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Precision of Glucose Measurements in Control Sera by Isotope Dilution/Mass Spectrometry: Proposed Definitive Method Compared with a Reference Method

Omer Pelletier and Cheryl Arratoon

This improved isotope-dilution gas chromatographic/mass spectrometric (GC/MS) method, in which [^{13}C]glucose is the internal standard, meets the requirements of a Definitive Method. In a first study with five reconstituted lyophilized sera, a nested analysis of variance of GC/MS values indicated considerable among-vial variation. The CV for 32 measurements per serum ranged from 0.5 to 0.9%. However, concentration and uncertainty values (mmol/L per gram of serum) assigned to one serum by the NBS Definitive Method (7.56 ±0.28) were practically identical to those obtained with the proposed method (7.57 ±0.20). In the second study, we used twice more [^{13}C]glucose diluent to assay four serum pools and two lyophilized sera. The CV ranged from 0.26 to 0.5% for the serum pools and from 0.28 to 0.59% for the lyophilized sera. In comparison, results by the hexokinase/glucose-6-phosphate dehydrogenase reference method agreed within acceptable limits with those by the Definitive Method but tended to be slightly higher (up to 3%) for lyophilized serum samples or slightly lower (up to 2.5%) for serum pools.

Additional Keyphrases: *quadrupole mass spectrometry · sugars · enzymatic methods compared*

In earlier work towards development of a Definitive Method for glucose in serum, we reported a gas chromatography/mass spectrometry (GC/MS) procedure in which the peak for glucose was fully resolved from peaks for other sugars that might be present in serum (*1*). The derivatization of sugars consisted of methyloximation of the carbonyl-containing sugars before trimethylsilylation of the hydroxyls to avoid variation in the ratios of α- and β-anomers obtained by a trimethylsilylation only. Thus each aldose or ketose produced a major and a minor peak representing *syn*- and *anti*-anomers from both α- and β-anomers. The procedure gave full resolution of known trace sugars that could be present in serum in addition to glucose. Characteristic ions from electron impact ionization were identified for these sugars. The ion at *m/z* 319 selected for glucose quantitation originates from the loss of trimethylsilanol from the fully silylated fragment C-3 to C-6 giving *m/z* 409 ions (*2*). Lyophilized serum samples were directly derivatized and the clear supernate was analyzed. The high specificity resulting from the high-resolution chromatography and selected ion monitoring was demonstrated by the analysis of a yeast-treated serum (*3*), which showed only traces of glucose and full analytical recoveries of glucose, galactose, mannose, fructose, and xylose added to that serum. In subsequent work (*4*) we applied this procedure to serum samples with deuterated glucose from an algal sugar mixture as a internal standard and a Hamilton Digital Dilutor for pipetting. However, the coefficients of variation we obtained, 1.2 to 1.4%, did not meet the 0.5% limit proposed for Definitive Methods (*5*). Using an approach that has been successful for the measurement of cholesterol in serum (*6*), we now present an improved method that utilizes an upgraded GC/MS system and the chromatographic procedure previously reported (*1, 4*). We also use an automatic pipette and an internal standard of ^{13}C-labeled glucose for preparing dilutions of serum samples and their bracketing standards. The method has been applied to measure glucose in lyophilized control sera and in serum pools, and we have compared the results with those obtained with a hexokinase/glucose-6-phosphate dehydrogenase method (*7*).

Health Protection Branch, Health and Welfare Canada, Tunney's Pasture, Ottawa, Ontario, Canada K1A OL2.

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Materials and Methods

Apparatus

GC/MS instrumentation. We used a Model 4000/4500 GC/MS system (Finnigan MAT, San Jose, CA 95134) for multiple-ion detection. The gas chromatograph was equipped with a 50 m × 0.2 mm (i.d.) fused silica column, deactivated with Carbowax 20 M and coated with SP 2100 (dimethyl silicone) (Hewlett-Packard, Palo Alto, CA). The column inlet was inserted into the injector about 10 mm from the bottom of the glass insert tube. The column outlet was led directly through the GC/MS transfer line to the entrance of the ion volume of the ion source. We conditioned new columns under helium pressure of 207 kPa by raising the temperature at 5 °C/min from 50 to 220 °C and holding at 220 °C for 3 h before the column outlet was introduced in the GC/MS transfer line. The mass spectrometer was calibrated at 45 eV with FC-43 (perfluorotributylamine) supplied by Finnigan MAT. The GC/MS temperature settings were as follows: injector 250 °C, oven 190 °C, separator oven 190 °C, and transfer line 190 °C, ionizer 130 °C, and manifold 90 °C. The helium head pressure was maintained at 207 kPa. We used the injector in the split mode at 10 mL/min while the sweep was set at 10 mL/min and the column flow about 1 mL/min (linear velocity = 53 cm/s). The preamplifier sensitivity was set at 10^{-7}, electron energy at 40 eV, emission current at 0.25 mA, and the electron multiplier in the range 1000 to 1900 V, as required to maintain sufficient sensitivity. Autosampler 8000 (Varian Instrument Group, Palo Alto, CA 94303) was set to make 1-μL injections. Acquisitions of ions at *m/z* 319.2 (±0.5) and at *m/z* 323.2 (±0.5) were done successively for 0.105 s at each *m/z* during scan cycles of 0.22 s.

Pipetting apparatus. We used a Model 25000 automatic pipette (Micromedic Systems, Inc., Huntsville, AL) to sample aliquots of serum and standards and to dispense them with a benzoic acid solution containing labeled internal standard. The automatic pipette was equipped with a footswitch, a 100-μL sampling piston (0.25 mm i.d. Teflon delivery tip), and a 1-mL piston.

Reagents

The water used in preparing the reagents and reconstituting sera was further demineralized by passing it through a

high efficiency column (Barnstead Blue 00803; Fisher Scientific). To sterilize the water, we boiled it for 30 min, allowing it to cool before use. All reagents were of analytical grade. All aqueous solutions were prepared under sterile conditions with use of autoclaved class-A glassware. To eliminate possible errors from inaccurate volume measurements while preparing standard solutions, we determined the net weights of solutions and calculated their volumes on the basis of their density.

Benzoic acid solution. Dissolve 0.5 g of benzoic acid in boiling hot water, allow it to cool to 23 ±0.5 °C, and bring to volume in a preweighed (W_a) 1-L volumetric flask (weighed to 10^{-2} g). Again weigh the flask (W_b) and calculate the density of the benzoic acid solution (DB) as follows:

$$DB = (W_b - W_a)/\left[\frac{W_b - W_a - 0.5}{0.997568} + \frac{0.5}{1.3211}\right]$$

Stock glucose standard solution, 10.00 g/L. This solution was prepared in a 1-L volumetric flask preweighed to 10^{-2} g (W_0) with 10 $\pm 10^{-3}$ g of NBS (U.S. National Bureau of Standards) Clinical Standard Glucose brought to 1 L with saturated benzoic acid solution. The filled flask was weighed again (W_t). The density (DG) and concentration (C_1) were calculated as follows:

$$DG = (W_t - W_0)/\left[\frac{W_g - W_0 - 10}{DB} + \frac{10}{1.562}\right]$$

$$C_1\ (g/L) = 10^4/(W_t - W_0)/DG$$

Bracketing standard solutions of glucose. Prepare two bracketing glucose standard solutions for each serum, one at 90 to 97% and another at 103 to 110% of the glucose concentration of serum as previously measured by an enzymatic comparison method (*7*). To prepare a solution of desired concentration (Cs), first add directly into preweighed [to 10^{-4} g (W_1)] 125-mL Nalgene bottles the required volume of stock glucose solution (V_1), according to the following scheme:

Cs, g/L	V_1, mL	Cs, g/L	V_1, mL
0.45–0.55	5	1.30–1.65	15
0.55–0.65	6	1.65–2.20	20
0.65–0.75	7	2.70–2.75	25
0.75–0.85	8	2.75–3.25	30
0.85–0.90	9	3.25–3.75	35
0.90–1.10	10	3.75–4.25	40
1.10–1.30	12	4.25–5.00	45

Reweigh the bottle (W_2) and calculate the required volume of benzoic acid solution (V_b) for dilution as follows:

$$V_b = (V_1 \times C_1/C_s) - V_1$$

Add V_b from a 100-mL buret and weigh the bottle again (W_3). Calculate actual concentration (CS) as follows:

$$CS\ (g/L) = C_1 \times \frac{(W_2 - W_1)}{DG}/\left[\frac{(W_2 - W_1)}{DG} + \frac{(W_3 - W_2)}{DB}\right]$$

Store 5-mL aliquots of the bracketed standards in 6-mL "Hypo-Vials" (Pierce Chemical Co.) at 5 °C.

[^{13}C]Glucose primary solution, 0.60 g/L. We prepared this solution with [^{13}C]-D-glucose (97.6% ^{13}C; MSD Isotopes, Merck Frosst, Montreal, Quebec, Canada), making the volume of solution equal to 10% of the volume of [^{13}C]glucose diluent solution required for a particular set of measurements plus 5.5 mL. The required weight of [^{13}C]glucose was transferred into a Teflon-lined screw-capped vial with the required volume of benzoic acid solution. When the solution was not used on the same day we stored it at 5 °C. We measured the mean (M) ratio of the GC area for this solution against a 100 g/L solution of D-glucose at *m/z* 323/319 for four mixtures of both solutions, treated as described under *Procedures*, except that we added 100-μL aliquots with a micropipette and diluted them with 1 mL of benzoic acid solution.

[^{13}C]Glucose diluting solution. After measuring the ratio (M) of the [^{13}C]glucose primary solution as described above, mix the remaining (RmL) of primary solution with the calculated required volume (CmL) of benzoic acid solution according to the following formula:

$$CmL = (10 \times RmL \times M) - RmL$$

Other chemicals. Methoxyamine hydrochloride, 10 g/L in pyridine (Fisher Scientific Co., Fair Lawn, NJ), and *N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co., Rockford, IL) were used for the derivatization procedures. We cleaned the injector syringes with 2,2,4-trimethylpentane, distilled in glass, and used pure ethanol for the final cleaning of the Micromedic pipette system.

Procedures

Lyophilized sera. Lyophilized sera were obtained from various representative commercial sources as follows:

1. Monitrol, E.S. Level I Chemistry Control: American Dade, Miami, FL.
2. SeraChem, Clinical Chemistry Control Chemistry Control Serum, Level I: Fisher Diagnostics, Orangeburg, NY.
3. Ortho Normal Control Serum: Ortho Diagnostics, Don Mills, Ontario.
4. Standard Reference Material 909: National Bureau of Standards, Washington, DC.
5. Ortho Abnormal Control Serum: Ortho Diagnostics, Don Mills, Ontario.

6 and 7. Two different lots of Wellcomtrol Unassayed Control Serum: Wellmark Diagnostics Ltd., Guelph, Ontario.

We reconstituted lyophilized sera as described for the proposed cholesterol Definitive Method (*6*). The actual volume (V_a, in milliliters) of water added from a pipet was calculated from its net weight. The weight of dry serum was measured to 10^{-5}g (*6*).

Dilution of serum and glucose standards with [^{13}C]glucose diluting solution. The required volumes of samples, bracketing standards, and diluting solutions were calculated by preliminary measurements made with a semi-automated version of the Reference Method with use of a Micromedic pipette (*5*). Because dilutions were considered the greatest source of variation, dilutions were made continuously, without interruption.

The dilutions of one type of serum and its bracketing standards were prepared and given numbers in the following sequence:

1–6 (low standard), 7–10 (serum sample 1), 11–13 (serum sample 2), and 14–18 (high standard) when using two samples; 1–4 (low standard), 5–8 (serum), and 9–11 (high standard) for a single serum sample.

For sera with an expected concentration (EC) >2.00 g/L, the volume of diluent (VD) was set at 2000 μL and the volume, in microliters of serum, and its bracketing standards (VS) was adjusted according to EC:

$$VS = (2.00/EC) \times 100\ \mu L$$

In this case, each dilution was made in a 12 × 75 mm test tube, and 1.5 mL was transferred to a 1.5-mL Varian Autosampler vial.

For sera with EC 1.00–2.00 g/L, VD was set at 1000 μL and dilutions were dispensed directly into 1.5-mL vials. VS was adjusted according to EC:

$$VS = (1.00/EC) \times 100\ \mu L$$

Sera with EC 0.70–1.00 g/L had the VS set at 100 μL and the VD adjusted as follows:

$$VD = (EC/1.00) \times 1000\ \mu L$$

For sera with EC ≤0.70 g/L, two aliquots of serum were added to each 1.5-mL vial. VD for each aliquot was 500 μL and VS of each aliquot was adjusted as follows:

$$VS = (50/EC) \times 100\ \mu L$$

The diluent container of the Micromedic System automatic pipette is shaped such that at least 1 cm of diluent remains at the bottom after the last dilution. We primed the automatic pipette with 20 mL of the diluent solution. The serum in the vials was mixed very gently by rolling and tilting the vials before dilution. If a vial consistently showed foaming, about 1–2 mL of the lower portion was transferred with a Pasteur pipette into a 12 × 75 mm test tube. The delivery tip of the pipette was then immersed about 5 mm into the sample in an area free of air bubbles. Immediately after filling the pipette, we withdrew the tip, sliding it gently along the wall of the vial; then we wiped the tip towards its end, in a rapid motion, with a disposable tissue. The standard or samples aliquots and diluent were dispensed after placing the tip in the center of the tube or vial about 1 cm above the expected final level of liquid. Again we withdrew the tip by moving it to touch the wall of the tube, sliding it gently and wiping it as described above.

After all dilutions for one experiment were completed, we cleaned the pump tubing and syringes, using a surface-active cleaning agent three or four times for the pick-up, and pumping, with the pistons at full capacity, about 75 mL of water followed by 25 mL of 70% ethanol and another 25 mL of absolute ethanol. We dried the tubings, pistons, and chambers by first pumping air to empty them and, after dismantling, by wiping the pistons and blowing nitrogen through the tubes and chambers.

Lyophilization. After overnight equilibration at room temperature, the diluted sera and bracketing standards in 1.5-mL vials were lyophilized for 48 h in a Labconco Tray Dryer (Labconco Corp., Kansas City, MO). We stored the lyophilized vials tightly capped at 5 °C, and, before derivatization, we allowed 1 h at room temperature for equilibration.

Derivatization. Before proceeding with derivatization for quantitative analysis, exclusively for ascertaining the GC/MS sensitivity, we derivatized three vials (dilutions numbered 1, 7, and 14 when analyzing two samples of a serum, or numbered 1, 5, and 9 when using a single sample). We derivatized batches of two samples of one serum to be run twice within 20 h in the following order: vials from dilution no. 4 (low standard), 9 (sample 1), 16 (high standard), 12 (sample 2), 5 (low standard), 17 (high standard), 10 (sample 1), 6 (low standard), 13 (sample 2), and 18 (high standard). For single serum samples, we derivatized in the following order: vials from dilutions no. 3 (low standard), 7 (serum), 11 (high standard), 8 (serum), and 4 (low standard).

Proceeding with a single vial at a time, we added 1 mL of methoxyamine hydrochloride solution with a Centaur micro–macro pipette. We immediately capped the vial and mixed by inversion before heating and every 20 min after while heating at 80 °C for about 2 h.

Without allowing the vials to cool, we added 300 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide with a Hamilton 500-μL gas-tight syringe through the septum. We mixed the contents of the vials by inversion before and every 5 min during incubation at 80 °C for 15 min.

After the samples had cooled to room temperature, with a Pasteur pipette we transferred the clear supernate from each vial containing serum to a clean vial.

Analysis. Because the [^{13}C]glucose derivatives gave the same type of fragmentation pattern as glucose (*4*), the corresponding ions resulting from the last 4-C fragment at *m/z* 323.2 were absent in glucose or serum samples when [^{13}C]glucose was omitted.

Before starting the measurements, we tested the eV settings with 1-μL injections of one of the vials derivatized for that purpose at 35, 40, 45, and 50 eV and selected the one that gave maximal response at *m/z* 319.2 and 323.2. A minimal area of 300 000 arbitrary units at *m/z* 323.2 was required before we proceeded with the analysis.

We injected 1-μL samples in the order of derivatization, each serum being bracketed with a low and high standard. For automated analysis each vial of sample and standard was separated by a wash vial containing trimethylpentane.

We completed two cycles of injections. We measured areas at *m/z* 319.2 and 323.2 after each chromatogram, using the cross-hairs and viewing the baseline amplified in the log scale. The baseline was based on approximately 50 scans on the left (ascending) side only of the peak. The final scan of the peak was set as the first scan to the right of the peak with a height of less than 2% of the maximal peak height. As a rule, the three injections comprising a sample and its two bracketing standards were rejected and repeated if the area at *m/z* 323.2 for [^{13}C]glucose for any of the three was less than half that of any of the other two areas at *m/z* 323.2.

Concentration determination. The calculated ratio (R) of the area of *m/z* 319.2 to that of *m/z* 323.2 for each injection was used to calculate the concentration of the glucose in serum (mmol/L) by the following formula:

$$\text{mmol/L} = A + \left[\frac{(B - A) \times (R_s - R_a)}{R_b - R_a}\right] \times 5.551$$

where A and B are concentrations (g/L) of the lower and higher standards, respectively; R_a, R_b, and R_s are the area ratios for the lower and higher standards and for the serum, respectively; and 5.551 is the factor converting g/L to mmol/L.

We also used another factor (mmol/Le) to correct for any error in the volume of water added while reconstituting lyophilized serum:

$$\text{mmol/Le} = \text{mmol/L} \times (V_a/V_e)$$

where V_a is the actual volume of water added for reconstitution and V_e is the exact specified volume. The correction factor V_a/V_e was derived from the more exact factor (V_a +

$V_d)/(V_e + V_d)$, where V_d, the volume occupied by dry serum, contributed only a negligible difference.

Finally, we corrected not only for the volume of water of reconstitution but also for any variation in the mass (g) of lyophilized serum from vial to vial.

Validation of measurements within a serum vial. In rare cases, the ranges of concentrations for two series of injections of one aliquot exceeded 1%. In such cases, after verifying the areas and calculations, we carried out two more series of injections and used the latter if they checked within 1%. If these did not check, we performed a third series and used the mean of the three sets. In rare cases where the CV of the four measurements from two aliquots of one serum sample exceeded 0.5%, we completed two runs of injections of an extra aliquot and bracketing standards and used the new values with those from one of the two other aliquots in closer agreement. If the CV was still >0.5%, all values were discarded because of imprecision in-between aliquots.

Results and Discussion

In the first study with five types of reconstituted lyophilized sera, the GC/MS procedure was as described above except for use of half the volume of diluent containing [^{13}C]glucose finally recommended. On each of four different days (starting times only), two vials of each of five sera were analyzed, with two dilutions per vial and two measurements per dilution. Analysis of the eight vials of a serum was spread over 11 weeks. We analyzed the data from each serum by a nested analysis-of-variance (ANOVA) procedure (*8*), using a statistical analysis system (*9*).

The serum dry-weight measurements and variation (CV) are shown in Table 1. The range of 0.34 to 0.78% total CV obtained in this study exceeded that in a similar study using a proposed Definitive Method for cholesterol (*6*), where a range of 0.20 to 0.44% was obtained. The relatively large variation of serum dry-weights in the present study could arise in the weight measurements or from lack of uniformity in the volume of serum initially dispensed by the manufacturer into each vial before lyophilization.

The ANOVA data for serum glucose in serum 4 (NBS human serum, certified Standard Reference Material no. 909) are given in Table 2. The among-vial variability was estimated to be 68.8% (CV = 0.49%) of the total variance of a single measurement. A statistical difference detected among vials based on the *F*-test at $\alpha = 0.05$ implies non-homogeneity of the material among vials. This contrasts with the apparent homogeneity observed with different vials of the same serum as part of cholesterol measurements (*6*), where the among-vial variance was 27.1% (CV = 0.18%) of the total variance. The among-day and among-aliquot variances (Table 2) were estimated to be 0 and 5.9% (CV = 0.15%), respectively, with the remaining 25.3% (CV = 0.30%) of a single measurement being attributed to random error. The overall reproducibility CV for a single measurement of this NBS serum was estimated to be 0.59%.

The glucose measurements and statistical analysis of precision for the five reconstituted sera are summarized in Table 3. When the results were expressed as millimoles per liter of reconstituted serum, the total reproducibility CV ranged from 0.59 to 1.26%. When a correction was applied to compensate for actual variation from the exact volume specified for reconstituting a serum, the reproducibility CV ranged from 0.49 to 0.92%. This mode of calculation clearly improved the variation of glucose concentration in the last four sera. Finally, when expressing concentrations per gram of dry serum, the total CV ranged from 0.44 to 0.83%, with a marked improvement for serum 5 consistent with a direct relationship between glucose content and dry weight. The major source of variation detected among vials implies non-homogeneity of the material among vials.

An important finding of this study was the agreement of glucose concentrations in serum 4 with the value certified by NBS on November 28, 1984, at about the same time as measurements were completed by the present method. In fact, the concentration and uncertainty value (mmol/L ± 95/99% statistical tolerance intervals reflecting the combined effects of measurement imprecision and the among-vial variability in the mass of dry serum) assigned by the NBS Definitive Method (*5*) was 7.56 ±0.28 as compared to 7.57 ±0.20 by the proposed method on the basis of the mean values in each of eight serum vials.

At this point, it appeared that the method was just barely qualifying as a Definitive Method for sera 2–4 with regard to an upper limit of 0.5% for the total reproducibility CV (*5*). We believed that the inherent non-homogeneity attributable to serum vials was contributing to the error *per se* and that a better evaluation could be made by using serum pools. We also decided to increase the volume of diluent containing [^{13}C]glucose, to minimize the among-aliquot variability which might be caused by a possible but not technically demonstrable carryover from one sample to another in the previous study.

In the second study, utilizing the method as described, we measured glucose in four serum pools and two additional reconstituted sera, using a single vial of each pool on eight different days with two dilutions per vial and two measurements per dilution. Table 4 summarizes the statistical analysis of precision of that study. In the four serum pools, the total reproducibility (CV) ranged from 0.26% to 0.50%, while in two reconstituted sera it ranged from 0.28% to 0.59%, with little improvement after adjusting values for an exact volume of water for reconstitution. The lower among-aliquot variation with four of the six sera (Table 4) appears

Table 1. Measurements and Variation in Dry Weight of Serum

Serum	Mean mass, g[a]	CV, %[b] Among-day	Within-day	Total
1	0.45046	0.08	0.33	0.34
2	0.49541	0.35	0.11	0.37
3	0.38476	0	0.78	0.78
4	0.84760	0.36	0.12	0.38
5	0.35283	0	0.76	0.76

[a] For eight serum vials of each. [b] df among-day = 3; within-day = 4; total = 7.

Table 2. Nested ANOVA of Glucose Data for Serum-4[a]

Source	df	Mean square, × 10^{-4}	Component of variance	% of the overall variance	CV, %
Among-day	3	9.160	0	0	0
Among-vial	4	4.452	9.824×10^{-4}	68.8	0.49
Among-aliquot	8	5.282	8.350×10^{-5}	5.9	0.14
Within-aliquot	16	3.614	3.614×10^{-4}	25.3	0.30
Total	31	4.633	1.427×10^{-3}		

[a] $\bar{X}$ = 6.422 mmol/L

Overall standard deviation (single measurement) = SD overall = $\sqrt{s^2 \text{ within-aliquot} + s^2 \text{ among-aliquot} + s^2 \text{ among-vial} + s^2 \text{ among-day}}$ = 3.7779×10^{-2} mmol/L; CV overall = 0.59%.

Table 3. Statistical Analysis of Precision Study for Glucose in Serum as Reconstituted (mmol/L) and after Adjusting to an Exact Reconstitution Volume (mmol/Le), and mmol/Le per Gram of Dry Serum[a]

			CV, %[b]				
Serum	Units	Mean	Among-day	Among-vial	Among-aliquot	Within-aliquot	Total[c]
1	mmol/L	4.14	.60	.56	.32	.26	.81
	mmol/Le	4.14	.57	.39	.32	.26	.81
	mmol/Le per gram	9.20	.27	.66	.32	.27	.83
2	mmol/L	4.07	.45	.54	.16	.33	.79
	mmol/Le	4.06	.13	.31	.16	.32	.49
	mmol/Le per gram	8.19	.02	.22	.16	.32	.42
3	mmol/L	5.07	0	.49	.22	.33	.63
	mmol/Le	5.06	.09	.32	.22	.33	.52
	mmol/Le per gram	13.15	.16	.37	.22	.33	.56
4	mmol/L	6.42	0	.49	.14	.30	.59
	mmol/Le	6.42	0	.38	.14	.30	.50
	mmol/Le per gram	7.57	.34	.39	.14	.30	.61
5	mmol/L	16.88	0	1.24	.15	.23	1.26
	mmol/Le	16.86	0	0.88	.15	.23	.92
	mmol/Le per gram	47.78	0	0.28	.15	.23	.44

[a] Volume of [^{13}C]glucose diluting solution was half that used in the final procedure.
[b] df among-day = 3; among-vial = 4; among-aliquot = 8; within-aliquot = 16.
[c] CV total based on overall standard deviation of a single measurement, SD overall = $\sqrt{s^2 \text{ within-aliquot} + s^2 \text{ among-vial} + s^2 \text{ among-day}}$.

related to a more uniform dispensing of serum aliquots with a larger volume of diluting solution than in the first study (Table 3). However, the fact that two of the six sera (Table 4) gave among-aliquot variation within the range of the preceding study (Table 3) suggests that the among-aliquot variation may also depend on the homogeneity characteristics of the sera during the repeated pipetting of aliquots. The among-day variations, which in this study (Table 4) also include among-vial variations, were relatively low (CV <0.20% in four cases). The generally better reproducibility with the serum pools as compared with reconstituted sera could be expected on the basis that the serum pools were expected to have greater uniformity of the actual glucose content from vial-to-vial. The total reproducibility CV for glucose measured in four serum pools were within the limit of <0.5% advocated for Definitive Methods (*5*).

During the time that the preceding studies were conducted, we analyzed the same sera by a semi-automated version (using a Micromedic Pipette) of the hexokinase/glucose-6-phosphate dehydrogenase Reference Method (*7*), utilizing the same bracketing standard solutions as in the GC/MS method.

For the first study for eight vials of five reconstituted sera by the GC/MS method (Table 3), mean concentrations (mmol/L) by the Reference Method were: serum 1, 4.277; serum 2, 4.093; serum 3, 5.083; serum 4, 6.481; and serum 5, 16.990. Although the values by the Reference Method were 0.32% to 0.92% greater for four sera and 3.23% greater for the remaining serum, none of these differences was statistically significant.

In the second study (Table 5) the mean concentration obtained by the Reference Method for one of the two reconstituted sera was 1.23% higher (P <0.01) than that of the GC/MS method, while it was 1.51% lower (P <0.05) for the other reconstituted serum. The four serum pools gave concentrations 0.24% to 2.49% lower by the Reference Method, with differences statistically significant for pools 3 and 4.

Although the results of these comparisons with the GC/MS method indicated that the Reference Method meets the ±3% criterion of acceptability (*11*), there seems to be a trend for the Reference Method to produce higher values for lyophilized sera and lower values for serum pools. For serum

Table 4. Statistical Analysis of Precision Study for Glucose in Serum Pools (mmol/L), and in Lyophilized Sera as Reconstituted (mmol/L) and after Adjusting to an Exact Reconstitution Volume (mmol/Le)

	CV, %[a]			
Mean glucose, mmol/L	Among-day	Among-aliquot	Within-aliquot	Total[b]
Pooled sera				
5.77	0.19	0.12	0.14	0.26
4.62	0.15	0	0.27	0.31
5.39	0.40	0	0.31	0.50
8.11	0.14	0.17	0.16	0.28
Lyophilized sera				
4.71	0.19	0	0.20	0.28
4.70[c]	0.24	0	0.30	0.38
2.85	0.45	0.33	0.20	0.59
2.84[c]	0.39	0.32	0.20	0.55

[a] df = among-day = 7; among-aliquot = 8; within-aliquot = 16; total = 31.
[b] CV total based on overall standard deviation of single measurement (n = 32), SD overall = $\sqrt{s^2 \text{ within-aliquot} + s^2 \text{ among-aliquot} + s^2 \text{ among-day}}$.
[c] mmol/Le.

Table 5. Comparison of Glucose Concentrations[a] as Measured by the GC/MS Method and the Reference Method in Serum Pools and in Reconstituted Sera

	GC/MS (A)		Comparison (B)			
Serum	mmol/L	CV, %	mmol/L	CV, %	%(B−A)/A	Prob. t-test[b]
Pool 1	5.769	0.26	5.755	1.87	−0.24	ns
Pool 2	4.621	0.31	4.595	1.83	−0.56	ns
Pool 3	5.387	0.50	5.253	0.92	−2.49	***
Pool 4	8.112	0.28	8.032	0.85	−0.99	**
Lyo. 1	4.713	0.28	4.771	0.72	+1.23	**
Lyo. 2	2.848	0.59	2.805	1.63	−1.51	*

[a] Mean and CV for all measurements in eight different vials on different days except for Pool-1 and Lyo.-2 (seven vials). In each vial, there were two measurements on each of two aliquots by GC/MS and single measurements on two aliquots by the comparison method.
[b] Probability t-test, based on two sample means with unequal variances: * = 0.01 < P < 0.05; ** = 0.001 < P < 0.01; *** = P < 0.001; ns = not significant (P > 0.05).

pools the lower values may be related to incomplete extraction of glucose from the precipitated protein, while for lyophilized sera the higher values may result from release of protein-bound glucose by the barium hydroxide treatment used in the protein-precipitation procedure. Glucose is believed to bind to serum proteins during the preparation of the control product (*12*) and also to a large extent during storage, depending on the temperature, storage interval, and batch of serum (*13*).

An evaluation of the glucose Reference Method with the first NBS Definitive Method (*9*), using conversion of glucose into 1,2:5,6-di-*O*-isopropylene-α-D-glucofuranose, commonly known as "diacetone glucose," also showed a trend for lower values in serum pools by the Reference Method, but no data were published comparing those two methods for lyophilized serum. The most recent NBS Definitive Method (*10*), in which glucose is converted to 1,2:3,5-bis(butylboronate)-6-acetate has been compared with the diacetone glucose method for the analysis of serum pools. Since the former (*10*) method showed a trend for slightly lower values (<1%) in serum pools as compared to the latter method, our proposed GC/MS method would appear to agree more closely with the diacetone glucose method for assay of serum pools.

The excellent agreement obtained between our GC/MS method and the former method (*10*) for the glucose concentration in serum 4 (Table 3) would indicate that the use of ethanol to deproteinize in that procedure does not liberate protein-bound glucose, or that little protein-bound glucose was present in that serum.

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