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Short Communication

Determination of plasma [6,6-$^2H_2$]glucose enrichment by a simple and accurate gas chromatographic–mass spectrometric method

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ABSTRACT

An improved method for the evaluation of glucose turnover rate in humans, using a prime-continuous infusion of [6,6-$^2H_2$]glucose, was developed. Deproteinization of plasma and conversion of glucose into the aldononitrile pentaacetate derivative are the only sample manipulations required prior to the gas chromatographic mass spectrometric analysis. In six normal adults (prime = 5 mg kg^{-1}; continuous infusion = 0.05 mg kg^{-1} min^{-1}) the hepatic glucose output calculated at steady state by the procedure described here was 2.1 ± 0.2 mg kg^{-1} min^{-1}, the isotopic enrichment being determined with a coefficient of variation of *ca.* 2%. In six additional subjects, with half of the above-mentioned doses of labelled glucose, the mean hepatic glucose output was exactly the same (3.2% coefficient of variation for the isotopic enrichment measurement). The method described allows the hepatic glucose output to be precisely determined with savings both of time and of labelled glucose.

INTRODUCTION

Glucose turnover in different animal species, including humans, has been studied for many years [1] using various isotopic tracers, labelled with either radioactive or stable isotopes [2,3]. For ethical reasons, radioactive isotopes have been substituted by stable ones, such as ^{13}C and ^2H, in clinical studies not only in infants and pregnant women but also in adults [4]. In addition to being safer than radiotracers, these isotopes produce more precise results owing to the specificity of the quantitation procedure, which combines gas chromatographic (GC) separation with mass spectrometry (MS); in particular, operating in the selected-ion monitoring (SIM) mode, signals are monitored that originate with high probability only from the compound to be analysed.

Various methods have been developed to derivatize glucose before the GC–MS analysis [4–6]. These procedures include a purification step, and the MS analysis is usually carried out in the chemical ionization (CI) mode. The use of CI increases the sensitivity of the method to measure the glucose enrichment because most of the ion

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current is accounted for by the molecular ion. We have modified a derivatization procedure previously described for the analysis of sugars in gum hydrolysates [7], and have used the aldononitrile pentaacetate derivative to quantify the plasma enrichment after infusion of [6,6-$^2H_2$]glucose in order to measure the glucose turnover.

EXPERIMENTAL

Materials

Pyridine (99.8% pure), acetic anhydride (higher than 98% pure) and hydroxylamine hydrochloride (98% pure) were purchased from Fluka (Buchs, Switzerland). [6,6-$^2H_2$]Glucose (99.4 atom % excess) was obtained from MSD Isotopes (Merck Sharp & Dohme, Munich, Germany). All other solvents were of analytical grade.

Sample preparation

Plasma aliquots (50 μl) were deproteinized with 0.8 ml of acetonitrile. After centrifugation, the supernatant was separated and evaporated to dryness under vacuum. Glucose was then converted into aldononitrile by treatment with hydroxylamine hydrochloride (2 mg) in 150 μl of pyridine at 90°C for 30 min. The solution was then treated with acetic anhydride (200 ml) followed by additional heating for 30 min at 90°C. The derivatized samples were dried under vacuum using a Savant centrifuge, residues were combined with 0.5 ml of dichloromethane, and 1–2 μl of the resulting solution were injected for GC–MS analysis. A calibration curve was prepared by adding known amounts of D-[$^2H_2$]glucose to 50 μl of pooled plasma of healthy volunteers to obtain deuterated glucose with mole % excess (MPE) in the 0–17% range. The glucose concentration of the plasma pool was preliminarily determined by GC–MS using [$^{13}C_6$]glucose as the internal standard and carrying out the analysis by the above described derivative. A calibration curve was also prepared mixing unlabelled and dideuterated standard glucose to obtain samples with a deuterium % excess in the same range of that of the curve prepared in plasma.

Calculations

The ratio (R_B) of h_{189}/h_{187} was determined by SIM (where h_{187} and h_{189} are the peak heights (or areas) at the glucose derivative retention time on the traces for the ions at m/z 187 and 189, respectively) in basal plasma extracts and standard glucose. For plasma samples spiked with known amounts of [$^2H_2$]glucose, and for standard mixtures of the labelled and unlabelled glucose, based on the R_B found for the derivative of authentic compound, the contribution of the natural glucose to the m/z 189 height was subtracted from that found in the analysis. The corrected value of h_{189} was used to calculate the $h_{189}/(h_{187}+h_{189})$ percentage ratio in samples of the calibration curve [8] as follows:

$$R_s = 100\,[h_{189}-h_{187}R_B]/[h_{187}+h_{189}-h_{187}R_B]$$

R_s (deuterium enrichment) values were then plotted against $W_D100/[W_N+W_D]$ percentage ratios (where W_N and W_D are the nanomoles of natural and dideuterated glucose species in the sample, respectively) and the lines $y = a+bx$ were calculated by regression analysis for both types of calibration curve.

R_s in plasma of subjects submitted to [6,6-$^2H_2$]glucose infusion was determined as described above, using the R_B value measured in plasma collected from the studied subject before infusion with the deuterium-labelled glucose. The MPE of deuterated glucose, $W_D100/[W_N+W_D]$, was then computed on the basis of the slope of the standard curve prepared in plasma. The hepatic glucose output (HGO) was calculated as previously described [9]:

$$HGO = [(100/MPE)-1]I$$

where I is the infusion rate (mg kg^{-1} min^{-1}).

GC–MS analysis

The isotopic enrichment of plasma glucose was determined using either an LKB 2091 GC–MS system or a Finnigan 4021. GC was performed using an SPB-35 wide-bore glass column, 30 m × 0.72 mm I.D., 0.35 μm layer thickness (Supelco, CA, USA). The injector temperature was 270°C, the oven temperature 210°C, and the carrier gas was helium at 55 mbar. MS conditions for the LKB 2091 were: ion energy, 70 eV; ion accelerating voltage, 3.5 kV with a resolution of 50 mV and an accuracy of 200 mV; multiplier voltage,

350 V; trap current, 50 mA; transfer line temperature, 280°C; acquisition lasted 20 and 60 ms for ions at m/z 187 and 189, respectively. For the Finnigan instrument, conditions were: ion energy, 70 eV; emission current, 0.23 mA; conversion dinode, +3.0 kV; preamplifier sensitivity, 10^{-7}; transfer line temperature, 290°C; manifold temperature, 60°C; acquisitions of the ions were performed with a 0.5 mass unit window for 0.1 s at each m/z value. The analyses were carried out by selected-ion recording (SIR), monitoring ions at m/z 187 and 189 for unlabelled and labelled glucose, respectively. The peak height (or area) at m/z 187 and 189 was measured. For the determination of the glucose level in the plasma used for the standard curve, the ions at m/z 314 and 319 for unlabelled and [^{13}C]glucose were monitored, respectively.

Clinical studies

The HGO was measured in twelve healthy volunteers. Briefly, [6,6-$^{2}H_2$]glucose was infused according to the prime constant rate infusion technique [10]. In six subjects the prime was of 5 mg/kg with a constant infusion of 0.05 mg kg^{-1} min^{-1}, and in six other subjects half of the above doses were used. Plasma samples were collected for GC–MS analysis at the steady-state period, between 100 and 120 min.

RESULTS AND DISCUSSION

The present method of measuring the plasma glucose enrichment, based on the analysis of the aldononitrile pentaacetate derivative, presents advantages over other reported procedures [4–6] without loss of specificity and precision. Laborious purification is avoided, and the chosen derivative allows analysis with most commercial GC–MS instruments, which are usually equipped only with the electron-impact ionization source. The method displays a good precision, the coefficient of variation (C.V.) being *ca.* 2 and 3.2% ($n=5$) at 2.5 and 1.2% of glucose isotopic enrichment, respectively. As a result of the simplicity and rapidity of both the purification and the derivatization steps and of the GC–MS analysis, up to 50 samples can be processed per day. The deproteinization step with acetonitrile gives an extract that is pure enough to give rise to a SIM chromatogram without any peak interfering with glucose analysis. On the other hand, as already described by others [7], the GC conditions allow separation of the glucose derivative from those of other sugars that may be present in plasma. Fig. 1 shows a SIM chromatogram of a typical plasma sample with a single peak originating from the glucose derivative.

The calibration standard curves were linear in the tested range (0–17 MPE), with $r = 0.9998$ for both the curve prepared in plasma and that obtained by simply mixing authentic labelled and unlabelled glucose; slopes of the curves did not differ significantly ($p>0.05$) from each other. Equations of the two regression lines ($y=a+bx$; where y is the R_S ratio described in Experimental and x is the isotopic enrichment for plasma and glucose curves) determined using the LKB spectrometer were $y=-0.25+1.01x$ and $y=-0.05+1.00x$, respectively. Intercepts of the lines on the y-axis did not differ significantly from zero ($p>0.05$). The similarity between slopes confirms the above-mentioned absence of any interference in the GC–MS analysis of this glucose derivative. With the Finnigan instrument, equations were $y=0.14+0.97x$ and $y=0.1+0.96x$, with no significant difference between the two spectrometers. The high accuracy of the method is clear from the equations of the calibration curves obtained with two different GC–MS instruments, which are not statistically different from each other or from the expected equation ($y=0+1x$).

Different groups working in this field have debated the best way to calculate the MPE from the GC–MS data [4]. We completely agree with Tserng and Kalhan [9] about the use of an appropriate standard curve for the calculation of the HGO. In fact, we use the equation HGO = [(100/MPE) − 1]I, with plasma glucose enrichment data expressed as the MPE and infusion data (I) as mg kg^{-1} min^{-1}. However, particular care should be used when the ratio $h_{189}/[h_{187}+h_{189}]$ is calculated from GC–MS analysis data. As described by Pickup and McPherson [11], if the height (or area) of the peak selected for the evaluation of the labelled material is used without any correction for the contribution of the natural enrichment one should not expect to obtain a straight line,

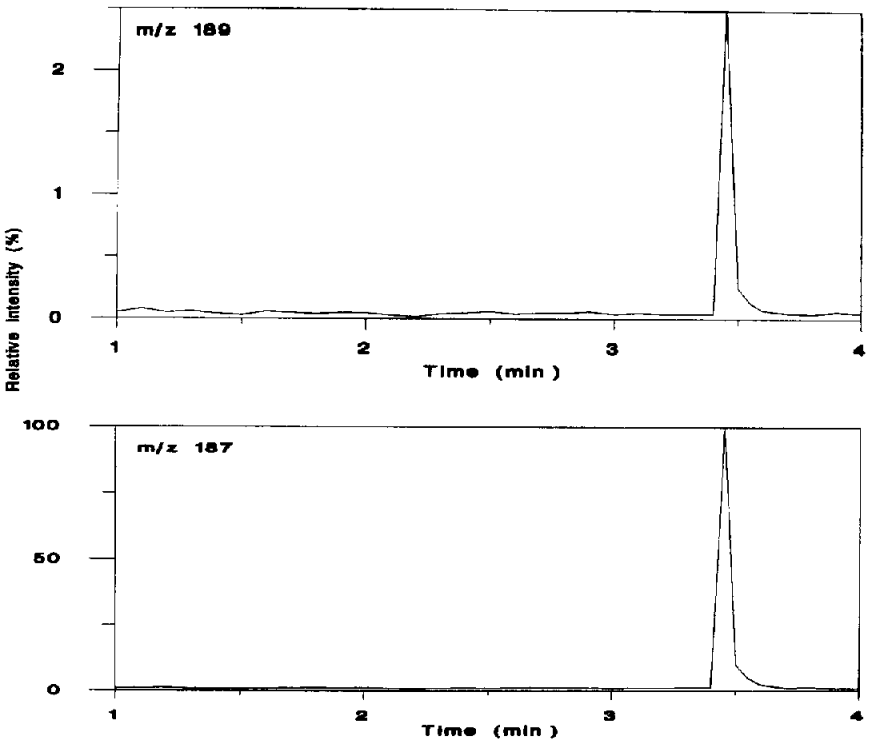

Fig. 1. Ion chromatogram of a typical extract of plasma enriched with [6,6-$^2H_2$]glucose.

unless both the interference of natural material at the m/z selected for the evaluation of the labelled species and that of the labelled material at the m/z used for the natural species are excluded or irrelevant. Under the conditions selected by us the latter interference can be considered to be zero, because the deuterated glucose enrichment was 99.4%; the former was more relevant, as R_B in standard glucose is 0.0141. If the relationship between MPE and the height ratios of the labelled (h_D) and total species ($h_D + h_N$) is considered to fit a line $y = a + xb$, the correlation coefficient will never become 1.000 even if significance of the fitting is reached. The discrepancy between the theoretical points and those of the fitted line is apparent in Fig. 2. Using the fitted line to calculate the MPE from the uncorrected ratios, erroneous results would be obtained with a greater error for low MPE values. If h_{189} is corrected for the contribution of the natural isotopes, as described in Experimental, the correlation between the MPE and R_S becomes a line without intercept on the y-axis and with the slope value of 1.0 ($y = x$) and the MPE is therefore correctly calculated using the fitted line.

Following the above-mentioned considerations, data obtained from the two clinical experiments were used to calculate the glucose turnover rate in adult humans. In the first study, with a whole dose, under steady-state conditions the plasma glucose isotopic enrichment (MPE) was $2.4 \pm 0.1\%$ (mean ± S.D.), from which the hepatic glucose production was calculated to be 2.1 ± 0.2 mg kg^{-1} min^{-1}, in good agreement with previously reported values [4–6]. In the second experiment, using half the dose of labelled glucose, the sensitivity was still adequate because the percentage peak-height ratio was at least twice that found in the plasma of the same subjects before treatment with the deuterated glucose. The MPE at steady state was 1.2 ± 0.1%, and the HGO determined with these data was 2.1 ± 0.2 mg kg^{-1} min^{-1}.

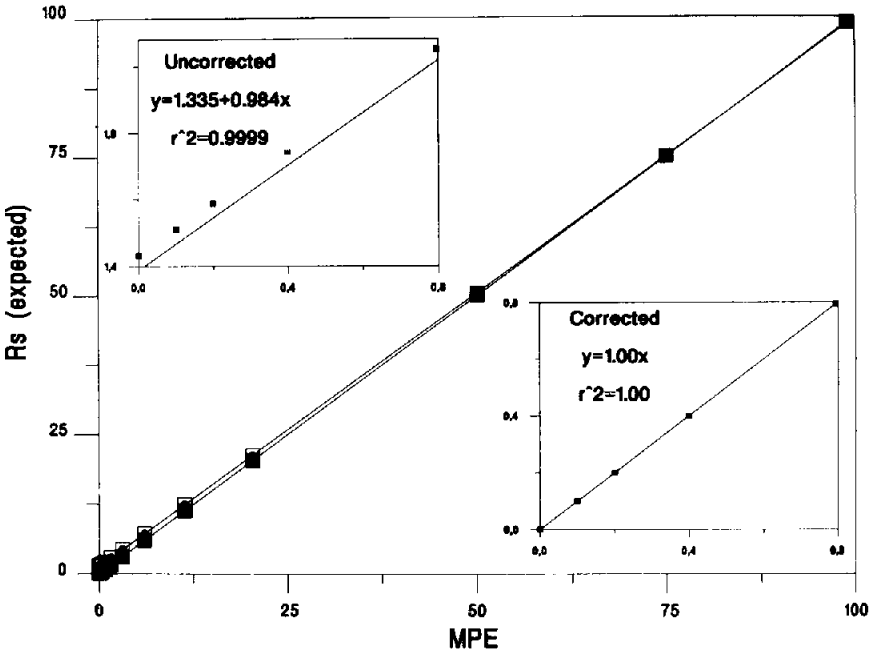

Fig. 2. Plot of the peak-height ratios (R_S) against isotopic enrichment (MPE). Values either uncorrected or corrected for the contribution of natural enrichment (calculated as described in the text) were fitted to a $y = a + bx$ equation. Compared with the corrected values (lower square) uncorrected data (see text) show a discrepancy with the fitted line (upper square).

CONCLUSION

The described method allows the precise determination of the glucose turnover rate in humans, even at low plasma isotopic enrichment, avoiding laborious sample purification procedures and prolonged GC–MS analyses. Less labelled glucose is required because a lower enrichment than that previously described [4–6] is sufficient for clinical studies to be performed.

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