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Determination of Serum Glucose by Isotope Dilution Mass Spectrometry: Candidate Definitive Method

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We report a rather simple method to determine glucose concentration in serum, using isotope dilution mass spectrometry and [$^{13}C_6$]glucose as internal standard. The procedure involves a single step of sample purification and the conversion of the analyte into its aldononitrile pentaacetate. The between-day and within-day contribution to total variance for a single measurement was determined by assaying Standard Reference Material (SRM) 909 serum. The method was then applied to measurement of glucose concentration in three lyophilized sera: SRM 909 and two other commercially available sera. In the two studies, the concentration of SRM 909 serum was found to be 0.8% above and 0.3% below the reported value (6.25 mmol/L), respectively; the overall coefficient of variation for determinations in all sera ranged from 0.37% to 0.56%. The precision and the accuracy of the method satisfy the requirements for a Definitive Method.

Several routine methods are used in clinical laboratories for the quantification of blood glucose (*1*). For their accuracy to be established, these methods must be compared with a Reference Method, which is more accurate and precise and can occasionally be performed in most clinical laboratories to calibrate the routine analysis procedure. The accuracy and precision of Reference Methods are evaluated by direct comparison with a Definitive Method, which measures the analyte concentration with the greatest accuracy and precision (CV $<\pm 0.5\%$) of all methods (*2*). In Definitive Methods, systematic errors are not only negligible but also identified. The technique that allows investigators to set up methods satisfying these criteria is isotope dilution mass spectrometry (ID-MS), which is based on the addition to the sample of an internal standard that consists of the analyte labeled with stable isotopes.[3] Because the chemical behavior of the internal standard and the analyte to be measured are 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.

For determination of serum glucose, two ID-MS methods have been described, both showing good accuracy. The first (*3*), developed at the U.S. National Institute of Standards and Technology (NIST, Gaithersburg, MD), involves conversion of glucose into either isopropylidene-α-D-glucofuranose or bis(butylboronate)-α-D-glucofuranose; with either derivative the CV of the analysis of NIST Standard Reference Material (SRM) serum with a custom-modified mass spectrometer is ±0.3%. The second method (*4*), carried out with a commercial mass spectrometer, involves conversion of glucose into its methyloxime trimethylsilyl derivative. Despite the presence of two peaks, arising from the two anomeric glucose forms, the CV for determinations of glucose in lyophilized sera was 0.28–0.59% by this procedure.

We present here another method based on ID-MS to measure glucose in lyophilized control sera; this method combines high accuracy and precision with a simple and time-saving procedure of sample preparation.

Materials and Methods

Materials

Reagents. Pyridine (99.8% pure), acetic anhydride (>98% pure), and hydroxylamine hydrochloride (98% pure) were purchased from Fluka Chemie (Buchs, Switzerland). D-Glucose (SRM 917a), with a certified purity of 99.8 ± 0.2%, and two sets of human serum Standard Reference Material (SRM 909) were purchased from NIST: lot 909, glucose concentration 6.40 mmol/L, total uncertainty ±0.28; revision March 1985 (one vial); and lot 909, 6.25 mmol/L, total uncertainty ±0.22; revision December 1990 (three vials). D-[$^{13}C_6$]Glucose (>98.9 atom %) was obtained from MSD Isotopes (Merck Sharp & Dohme, Munich, F.R.G.). All other chemicals were of analytical grade. Fresh doubly distilled water was used to prepare the standard solutions.

Standard solutions. D-Glucose (SRM 917a) solution was prepared by dissolving about 10 mg of the analyte, weighed with a microbalance (Cahn Instruments, Cerritos, CA; imprecision 10^{-3} mg), in water weighed with an electronic balance (Model E50; Gibertini, Milan, Italy; weight at 10^{-4} g), with imprecision of about 0.29% at 5 mg and <1% at 1 mg (n = 10). The accuracy of the semimicrobalance was checked with calibrated weights. D-[$^{13}C_6$]glucose was weighed and dissolved in water according to the same procedure.

Both standard solutions were either used immediately for the preparation of bracketing diluted standards and serum samples or kept at 4 °C for no more than two weeks. Density of the two standard solutions

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[3] Nonstandard abbreviations: ID-MS, isotope dilution mass spectrometry; GC-MS, gas chromatography–mass spectrometry; NIST, National Institute of Standards and Technology; and SRM, Standard Reference Material.
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was determined by weighing with a semimicrobalance ten 50-μL aliquots of each solution, taken with an SGE syringe (Scientific Glass Engineering, Villeneuve St. George, France). The syringe was conditioned by filling and discharging the contents four times, after which we weighed aliquots of the standard glucose solutions. We used the same syringe for both standards to avoid possible variation between syringes. Density was 1.003 ± 0.003 kg/L (CV 0.29%) for D-[$^{13}C_6$]glucose and 1.003 ± 0.001 kg/L (CV 0.14%) for unlabeled glucose. The syringe imprecision, determined by weighing 50 μL of distilled water at 21.5 °C, was <0.2%.

Bracketing solutions. Glucose standard solutions were prepared by mixing the SRM 917a D-glucose solution with the D-[$^{13}C_6$]glucose solution to provide a series of mixtures with known abundance ratios of the ions selected for the analysis in the range 0.8–1.2. Each point of the bracketing standard curve was obtained by weighing the amounts of glucose and labeled glucose solutions with the semimicrobalance. The calibrated syringe (SGE) was preconditioned four times with the solution to be aliquoted, as described above, and an aliquot of the standard solution was then collected for the analysis. Again, a single syringe was used for preparation of standards and serum samples.

Procedures

Weighing procedures. The weight of each aliquot was determined by weighing the water standard solutions (or serum samples) at 45 and 90 s after sampling, and the original weight at 0 s was extrapolated as reported elsewhere (*5*). The weight ratios were then calculated for each standard point, according to the density and the concentration of the two standard solutions.

Serum samples. Human lyophilized serum (SRM 909) was reconstituted as described in the instruction sheet from NIST (weight at 10^{-4} g). Serum density, measured as described above, was 1.023 ± 0.002 kg/L (CV 0.16%) for the oldest SRM specimen and 1.024 ± 0.002 kg/L (CV 0.19%) for the other three SRM 909 serum vials.

A first study, carried out with the SRM control serum purchased in 1987, was used to determine the precision and accuracy of the method. The second experiment, with the more recently purchased SRM 909 serum set, was performed to determine the reproducibility of the procedure, so its accuracy and precision were again measured. Other lyophilized sera (Precinorm U, lot no. 160565, glucose concentration 6.88 mmol/L by the hexokinase method; and Precipath U, lot no. 171760, glucose concentration 13.4 mmol/L) were purchased from Boehringer (Mannheim, F.R.G.) and treated as described above.

Aliquots of each serum were first analyzed by gas chromatography (GC), with D-allose (1 g/L in serum) as the internal standard, to determine the approximate glucose concentration. To weighed amounts of each serum we added a weighed aliquot of D-[$^{13}C_6$]glucose, obtaining an approximately equimolar ratio of [^{12}C]glucose/[^{13}C]glucose. After stirring, the mixtures were kept at 4 °C for 24 h to allow equilibration before protein precipitation. Four aliquots from each serum specimen were prepared for GC-MS analysis.

Sample derivatization. Serum samples (20–50 μL) were deproteinized with 0.8 mL of acetonitrile. After centrifugation, the supernate was separated and evaporated to dryness under reduced pressure. The glucose in the residue was then converted into aldononitrile by treatment with hydroxylamine hydrochloride, 2 mg in 150 μL of pyridine, at 90 °C for 30 min as already described (*6*). Acetylation was then carried out by adding acetic anhydride (200 μL) and heating for 30 min at the same temperature. The derivatized samples were dried and residues were dissolved in 0.5 mL of dichloromethane for GC-MS analysis. The bracketing standards were also derivatized as described. Because the aldononitrile pentaacetate derivative was stable for at least two months at 0–4 °C, we could analyze the sample over a long period without any change in the isotope ratio or loss of sensitivity resulting from sample degradation.

GC/MS conditions. The isotopic ratio of serum glucose was determined by using a Model 2091 GC-MS system (LKB, Bromma, Sweden). For GC we used an SPB-35 wide-bore glass column (Supelco Inc., Bellefonte, PA) 30 m × 0.72 mm (i.d.). The injector temperature was 270 °C, the oven temperature was 210 °C, and the carrier gas was helium at 45 kPa. Mass spectrometry conditions for the LKB 2091 were ion energy 70 eV; ion accelerating voltage 3.5 kV (stability >0.005% in 10 min) with a resolution of 50 mV and an accuracy of 200 mV; multiplier voltage 350 V; trap current 50 mA; source temperature 270 (±1) °C; and transfer line temperature 280 °C. Resolution was set at 300. Acquisition, with a time-constant of the filter set at 8 s, lasted 20 ms for each channel, giving about 100 measurements during glucose elution from the chromatographic column. This number of measurements was found to be enough to define the peak profiles and to minimize the variations of the peak-height ratio between consecutive injections of the same sample. The analyses were carried out by selected ion recording, monitoring ions at *m/z* 314 and 319 for unlabeled and labeled glucose, respectively. The amplifiers for the two channels were intercalibrated with an accuracy higher than 1%. The gain for both channels ranged from 50 to 500 mV. The baseline was drawn manually from the beginning to the end of the peak and the peak height was measured to 0.1 mm with a caliper.

Analysis. Extracts from four aliquots of each serum sample were derivatized and 1 μL was injected. Each sample was injected twice, a third injection being carried out if the difference in peak height ratio (*m/z* 314 to 319) exceeded 1%; the mean of the values was used for calculations. Injections of samples were alternated with duplicate analysis of standard calibrators having ^{12}C/^{13}C ratios of 0.8, 1.0, 1.2, 1.0, and 0.8, which we injected in this sequence. In rare cases one aliquot had to be rejected because the CV exceeded 0.5%, attributable to either human or instrumental errors. To be able to

analyze data by the nested ANOVA for all vials, we considered only the three closest data in all cases.

Aliquots of one SRM 909 serum were injected with two sets of the bracketing standards, to determine the variability between the different bracketing calibration curves. The within-day and between-day imprecision was determined by measuring the older SRM 909 serum three times a day for two days and once on a third day. The reproducibility and accuracy of the method were evaluated by using the three vials of the SRM 909 serum purchased later.

Data calculation. To calculate the concentration of glucose in serum, we computed the weight ratio of [^{13}C]- to [^{12}C]glucose in serum (ER_S) as follows:

$$ER_S = [(MR_S - MR_L) \times (ER_H - ER_L)]/(MR_H - MR_L) + ER_L$$

from peak-height ratios (MR) measured in serum (S) and in higher (H) and lower (L) bracketing standards and from the known weight ratios in the same standards (ER_H and ER_L, respectively) (*2*).

The concentration of glucose in the serum (mmol/L) was then obtained as $(W_{13C}) \cdot (D_S) \cdot P/(M_S \cdot ER_S \cdot 180.16)$, where W_{13C} is the amount (mg) of D-[$^{13}C_6$]glucose added to the analyzed sample, M_S is the weight (mg) of the aliquot, D_S is the density (kg/L) of the serum determined as described above, P is the percent purity of the SRM 917a glucose used, and 180.16 is the molecular mass of glucose.

Statistical analysis. Data were analyzed by multifactor analysis of variance (ANOVA) for balanced and unbalanced data and by the nested ANOVA with a commercial software for IBM computers. The statistical level of significance was set at 95%.

Results

GC-MS Data

The electron ionization mass spectra of the aldononitrile pentaacetate of the two isotopic specimens of glucose used are reported in Figure 1. The selected ion at *m/z* 314 originates from the loss of $C_3H_5O_2$ fragment; the remaining fragment from [$^{13}C_6$]glucose contains five ^{13}C atoms (*m/z* 319). Under the described conditions glucose was well separated from the other hexoses (*6, 7*). The measured isotope purity of the [$^{13}C_6$]glucose was >98.8 atom %.

Precision Study

Bracketing standard set precision. Two sets of bracketing calibration mixtures were prepared and tested for internal consistency. In the same day the different calibration sets were used to measure the glucose concentration in the SRM 909 human serum. Aliquots (n = 3) collected from a single vial gave a mean (± SD) value of 6.30 (± 0.02) mmol/L and 6.31 (± 0.02) mmol/L with the two sets of standard. The two means were not statistically different.

Within-day imprecision. Each aliquot of the same serum (the oldest SRM 909 serum) was analyzed in duplicate three times a day for two days. The CV never exceeded 0.5%, the mean values for the two days of analysis being 0.13% and 0.17%. As reported in Table 1, CV values calculated on the basis of the nested ANOVA indicate that the within-day variance contributed only 28% to the total variance. No statistical differences were revealed by the *F*-test.

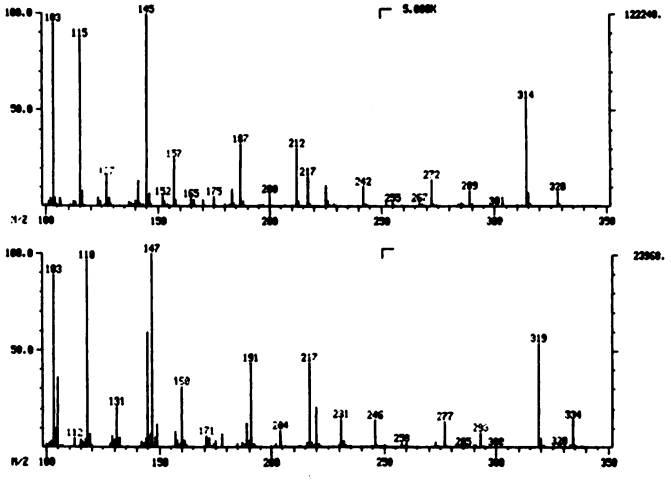

Fig. 1. Mass spectra of aldononitrile pentaacetate derivative of natural D-glucose (*top*) and D-[$^{13}C_6$]glucose (*bottom*)

Between-day imprecision. Aliquots taken from a single SRM 909 serum vial, the same as that for the within-day imprecision evaluation, were analyzed for three days; the data are shown in Table 1. The ANOVA did not indicate statistical differences among results of the three consecutive days of analysis.

Reproducibility Study

Three aliquots of three vials of SRM 909 serum were analyzed after about two months from the first study to determine the accuracy of the method at different times. The results are summarized in Table 2. All values, including the old one, were within the limits of uncer-

Table 1. Within-Day and Between-Day Imprecision

Day	Glucose, mmol/L[a]
1 Morning	6.303 (0.019)
Mid-day	6.308 (0.029)
Afternoon	6.292 (0.019)
Mean	6.301
2 Morning	6.302 (0.033)
Mid-day	6.295 (0.021)
Afternoon	6.280 (0.011)
Mean	6.292
3 Morning	6.297 (0.022)

Variance analysis	CV, %
Total[b]	0.42
Within aliquots[b]	0.30
Between aliquots[b]	0.29
Within day	0.23
Between days	0.00

[a] Mean (SD) of duplicate injections of three aliquots.
[b] Based on the data from days 1 and 2.

tainty given by NIST (revision December 1990).

We applied the method also to Precinorm U and Precipath U to verify the validity of the methodology with different matrixes at different concentrations. Results are shown in Table 2.

Discussion

The present method to determine glucose concentration in lyophilized sera displays high precision and good accuracy despite the simple procedure for sample preparation. To achieve the criteria for a Definitive Method, we determined the variation introduced by each operation of the described method. The procedure is essentially based on a double measurement at each step of the samples and standards preparation. In the initial phase, to reach sufficiently high precision, we use a calibrated Class A glass pipette and an SGE syringe. Variation (CV) of volume measurement with these tools was <0.2% with both water and serum. The accuracy determined with distilled water was within 0.3% when expressed as the percentage deviation from the expected value calculated from the water density at the measured water temperature and from the collected volume.

The second step involves weighing the aliquots collected with the above-described precision. The volume and weight values were compared and only seldom did they not completely overlap when converted into one another by use of the mean density. The weight of the aliquots was then used to calculate the collected volume, based on the density of the solutions. With this procedure the method was applied to determine the glucose concentration of SRM 909 serum and of other commercial sera.

The validity of the bracketing standards was confirmed by comparison of the glucose concentration measured with two sets of standards, which did not differ from each other ($P > 0.05$; F-test). The between-day and within-day imprecision was determined by using one of these standard calibrator sets. Statistical analysis of the data obtained in the first experiment showed that changing the day of analysis does not contribute to the variance (Table 1) and only 28% of the total imprecision is due to the within-day variation (CV <0.23%). In both cases the ANOVA for balanced data did not detect significant difference at the 95% confidence level, even though a small trend toward low data was observed late in the day. The remaining imprecision of the method was associated with between-aliquot (35% of the overall variability) and within-aliquot variations (about 37%), which may be due to errors associated with the weighing of sera and of standard [^{13}C]glucose added to the sample (balance measurement variability) or to a small nonhomogeneity of the serum. Despite the fact that this variability could not be decreased even by calculating the glucose concentration on the basis of the weight of each single aliquot, the overall CV was <0.5%.

The glucose concentration determined in the first experiment (6.30 mmol/L) was only 0.8% over the value reported by NIST on December 1990. Two months later, the concentration measured in three SRM 909 serum vials was 6.23 mmol/L (CV <0.27%), only 0.32% less than the 6.25 mmol/L measured by NIST. In this second experiment about 42% of the total variability was due to the between-vial variance (CV <0.24%) and only 9% to that between aliquots (CV <0.11%). In contrast to previously reported results with this serum (*4*), the multifactor ANOVA revealed no significant difference between vials, in good agreement with the NIST results (*3*), even though one of the three SRM 909 serum vials gave a lower glucose content. However, when we expressed the data per gram of dry serum, the between-vial variation became smaller, with the CV decreasing from 0.27% to 0.17%, suggesting that the apparent difference between vials may be due to a different mass of the lyophilized material. The CV for a single measurement in the second experiment was slightly lower (<0.37%) than that found in the first experiment for the same serum type (<0.42%), indicating either a better homogeneity of the serum in the vials used or an improved sample preparation. The overall CV of the described method for the glucose concentration measurement in SRM 909 serum did not reach that reported by White et al. (*3*), who used a custom-modified mass spectrometer, but is better than that of the procedure described by Pelletier and Arratoon (*4*).

For the other commercial sera tested, the overall CV was slightly higher, 0.53% and 0.56% for Precinorm and Precipath, respectively (Table 2). For the serum with a low glucose concentration, the multifactor ANOVA pointed out a significant difference at the 95% confidence level between vials, which contributed 62% to the total variance. This high variability can be attributed to one of the three Precinorm vials, for which the glucose concentration was about 8% greater than that of the other two vials. This discrepancy between vials again probably results from a different mass of the lyophilized material in the vials; however, this could not be ascertained because we did not weigh the dry-serum content of the commercial serum vials. In contrast, the Precipath serum showed homogeneity between vials (CV =

Table 2. Imprecision with Various Sera

Serum	Glucose concn, mmol/L per vial[a]	CV, % Between vials	Between aliquots	Within aliquots	Total
SRM	6.237 (0.014)				
	6.207 (0.019)				
	6.234 (0.009)				
Mean	6.226 (0.017)	0.24	0.11	0.26	0.37
Precipath	13.473 (0.101)				
	13.464 (0.068)				
	13.471 (0.024)				
Mean	13.469 (0.005)	0.00	0.51	0.21	0.56
Precinorm	6.152 (0.011)				
	6.097 (0.011)				
	6.108 (0.027)				
Mean	6.119 (0.029)	0.42	0.27	0.18	0.53

[a] Mean (SD) of duplicate injections of three aliquots from each vial.

0%), but a large contribution to the overall CV was the between-aliquot variability (85%), yielding a CV of 0.51%.

In conclusion, the described method for determination of glucose concentration in serum displays total reproducibility and accuracy that satisfy the criteria for a Definitive Method (*2*).

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References

1. Kaplan LA. Glucose. In: Pesce AJ, Kaplan LA, eds. Methods in clinical chemistry, St. Louis: CV Mosby Co., 1987:105–11.
2. Cohen A, Hertz HS, Mandel J, et al. Total serum cholesterol by isotope dilution/mass spectrometry: a candidate Definitive Method. Clin Chem 1980;26:854–60.
3. White EV, Welch MJ, Sun T, Sniegoski LT, Hertz HS, Cohen A. The accurate determination of serum glucose by isotope dilution mass spectrometry—two methods. Biomed Mass Spectrom 1982;9:395–404.
4. Pelletier O, Arratoon C. Precision of glucose measurement in control sera by isotope dilution/mass spectrometry: proposed Definitive Method compared with a Reference Method. Clin Chem 1987;33:1397–402.
5. Siekmann L, Brener H. Determination of cortisol in human plasma by isotope dilution mass spectrometry. J Clin Chem Clin Biochem 1982;20:883–92.
6. Varma R, Varma RS, Wardi AH. Separation of aldononitrile acetates of neutral sugar by gas–liquid chromatography and its application to polysaccharides. J Chromatogr 1973;77:222–7.
7. Magni F, Monti L, Brambilla P, Poma R, Galli Kienle M. Determination of plasma [6,6-$^2H_2$]glucose enrichment by a simple and accurate GC-MS method. J Chromatogr 1992; in press.