Quantitative Determination of Endogenous Glucose in Human Nerve Tissues and Erythrocytes by Turboionspray LC/MS/MS

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OVERVIEW

Purpose

To develop and validate an LC/MS/MS method for accurate determination of endogenous glucose and other monosaccharides/polyols over a broad linear range in different biological matrices.

Methods

Glucose and other endogenous monosaccharides/polyols were extracted from lysed erythrocytes or homogenized nerve tissue by protein precipitation with acetonitrile. Samples were analyzed on a MDS Sciex API3000 equipped with a Shimadzu 10ADvp series LC system. Endogenous glucose concentrations were quantified against internal calibration curves generated by stable isotope-labeled glucose (D-[1,6-$^{13}C_2$-6,6′-$^{2}H_2$]-glucose) using another stable isotope-labeled glucose (D-[1,2-$^{13}C_2$]-glucose) as an internal standard (IS).

Results

A simple and high throughput method was developed to accurately quantify glucose and other endogenous monosaccharides/polyols in biological matrices.

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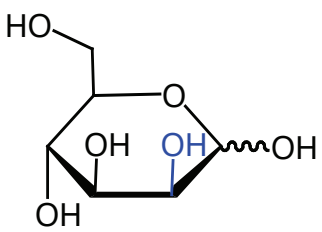
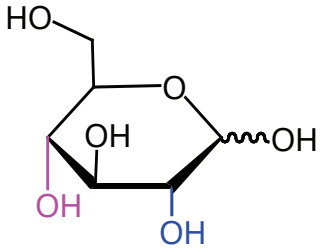
INTRODUCTION

Abnormal levels of glucose, fructose, sorbitol, and other monosaccharides/polyols in different tissues and organs are important indications for a variety of diseases.[1-2] It is critical to monitor the concentration changes of these compounds in target tissues and organs for certain pathological and pharmacological studies. To actually determine the concentrations of these endogenous compounds over a broad linear range in different biological matrices, a simple and high throughput LC/MS/MS method was developed. On a Transgenomic CARBOSep CHO-620 CA column, glucose was well resolved from other tested monosaccharides/polyols, including galactose, mannose, fructose, myo-inositol, adonitol, i-erythritol, L-arobitol, galacitol, and sorbitol. To accurately quantify endogenous glucose, internal calibration curves were generated using "equivalent" isotope-labeled glucose. A separate isotope-labeled glucose was used as an internal standard. The chemical structures of the natural glucose and its typical isomers and analogs are shown in Figure 1.

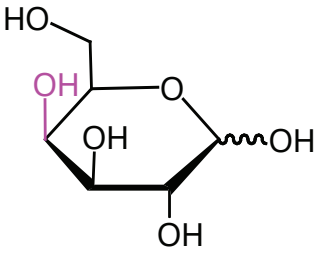
Figure 1. Chemical Structures of Glucose and Its Isomers/Analogs. "*" Represents ^{13}C.



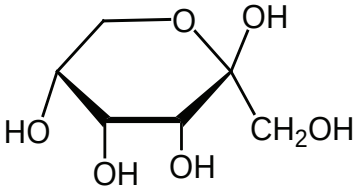
D-Mannose



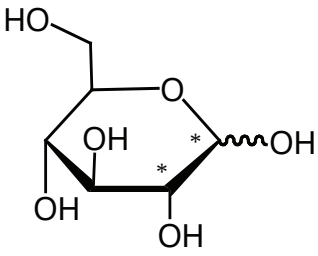
D(+)-Glucose



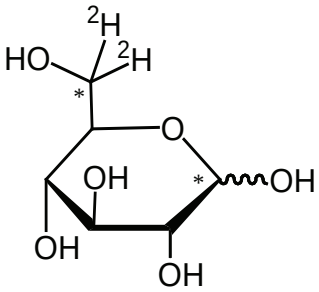
D(+)-Galactose



D-Fructose



D-[1,2-$^{13}C_2$]-glucose



D-[1,6-$^{13}C_2$-6,6'-$^{2}H_2$]-glucose

METHOD

Sample Preparation

1. Lysed red blood cell samples (100 µL) are diluted with 4.90 mL of water. Two hundred (200) µL of the diluted samples are aliquoted into a 13 x 100 mm polypropylene tube, followed by the addition of 200 µL of the internal standard (25,000 ng/mL D-[1,2-$^{13}C_2$]-glucose in water) and 400 µL of water.
2. Vortex mix samples.
3. Add 4.00 mL of acetonitrile to each sample to precipitate the proteins.
4. Vortex mix and centrifuge samples.
5. Transfer the clear supernatant to a clean test tube and evaporate.
6. Reconstitute the sample residue into 0.500 mL of water.
7. Filter the sample through a Varian Captiva 96-well filter plate (Part# A5960045) into a 96-well plate.
8. Store the samples at 1-8°C for analysis.

Chromatographic Conditions

Column:	Transgenomic CARBOSep CHO-620 CA, 6.5 x 300 mm, Part# CHO-99-9753
Mobile Phases:	A: water, 500 µL/min (isocratic) C: water, 500 µL/min (make-up solvent for diversion)
Injection Volume:	10 – 50 µL
Column Temperature:	90°C
AS Temperature:	1-8°C
Divert Time:	6.0 – 11.8 minutes into MS

Mass Spectrometer Conditions

Instrument:	MDS API 3000, Analyst 1.2
Ionization Mode:	Turboionspray, Negative Ion Mode
Source Temperature:	450°C
SRM Transitions:	D(+)-glucose (analyte) 179.1→119.1 D-[1, 6-$^{13}C_2$-6, 6'-$^{2}H^2$]-glucose (calibration curve) 183.3→122.1 D-[1, 2-$^{13}C_2$]-glucose (internal standard) 181.2→119.1

RESULTS

Method Development

MRM Transition Selection

The product ion spectra of glucose and D-[1, 6-$^{13}C_2$-6, 6′-$^{2}H_2$]-glucose are shown in Figures 2 and 3, respectively. The most abundant product ions for D-glucose and D-[1, 6-$^{13}C_2$-6, 6′-$^{2}H_2$]-glucose are 89.1 and 91.0 m/z, respectively. However, these transitions are not the ideal transitions for accurate quantitation of endogenous glucose using calibration curves generated by D-[1, 6-$^{13}C_2$-6, 6′-$^{2}H_2$]-glucose, as these transitions demonstrated non-equivalent ion abundance compared to unlabeled glucose under the experimental MS/MS conditions. Instead, 179.1→119.1 for glucose and 181.2→119.1 for D-[1, 6-$^{13}C_2$-6, 6′-$^{2}H_2$]-glucose were used because of their equivalent ion abundance (Table 1). For internal standard D-[1, 2-$^{13}C_2$]-glucose, the transition 181.2→119.1 was used accordingly (Figure 4).

Table 1. Equivalence of Ionization for D(+)-glucose and D-[1, 6-$^{13}C_2$-6, 6′-$^{2}H_2$]-glucose

Area Ratio (Analyte/IS)	Mean	S.D.	Precision%	n	Mean of Two Weighings
D-Glucose/IS					
Weighing 1	1.06	0.0158	1.5	6	1.04
Weighing 2	1.03	0.00779	0.8	6	
D-[1, 6-$^{13}C_2$-6, 6′-$^{2}H_2$]-Glucose/IS					
Weighing 1	0.997	0.0161	1.6	6	1.004
Weighing 2	1.01	0.0147	1.5	6	
Total equivalency between D-glucose and D-[1, 6-$^{13}C_2$-6, 6′-$^{2}H_2$]-glucose: 96.5					

Identical solution concentrations were prepared in water in duplicates for D-glucose and D-[1, 6-$^{13}C_2$-6, 6′-$^{2}H_2$]-glucose, respectively. Each solution was mixed with equal volumes of the same concentration of D-[1,2-$^{13}C_2$]-glucose (IS). Samples were analyzed under optimized conditions for six replicates.

Liquid Chromatography Development

Many endogenous mono-saccharides (such as glucose, galactose, mannose, fructose, etc.) have exactly the same MS/MS transitions and, thus, it is important to separate the target analyte(s) from these isomers.[3] Several amino columns and carbohydrate affinity columns were investigated to separate glucose from other endogenous mono-saccharides/polyols. The best separation was obtained on a Transgenomic CARBOSep CHO-620 CA column. Under optimized conditions, glucose was well resolved from the other tested monosaccharides/polyols (Figure 5).

Method Selectivity and Specificity

Six different lots of extracted human red blood cell and human nerve tissue samples were analyzed as blanks and QCs. There were no measurable peak areas detected in the blanks or QCs for analyte or internal standard that could affect the quantitation.

Validation

Linear, Precision, Accuracy & Extraction Recovery

The linear, precision, accuracy, and extraction recovery was fully validated per GLP. The method showed excellent reproducibility (overall CV% ≤ 5.9% for erythrocytes, ≤ 9.0% for nerve tissues) and accuracy (overall bias ≤ 9.3% for erythrocyte assay, ≤ 9.3% for nerve tissue assay). Tables 2-4 show the statistic data for erythrocyte assay validation. The overall extraction recovery was 94.9% for erythrocyte assay and 103% for nerve tissue assay.

Table 2. Summary of Calibration Curve Parameters for D-[1, 6-$^{13}C_2$-6, 6'-$^{2}H_2$]-glucose in Human Erythrocyte Assay (Linear Weighted 1/x)

	Slope	Intercept	R-Squared
Mean	0.000720	-0.000010	0.9995
S.D.	0.000016	0.000537	0.0003
%CV	2.2	-5370.0	0.0
n	3	3	3

Table 3. Back-Calculated Concentrations of Calibration Standards for D-[1, 6-$^{13}C_2$-6, 6'-$^{2}H_2$]-glucose in Human Erythrocyte Assay (Linear Weighted 1/x)

Concentration (µg/mL)	Mean	S.D.	%CV	%Bias	n
20.0	20.3	0.471	2.3	1.5	6
50.0	50.8	1.75	3.4	1.6	6
250	248	6.28	2.5	-0.8	6
500	494	17.4	3.5	-1.2	6
1,000	987	9.15	0.9	-1.3	6
2,500	2,490	66.8	2.7	-0.4	6
4,250	4,250	103	2.4	0.0	6
5,000	5,030	99.7	2.0	0.6	6

Table 4. Intra- and Inter-assay Accuracy & Precision for D-[1, 6-$^{13}C_2$-6, 6'-$^{2}H_2$]-glucose Quality Control Samples in Human Erythrocyte Assay

Nominal Concentration (µg/mL)	LLOQ QC 20.0 µg/mL	Low QC 60.0 µg/mL	Medium QC 2,760 µg/mL	High QC 4,000 µg/mL
Mean Observed Concentration	19.1	54.4	2,800	4,100
%Bias	-4.5	-9.3	1.4	2.5
Between Run Precision (%CV)	0.0	1.4	0.0	0.0
Within Run Precision (%CV)	6.3	5.6	2.3	3.1
Total Variation (%CV)	5.9	5.8	2.2	3.0
n	18	18	18	18
Number of Runs	3	3	3	3

Stability

As shown in Table 5, D-[1, 6-$^{13}C_2$-6, 6'-$^{2}H_2$]-glucose is stable in lysed human erythrocytes or homogenized human nerve tissues after four (4) freeze-thaw cycles in a wet-ice bath or under batch-top storage conditions for at least six (6) hours in a wet-ice bath. It is also stable in processed extracts (reconstituted in water) when stored at 1-8°C for at least 78 hours.

Table 5. Freeze/Thaw Stability and Bench-Top Storage Stability on Wet-Ice Bath for D-[1, 6-$^{13}C_2$-6, 6'-$^{2}H_2$]-glucose in Human Erythrocyte Assay

	Mean	S.D.	%CV	%Theoretical	%Bias	n
Freeze/Thaw Stability						
LLOQ QC 60.0 µg/mL, 4 Cycles	57.0	2.83	5.0	95.0	-5.0	6
Low QC 60.0 µg/mL, 4 Cycles	57.0	2.83	5.0	95.0	-5.0	6
Bench-Top Stability on Wet-Ice						
Low QC 60.0 µg/mL, ~ 8 Hours	52.8	1.53	2.9	88.0	-12.0	6
High QC 4,000 µg/mL, ~ 8 Hours	4,060	142	3.5	101.5	1.5	6

Figure 2: Product Ion Scan of D(+)-Glucose

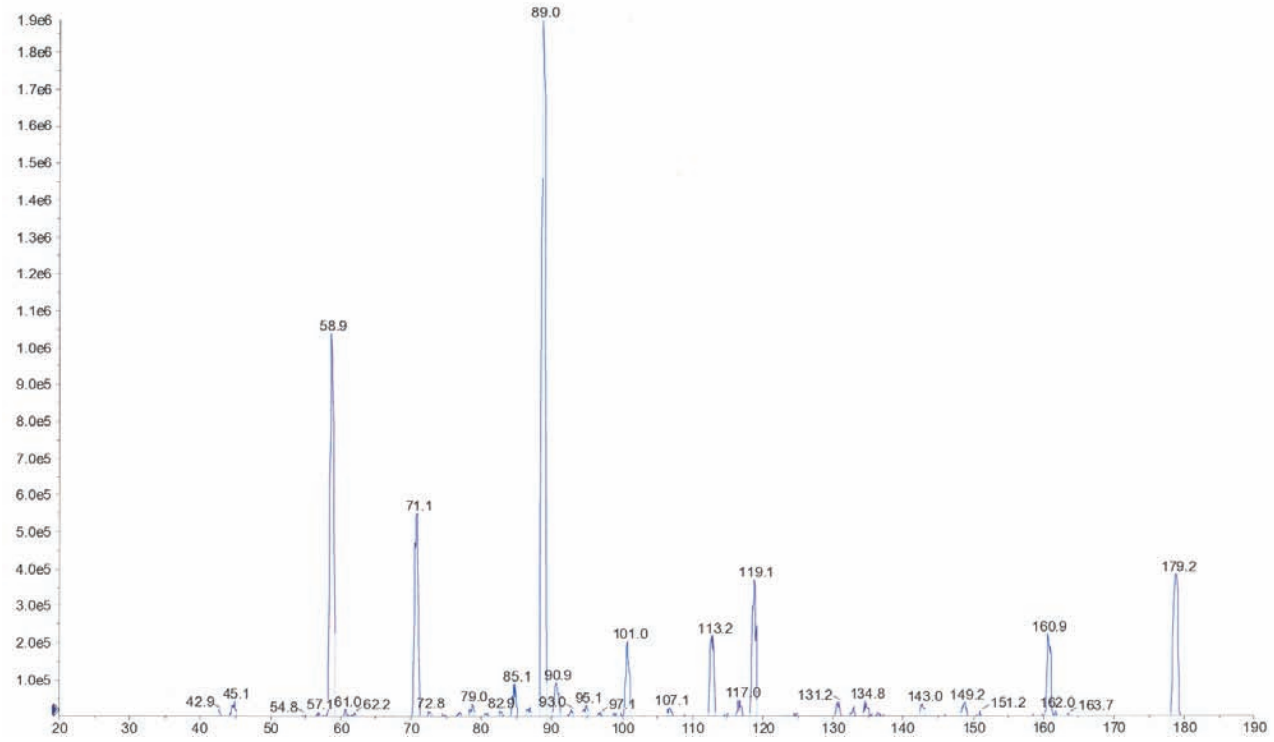


Figure 3: Product Ion Scan of D-[1, 6-$^{13}C_2$-6, 6′-$^{2}H_2$]-Glucose

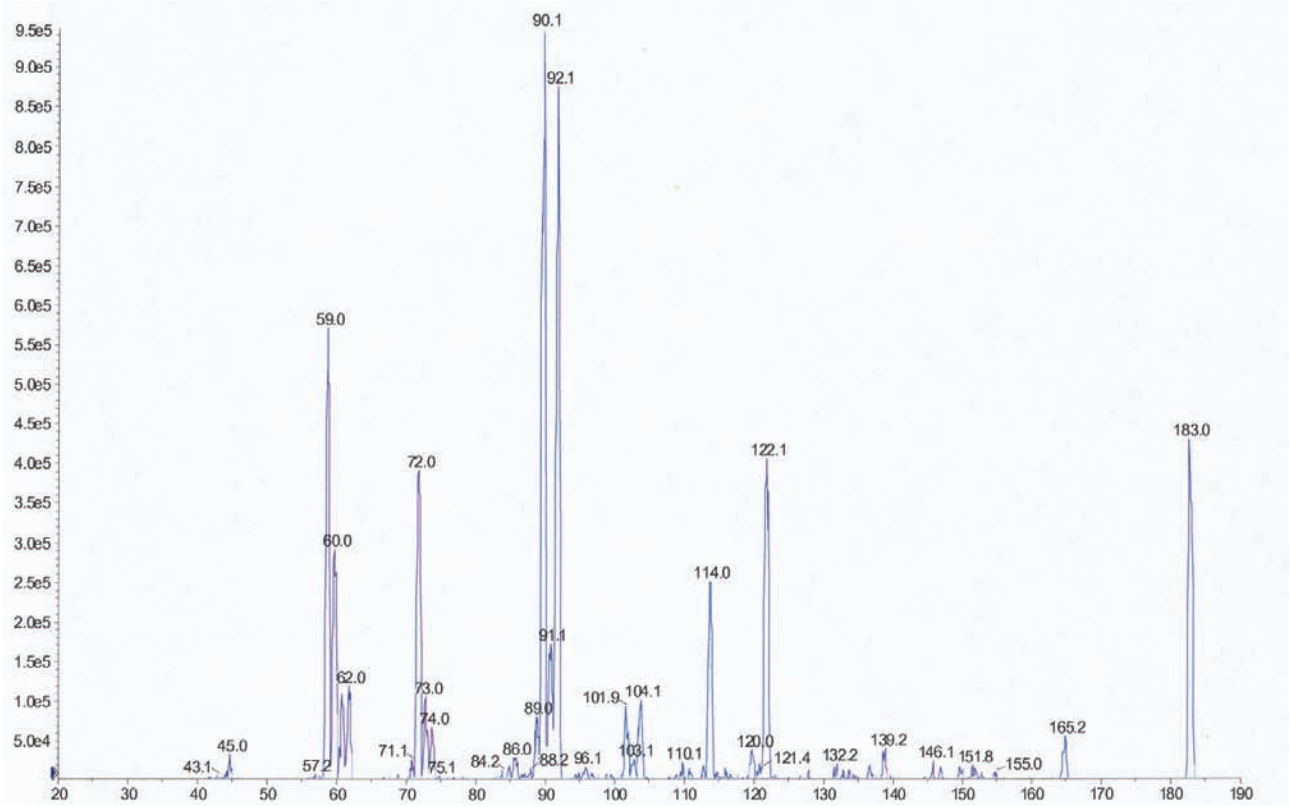


Figure 4: Chromatograms of D(+)-Glucose and Isotope-Labeled Glucoses Used in Validation and Sample Analysis Assays

Top Trace (Red): D-[1, 2-$^{13}C_2$]-Glucose (181.2 → 119.1, Internal Standard); Middle Trace (Blue): D(+)-Glucose (179.1 → 119.1, Analyte)

Bottom Trace (Green): D-[1, 6-$^{13}C_2$; 6, 6′-$^{2}H_2$]-Glucose (183.3 → 119.1, Calibrator)

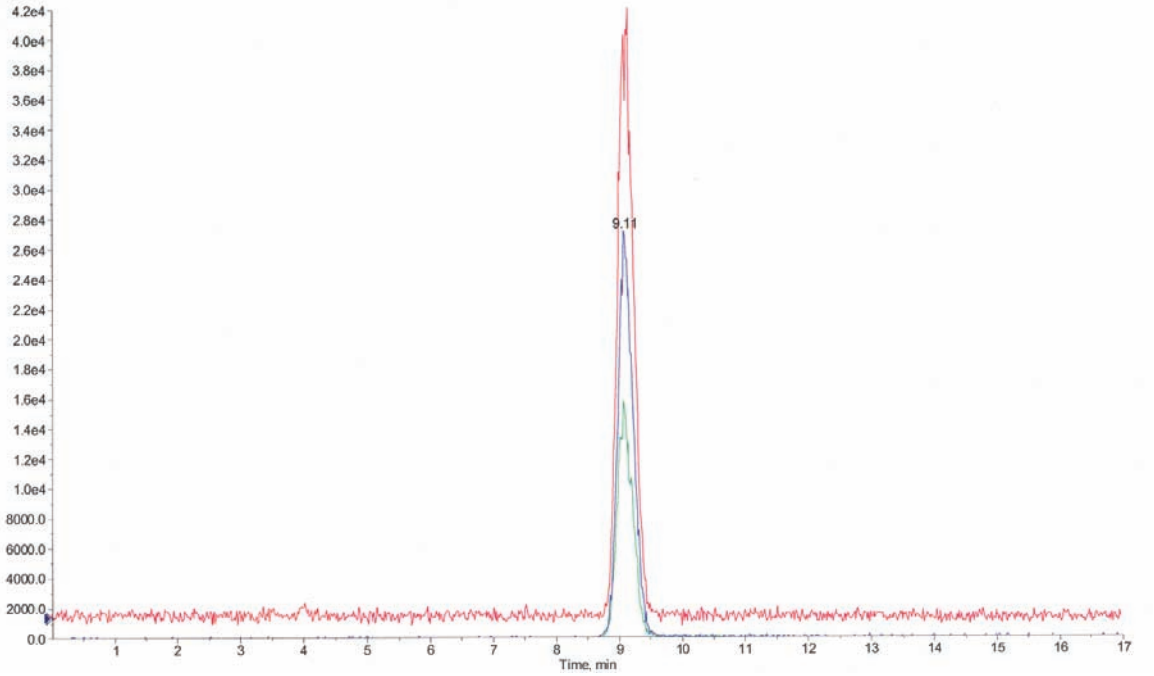
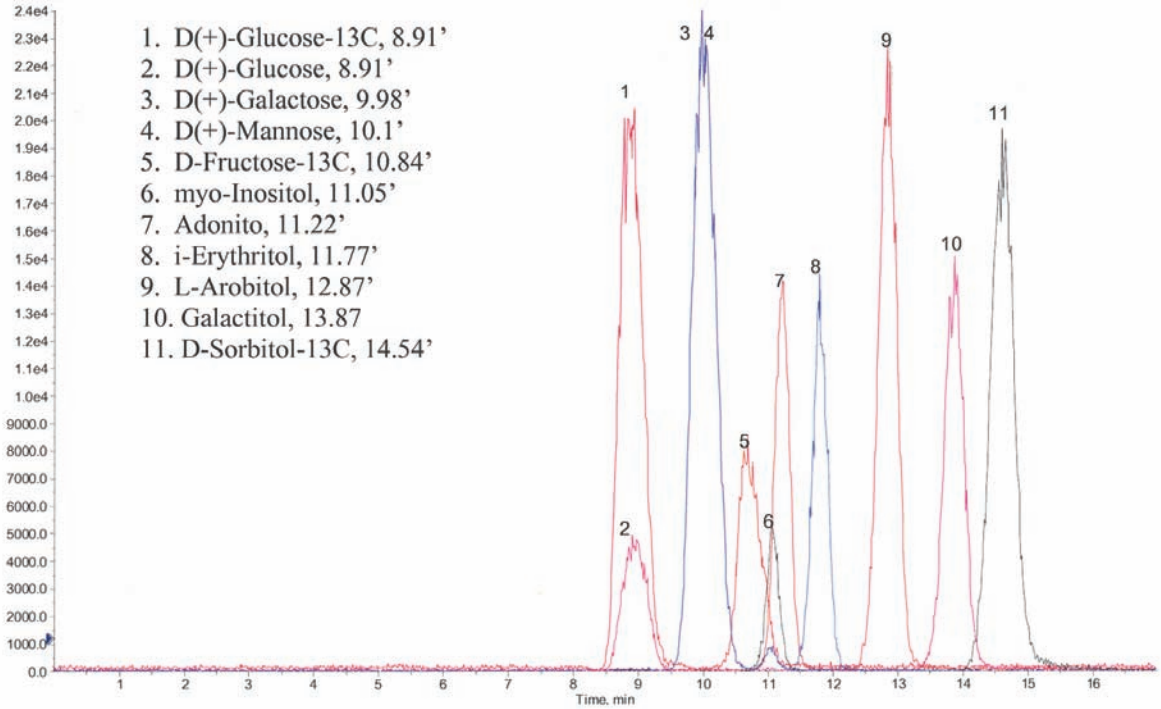


Figure 5: A Typical Overlaid SRM Chromatogram of a Sample Mixture Containing 11 Test Compounds.

Liquid chromatography conditions were optimized using natural or isotope-labeled mono-saccharides/polyols as indicated.



CONCLUSIONS

- LC/MS/MS has been successfully applied to the quantitation of endogenous glucose using internal calibration curves generated by stable isotope labeled analyte in human erythrocytes and human nerve tissues.
- The validated LC/MS/MS assays offer high throughput, improved accuracy, selectivity, specificity, ruggedness and linear range compared to existing methods for analysis of endogenous glucose and other mono-saccharides/polyols.

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3. Liang HR, et al. Quantitative determination of endogenous sorbitol and fructose in human erythrocytes by atmospheric-pressure chemical ionization LC tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2005; 824(1-2):36-44.