine peak due to its larger size. The β-glucosamine charge at pH 10.01 is therefore ~−1.3 (−1 from the fluorescamine carboxylic acid and −0.3 from the glucosamine).