Modifications to the NIST Reference Measurement Procedure (RMP) for the Determination of Serum Glucose by Isotope Dilution Gas Chromatography/ Mass Spectrometry

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Abstract

NIST has been providing a Standard Reference Material (SRM) for glucose in human serum for many years to support accuracy in health care measurements. The definitive method (DM), now known as the reference measurement procedure (RMP), for the analysis of glucose in serum was originally published in 1982 by NIST. Over the years the method has been subject to a number of modifications in order to adapt to newer technologies and simplify sample preparation. Recently this method has undergone the elimination of a deionization step, which is the most significant change made to date. In addition, high performance capillary columns have replaced the support-coated open tubular (SCOT) columns used in the original work and better electronics have enabled bench top quadrupole mass spectrometers to perform measurements with precisions that approach those only attainable in the past with large, complex magnetic sector mass spectrometers. We discuss here an adaptation of the method associated with serum glucose measurements using a modified isotope-dilution gas chromatography / mass spectrometry (ID-GC/MS) method. As before the glucose was deproteinized with ethanol and derivatized to form α-D-glucofuranose cyclic 1,2:3,5-bis(butylboronate)-6-acetate (glucose BBA). The method uses uniformly labeled (^{13}C) glucose as the internal standard and samples were analyzed by GC/MS. This modified method demonstrates both good precision and accuracy in addition to reducing the sample preparation time by a day and a half. NIST has used this modified method to certify the concentrations of glucose in SRM 965b, Glucose in Frozen Human Serum and SRM 1950, Metabolites in Human Plasma.

Keywords Glucose · Serum · Isotope dilution · Gas Chromatography / Mass Spectrometry · GC/MS

Introduction

The National Institute of Standards and Technology (NIST) has a long-running program to develop higher order reference measurement procedures (RMPs),[1] for the determination of health status markers in serum, formerly called definitive methods (DMs). These RMPs are generally based upon isotope dilution mass spectrometry. For organic analytes, mass spectrometry is coupled with either gas or liquid chromatography (GC/MS or LC/MS). NIST uses these RMPs to certify analyte concentrations in serum-based matrix Standard Reference Materials (SRMs). In 1982, NIST published descriptions of two DMs for glucose in serum,[2] involving isotope dilution GC/MS. Of these two methods, only the method involving derivatization with butylboronic acid was used for measurements.

Over the years, this method has been modified in a number of ways. Part of the incentive for making changes was to adapt to newer technology. High performance fused silica capillary columns have replaced the SCOT columns used in the original work. Better electronics have enabled bench top quadrupole mass spectrometers to perform measurements with precisions that approach those only attainable in the past with large, complex magnetic sector mass spectrometers. Another incentive was to simplify the sample preparation, the limiting step in sample throughput, to permit larger sets of samples to be measured. The original RMP involved spiking, equilibrating, deproteinizing, centrifuging, deionizing with mixed cation and anion exchange resin, concentrating, freeze drying, derivatizing, and the final analysis carried out via GC/MS. This whole procedure took 4 to 5 days to complete. Adjustments to the NIST RMP for measurement of glucose in serum were made during value assignment of SRM 965a Glucose in Frozen Human Serum (2004) and then more recently during the certification of SRM 965b (2007). NIST's SRM materials all bear distinguishing names, numbers, and letters by which they are permanently and uniquely identified. Each SRM is attributed a number as the primary identifier and subsequently a letter (beginning with a = first) for

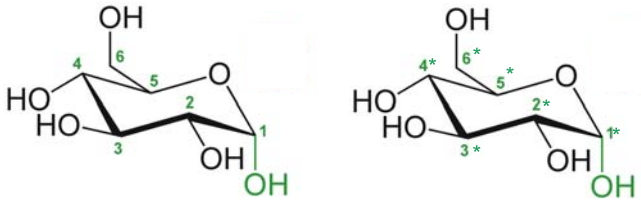
each renewal identifier. For example; the release of SRM 965 was followed by 965a, and then by 965b.

This paper describes the changes that have been made in this RMP and documents the application of the updated RMP to determine glucose concentration in two candidate SRMs, 965b Glucose in Frozen Human Serum and 1950 Metabolites in Human Plasma. Comparison of results from the revised method with certified values for existing SRMs demonstrated that these modifications have not affected the quality of the measurements.

Experimental

Materials

All SRMs were obtained from NIST (Gaithersburg, MD). The concentration of glucose in SRM 965b was reported at 4 different levels; 36, 82, 126 and 307 mg/dL for levels 1 – 4 respectively. SRM 1950, Metabolites in Human Plasma, is currently in development at NIST. SRMs 917a and 917b, D-Glucose, used to prepare the calibration solutions have certified mass purity's of 99.8 ± 0.2 and 99.7 ± 0.2 respectively. Structures of α-D-Glucose and α-D-$^{13}C_6$-Glucose are shown in Figure 1. A DB-5MS 30 m capillary column was obtained from Agilent Technologies (Wilmington, DE). All other chemicals used were reagent grade.



α-D-Glucose α-D-$^{13}C_6$-Glucose

Fig. 1 Structures of α-D-Glucose and α-D-$^{13}C_6$-Glucose (* ^{13}C labeling).

Preparation of calibration solutions

Two independently weighed standard stock solutions of glucose were prepared for each set. Approximately 25 mg of either SRM 917a or 917b was accurately weighed on an analytical balance and dissolved in distilled water in a 25-mL volumetric flask. A

solution of isotopically labeled internal standard, $^{13}C_6$-labeled glucose was prepared in the same way as the two unlabeled glucose solutions at a concentration of about 1 mg/g.

Two aliquots from the solution prepared with SRM 917a and three aliquots from the solution prepared with SRM 917b were spiked with $^{13}C_6$-labeled glucose yielding 5 calibrants with the mass ratios of unlabeled to labeled compound ranging from 0.8 – 1.2. The calibrants were freeze-dried. Freeze-drying was begun with a shelf temperature of -30 °C, and the temperature was gradually raised to 0 °C and left at that temperature overnight. The shelf temperature was raised to 20 °C for 1 h before samples were removed and brought to room temperature in a desiccator. The calibrants were then derivatized by heating at 95 °C for 1 h with 0.25 mL of butylboronic acid in pyridine (15 mg/mL). A 50 µL aliquot of acetic anhydride was added and the solutions were allowed to stand for 2 h. Pyridine was removed by evaporation under nitrogen at 45 °C and the residue was reconstituted in 200 µL of isooctane containing 1% acetic anhydride. The standards were further diluted with isooctane-acetic anhydride for GC/MS analysis.

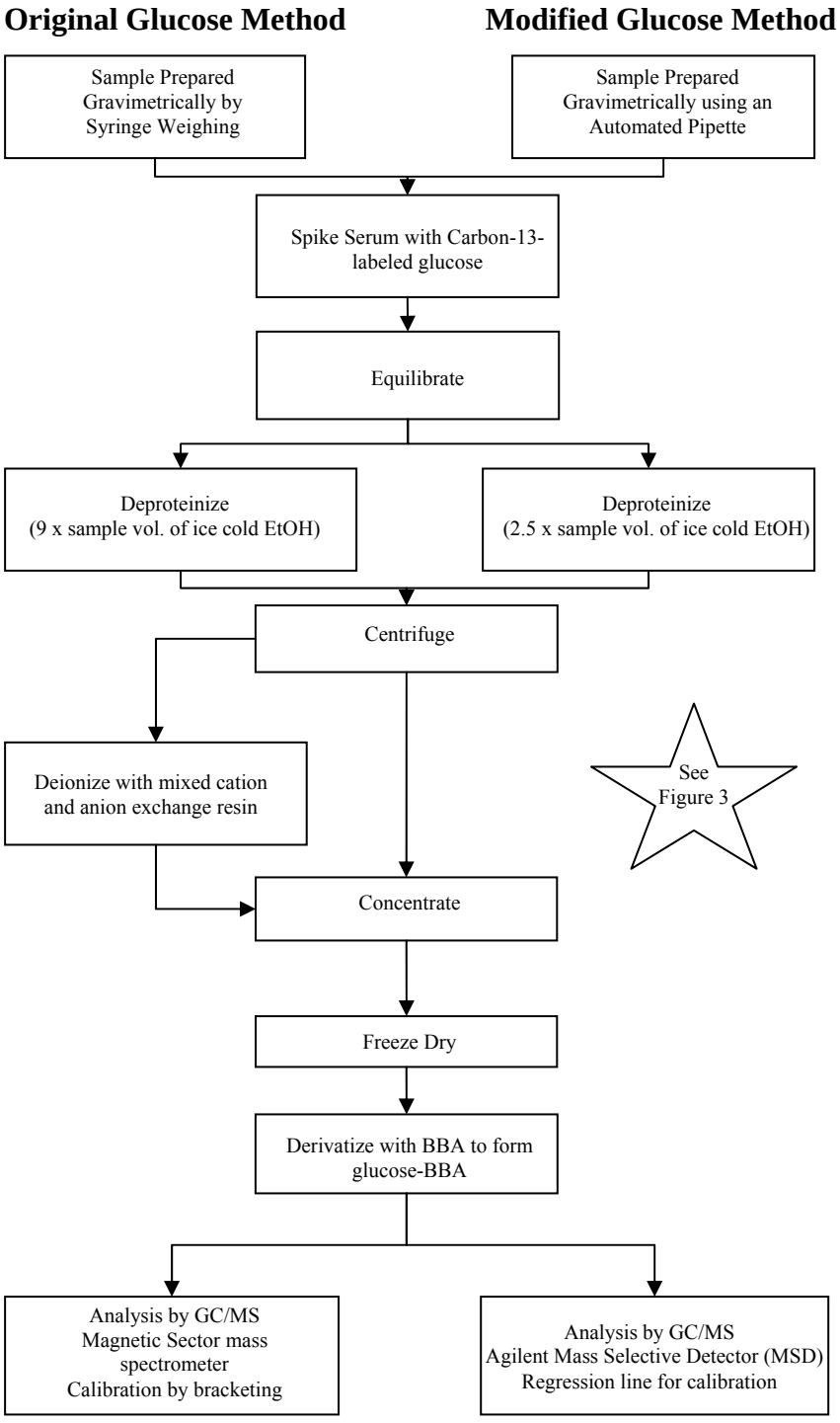
Sample preparation

Samples were prepared in six different sets, (each prepared on a different day) 12 samples per set. Each set consisted of a single aliquot from two vials of each of the four concentrations of glucose in SRM 965b and two vials of SRM 1950. A single aliquot was taken from the two concentrations of glucose in SRM 965a and these samples were used as controls since the certified values for glucose had been previously measured.[3]

Aliquots of the $^{13}C_6$-labeled glucose calibrant solution were sampled (200 µL for Levels I & II, 250 µL for level 3 and 600 µL for level 4) using a automated pipet, and weighed into 15-mL plastic centrifuge tubes. The selected vials of the frozen serum were allowed to reach room temperature and rotated periodically to ensure complete vial homogeneity. Aliquots of serum were sampled using a Rainin pipet, and weighed into the plastic tubes containing the aliquots of $^{13}C_6$-labeled glucose, to get an approximately 1:1 ratio of analyte to internal standard. A 0.1 mL aliquot of sodium azide solution (1.5g / 100 mL aqueous solution) was added and each sample was allowed to equilibrate at room temperature overnight. For deproteinization absolute ethanol, cooled to 0 ^{o}C, was added to each tube, approximately 2 to 2.5 times the aqueous volume. Samples were mixed on a

vortex mixer and then centrifuged for 15 min at 2500 rpm. The supernatant liquid was transferred into glass screw-capped centrifuge tubes and ethanol was removed by evaporation under nitrogen at 45 °C. A 1 mL aliquot of water was added and the samples were frozen. The samples were freeze-dried following the same procedure as the calibrants. The samples were derivatized by heating at 95 °C for 1 h with 0.25 mL butylboronic acid in pyridine (15 mg/mL), 0.5 mL for samples from SRM 965a and 965b Level 4. A 50 µL aliquot of acetic anhydride, 100 µL for samples from SRM 965a and 965b Level 4 was added and the solutions were allowed to stand for 2 h. Pyridine was removed by evaporation under nitrogen at 45 °C and the residue was reconstituted in 200 µL isooctane containing 1% acetic anhydride. The samples were further diluted with isooctane-acetic anhydride for GC/MS analysis, giving an approximate ratio of 1:1 for the analyte to calibrant peak area. Figure 2 shows the modifications between the old and new glucose methods and highlights the removal of the deionization step.

Fig. 2 Schematic outline of the Glucose method, highlighting the modifications.



GC/MS conditions

Analysis was performed on an Agilent HP 5973 Mass Selective Detector (Wilmington, DE). The samples were introduced with an HP 7683 auto sampler through an HP 6890N gas chromatograph equipped with a splitless injector and a 30 m non-polar capillary column (DB5-MS). The GC conditions were as follows: Injector 200 °C, MS quad 150 °C, MS source 230 °C. The column temperature was 150 °C, one minute hold time, 40 °/min to 200 °C, 10 min hold time. The retention time for the derivative measured, α-D-glucofuranose cyclic 1,2:3,5-bis(butylboronate)-6-acetate (glucose BBA) under these conditions was 9.1 min. The ions monitored were m/z 297 and m/z 303 for glucose and $^{13}C_6$-labeled glucose respectively. Samples were diluted with isooctane-acetic anhydride, and 1 μL was injected.

The following measurement protocol was used for GC/MS analysis. Five calibrants were analyzed along with the samples. The standards were analyzed first, followed by the samples, then the samples and standards in reverse order. Instrumental response was determined from a linear regression fit of the calibration data using a $y = mx + b$ regression model.

Results and Discussion

Reference measurement procedures (RMPs) can serve a number of purposes, including the value assignment of SRMs. The Joint Committee for Traceability in Laboratory Medicine (JCTLM) maintains a database of recognized RMPs for clinical analytes. Many of these methods are based upon isotope-dilution mass spectrometry (ID-MS). In this approach, a stable isotope labeled form of the analyte serves as the internal standard. Because the chemical behavior of the internal standard and the analyte to be measured are essentially the same, the ratio between the unlabeled and labeled substances remains unchanged during the overall procedure and no correction for sample recovery is needed. Moreover, this technique is highly specific: gas-chromatographic separation can be combined with the monitoring of selected ions.[4] ID-MS is an ideal technique for RMP analysis since the analytical determination is not dependent on sample recovery.[5]

Because of changes in technology, RMPs may occasionally need to be re-evaluated and modifications made to reflect current practices. In this work, a number of experiments were carried out to assess the sample preparation procedure and determine if any of the original steps could be eliminated. As noted earlier, the existing RMP includes a labor-intensive sample preparation protocol. The SRMs from previous lots denoted by identical numbers but increasing letters, served as controls and provide validation to any changes made to the method. Simultaneous analysis of samples from SRMs 965 and 965a were carried out during the first revision and simultaneous analysis of samples from SRMs 965a and 965b during the second revision. The accumulation of modifications over the past several years warranted publication, and thus this report on then accurate abridged method for the analysis of glucose in serum was prepared.

Alterations to the existing method that were investigated included an alternative method for weighing samples, reduced solvent volumes, and the most significant change, the removal of the deionization step over the mixed cation and anion exchange resin. Final analyses was performed on a bench top quadrupole mass spectrometer and quantification of samples was changed from the bracketing procedure described by Cohen et al [6] to the use of a linear regression line calculated from five calibration standards to determine the instrumental response using a $y = mx + b$ regression model.

In the original RMP, samples were prepared gravimetrically by syringe weighing. This technique can be problematic because of the varying size of air bubbles within the syringe. This makes it more difficult to obtain target weights, necessitating the use of larger sample volumes. Because the serum solutions are aqueous, using an automated pipette and weighing the tubes before and after the addition of the solution is a suitable and trouble-free alternative to the syringe weighing technique.

As done previously, the samples were left to equilibrate overnight to ensure complete equilibration of glucose in serum with the spiked internal standard, which is necessary for accurate measurement. Also unchanged in the method was the addition of sodium azide. It is a bacteriostatic agent that prevents the growth of bacteria which can consume glucose, thus acting as a preservative within the samples. Experiments have shown that deproteinization was a necessary step to obtain a reasonably clean derivative for GC/MS analysis, so eliminating this step was not a possibility. However, the use of smaller

sample volumes of ice-cold ethanol was evaluated, and samples were deproteinized with only 2-2.5 times the sample volume of ice-cold ethanol compared with nine times the sample volume of ice-cold ethanol in the original RMP.

The biggest modification to the RMP involves the elimination of the mixed cation and anion exchange resin. Packing and using these resin columns is both a tedious and time consuming process, especially when dealing with large sample sets. At the time of initial publication this was the only resin material available, however there are currently a high number of pre-packed columns commercially available. Thus, it was desired to find a suitable alternative cartridge that could replace the use of the hand packed resin columns. The original method[2] was followed until the samples were deproteinized and then the use of various commercially available pre-packed columns was investigated.

The first alternative to the hand packed mixed cation and anion exchange resin was the use of both a SCX (strong cation exchange) and SAX (strong anion exchange) cartridges. The mixed resin columns used in the original RMP contained both a strong cation exchange and a weak anion exchange resin. However, it was thought that the glucose molecule would not be in contact with the SAX long enough for degradation to occur. The eluant collected from the SAX cartridges was somewhat basic, so acetic acid was added to the eluant to acidify the solution. However, due to the instability of glucose in alkaline conditions, degradation occurred during the concentration of the eluent.

The second approach used a polyacrylamide size exclusion gel. The protein solution containing the serum and labeled glucose was poured directly onto the gel. The gel is extremely hydrophilic and essentially free of charge. Glucose recovery was about 80% in the 5 mL rinse and 99% in the 8 mL rinse; however, this was a time-consuming separation.

Another cartridge that was investigated was a mixed-mode SPE cartridge consisting of strong cation exchange and strong anion exchange sorbents packed into one bed. Glucose recovery was 87%, showing that no degradation to the glucose occurred during the concentration step. However, HCl was found to be present in the eluant, making it acidic. A C18 cartridge and a strong cation exchange cartridge were also evaluated; however, these did not retain glucose at all and were not effective as a clean up step.

Next we investigated the omission of the ion exchange step altogether. A set of samples were run for SRM 1950 and SRM 965a Level 2, using three approaches: the standard glucose approach (hand packed mixed resin), mixed mode pre-packed resin SPE cartridge, and protein precipitation but no resin treatment. Table 1 shows the glucose concentrations found in both SRMs via each of the three methods. The results from all three approaches are consistent and show good precision and accuracy. The results from control SRM 965a fall within the certified range validating all three approaches. This can also be seen in Figure 3 giving a visual representation of the numerical values from the three different clean up methods. This Experiment indicated that the omission of the ion exchange step did not interfere with the derivatization process or with the mass spectrometry analysis and thus the step was removed from the method.

Table 1 Determination of glucose in SRMs 1950 and 965a Level 2 using different cleanup methods.

		Mean	**SD[a]**	**CV[b]**	**Overall**				
		mg/dL	**mg/dL**	**(%, n=2)**	**Mean**	**SD[c]**	**CV (%, n=3)**	**Target**	**Certified Range**
					mg/dL	**mg/dL**		**mg/dL**	
SRM 1950	Hand Packed Mixed Resin	82.450	0.085	0.1	82.318	0.141	0.2	81	unknown
	Pre-Packed Mixed Resin	82.335	0.092	0.1					
	No Resin	82.170	0.226	0.3					
SRM 965a Level 2	Hand Packed Mixed Resin	78.640			78.530	0.095	0.1	78.5	77.64 - 79.36
	Pre-Packed Mixed Resin	78.470							
	No Resin	78.480							

[a] Standard deviation of a measurement using a specific clean up method
[b] CV coefficient of variation
[c] Standard deviation of the mean for that SRM

Fig. 3 Glucose concentration (mg/dL) in SRM 1950 & 965a level 2 using different types of resin in the ion exchange step.

After freeze drying, the samples were then derivatized. In the previous method samples were heated with 1-butylboronic acid in anhydrous pyridine to convert D-glucose into its dibutylboronate derivative. Acetic anhydride was then added and samples were left for 1 or 2 h to acetylate the hydroxyl group at the C-6 to form α-D-glucofuranose cyclic 1,2:3,5-bis(butylboronate)-6-acetate (glucose BBA). The pyridine was evaporated under vacuum and tubes were left overnight, under vacuum, in a dessicator containing phosphorous pentoxide and anhydrous calcium sulfate to remove the final traces of pyridine. Finally iso-octane containing 1% acetic anhydride was added to dissolve the sample for analysis by GC/MS. To reduce the sample preparation time for this derivatization step, samples were heated with the butylboronic acid in anhydrous pyridine, the acetic anhydride was added and was allowed to stand for a few hours, previously overnight, to form the glucose BBA. The pyridine was then removed by

evaporation under a stream of nitrogen and residues were taken up in isooctane containing 1% acetic anhydride.

The measurements using the original method were performed on a Varian MAT CH 7A magnetic sector mass spectrometer equipped with a combined chemical ionization / electron impact ion source, a multi-ion selection device (MIS) and a Varian 2740 gas chromatograph. Quantification was carried out using the bracketing procedure. In the current method the analysis was performed on an Agilent HP 5973 Mass Selective Detector. The samples were introduced with an HP 7683 auto sampler through an HP 6890N gas chromatograph equipped with a splitless injector and a 30 m non-polar capillary column (DB5-MS). Instrumental response was determined from a linear regression fit of the calibration data using a $y = mx + b$ regression model. By replacing the bracketing procedure used in the original method with the linear regression model we reduced the number of calibrant solutions required while maintaining linear response that closely encompassed the range of the samples.

Repeatability (within-set precision) and intermediate precision (between-set precision) were investigated for this GC/MS method. The method was applied to frozen serum samples from SRMs 1950, 965a, and 965b with glucose concentrations ranging from 34.2 to 296.1 mg/dL. Six sets of samples were analyzed, each set consisting of 12 samples. Each set was made up of a single aliquot from two vials of SRM 1950, a single aliquot from two vials of each of the four concentrations of glucose in SRM 965b, and a single vial from two concentrations of glucose in SRM 965a as controls. The between-set precision of the GC/MS measurements of glucose in SRMs 1950, 965b, and 965a are shown in tables 2, 3, and 4 respectively. These results will be used in the certification of SRMs 1950 and 965b. Repeatability with within-set coefficients of variation (CVs) ranging from 0.1 to 3.3 % and intermediate precision with between-set CVs ranging from 0.3 to 1.3 % was demonstrated.

There are thee values, one each for levels 1, 2, and 3 of SRM 965b that are statistical outliers by the Dixon Q-Test. However, we believe these values should be included as there is a possibility that there is some inhomogeneity among the vials. The position in the filling order of these vials with discordant values appears to be random.

The results tended to be low for the control samples (SRM 965a) and were just outside of the certified values for Levels 1 and 2, while the values for levels 3 and 4 were at the lower limits of the certified range. It is possible that some degradation has occurred in SRM 965a, however we do not plan on making any changes to the certificate of analysis as this material has been depleted from our stock.

Excellent linearity was obtained for all linear regression lines. A typical regression line was: y = 1.150989*x* + 0.043730 (*R* = 0.9996; standard error = 0.0051; n = 15). Selected ion chromatograms for glucose and $^{13}C_6$-labeled glucose from a serum sample from SRM 965a at a concentration of 76.639 mg/dL are shown in Figure 4.

Table 2: Between-set precision of GC/MS measurements of Glucose in Serum, SRM 1950.

Serum	Set	Mean (mg/dL)	SD[a] (mg/dL)	CV (%, n=2)	Overall Mean (mg/dL)	Overall SD[b] (mg/dL)	Overall CV (%, n=6)
1950	1	81.625	0.410	0.5	82.162	0.263	0.3
	2	82.488	0.103	0.1			
	3	82.139	0.045	0.1			
	4	81.991	0.131	0.2			
	5	82.720	0.750	0.9			
	6	82.010	0.255	0.3			

[a] Standard deviation of a single measurement with a set
[b] Standard deviation of the mean for that level

Table 3: Between-set precision of GC/MS measurements of glucose in serum, SRM 965b.

Serum	Set	Mean (mg/dL)	SD[a] (mg/dL)	CV (%, n=2)	Overall Mean (mg/dL)	Overall SD[b] (mg/dL)	Overall CV (%, n=6)
965b Level 1	1	32.780	0.074	0.2	33.077	0.418	1.3
	2	32.885	0.210	0.6			
	3	33.121	0.057	0.2			
	4	32.954	0.154	0.5			
	5	33.845	1.110	3.3			
	6	32.880	0.014	0.0			
965b Level 2	1	74.114	1.367	1.8	75.599	0.421	0.6
	2	75.739	0.222	0.3			
	3	76.142	0.600	0.8			
	4	76.282	0.572	0.7			
	5	76.040					
	6	75.275	0.573	0.8			
965b Level 3	1	116.855	0.477	0.4	118.445	0.627	0.5
	2	117.867	0.264	0.2			
	3	117.418	0.684	0.6			
	4	121.352	1.968	1.6			
	5	119.070	1.329	1.1			
	6	118.110	1.103	0.9			
965b Level 4	1	293.145	0.386	0.1	294.505	1.164	0.4
	2	294.731	0.878	0.3			
	3	292.433	0.781	0.3			
	4	294.995	0.683	0.2			
	5	297.150	3.210	1.1			
	6	294.575	2.539	0.9			

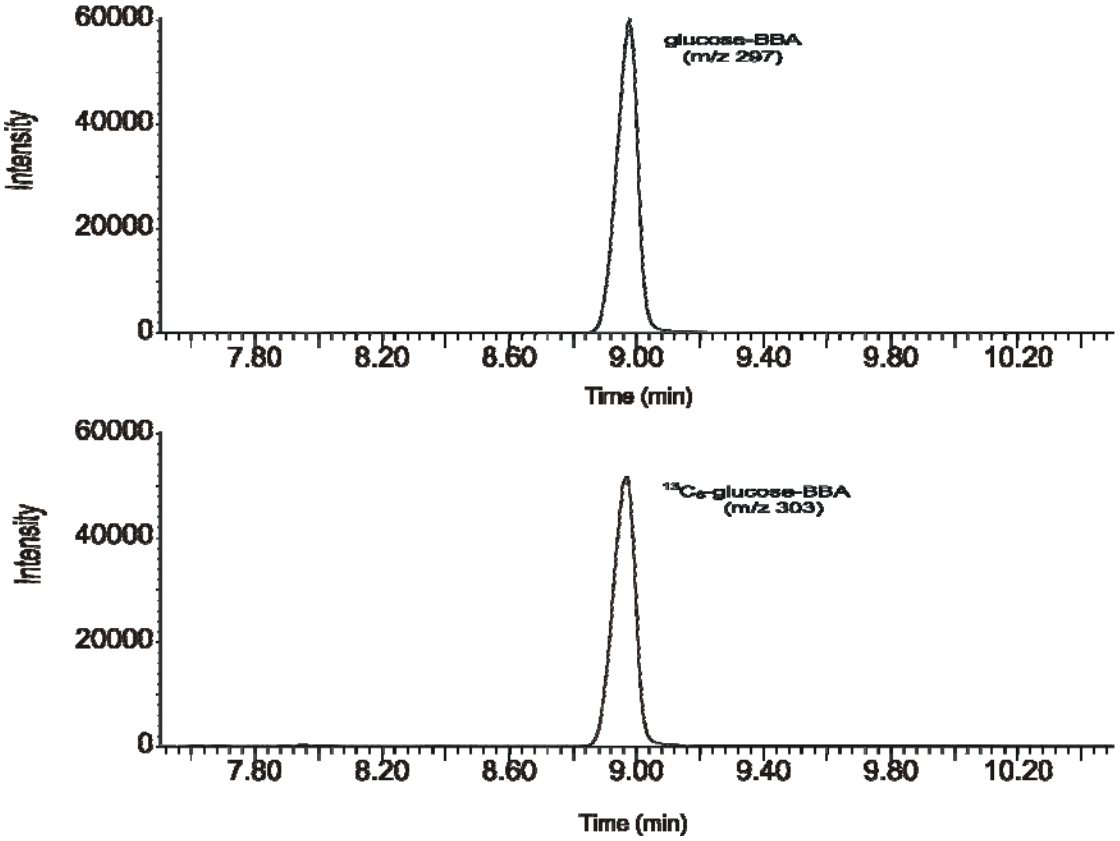
[a] Standard deviation of a single measurement with a set
[b] Standard deviation of the mean for that level

Table 4: Between-set precision of GC/MS measurements of glucose in serum, SRM 965a.

Serum	Set	Conc. (mg/dL)	Overall Mean (mg/dL)	Overall SD[b] (mg/dL)	Overall CV (%, n=3)	Certified Range (mg/dL)
965a Level 1	3	33.940	33.947	0.031	0.090	34.20 - 34.92
	4	33.980				
	6	33.920				
965a Level 2	1	76.850	77.497	0.813	1.059	77.64 - 79.36
	2	77.230				
	5	78.410				
965a Level 3	3	121.660	121.773	0.110	0.091	120.80 - 123.40
	4	121.880				
	6	121.780				
965a Level 4	1	288.970	290.007	1.463	0.506	289.10 - 296.10
	2	291.680				
	5	289.370				

[b] Standard deviation of the mean for that level

Fig. 4 Selected ion chromatograms by GC/MS for glucose and $^{13}C_6$-labeled glucose from a serum sample from SRM 965a Set 1 L2 at concentration of 76.639 mg/dL.



Conclusions

This modified ID-GC/MS method for serum glucose demonstrates good precision. Several pre-packed columns were investigated in place of the hand-packed resin used in the original RMP. Results from these experiments led to the elimination of the deionizing step altogether, which had the largest impact on reducing the sample preparation time. The sample preparation is less extensive than the previous RMP and the time has been reduced by approximately a day and half. NIST had used this method to certify the concentrations of glucose in SRM 965b and SRM 1950 which can provide an accuracy base to which routine methods for glucose can be compared.

References

1. Schaffer, R., G.N. Bowers, and R.S. Melville, *History of NIST's Contributions to Development of Standard Reference Materials and Reference and Definitive Methods for Clinical-Chemistry.* Clinical Chemistry, 1995. **41**(9): p. 1306-1312.
2. White, E., et al., *The Accurate Determination of Serum Glucose by Isotope-Dilution Mass-Spectrometry - 2 Methods.* Biomedical Mass Spectrometry, 1982. **9**(9): p. 395-405.
3. NIST. *Certificate of Analysis, Standard Reference Material 965a, Glucose in Frozen Human Serum*. 2008 29 September 2008 [cited; Available from: https://www-s.nist.gov/srmors/view_cert.cfm?srm=965a.
4. Magni, F., et al., *Determination of Serum Glucose by Isotope-Dilution Mass-Spectrometry - Candidate Definitive Method.* Clinical Chemistry, 1992. **38**(3): p. 381-385.
5. Phinney, C.S., et al., *Definitive method certification of clinical analytes in lyophilized human serum: NIST standard reference material (SRM) 909b.* Fresenius Journal of Analytical Chemistry, 1998. **361**(2): p. 71-80.
6. Cohen, A., et al., *Total serum cholesterol by isotope dilution/mass spectrometry: a candidate definitive method.* Clinical Chemistry, 1980. **26**(7): p. 854-860.