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Quantitative Analysis of Stable Isotopes of Glucose in Blood Plasma Using Quadrupole GC-MS

GC/MS Technical Report No.2

GC/MS Metabolomics & Life Science Project
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Abstract

In order to investigate the dynamics of glucose in living organisms, a stable isotope of glucose is administered, and its concentration in blood and tissues is measured with quadrupole GC-MS. In this study, we conducted a comparative investigation of the influence of the derivatization method on the results obtained in the quantitative analysis of D-glucose-$^{13}C_6$ using quadrupole GC-MS. As a result, it was discovered that aldonitrile-pentaacetylation gives a single derivatization product, and in mass separation performed with MS, it yielded good quantitative results with little influence by the natural isotopes of glucose that are present at high concentrations in living organisms.

Keywords: GC/MS, Glucose, Isotropic labeling, Plasma, Derivatization, Aldonitrile pentaacetate

Introduction

Glucose is a substance that is consumed and produced in the metabolic pathways of glycolysis and gluconeogenesis and is essential for the energy of life. In order to investigate these metabolic pathways and diseases such as diabetes, the concentration of isotopically labeled compounds is measured in blood and tissues as tracers. In measurement performed using GC-MS, stable isotopes such as ^{13}C and ^{2}H (D) are often used as labels instead of elements (i.e., carbon and hydrogen).

In the quantitative analysis of sugars, such as glucose, performed using GC-MS, the sugars are derivatized in order to make them volatile. There are many derivatization methods, which can be classified according to reaction. They include acylation, esterification, and silylation. Trifluoroacetylation is an acylation reaction and is often used for amine drugs, such as stimulants, as the derivatization method for amines, amino acids, and alcohols. Trimethylsilylation is a silylation reaction and is often used in the field of life science as the derivatization method for steroids, amino acids, sugars, and organic acids.

Methoxime-trimethylsilylation is used in a wide range of applications as a derivatization method that enables the batch analysis of amino acids, organic acids, and sugars in samples of living organisms. Acetylation performed with acetic anhydride is an esterification reaction and is used as the derivatization method for alcohols, fatty acids, and amino acids.[1]

An important aspect of the quantitative analysis of a stable glucose isotope in blood and tissues performed using GC-MS is how well the isotope is separated from the glucose that is present in high concentrations in the living organism itself. Because separation is difficult with GC alone, mass separation with MS is required.

The intensity of the monitor ions of the stable glucose isotope, however, is inevitably equal to the sum of the intensities for the stable glucose isotope and the natural glucose isotopes in the living organism itself. Therefore, in order to reduce the lower limit of detection, it is important to minimize the contribution of the natural isotopes to the monitor ion intensity. This contribution depends not only on the relative concentration of the natural isotopes with respect to the stable isotope, but also on the types of isotopically labeled elements and the number of isotopic labels among the monitor ions. The larger the number of substituted elements, the smaller the influence of the natural isotopes, and the easier it is to perform separation with MS. There is, however, a problem in that as the number of substituted elements increases, so does the cost of the reagent. The elemental composition of the monitor ions is determined by the chemical structure of the stable glucose isotope that is used as a label and the derivatization method.

For this application report, we used glucose, for which the reagent is relatively inexpensive (D-glucose-$^{13}C_6$), as a label and investigated which of the derivatization methods trifluoroacetylation, methoxime-trimethylsilylation, and aldonitrile-pentaacetylation is suitable for quantitative analysis.

Experiment

Reagents

D-glucose (25 g, Wako Pure Chemical Industries, Ltd.) and D-glucose-^{13}C$_6$ (ISOTEC) were used as standard reagents. D-glucose standard aqueous solutions were prepared by adding D-glucose to ultrapure water to concentrations of 1 and 10 µg/mL. D-glucose-^{13}C$_6$ standard aqueous solutions were prepared by adding D-glucose-^{13}C$_6$ to ultrapure water to concentrations of 1 and 10 µg/mL. Rat blood plasma (concentration of glucose in blood plasma: 371.3 mg/dL) was used as a biological sample.

Derivatization Reagents

Trifluoroacetylation

The MBTFA (N-methyl-bis-trifluoroacetamide) used for trifluoroacetylation was purchased from Wako Pure Chemical Industries, Ltd. (1 mL x 10 ampoules).

Methoxime-trimethylsilylation

The methoxyamine hydrochloride solution (20 mg/mL, pyridine solution) used for methoximation was prepared by dissolving methoxyamine hydrochloride (Wako Pure Chemical Industries, Ltd.) in pyridine (Wako Pure Chemical Industries, Ltd.) to a concentration of 20 mg/mL. The MSTFA (*N*-methyl-*N*-trimethylsilyl-trifluoroacetamide) used for trimethylsilylation was purchased from GL Sciences Inc.

Aldonitrile-pentaacetylation

The aldonitrile derivatization reagent solution (0.2 mol/L, pyridine solution) used for aldonitrilation was prepared by dissolving hydroxylammonium chloride (Wako Pure Chemical Industries, Ltd.) in pyridine (Wako Pure Chemical Industries, Ltd.) to a concentration of 0.2 mol/L. The acetic anhydride used for acetylation was purchased from Wako Pure Chemical Industries, Ltd.

Pretreatment Methods

Trifluoroacetylation

20 µL of D-glucose standard aqueous solution (10 µg/mL) was sampled and freeze-dried. Trifluoroacetylation was performed by adding 10 µL of pyridine and 10 µL of MBTFA (N-methyl-bis-trifluoroacetamide) to the dried sample, and then heating at 60 ºC for 60 minutes. The resulting solution was used as a mass spectrum confirmation sample.[2]

Methoxime-trimethylsilylation

150 µL of both D-glucose and D-glucose-^{13}C$_6$ standard aqueous solutions (1 µg/mL) were sampled and freeze-dried. Methoximation was performed by adding 50 µL of methoxyamine hydrochloride solution (20 mg/mL, pyridine solution) to the dried samples, and then heating at 30 ºC for 90 minutes. After that, trimethylsilylation was performed by adding 100 µL of MSTFA and then heating at 37 ºC for 30 minutes. The resulting solution was used as a mass spectrum confirmation sample.

A D-glucose-^{13}C$_6$–added blood plasma sample was prepared by adding D-glucose-^{13}C$_6$ to blood plasma to a concentration of 2.5 µg/mL. 80 µL of the prepared sample was sampled and 240 µL of ethanol was added, and the solution was stirred and subjected to centrifugal separation (10,000 rpm, 5 minutes) at room temperature. After that, 150 µL of supernatant was sampled and freeze-dried. Methoximation was performed by adding 31.25 µL of methoxyamine hydrochloride solution (20 mg/mL, pyridine solution) to the dried sample and then heating at 30 ºC for 90 minutes. After that, trimethylsilylation was performed by adding 62.5 µL of MSTFA and then heating at 37 ºC for 30 minutes. The resulting solution was used as a measurement sample (concentration of D-glucose in measurement sample: 1,392 µg/mL).[3]

Aldonitrile-pentaacetate derivatization

400 µL of both D-glucose and D-glucose-^{13}C$_6$ standard aqueous solutions (1 µg/mL) were sampled and freeze-dried. Nitrilation was performed by adding 150 µL of hydroxylammonium chloride (0.2 mol/L, pyridine solution) to the dried sample, and then heating at 90 ºC for 40 minutes. After that, acetylation was performed by adding 250 µL of acetic anhydride, and then heating at 90 ºC for 60 minutes. After that, the sample was dried in a nitrogen gas stream at 50 ºC, and then redissolved in 400 µL of ethyl acetate. The resulting solution was used as a mass spectrum confirmation sample.

A D-glucose-^{13}C$_6$-added blood plasma sample was prepared by adding D-glucose-^{13}C$_6$ to blood plasma to a concentration of 4 µg/mL. 300 µL of the prepared sample was sampled and 900 µL of ethanol was added, and the solution was stirred and subjected to centrifugal separation (10,000 rpm, 5 minutes) at room temperature. After that, 400 µL of supernatant was sampled and freeze-dried. The dried sample was subjected to aldonitrile-pentaacetate derivatization using the same procedure described above. The resulting solution was used as a measurement sample (concentration of D-glucose in measurement sample: 928 µg/mL).[4]

Instruments

GCMS-QP2010 Plus was used for GC-MS and GCMSsolution was used for data processing. The analytical conditions are shown in Table 1.

Table 1: Analytical Conditions

Instruments
GC-MS	: GCMS-QP2010 Plus
Auto-Injector	: AOC-20i + s
Column	: Rtx®-5MS (30 m x 0.25 mm I.D. df=0.25 µm, Restek Corporation)

Analytical Conditions
Trifluoroacetylation and Methoxime-Trimethylsilylation

GC
Injection temp.	: 250 °C
Injection mode	: Splitless
Sampling time	: 1.0 min
Column temp.	: 80 °C (2 min) - (15 °C/min) - 320 °C (6 min)
Carrier gas	: He (Constant Linear Velocity)
Linear velocity	: 36.8 cm/sec
Injection volume	: 1 µL

MS
Ion source temp.	: 200 °C
Interface temp.	: 250 °C
Scan range	: m/z 45 – 600
Event time	: 0.5 sec

Aldonitrile-Pentaacetylation

GC
Injection temp.	: 250 °C
Injection mode	: Split
Column temp.	: 80 °C (2 min) - (15 °C/min) - 320 °C (6 min)
Carrier gas	: He (Constant Linear Velocity)
Linear velocity	: 36.8 cm/sec
Split ratio	: 15
Injection volume	: 1 µL

MS
Ion source temp.	: 200 °C
Interface temp.	: 250 °C
Scan	
Scan range	: m/z 45 – 600
Event time	: 0.5 sec
SIM	
Monitoring ion	: m/z 191, 319
Event time	: 0.4 sec

Results and Discussion

Trifluoroacetylation

The chromatogram obtained by analyzing a D-glucose mass spectrum confirmation sample (10 µg/mL) is shown in Fig. 1. With trifluoroacetylation, four peaks were detected, each with a different intensity. Glucose is a reducing aldose, and the four peaks detected with trifluoroacetylation corresponded to alpha- and beta- furanose and pyranose anomers in the derivatization product, which has the structure shown in Fig. 2. When derivatization produces four derivatization products like this, the separation sensitivity decreases and data processing becomes complicated. Therefore, no further consideration was given to trifluoroacetylation.
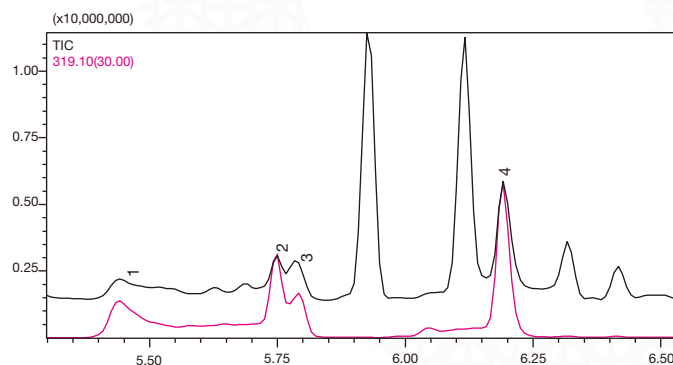
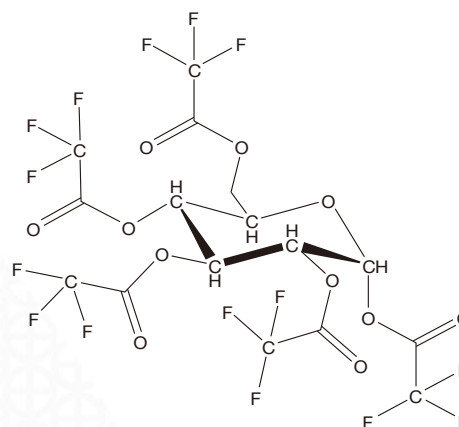Fig. 1: Chromatogram of Trifluoroacetylated Glucose

Fig. 2: Structural Formula of Trifluoroacetylated Glucose

Methoxime-Trimethylsilylation

The total ion chromatograms and mass chromatograms obtained by analyzing a D-glucose and D-glucose-$^{13}C_6$ mass spectrum confirmation sample (1 µg/mL) are shown in Fig. 3. Judging fr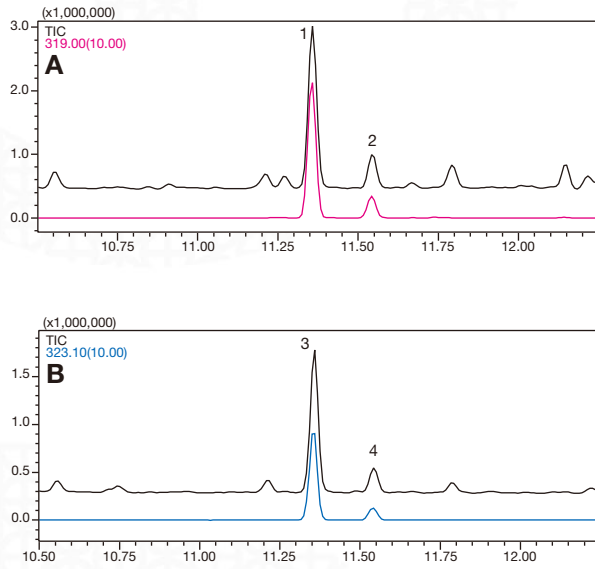om the mass spectra shown in Fig. 4, the detected peaks were probably D-glucose and D-glucose-$^{13}C_6$ methoxime-trimethylsilyl derivatization products (Fig. 5). Two peaks were detected in each case because syn and anti products resulted from the methoximation of carbonyl groups.
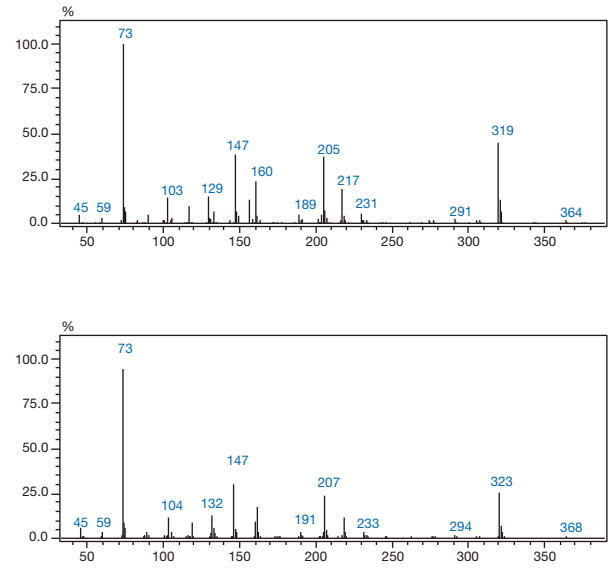
Fig. 3: Chromatograms of Methoxime-Trimethylsilylated Glucose
A: D-Glucose, B: D-Glucose-$^{13}C_6$

Fig. 4: Mass Spectra of Methoxime-Trimethylsilylated Glucose
A: D-Glucose, B: D-Glucose-$^{13}C_6$
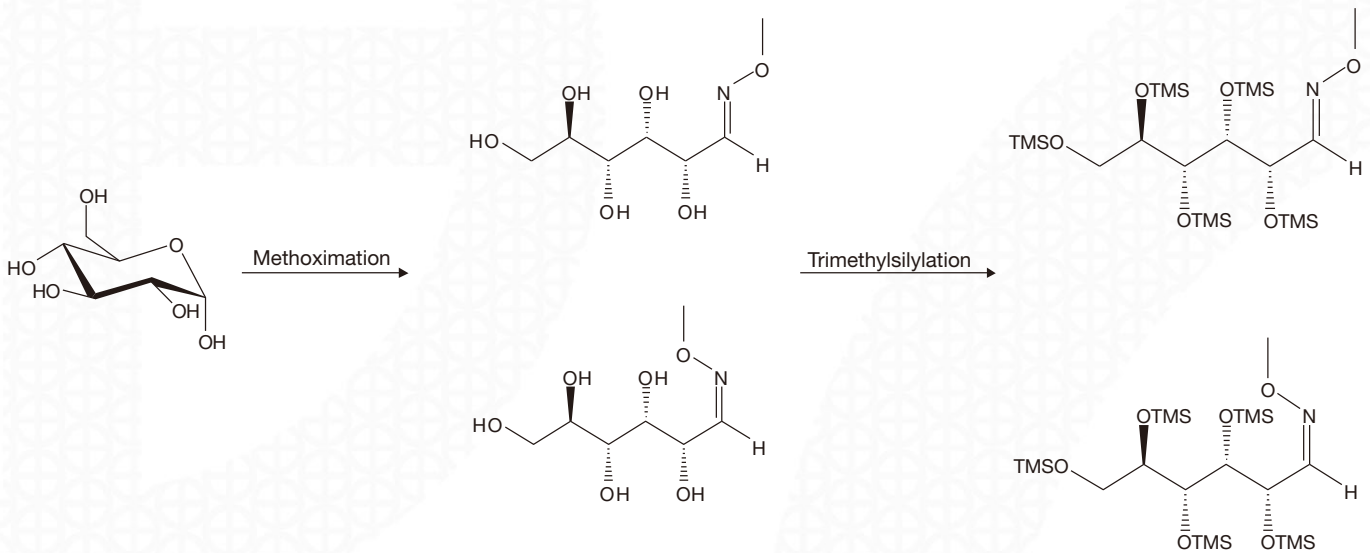
Fig. 5: Methoxime-Trimethylsilylation Reaction of D-Glucose

On the basis of the mass spectra for D-glucose and D-glucose-$^{13}C_6$ (Fig. 4), we decided to consider the ions m/z 319 and m/z 323, which have large mass numbers and relatively high intensities, as monitor ions. These ions probably contain 13 carbon (C) molecules, as shown in Fig. 6, of which four are ^{13}C molecules, and three silicon (Si) molecules.

The intensity of the labeled glucose monitor ion (m/z 323) is equal to the sum of the values for the labeled glucose and the natural glucose isotopes in the living organism itself. In particular, with blood plasma samples in which the concentration of glucose in the living organism itself is high compared to that of the labeled glucose, the contribution of natural isotopes must be considered. In order to estimate this contribution, the abundance ratios of natural isotopes in the range m/z 319 to m/z 325 were calculated for a glucose concentration of 1,329 µg/mL. The results are shown in Table 2. Calculation was based on natural abundance ratios of 98.90% for ^{12}C, 1.10% for ^{13}C, 92.27% for ^{28}Si, 4.68% for ^{29}Si, and 3.05% for ^{30}Si. As a result, it was calculated that, if m/z 323 is used as the monitor ion for the labeled substance, then there is a contribution of 0.69% by natural isotopes, which translates to a concentration of 6.4 µg/mL.
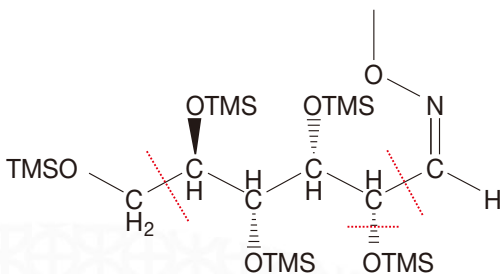
The total ion chromatogram and mass chromatogram obtained by subjecting a D-glucose-$^{13}C_6$ -added blood plasma sample (1 µg/mL) to methoxime-trimethylsilylation and then analyzing it are shown in Fig. 7. As with the results for the mass spectrum confirmation samples shown in Fig. 3, D-glucose and D-glucose-$^{13}C_6$ could not be separated by the column. Also, because there was a high concentration of D-glucose in the living organism, the column's load capacity was exceeded, and the peak form was adversely affected. In order to avoid this column overload due to high concentrations of D-glucose, split analysis is necessary.
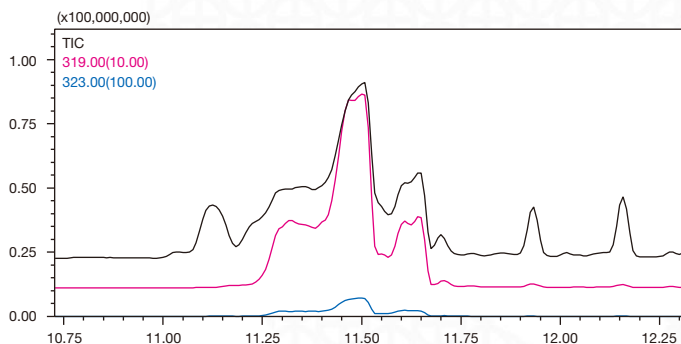

Fig. 7: Chromatograms of D-Glucose and D-Glucose-$^{13}C_6$ (1 µg/mL) in Blood Plasma Sample

Fig. 6: Fragmentation of m/z 319 and m/z 323 in Methoxime-Trimethylsilylated Glucose

With this derivatization method, for a glucose concentration of 1,329 µg/mL in blood plasma, the contribution to quantitative results is 6.4 µg/mL, making it difficult to attain the desired (µg/mL-order) lower limit of detection. Also, if the split ratio was increased to improve the peak form, judging from the chromatograms in Fig. 7, detection may become difficult. It became clear, then, that this derivatization method is not suitable for the kind of analysis being considered here.

Table 2 Influence of Fragment Ions in Methoxime-Trimethylsilylated Glucose and Natural Isotopes

m/z	Isotopic Abundance	Glcose [µg/ml] 1392.0
319	100.00%	934.7
320	30.23%	282.6
321	14.79%	138.2
322	2.99%	28.0
323	0.69%	6.4
324	0.07%	0.7
325	0.01%	0.1

Aldonitrile-Pentaacetate Derivatization

The SIM total ion chromatogram and mass chromatogram obtained by subjecting D-glucose-$^{13}C_6$ aqueous solution (1 µg/mL) to aldonitrile-pentaacetylation and then analyzing it are shown in Fig. 8. With aldonitrile-pentaacetylation, no isomers were produced by the derivatization reaction shown in Fig. 9, and a single peak was detected.
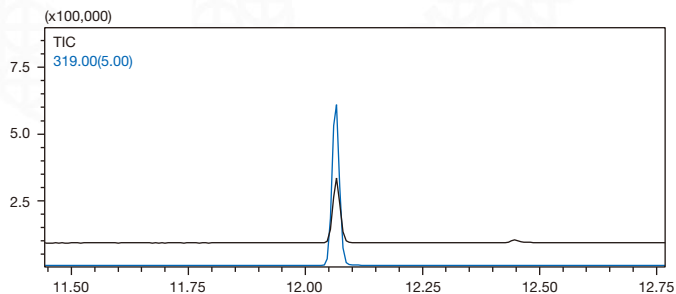

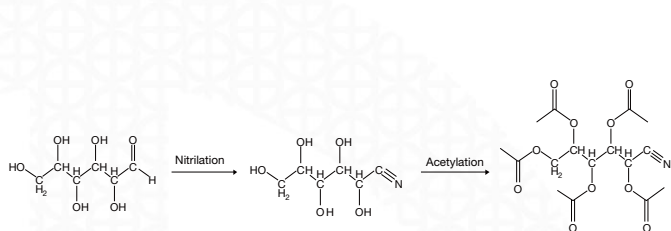Fig. 8: Chromatograms of Aldonitrile-Pentaacetylated D-Glucose-$^{13}C_6$ (1 µg/mL)

Fig. 10: Mass Spectra of Aldonitrile-Pentaacetylated Glucose A: D-Glucose, B: D-Glucose-$^{13}C_6$

Fig. 9: Aldonitrile-Pentaacetylation Reaction of D-Glucose
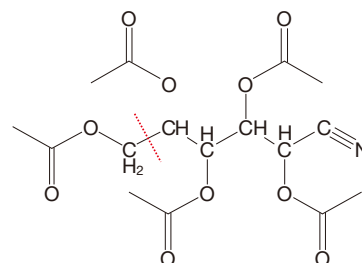
Fig. 11: Fragmentation of *m/z* 314 and *m/z* 319 in Aldonitrile-Pentaacetylated Glucose

On the basis of the mass spectra for D-glucose and D-glucose-$^{13}C_6$ shown in Fig. 10, we decided to consider the ions *m/z* 314 and *m/z* 319, which have large mass numbers and relatively high intensities, as monitor ions. These ions probably contain 13 carbon (C) molecules, as shown in Fig. 11, of which five are ^{13}C molecules. The influence of the abundance ratios of natural isotopes in the range *m/z* 314 to *m/z* 321 was calculated for a glucose concentration of 928 µg/mL in the blood plasma sample used in this study. The results are shown in Table 3. From the fact that the monitored fragment ions contain five carbon molecules and no silicon molecules, it was deduced that the contribution of natural isotopes is 0.00237%, which is approximately 1/300 of the equivalent figure for methoxime-trimethylsilylation.

Table 3 Influence of Fragment Ions in Aldonitrile-Pentaacetylated Glucose and Natural Isotopes

m/z	Isotopic Abundance	Glcose [µg/ml] 1392.0
314	100.00000%	783.68672
315	15.38088%	120.53792
316	2.70578%	21.20480
317	0.29485%	2.31072
318	0.03079%	0.24128
319	0.00237%	0.01856
320	0.00024%	0.00186
321	0.00001%	0.00009

6

The mass chromatograms obtained by subjecting a blood plasma sample (blank) and a D-glucose-$^{13}C_6$-added blood plasma sample (1 μg/mL) to aldonitrile-pentaacetylation and analyzing them are shown in Fig. 12. In order to prevent column overload due to high-concentration D-glucose, the split injection method was considered. With the split injection method, if the split ratio is increased, the volume of sample introduced into the column can be decreased but the sensitivity decreases by a corresponding degree. It was established that, for a split ratio of 15, the peak form is good and sufficient sensitivity can be attained. With the blood plasma sample (blank), D-glucose-$^{13}C_6$ was detected at a concentration of 0.022 μg/mL, which is very close to the concentration of natural isotopes given by D-glucose in Table 3. Also, a good quantitative value of 1.019 μg/mL was obtained for the D-glucose-$^{13}C_6$–added sample.

Blood Plasma Sample (Blank)
Quantitative Value: 0.022 μg/mL
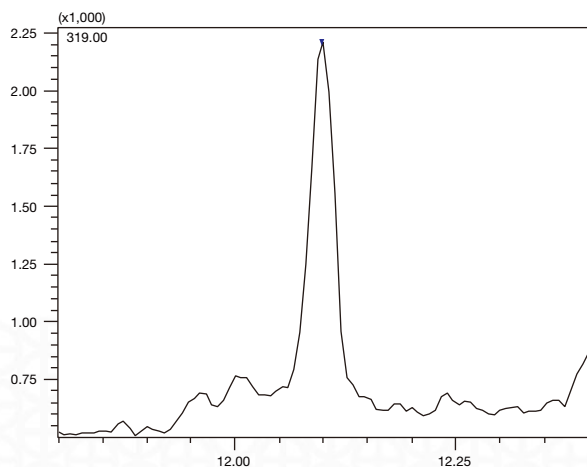
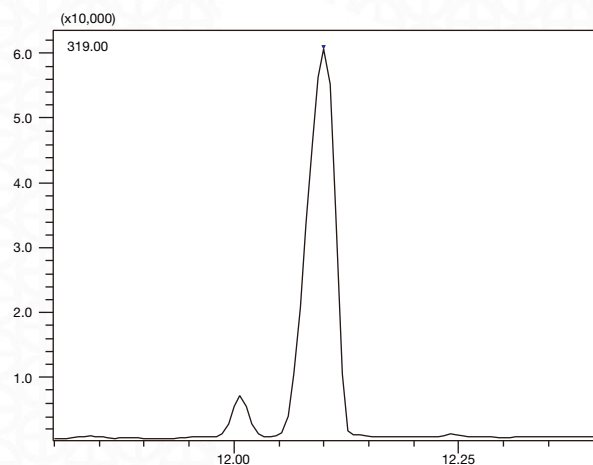D-Glucose-$^{13}C_6$-Added Blood Plasma Sample (1 μg/mL)
Quantitative Value: 1.019 μg/mL

Fig. 12: Mass Chromatograms of Blood Plasma Sample (Blank) and D-Glucose-$^{13}C_6$-Added Blood Plasma Sample (1 μg/mL)

Conclusion

The use of trifluoroacetylation, methoxime-trimethylsilylation, and aldonitrile-pentaacetylation as derivatization methods in the quantitative analysis of stable glucose isotopes in blood plasma performed with quadrupole GC-MS was investigated. As a result, it was discovered that aldonitrile-pentaacetylation was unlike other derivatization methods in that it produced only a single derivatization product and data analysis was simple.

Mass separation using MS is required to perform the quantitative analysis of D-glucose-$^{13}C_6$ without being influenced by glucose in the living organism itself because column separation is difficult. With methoxime-trimethylsilylation, the monitor ions suitable for quantitative analysis contain silicon, which has a high natural isotopic ratio, and results are influenced by natural isotopes of D-glucose in the living organism itself, making it difficult to perform quantitative analysis at levels of less than a few μg/mL. The degree of influence of natural isotopes on aldonitrile-pentaacetate derivatives, however, was found to be approximately 1/300 of the equivalent figure for methoxime-trimethylsilylation derivatives. In the quantitative analysis, then, of isotopes of target compounds in living organisms, the influence of natural isotopes must be considered, and improvising with the derivatization method, can facilitate micro-level quantitative analysis.

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Shimadzu GC-MS and Metabolomics

The Shimadzu GC-MS is used in advanced metabolomics research of congenital metabolic abnormalities, and is earning high acclaim internationally. A GC-MS must have the following functionality to be suitable for metabolite analysis and metabolomics analysis.

(1) Metabolites that deserve attention are not always present at high concentrations, so sufficiently high sensitivity for the detection of trace level metabolites is necessary.
(2) To enable an exhaustive metabolite search, it is important to minimize the loss of components during sample preparation. Sample cleanup is often omitted for this reason, resulting in analytical samples that contain significant interferences. This can be a problem when a GC-MS is used to analyze such samples due to contamination of the ion source. Therefore, an instrument that is resistance to contamination and which allows simple cleaning of the ion source even in the event of contamination is highly desirable.
(3) Since it is not uncommon for metabolites to have similar mass spectra, identification that is based on both the retention index and mass spectrum is required. Therefore, the data analysis software used for analysis should also support the use of retention indices.
(4) NIST and other mass spectral libraries do not contain entries for every metabolite. Therefore, specialized libraries for specific metabolites are required.

The Shimadzu GCMS-QP2010 Plus satisfies all of these conditions.

Gas Chromatograph / Mass Spectrometer GCMS-QP2010 Plus

Features of GCMS-QP2010 Plus
1. High sensitivity
2. Easy maintenance
3. Compound identification using retention indices

GC/MS Metabolites Spectral Database (Amino acids, fatty acids, organic acids)

The GC/MS Metabolites Spectral Database is a mass spectral library for the GCMSsolution workstation software which controls the GCMS-QP2010 series gas chromatograph / mass spectrometer. Use of a mass spectral library equipped with retention indices greatly reduces the number of candidate compounds to improve the reliability of search results.

This database consists of 4 different kinds of method files containing analytical conditions, mass spectra, retention indices, etc., and 4 kinds of libraries containing CAS numbers and other compound information, mass spectra and retention indices. A printed handbook containing the library information is also provided with the database.
The methods and libraries contain metabolite-related information for amino acids, fatty acids and other organic acids, including 261 electron ionization spectra and 50 chemical ionization spectra.

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