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Differentiation and Quantification of C1 and C2 ^{13}C -Labeled Glucose by Tandem Mass Spectrometry

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Abstract

The fragmentation patterns of various ^{13}C -labeled glucose molecules were analyzed by electrospray ionization tandem mass spectrometry. Derivatization of glucose to yield methylglucosamine makes the C-C bond between C1 and C2 a favored cleavage site. This is in contrast to underivatized glucose, which favorably undergoes loss of a fragment containing both C1 and C2. Based on the fragmentation pattern of methylglucosamine, we developed a method to distinguish and quantify C1 and C2 ^{13}C -labeled glucose by derivatization with methylamine followed by multiple reaction monitoring scans in a Q-trap mass spectrometer. Fragment ion ratios in the tandem mass spectra showed an isotope effect with ^{13}C or deuterium labeling, so a correction factor was introduced to make the quantification more accurate. The present approach can be applied to individually monitor the metabolic origin and fate of C1 and C2 atoms in ^{13}C -labeled glucose. This method provides a new means of quantifying glucose isotopomers in metabolic studies.

Keywords

labeled isotopes; ion fragmentation; mass spectrometry; derivatized glucose; multiple reaction monitoring scans

Introduction

Glucose, the major fuel source of most organisms, is a key compound in carbohydrate metabolism. In order to understand glucose metabolism in detail, it is highly desirable to develop a sensitive, rapid and reliable method that allows the individual carbon atoms in the glucose molecule to be distinguished. For example, we are particularly interested in studying the participation of glucose in ammonia metabolism in mosquitoes. By feeding mosquitoes with ^{13}C -glucose labeled at C1, C2, or C1 and C2, it should be possible to monitor their metabolic fate and quantify their concentrations in different tissues over a time course. The incorporation of specific ^{13}C atoms from labeled glucose into different metabolites, such as alanine labeled at specific positions, would allow us to investigate the metabolic pathways of glucose and its relation to ammonia metabolism.

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Gas chromatography coupled with mass spectrometry (GC-MS) has been widely used for study of monosaccharide metabolism [1]. However, the major disadvantage of this method is that isotopomers labeled by ^{13}C at different positions cannot always be distinguished. Moreover, these methods cannot perform accurate quantification of each isotopomer, although the intensities of the fragment peaks in electron ionization (EI) mass spectra can provide some relative ^{13}C enrichment information. Alternatively, nuclear magnetic resonance (NMR) is able to distinguish compounds that are ^{13}C labeled at different positions, thus it has also been applied to study dynamic carbohydrate metabolism [2]. However, the low inherent signal-to-noise ratio and the requirement for sample purification weakens its sensitivity and increases the time for data collection.

Tandem mass spectrometry (MS/MS) has been used as a sensitive, rapid, reliable and effective method to perform metabolic studies; for example, identification and quantification of amino acids in blood for the detection of diseases [3-8]. Performing MS/MS in the multiple reaction monitoring (MRM) scan mode allows identification and quantitation of multiple compounds with high sensitivity but without previous purification. This is a great advantage for studying complex biological samples, such as neonatal blood [9], intracellular folates [10], whole body [11], excreta [12] or tissues [13] from *Aedes aegypti* mosquitoes, the main vectors of Dengue and Yellow fever.

In order to elucidate the metabolic pathways that blood-fed mosquitoes use to avoid ammonia toxicity, we have studied the kinetics of incorporation of ^{15}N from labeled ammonium chloride into several nitrogen compounds in *A. aegypti* females by MRM. We observed that the labeled nitrogen of $^{15}\text{NH}_4\text{Cl}$ is initially fixed and assimilated in *A. aegypti* into [5- ^{15}N]-glutamine by a glutamine synthetase/glutamate synthase pathway, followed by the production of [^{15}N]-glutamate which is mainly converted to [^{15}N]-proline [11]. We have also shown that mosquitoes can use the ^{15}N from the amide group of two [5- ^{15}N]-glutamine molecules to produce one molecule of uric acid labeled at two nitrogen positions. This uric acid can either be excreted, or further metabolized via an amphibian-like uricolytic pathway that produce glyoxylic acid and two molecules of urea labeled at one position [12]. In addition, we have recently reported that a differential ammonia metabolism occurs in *A. aegypti* fat body and midgut tissues [13]. The use of stable labeled isotopes, MRM and RNAi techniques have allowed us to describe for the first time the multiple metabolic routes that ammonia follows in mosquitoes. Now, we are directing our efforts to the investigation of metabolic flux of ^{13}C compounds involved in ammonia metabolism in mosquitoes. However, there has not yet been an established MS/MS method to differentiate and quantify the glucose molecules that are ^{13}C labeled at different positions for the purpose of metabolic study.

This paper reports an investigation of the fragmentation patterns of various ^{13}C labeled glucose molecules. It is found that C1 and C2 ^{13}C -labeled glucose can be distinguished by MRM after a simple chemical derivatization. The method allows quantification of the $^{13}\text{C}1$ and $^{13}\text{C}2$ isotopomers using deuterium labeled glucose as the internal standard. To maximize quantitative accuracy, we developed correction factors for isotope effects that influence the fragment ion intensities of deuterium and ^{13}C -labeled glucose.

Materials and Methods

Reagents

The labeled isotopes D-Glucose-1- ^{13}C , D-Glucose-2- ^{13}C , D-Glucose-1,2- $^{13}\text{C}_2$, D-Glucose-D₇ (1,2,3,4,5,6,6-D₇), and D-Glucose-D₂ (6,6-D₂) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). D-Glucose (unlabeled), methylamine solution (ca. 40w/v %), borane-dimethylamine complex (97%), boric acid, sodium tetraborate, methanol and toluene were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Glucose Derivatization

The derivatization of glucose to form methylglucosamine (Glc-MA; Scheme 1) was performed based a method previously developed by Honda et al. [14] for ultramicroanalysis of reducing carbohydrates by capillary electrophoresis with laser-induced fluorescence detection. Stock solutions were prepared by dissolving unlabeled or labeled ^{13}C -Glc, Glc- D_2 or Glc- D_7 in 1 M methylamine solution containing 0.2 M dimethylamine-borane complex adjusted to pH 4.5 with acetic acid. Only 10 μL of the stock solutions (0.1 mM) were incubated at 40°C for 30 minutes and the solvents evaporated to dryness under nitrogen atmosphere at 60°C. The residue was treated with 100 μL of methanol and 100 μL of toluene and then the solvents were evaporated to dryness under nitrogen atmosphere at 60°C. The last operation was repeated twice more. The residue of methylglucosamine was stored at -20°C until utilized.

Low-Energy Collision-Induced Dissociation (CID)

Low-energy CID experiments were performed on an Applied Biosystem Q-trap 4000 mass spectrometer (Foster City, CA) with a nanoelectrospray ionization source operated in the positive ion mode. Underivatized and derivatized samples were dissolved in a solution of H_2O : MeOH (30:70, v/v) containing 1% acetic acid to reach a concentration of 20 μM . In some cases, an appropriate amount of Li_2CO_3 (10 μM final concentration) was added to the underivatized glucose solutions to improve ionization efficiency. The solutions were then electrosprayed into the mass spectrometer with a flow rate of ca. 2.0 $\mu\text{L}/\text{min}$. The electrospray voltage was between 2.2 kV and 2.5 kV, and the capillary temperature was maintained at 200°C. Nitrogen served as the collision gas with a collision cell pressure of about 8 millitorr, and a laboratory collision energy of 30 to 35 eV was used to produce extensive fragments from the precursor ion. Monoisotopic precursor ions were selected at unit mass resolution in order to avoid ambiguities from isotope contributions.

Results and Discussion

Fragmentation Spectra of Underivatized and Derivatized Glucose

It is well established that lithium adducts of various carbohydrates are readily dissociated via MS/MS. [15-20]. Thus, lithium carbonate was added to the spray solution, resulting in the exclusive production of the $[\text{Glc}+\text{Li}]^+$ ion (m/z 187). The MS/MS spectrum of this ion shows a clear fragmentation pattern of high quality (Figure 1a).

The MS/MS spectrum shows a major product ion resulting from the loss of 60 Da from the lithiated precursor ion. This is well known to correspond to the neutral loss of $\text{C}_2\text{H}_4\text{O}_2$ [16]. Because this neutral loss contains both C1 and C2, the MS/MS spectrum of unlabeled glucose alone does not provide enough information to distinguish C1 and C2. To illustrate this, ^{13}C labeled glucose isotopomers were fragmented under the same conditions. The MS/MS spectra of those three compounds confirm that the two carbon atoms in the loss of $\text{C}_2\text{H}_4\text{O}_2$ must be C1 and C2, because C1 or C2 ^{13}C -labeled glucose lost 61 Da ($188 \rightarrow 127$), while glucose ^{13}C -labeled at both C1 and C2 lost 62 Da ($189 \rightarrow 127$) (Figure 1 b-d). Under these circumstances, distinction of glucose ^{13}C labeled at C1 versus C2 is not possible.

In order to provide a means of distinguishing $^{13}\text{C}1$ and $^{13}\text{C}2$ glucose by MS/MS, methylglucosamine (Glc-MA), a derivative of glucose, was next considered. We hypothesized that addition of a methylamine group to C1 might lead to preferred cleavage of the C1-C2 bond, yielding the stable product ion $\text{CH}_2=\text{NHCH}_3^+$ (m/z 44) that would contain only one carbon atom (C1) derived from glucose. Indeed, the MS/MS spectrum of unlabeled Glc-MA shows a strong product ion peak at m/z 44 by loss of 152 Da ($\text{C}_5\text{H}_{12}\text{O}_5$) from the $[\text{M}+\text{H}]^+$ precursor ion m/z 196 (Figure 2a), implying that the chemical modification makes the cleavage of the C-C bond between C1 and C2 the most favored fragmentation pathway. The MS/MS

spectra of ^{13}C -labeled Glc-MA molecules further confirm that the major product ion $\text{CH}_2=\text{NHCH}_3^+$ contains C1 only and that the C-C bond between C1 and C2 is cleaved (Figure 2b, c, d). C1 ^{13}C -labeled Glc-MA (Glc-1- ^{13}C -MA) produces a fragment ion at m/z 45 from the precursor ion at m/z 197 upon activation. C2 ^{13}C -labeled Glc-MA (Glc-2- ^{13}C -MA) generates a fragment ion at m/z 44 from the precursor ion at m/z 197. Glc-MA labeled at C1 and C2 (Glc-1,2- ^{13}C -MA) shows a fragment ion at m/z 45 from its precursor ion at m/z = 198. This illustrates that derivatized glucose molecules ^{13}C labeled at C1 and C2 can be distinguished by tandem mass spectrometry. Glc-1,2,3,4,5,6,6-D $_7$ -MA or Glc-6,6-D $_2$ -MA can serve as an internal standard for quantification by MRM. When fragmented, Glc-D $_7$ -MA produces a strong fragment ion at m/z 45 from the precursor ion at m/z 203 (Figure 2e), while Glc-D $_2$ -MA produces m/z 44 from the precursor ion at m/z 198 (Figure 2f).

Differentiation and Quantification of C1 and C2 ^{13}C -Labeled Glucose by Multiple Reaction Monitoring Scans

The chemical modification performed on glucose makes the C-C bond between C1 and C2 more susceptible to cleavage, so Glc-1- ^{13}C -MA and Glc-2- ^{13}C -MA show distinct MS/MS spectra, although they have the same molecular weight. The different product ion masses make it possible to differentiate those two compounds from a mixture without separation or purification, and quantify them with a known amount of Glc-D $_7$ -MA or Glc-D $_2$ -MA added into the sample as the internal standard. In the MRM scan mode in a triple quadrupole type instrument, the first quadrupole mass analyzer is set to select the precursor ions, namely m/z 197 for Glc-1- ^{13}C -MA and Glc-2- ^{13}C -MA, m/z 203 for Glc-1,2,3,4,5,6,6-D $_7$ -MA, and m/z 198 for Glc-1,2- ^{13}C -MA and Glc-6,6-D $_2$ -MA; the third quadrupole mass analyzer transmits their corresponding neutral loss product ions, namely m/z 45 for Glc-1- ^{13}C -MA, Glc-1,2- ^{13}C -MA and Glc-1,2,3,4,5,6,6-D $_7$ -MA, m/z 44 for Glc-2- ^{13}C -MA and Glc-6,6-D $_2$ -MA (Table 1). In this way, MS/MS in MRM mode identifies those two isotopic species, and provides a strategy to monitor each of those two carbon atoms separately. In addition, the result also provides important quantitative information, because the resulting product ion peak intensity ratio between ^{13}C labeled Glc-MA and internal standard deuterium labeled Glc-MA is proportional to the original concentration ratio in the sample.

Deuterium-labeled compounds are commonly used as internal standards for amino acid quantification by MS/MS [21], but isotopic effects are not always taken into account because they are not significant if the deuteriums are not involved in the reaction. However, in the presence of an isotope effect, the accuracy of MS/MS based quantification can be significantly compromised [22,23]. This is because isotopic substitution may greatly change the rate of the fragmentation in MS/MS. These differences can be the result of differences in the reaction being monitored or a difference in another competing reaction. It is observed that when Glc-MA, Glc-1- ^{13}C -MA, Glc-2- ^{13}C -MA, Glc-1,2- ^{13}C -MA, Glc-D $_7$ -MA and Glc-D $_2$ -MA (with the same concentration in solution) were fragmented under the same conditions, the peak intensity ratios between the fragment ion $\text{CH}_2=\text{NHCH}_3^+$ (m/z value varies with labeling, Table 1) and the corresponding precursor ion show some differences (Figure 3a). This means that the concentrations of ^{13}C labeled species would be overestimated if Glc-D $_2$ -MA is used as the internal standard and the peak ratio differences shown in Figure 3a is not considered. To make the quantification more accurate, a “correction factor” is introduced. We consider the correction factor for Glc-D $_2$ -MA as 1 because this will be used as the internal standard. The correction factors for other species are 0.771 for Glc-MA, 0.815 for Glc-1- ^{13}C -MA, 0.916 for Glc-2- ^{13}C -MA, 0.783 for Glc-1,2- ^{13}C -MA, and 1.556 for Glc-D $_7$ -MA, which are calculated based on Figure 3a. To test the performance of the MRM method and the correction factor, a mixture containing the same amount of Glc-1- ^{13}C -MA, Glc-2- ^{13}C -MA, Glc-1,2- ^{13}C -MA and Glc-D $_2$ -MA was measured using the MRM method. Then the amount of Glc-1- ^{13}C -MA, Glc-2- ^{13}C -MA and Glc-1,2- ^{13}C -MA was calculated and corrected by the correction factor

using Glc-D₂-MA as the internal standard. The results are listed in Table 2, showing that the introduction of the correction factor improves the accuracy of the quantification. Experiments using different concentrations of each compound were also tested with the method (data not shown). It should be noted that the “correction factor” introduced above is a purely empirical adjustment of the MS/MS data, applied without consideration of thermodynamics or kinetics. To fully understand the isotopic effect in the fragmentation of Glc-MA, a more detailed investigation of every possible fragmentation pathway would be required. For this study, we checked the ratio between total fragment ion intensity and total ion abundance (Figure 3b) and the ratio between CH₂=NHCH₃⁺ ion intensity and the total ion abundance (Figure 3c). The peak ratio in Figure 3b is almost the same among all the Glc-MA isotopomers. However, the peak ratio pattern in Figure 3c is very similar to that in Figure 3a, indicating that at least one fragmentation pathway shows a kinetic isotope effect.

Conclusions

It is observed in this study that the C-C bond between C1 and C2 atoms in underivatized lithiated glucose is not cleaved, and C1 and C2 tend to be lost together. In this event, the fragmentation cannot differentiate C1 ¹³C-labeled glucose from C2 ¹³C-labeled glucose. To resolve this problem, we chemically modified the glucose molecule into methylglucosamine, making the C-C bond between C1 and C2 in glucose the most favored bond cleavage site upon CID by forming a stable product ion containing only C1. Therefore, the chemical derivatization makes it possible to distinguish the C1 and C2 ¹³C-labeled glucose molecules by tandem mass spectrometry, which allows the two carbon atoms to be followed separately in metabolic pathway studies. Based on the fragmentation patterns of derivatized glucose, we developed an MRM method to identify C1 and C2 labeled glucose molecules and quantify each isotopomer using Glc-D₂-MA as the internal standard. A correction factor is introduced in order to make the quantification more accurate. The high speed, high sensitivity and reliability of MS/MS make this a novel and promising method for following the metabolic pathways of carbon atoms in glucose and quantitatively studying the kinetics of the metabolic transformations of isotopomers in biological systems.

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Figure 1. MS/MS spectra of lithiated glucose. a) $[Glc+Li]^+$ (m/z 187); b) $[Glc-1-^{13}C+Li]^+$ (m/z 188); c) $[Glc-2-^{13}C+Li]^+$ (m/z 188); and d) $[Glc-1,2-^{13}C_2+Li]^+$ (m/z 189). The collision energy used was 35 eV.

Figure 2. MS/MS spectra of unlabeled and labeled methylglucosamine: a) $[\text{Glc-MA}+\text{H}]^+$ (m/z 196); b) $[\text{Glc-1-}^{13}\text{C-MA}+\text{H}]^+$ (m/z 197); c) $[\text{Glc-2-}^{13}\text{C-MA}+\text{H}]^+$ (m/z 197); d) $[\text{Glc-1,2-}^{13}\text{C-MA}+\text{H}]^+$ (m/z 198); e) $[\text{Glc-D}_7\text{-MA}+\text{H}]^+$ (m/z 203) and f) $[\text{Glc-D}_2\text{-MA}+\text{H}]^+$ (m/z 198). The collision energy used was 30 eV.

Figure 3.

Peak intensity ratio between: a) the fragment ion $\text{CH}_2\text{NHCH}_3^+$ (and labeled equivalent) and its precursor ion for protonated Glc-MA, Glc-1-¹³C-MA, Glc-2-¹³C-MA, Glc-1,2-¹³C-MA, Glc-D₇-MA and Glc-D₂-MA; b) total fragment ion abundance and total ion abundance for all fragments and precursor ions for protonated Glc-MA, Glc-1-¹³C-MA, Glc-2-¹³C-MA, Glc-1,2-¹³C-MA, Glc-D₇-MA and Glc-D₂-MA; c) $\text{CH}_2\text{NHCH}_3^+$ (and labeled equivalent) ion abundance and the total ion abundance for all fragments and precursor ions for protonated Glc-MA, Glc-1-¹³C-MA, Glc-2-¹³C-MA, Glc-1,2-¹³C-MA, Glc-D₇-MA and Glc-D₂-MA. Data are presented as mean $\pm$ standard error of three independent samples.

Scheme 1.

Derivatization of glucose and the structure of the product methylglucosamine (Glc-MA).

Table 1

m/z of precursor ions and neutral loss of unlabeled and labeled methylglucosamine

Methylglucosamine (Glc-MA) ^a	Precursor ion	Neutral loss	Precursor ion/Fragment ion pair
Glc-MA	196	152	196 → 44
Glc-1- ¹³ C-MA	197	152	197 → 45
Glc-2- ¹³ C-MA	197	153	197 → 44
Glc-1,2- ¹³ C-MA	198	153	198 → 45
Glc-D ₇ -MA	203	158	203 → 45
Glc-D ₂ -MA	198	154	198 → 44

^aThe quantification of unlabeled and labeled methylglucosamine can be performed by multiple reaction monitoring scans (MRM).

Table 2

Calculated amounts of labeled methylglucosamine using MRM with and without correction factor (CF)

Methylglucosamine (Glc-MA) ^a	True amount nmol	Calculated amount (without CF) nmol	Calculated amount (with CF) nmol
Glc-1- ¹³ C-MA	0.200	0.243 ± 0.006	0.198 ± 0.005
Glc-2- ¹³ C-MA	0.200	0.219 ± 0.002	0.200 ± 0.002
Glc-1,2- ¹³ C-MA	0.200	0.258 ± 0.002	0.202 ± 0.002
Glc-D ₂ -MA	0.200		

^aThe quantification of labeled methylglucosamine was performed by multiple reaction monitoring scans (MRM) using Glc-D₂-MA as internal standard. The values are presented as mean ± standard error of three independent samples.