A Rapid LC-MS/MS Alternative to the Isotope Dilution GC-MS NIST Reference Measurement Procedure for Determining Glucose in Human Plasma

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Overview

- Manufacture of glucose monitors requires a **reference measurement procedure** (RMP)
- Current NIST RMP for glucose in human serum involves **sample preparation requiring 2-3 days**
- An LC-MS/MS method requiring **about 1 hour for sample preparation** offers a potential alternative

Introduction

Accurate blood glucose monitoring is critical for managing diabetes mellitus. The manufacture of glucose monitors requires a glucose reference method to ensure lot-to-lot consistency. Mass spectrometric detection of glucose has long been the reference measurement technique of choice for direct measurement of glucose. The National Institute of Standards and Technology (NIST) primary reference measurement procedure (RMP) for glucose in human serum, published in 1982 and updated in 2010, utilizes isotope dilution with GC-MS detection. That method requires derivatization, with **sample preparation taking 2-3 days**, limiting throughput and precluding the use of the assay for routine sample assessment. Presented here is an alternative LC-MS/MS method combining high accuracy and precision with the efficiency and throughput desired to enable routine use.

Method

The concentration range of interest is 0.150 to 6.50 mg/mL in human plasma and serum. One challenge in analyzing glucose via LC-MS/MS is that glucose is not easily ionized. The most significant challenge for an LC-MS/MS glucose assay is that the accuracy and precision of the current NIST RMP are at least **10-fold more stringent** than the 15% acceptance criteria common in bioanalytical methods.

Calibration

Whole blood is not a viable matrix for preparing glucose standards because the glucose continues to be metabolized by erythrocytes *in vitro*. For subject samples, plasma must be harvested immediately upon collection of whole blood to stabilize glucose levels. Ideally, the calibration standards would also be prepared in plasma. However, consistently and completely stripping plasma of endogenous glucose to generate sufficiently blank matrix for spiking standards proved difficult. As a result, **aqueous calibration standards were used**. This is the same approach used in the current NIST RMP.

Prior to the work presented here, equivalency between water, human serum, and human plasma was established across the concentration range of interest. This method utilizes aqueous calibration standards and serum standard reference material from NIST (SRM965b) as quality control (QC) samples to quantitate plasma samples.

Instrumentation

- Thermo Scientific TSQ Vantage mass spectrometer (Negative Ion Electrospray)
- Waters Acquity UPLC™ (with BEH Amide column, 2.1 x 100 mm, 1.70 µm)
- Tomtec Quadra 96® Model 320
- Hamilton Microlab® Star liquid handling system (**Figure 1**)

Figure 1. Hamilton Microlab® Star

Note: The Hamilton liquid handling system prepared all the calibration standards fresh from a stock solution. It also then aliquotted all the samples and added the internal standard to each. It was configured to accurately dispense water, serum, and plasma.

Sample Preparation

- 100 µL sample aliquots, 50 µL internal standard added by Hamilton Microlab® Star.
- Tomtec Quadra 96® added 600 µL acetonitrile (ACN). Vortex mixed and centrifuged.
- Tomtec Quadra 96® transfers 15.0 µL of supernatant. Evaporated under nitrogen at 40 °C.
- Tomtec Quadra 96® reconstitutes with 900 µL Mobile Phase A. Vortex mixed and centrifuged.

Liquid Chromatography

Mobile Phase A
5:95:0.1, H_2O:ACN:NH_4OH, vol:vol:vol

Mobile Phase B
5:95:0.1, H_2O:MeOH:NH_4OH, vol:vol:vol

Flow Rate: 0.75 mL/min
Injection Volume: 10.0 µL
Column Temperature: 70°C

Table 1. Mobile Phase Gradient

Time (min)	%MP A	%MP B
Initial	100	0.0
0.13	100	0.0
2.00	93.0	7.00
2.33	70.0	30.0
2.80	70.0	30.0
3.00	20.0	80.0
3.27	20.0	80.0
3.67	100	0.0
4.00	100	0.0

Detection

Negative Ion Electrospray, unit resolution

Compound	Precursor Ion (Q1 m/z)	Product Ion (Q3 m/z)	Collision Energy (eV)	Approx. Retention Time (min)	Scan Time (sec)
Glucose	179.1	89.1	8	2.5	0.275
Glucose-$^{13}C_6$	185.1	92.1	10	2.5	0.275

Results

A calibration curve was prepared in duplicate from aqueous calibration standards (CS) at six concentration levels spanning 0.150 to 6.50 mg/mL, prepared from NIST SRM 917c. A linear regression was performed with 1/x weighting. The resulting R-Squared value was 0.9997 with a mean %Bias of the back-calculated CS values of 2.0%. This calibration curve was used to assess six replicates each of the four levels of glucose in standard serum material from NIST (SRM 965b). The results are listed in **Table 2**.

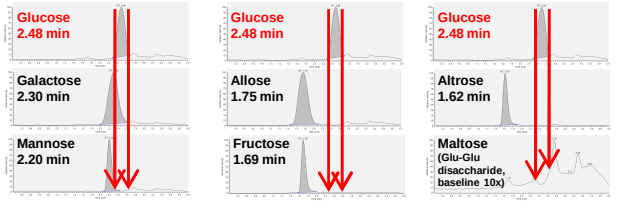
Table 2. Precision and Accuracy Summary for NIST Serum Standards

	Level 1 0.331 mg/mL	Level 2 0.756 mg/mL	Level 3 1.19 mg/mL	Level 4 2.95 mg/mL
n	6	6	6	6
%CV	0.7	0.5	0.3	0.8
%Bias	3.1	0.0	-1.0	-0.7
Max/Min %Bias	4.5/2.5	0.5/-0.6	-1.2/-0.5	0.5/-1.6

These precision and accuracy results were acquired after having the **Hamilton Microlab® Star prepare the curve fresh from stock, perform all the aliquotting, and add the internal standard**.

Other naturally occurring isobaric sugars are well-resolved from glucose except for galactose (**Figure 2**). However, **endogenous levels of galactose are at least three orders of magnitude lower than glucose**, so this should not impact method performance. Polysaccharides do not co-elute with glucose, and would not interfere even if source fragmentation yielded glucose.

Figure 2. Chromatographic Resolution of Glucose vs. Isobaric Monosaccharides & Maltose



Conclusions

The LC-MS/MS method presented here has practical advantages over the current NIST RMP for determining glucose in human serum, with **sample preparation taking roughly 1 hour rather than 2-3 days**. This method requires no derivatization and involves simple, fully automated, sample preparation. The precision and accuracy demonstrated during this initial characterization are encouraging, and suggest further optimization. After completion of additional characterization studies aimed at increasing accuracy and precision further, full validation is planned.