

A Liquid Chromatography–Mass Spectrometry Method to Measure Stable Isotopic Tracer Enrichments of Glycerol and Glucose in Human Serum

T. S. McIntosh, H. M. Davis, and D. E. Matthews*

U.S. Clinical Pharmacology Unit, GlaxoSmithKline, Philadelphia, Pennsylvania 19104; and

**Departments of Medicine and Chemistry, University of Vermont, Burlington, Vermont 05405*

Received May 16, 2001; published online November 26, 2001

Stable isotopes are commonly used as tracers for the measurement of glycerol and glucose kinetics in metabolic studies. Traditionally, the analysis of these isotopes has been performed using gas chromatography–mass spectrometry, which requires that the analytes first be derivatized. The derivatization process adds considerable complexity to the method. Liquid chromatography–mass spectrometry (LCMS) can measure many metabolites directly with limited sample preparation. We present a novel analytical method for the measurement of [1,1,2,3,3-²H₅]glycerol (*d*₅-glycerol) and [6,6-²H₂]glucose (*d*₂-glucose) isotopic tracer enrichments in human serum in a single run by LCMS. After a simple extraction step, the sample is separated isocratically by HPLC, and the isotopes are measured using positive electrospray ionization with selected ion monitoring of the sodium–adduct ions. The method is linear over a wide range of *d*₂-glucose and *d*₅-glycerol enrichments. The within-day standard deviation of measurement of serum samples was 0.05 mole% excess (MPE) for *d*₂-glucose and 0.25 MPE for *d*₅-glycerol. The variation of tracer enrichment among days was about double that measured within 1 day. © 2001 Elsevier

Science

Key Words: stable isotopes; deuterated tracers; tracer kinetics.

The kinetics of glucose and glycerol have been measured *in vivo* for many years using both radioactive and stable isotopically labeled tracers. A variety of methods have been published to measure the stable isotopic enrichments of tracers for these and other metabolites. Almost all of the methods have been based around use of gas chromatography–mass spectrometry

(GCMS).¹ For both glucose and glycerol, the GCMS methods require extensive sample pretreatment and derivatization before injection into the GCMS (1–4).

The primary tracer that has been used to measure *in vivo* production of glucose is [6,6-²H₂]glucose (abbreviated *d*₂-glucose) (2–4). Several different approaches to derivatizing glucose have been developed and are commonly used today: the butaneboronate acetate derivative (5), the pentaacetate derivative (4, 6), and several others. These derivatization schemes have been used to measure a variety of glucose tracers besides [6,6-²H₂]glucose.

Although only one tracer is commonly used to measure glycerol kinetics, i.e., [1,1,2,3,3-²H₅]glycerol (abbreviated *d*₅-glycerol) (4), measuring this particular glycerol tracer is difficult. The most commonly used methods of derivatization of glycerol are the formation of the triacetate ester derivative (7) or tris-trimethylsilyl derivative (4). Unfortunately, both of these derivatives produce fragment ions missing one of the three-glycerol carbons and two of the glycerol hydrogens (i.e., two ²H of the *d*₅-glycerol are lost). Gilker *et al.* (8) used negative chemical ionization GCMS and a trisheptafluorobutyl ester derivative to keep the glycerol molecule intact during measurement. Ackermans used a similar approach with positive chemical ionization and the triacetate ester derivative (9). Magni *et al.* (10) and Flakoll *et al.* (11) used the tris-*t*-butyldimethylsilyl derivative and monitored the [M-57]⁺ ion to measure the entire glycerol molecule. All of these methods take significant care to make and measure the derivatives by GCMS.

¹ Abbreviations used: GCMS, gas chromatography–mass spectrometry; LCMS, liquid chromatography–mass spectrometry; ESI, electrospray ionization; MPE, mole% excess; SIM, selective ion monitoring; SEE, standard error of the estimate.

An alternative to GCMS is liquid chromatography–mass spectrometry (LCMS) using either electrospray ionization (ESI) or atmospheric pressure chemical ionization. Although LCMS techniques have been available for several years and commercial instruments are sold in several different configurations, application of LCMS to measurement of stable isotopic enrichment of tracers has been very limited. The primary advantage of using LCMS is that sample preparation is reduced and no derivatization needs to be performed. Three reports have appeared using thermospray LCMS for measurement of glucose. Esteban *et al.* (12) measured glucose and [U-¹³C]glucose, monitoring the [M + NH₄]⁺ ion. The [M + NH₄]⁺ ion was obtained by adding ammonium acetate to the mobile phase. They indicated that the method was sensitive and could measure [U-¹³C]glucose in the 0.1–1% enrichment range, but provided no results for real samples. Reid *et al.* reported a method to measure glucose and [1-²H]glucose tracer enrichments by thermospray LCMS using the [M – H][–] ion at *m/z* = 179 (13). Little detail concerning detection limits was given, and no subsequent reports have appeared. Takatsu and Nishi reported in 1991 use of LCMS and a [U-¹³C]glucose internal standard as a reference method for measuring serum glucose concentration with a ±1% precision of measurement (14).

None of these methods applied recent ESI-LCMS techniques to measurement of glucose. We have tried ESI-LCMS using commonly available commercial instrumentation and found it suitable for the measurement of both glycerol and glucose. In addition, both glycerol and glucose and their isotopic enrichments can be obtained from a single LCMS run. Because both glucose and glycerol tracers are commonly administered simultaneously in metabolic studies (15–20), measuring both enrichments of glucose and enrichments of glycerol from the same serum sample greatly reduces the time required for sample analysis. The ESI-LCMS method we describe takes advantage of production of a sodium-adduct ion for both glucose and glycerol without the addition of sodium salts to the mobile phase. This ion is suitable for selected ion monitoring of glucose and glycerol and their stable isotopes.

MATERIALS AND METHODS

Materials. HPLC water, acetonitrile, glucose, and glycerol were obtained from Sigma Chemical Co. (St. Louis, MO). Acetone was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). [1,1,2,3,3-²H₅]Glycerol and [6,6-²H₂]glucose were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA).

Equipment. The LCMS was an Agilent Technologies Model LC/MSD supplied with Chem Station 1100 software (Wilmington, DE). Nitrogen gas was supplied to the LC/MSD using a Jun-Air Model 2000-25M air

compressor (Buffalo Grove, IL) connected to a Whatman Model 75-72-K727 nitrogen generator (Haverhill, MA). The HPLC column used was a Shodex Asahipak NH₂P-50 4E, 4.6 × 250 mm (Thompson Instrument Co., Clear Brook, VA).

Enrichment standards. Standards of defined *d*₂-glucose and *d*₅-glycerol enrichment were prepared by making stock solutions of both deuterated and natural materials in water. These stock solutions were then aliquotted to give defined mole ratios (*n*_{*i*}/*n*₀) of labeled (*i*) vs unlabeled (0) materials, where *n* represents the number of moles and *i* indicates the degree of labeling (i.e., *i* = 0 for unlabeled, 5 for *d*₅-glycerol, and 2 for *d*₂-glucose). The mole ratio values were also transformed into standard mole fractions of enrichment: $E = 100 \cdot n_i/n_0/(1 + n_i/n_0)$ where the factor of 100 converts mole fraction to mole% enrichment (MPE). The concentrations of glucose and glycerol in these standards were set to approximate normal values in human serum (i.e., 5.5 mM for glucose and 100 μM for glycerol). The standards ranged in enrichment from 0–30 MPE for *d*₅-glycerol and from 0–5 MPE for *d*₂-glucose.

Preparation of serum samples. Whole blood was collected from healthy overnight-fasted human volunteers in Vacutainer serum separator tubes. After the samples had set for 15 min at room temperature, the tubes were centrifuged at 2000*g* for 10 min at 4°C to separate the serum. The serum was transferred to polypropylene tubes and stored at –80°C until later measurement. To test for stability of the method for measuring physiological samples over time, normal fasting volunteer serum was pooled and was spiked with both *d*₂-glucose (1 and 5 MPE) and *d*₅-glycerol (3 and 9 MPE). The amount of labeled material added was based upon assumed normal serum concentrations of 5.5 mM for glucose and 100 μM for glycerol. These serum standards were stored at –80°C until analysis.

Sample analysis. Serum samples were thawed in a 37°C water bath. Aliquots of serum (0.3 ml) were placed in 1.7-ml microcentrifuge tubes containing 1 ml of ice-cold acetone. The tubes were vortexed and placed on ice for 10 min and then centrifuged at 17,000*g* for 2 min. The supernatant was pipetted into 12 × 75-mm glass tubes and concentrated to dryness under a stream of nitrogen at 37°C. Into each tube, 0.3 ml of a 75% acetonitrile/25% water mixture was added. The tubes were vortexed for 15 s and filtered through a 0.45-μm syringe filter into HPLC vials. These vials were placed into the LCMS autosampler and 20 μl was injected into the LC/MSD. The liquid chromatograph conditions were 1.0 ml/min isocratic 65% acetonitrile/35% water and a column temperature of 35°C. The mass spectrometer conditions were electrospray ionization in a positive ion mode; fragmentor voltage 40 V;

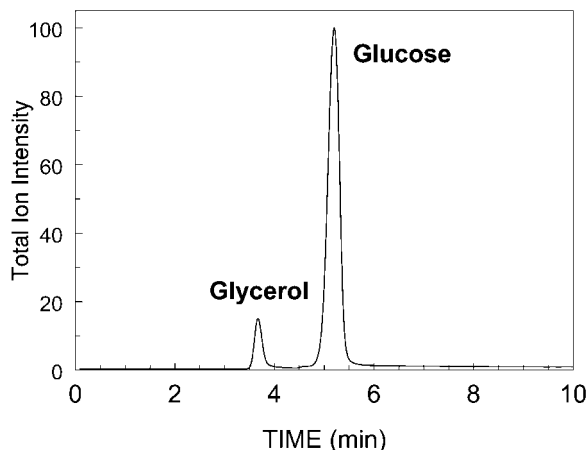


FIG. 1. Ion chromatogram of glycerol and glucose standards under positive electrospray conditions. The ions $m/z = 115$ for glycerol and $m/z = 203$ for glucose were monitored and their intensities summed and plotted as a function of time. The amount injected ($20 \mu\text{l}$) contained equal concentrations ($500 \mu\text{M}$) of glycerol and glucose in water.

drying gas temperature 300°C ; drying gas flow 13 L/min ; capillary voltage 5500 V ; and nebulizer pressure 60 psi . The mass spectrometer conditions were first optimized using flow injection analysis on the standards, without the column. Scanning mode was used to obtain the spectra shown in the results, but the LCMS was operated in selective ion monitoring (SIM) mode for measuring all isotopic enrichment ratios. The ions monitored by SIM were the $[\text{M} + \text{Na}]^+$ ion that corresponds to $m/z = 203$ and $m/z = 205$ for unlabeled and d_2 -glucose, respectively, and $m/z = 115$ and $m/z = 120$ for unlabeled and d_5 -glycerol, respectively. Duplicate injections were made for each sample.

Calculations. The area under the curve was determined for each ion and peak eluting in each sample measurement using the Chem Station 1100 software. The ratio of these areas were taken to determine the ratio of labeled/unlabeled material: $R_{2/0} = A_{205}/A_{203}$ for glucose and $R_{5/0} = A_{120}/A_{115}$ for glycerol, where A is the measured peak area of the indicated ion. These peak area ratios were compared against the measured standards of known enrichment to calibrate the instrument, as previously described for GCMS (8):

$$R_{i/0} = R_{i/0(0)} + k_i \cdot (n_i/n_0),$$

where i indicates the label (either $i = 2$ for glucose or $i = 5$ for glycerol), k_i is the slope of the measured ratio ($R_{i/0}$) vs labeled to unlabeled mole ratio (n_i/n_0). The $R_{i/0(0)}$ term is the ratio of ion i to 0 in the mass spectrometer when unlabeled material is injected and represents the natural isotopic background. The term

$R_{2/0(0)}$ is $\sim 1.3\%$ for d_2 -glucose, while $R_{5/0(0)}$ is negligible for d_5 -glycerol measurement.

RESULTS

Figure 1 shows the LCMS chromatogram of the ions monitored for glycerol and glucose. This chromatogram corresponds to an equimolar injection of glycerol and glucose standards. Glycerol elutes at $\sim 3.7 \text{ min}$ and glucose elutes $\sim 1.5 \text{ min}$ later. The entire run is completed in 10 min or less. Spectra were obtained for these peaks for samples of unlabeled glycerol and glucose and for similar d_5 -glycerol and d_2 -glucose.

Figure 2 shows the ESI mass spectra for glucose and d_2 -glucose obtained from injection of standards. The prominent peaks in the glucose spectrum correspond to monomer and dimer ions with sodium. There is a strong $[\text{M} + \text{Na}]^+$ ion at $m/z = 203$ for glucose and $m/z = 205$ for d_2 -glucose and, interestingly, a strong $[2\text{M} + \text{Na}]^+$ ion at $m/z = 383$ for glucose and $m/z = 387$ for d_2 -glucose. The increase in mass by 4 Da going from unlabeled ($m/z = 383$) to d_2 -glucose ($m/z = 387$) confirms that this ion contains two glucose molecules coupled by a sodium atom. There is the presence of a $[\text{M} + 18]^+$ ion adduct in the spectrum of both glucose and d_2 -glucose.

Figure 3 shows the ESI spectra for glycerol and d_5 -glycerol. The ion of greatest abundance is the $[\text{M} + \text{Na}]^+$ ion at $m/z = 115$ for glycerol and 120 for d_5 -glycerol. There is almost no glycerol-dimer ion containing a sodium, but there is a lower intensity dimer containing a potassium at $m/z = 223$ and 233 for

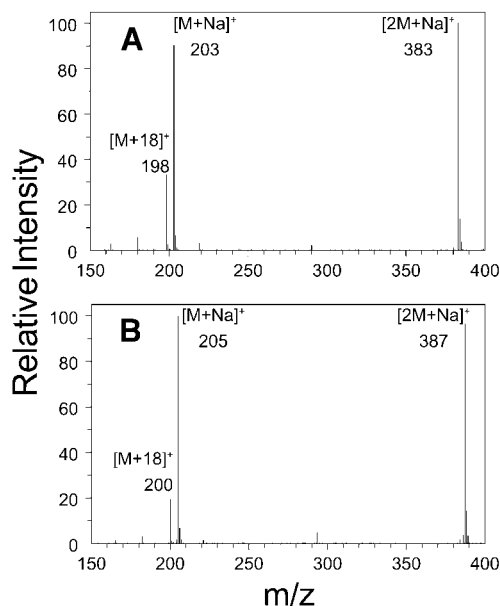


FIG. 2. Positive electrospray spectra of glucose (A) and $[6,6\text{-}^2\text{H}_2]$ glucose (d_2 -glucose, B). The LCMS was set to scan from $m/z = 50\text{--}500$. The spectra are background subtracted.

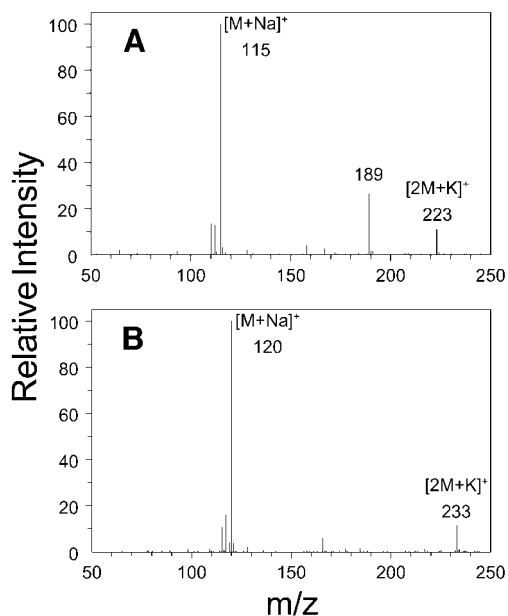


FIG. 3. Positive electrospray scanning spectra of glycerol (A) and [1,1-2,3,3-²H₅]glycerol (*d*₅-glycerol, B). The LCMS was set to scan from $m/z = 50$ –500. The spectra are background subtracted.

glycerol and *d*₅-glycerol. The 10-Da increase for this ion going from glycerol to *d*₅-glycerol defines the dimer. The $m/z = 189$ ion was present in the glycerol, but not

present in the *d*₅-glycerol and is assumed to be a contaminant of the glycerol. There is a small (1–2%) [M + H]⁺ ion at $m/z = 193$ and 198 for glycerol and *d*₅-glycerol, respectively, and ions of 10–15% abundance at [M + 18]⁺ and [M + 20]⁺ in the spectra of both the unlabeled and the *d*₅-glycerol. As per glucose, the [M + Na]⁺ ion was chosen for SIM measurement of glycerol isotopes.

Standards over a defined *d*₂-glucose and *d*₅-glycerol enrichment were injected in duplicate and measured using SIM of the [M + Na]⁺ ions for each. Figure 4 shows the standard curve for *d*₅-glycerol enrichment in the tracer range (i.e., less than about 10 MPE). The regression equation and standard error of the estimate (SEE) for these data are shown in the figure legend. The intercept is not significantly different from zero, and slope of the line is linear. Because there is essentially no natural isotopomer combination to produce a 5-Da increase at $m/z = 120$ of glycerol, the curve passes through the origin. The enrichment curve was extended to 30 MPE *d*₅-glycerol (43% mole ratio) to demonstrate extended linearity. Results of adding two additional samples to the standard curve are shown in the insert graph in Fig. 4. These results indicate that the *d*₅-glycerol could be used as an internal standard for quantitation of glycerol from physiological samples

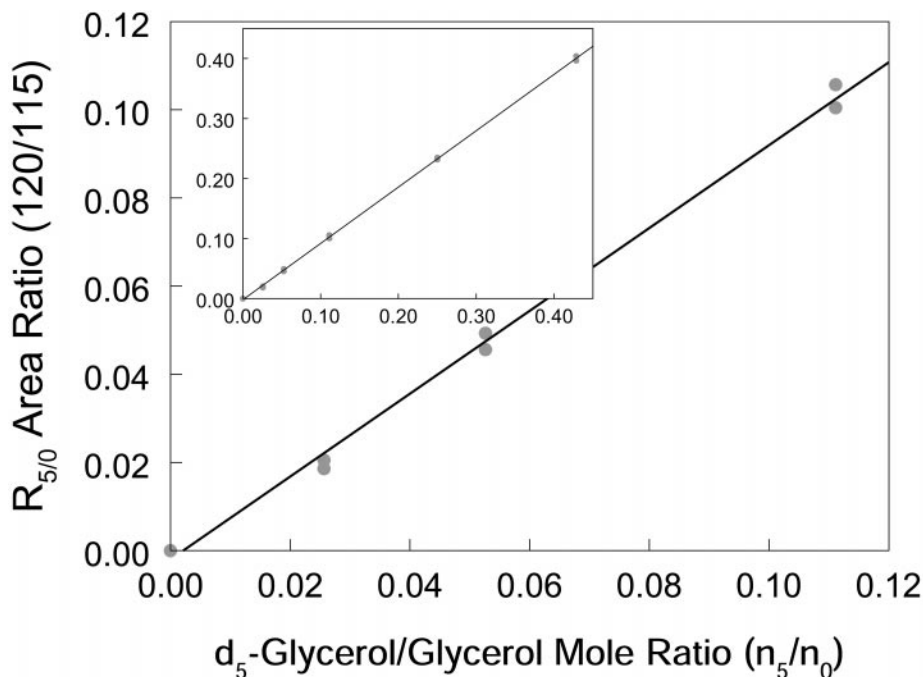


FIG. 4. Measurement of a *d*₅-glycerol standard curve of known *d*₅-glycerol content. The x-axis is the mole ratio (n_5/n_0) of *d*₅-glycerol/natural glycerol. The y-axis is the measured ion abundance ratio ($R_{5/0}$) of $m/z = 120$ and 115. The regression line through the points is $R_{5/0} = -0.002 \pm 0.011 + 0.939 \pm 0.005 \cdot n_5/n_0$. The regression line is linear ($r^2 = 0.996$). The standard error of the estimate is 0.277%. (Inset) Additional samples beyond the tracer dose range (0–40+% *d*₅-glycerol/glycerol). These samples show an extension of the linear range beyond the tracer range. The regression equation of the insert is not different from the regression equation of the limited range.

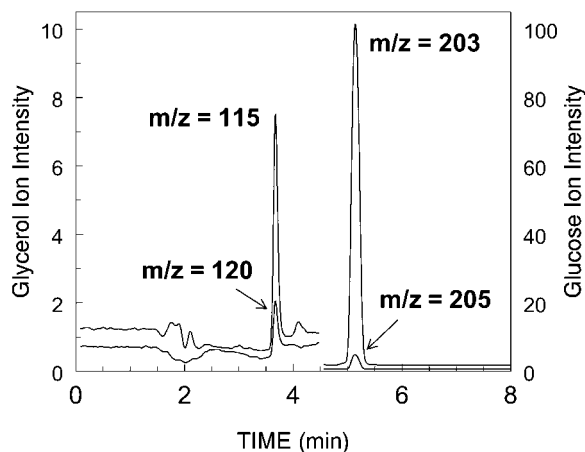


FIG. 5. Selected ion monitoring chromatograms of a serum sample for glycerol and glucose. The ions at $m/z = 115$ and 120 were monitored for glycerol and d_5 -glycerol for the first 4.5 min and $m/z = 203$ and 205 were monitored for glucose and d_2 -glucose from 4.5 min on. Glycerol eluted at 3.7 min, and glucose eluted at 5.1 min. The most intense ions are at $m/z = 115$ and 203 . The scale has been increased (left-most y -axis) 10-fold to display the glycerol ions relative to the more intense glucose ions (right-most y -axis). The sample was a human serum sample that had an enrichment of ~ 15 MPE d_5 -glycerol and ~ 2 MPE d_2 -glucose.

using LCMS in a fashion similar to what we have demonstrated previously for GCMS (8).

The standard curve for d_2 -glucose (not shown) was plotted as the observed $205/203$ area ratio of the d_2 -glucose/glucose ions ($R_{2/0}$) vs the known mole ratio of d_2 -glucose/glucose (n_2/n_0). The d_2 -glucose enrichments range from 0 to 5 MPE. The regression equation was $R_{2/0} = 0.014 \pm 0.003 + 0.917 \pm 0.11 \cdot n_2/n_0$ and indicates that the relationship with measured ion abundance ratio by LCMS is linear with increasing enrichment of d_2 -glucose. The regression line is linear ($r^2 = 0.999$). The SEE was 0.066%. The slope of the line is 0.92 ± 0.01 , indicating a slope close to unity. The less than unity slope may be a function of the d_2 -glucose used, which may have been less than 100% pure—as often is the case. The intercept ($1.4 \pm 0.3\%$) is appropriate for the natural abundance isotopes of glucose at this mass.

Serum samples were extracted and injected into the LCMS, and glycerol and glucose were measured by SIM. A representative SIM chromatogram of a serum sample measured for glycerol and glucose and their isotopes is shown in Fig. 5. The SIM chromatogram is typical of serum measurements and indicates that the glycerol ion abundance is significantly lower than that of glucose. The glycerol is of lower peak intensity because glycerol is approximately 50-fold lower in concentration in plasma and because the glycerol $[M + Na]^+$ ion is less intense in general compared to the glucose $[M + Na]^+$ ion.

To demonstrate the precision of d_2 -glucose and d_5 -glycerol of serum samples, serum samples were prepared from pooled serum with addition of known amounts of d_2 -glucose and d_5 -glycerol. These samples were then measured once each day for 3 days. The results of these measurements are shown in Table 1. The precision of measurement within a single day's measurement was similar across the 3 measurement days for each sample type (d_2 -glucose and d_5 -glycerol) and between the lower and higher enrichment samples. The average daily standard deviation was 0.05 MPE (CV = 2%) for d_2 -glucose serum sample measurement and 0.25 MPE (CV = 5%) for d_5 -glycerol. The precision across days (shown in the lower part of Table 1) was about double the within-day precision. The d_2 -glucose enrichment measured across days averaged 0.15 MPE for the two samples (CV = 8%), and the d_5 -glycerol enrichment was 0.5 MPE for the two samples (CV = 12%) across days.

DISCUSSION

Measurement of low-molecular-weight metabolites has been a challenge by ESI-LCMS because of potential interferences from solvent and other ions. We report here a method to measure two key metabolites commonly followed in metabolic studies in humans: glucose, indicative of sugar metabolism, and glycerol, indicative of fat metabolism. Our method using the $[M + Na]^+$ ion with ESI-LCMS and a standard “bench top” quadrupole instrument will measure glucose, glycerol, and the commonly used stable isotopically labeled tracers of these compounds, d_2 -glucose and d_5 -glycerol (4). Reports have been published measuring glucose using the older “thermospray” version of ESI for measurement of glucose (12–14), but none have measured glycerol. To our knowledge, this report is the first one for measurement of glycerol and its stable isotopic tracer enrichments by LCMS.

It was a challenge to measure both glycerol and glucose in the same sample from the same LCMS injection. Not only is the concentration of glycerol in serum ~ 50 -fold lower than that of glucose, but also we find that glycerol does not produce nearly as intense an ESI spectrum as does glucose. Glycerol does not ionize as well as glucose under the conditions of our assay (e.g., the glycerol peak in Fig. 1 is $\sim 10\%$ as large as the glucose peak). To compensate for these differences, we used a larger injection volume ($20 \mu\text{l}$) than was needed for glucose to measure the glycerol enrichment. If only glucose were measured, a considerably smaller sample size and sample injection volume could have been used with no loss of sensitivity. Although we have not shown the data, the acetone extract can be injected directly, eliminating the dry-down step—if only glucose is measured and only $1\text{--}2 \mu\text{l}$ are injected. This approach pro-

TABLE 1
Measurement of d_2 -Glucose and d_5 -Glycerol Enrichments in Serum Samples
to Demonstrate Measurement Precision and Reliability

Sample	Replicate	d_2 -Glucose (MPE)			d_5 -Glycerol (MPE)		
		1	2	3	1	2	3
A	1	1.250	1.509		3.36	3.29	2.96
	2	1.254	1.610	1.236	3.33	3.12	3.02
	3	1.258	1.743	1.248	3.30	2.65	2.78
	Mean	1.254	1.621	1.242	3.33	3.02	2.92
	SD	±0.004	±0.117	±0.008	±0.03	±0.33	±0.13
B	1	5.194	5.357	5.181	8.54	9.02	9.07
	2	5.347	5.243	5.150	9.10	8.11	9.03
	3	5.190	5.400	5.166	8.66	8.25	8.71
	Mean	5.244	5.333	5.166	8.76	8.46	8.94
	SD	±0.089	±0.081	±0.015	±0.30	±0.49	±0.20
Mean values for all 3 days							
Sample	d_2 -Glucose (MPE)		d_5 -Glycerol (MPE)				
	Mean	SD	Mean	SD			
A	1.372	±0.202	3.09	±0.57			
B	5.248	±0.095	8.72	±0.40			

Note. Known amounts of d_5 -glycerol and d_2 -glucose were added two aliquots of a pooled serum sample. The samples were then split into three aliquots and measured three times each on three different days. There was one missing value for sample A measurement day 3.

vides significant savings in sample processing time for studies that only use a glucose tracer.

The formation of sodium adduct in the spray chamber of the mass spectrometer relies on the presence of trace amounts of sodium in the mobile phase, presumably from the glass solvent containers. In our hands with our instrument, this approach works satisfactorily and reproducibly. We did attempt to standardize the amount of sodium in the system by adding low concentrations of sodium acetate to the mobile phase, but the result was a decrease in signal for all ions (data not shown). We did not try to force formation of an $[M + H]^+$ ion over the $[M + Na]^+$ by adding any acid to the mobile phase. The success we had with the $[M + Na]^+$ ion was sufficient.

The primary advantage of LCMS is that sample preparation is greatly reduced. We are able to inject deproteinized extracts of serum samples directly into the LCMS system. In contrast, GCMS requires derivatization of both glucose and glycerol (2–4, 6, 8–11). There is no simple derivative that is useful for both without disadvantages of measurement of both. Furthermore, several of the commonly used derivatives of glycerol suffer because the ion monitored by GCMS is a fragment ion that does not contain all five of the 2H in the tracer (4). We (8) and others (10, 11) have applied specialized techniques to obtain ions that do contain all five 2H in the d_5 -glycerol for measurement, but all of

these methods still require derivatization of the glycerol in the sample. In normal practice, the formation of the derivative takes 1 day for each group of samples that can be measured by GCMS in 1 day. Thus, measurement of a group of samples for both d_2 -glucose and d_5 -glycerol would take ~4 days, whereas the LCMS method described here can accomplish the same task in less than half of the time.

The LCMS precision of measurement of d_2 -glucose enrichment (~0.05 MPE within 1 day and ~0.15 MPE among days) is comparable to the best of the measurement methods by GCMS. The precision of d_5 -glycerol measurement (~0.25 MPE within 1 day and ~0.5 MPE among days) is about half as good as we can do using GCMS (8). In this regard, the existing GCMS methods are superior for measurement precision of d_5 -glycerol. Further work is needed to refine our ability to measure d_5 -glycerol by LCMS. However, the variability of d_5 -glycerol enrichment by LCMS is well within the variability of biological variation of glycerol flux. For example, we find that the coefficient of variation of glycerol appearance rate among individuals in the fasting state is ~30% (21). Our LCMS measurement precision, even among days, is still less than half of the biological variation among individuals.

In summary, we present a novel method for measuring the stable isotopic tracer enrichments of glycerol and glucose in human serum by LCMS in a single run.

The method involves a simple liquid extraction of the sample, isocratic separation on a column designed for analysis of sugars, and detection of sodium adduct ions by positive electrospray mass spectrometry with selected ion monitoring. None of the equipment used needs sophistication. The LCMS method is superior to former GCMS methods because no complicated derivatization schemes of the samples are required and because both analytes can be measured simultaneously.

ACKNOWLEDGMENTS

The authors thank Jim Lau of Agilent Technologies for technical advice. This work was supported in part by a National Institutes of Health Grant RR-00109 (D.E.M.).

REFERENCES

1. Matthews, D. E., and Bier, D. M. (1983) Stable isotope methods for nutritional investigation. *Annu. Rev. Nutr.* **3**, 309–339.
2. Kalhan, S. C. (1990) Stable isotope tracers for studies of glucose metabolism. *J. Lab. Clin. Med.* **116**, 615–622.
3. Wolfe, R. R. (1990) Isotopic measurement of glucose and lactate kinetics. *Ann. Med.* **22**, 163–170.
4. Wolfe, R. R. (1992) Radioactive and Stable Isotope Tracers in Biomedicine: Principles and Practice of Kinetic Analysis, Wiley-Liss, New York.
5. Bier, D. M., Arnold, K. J., Sherman, W. R., Holland, W. H., Holmes, W. F., and Kipnis, D. M. (1977) In-vivo measurement of glucose and alanine metabolism with stable isotopic tracers. *Diabetes* **26**, 1005–1015.
6. Guo, Z., Lee, W. N. P., Katz, J., and Bergner, A. E. (1992) Quantitation of positional isomers of deuterium-labeled glucose by gas chromatography/mass spectrometry. *Anal. Biochem.* **204**, 273–282.
7. Beylot, M., Martin, C., Beaufrère, B., Riou, J. P., and Mornex, R. (1987) Determination of steady state and nonsteady-state glycerol kinetics in humans using deuterium-labeled tracer. *J. Lipid Res.* **28**, 414–422.
8. Gilker, C. D., Pesola, G. R., and Matthews, D. E. (1992) A mass spectrometric method for measuring glycerol levels and enrichments in plasma using ^{13}C and ^2H stable isotopic tracers. *Anal. Biochem.* **205**, 172–178.
9. Ackermans, M. T., Ruiten, A. F. C., and Endert, E. (1998) Determination of glycerol concentrations and glycerol isotopic enrichments in human plasma by gas chromatography mass spectrometry. *Anal. Biochem.* **258**, 80–86.
10. Magni, F., Arnoldi, L., Monti, L., Piatti, P., Pozza, G., and Galli, K. M. (1993) Determination of plasma glycerol isotopic enrichment by gas chromatography-mass spectrometry: An alternative glycerol derivative. *Anal. Biochem.* **211**, 327–328.
11. Flakoll, P. J., Zheng, M., Vaughan, S., and Borel, M. J. (2000) Determination of stable isotopic enrichment and concentration of glycerol in plasma via gas chromatography-mass spectrometry for the estimation of lipolysis in vivo. *J. Chromatogr. B Biomed. Sci. Appl.* **744**, 47–54.
12. Esteban, N. V., Liberato, D. J., Sidbury, J. B., and Yergey, A. L. (1987) Stable isotope dilution thermospray liquid chromatography/mass spectrometry method for determination of sugars and sugar alcohols in humans. *Anal. Chem.* **59**, 1674–1677.
13. Reid, S., Shackleton, C., Wu, K., Kaempfer, S., and Hellerstein, M. K. (1990) Liquid chromatography/mass spectrometry of plasma glucose and secreted glucuronate for metabolic studies in humans. *Biomed. Environ. Mass Spectrom.* **19**, 535–540.
14. Takatsu, A., and Nishi, S. (1991) Stable isotope dilution method for the determination of serum glucose using discharge-assisted thermospray liquid chromatography/mass spectrometry. *Biol. Mass Spectrom.* **20**, 415–418.
15. Shaw, J. H. F., and Wolfe, R. R. (1989) An integrated analysis of glucose, fat, and protein metabolism in severely traumatized patients: Studies in the basal state and the response to total parenteral nutrition. *Ann. Surg.* **209**, 63–72.
16. Jahoor, F., Klein, S., and Wolfe, R. R. (1992) Mechanism of regulation of glucose production by lipolysis in humans. *Am. J. Physiol. Endocrinol. Metab.* **262**, E353–E358.
17. Romijn, J. A., Coyle, E. F., Sidossis, L. S., Gastaldelli, A., Horowitz, J. F., Endert, E., and Wolfe, R. R. (1993) Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. *Am. J. Physiol. Endocrinol. Metab.* **265**, E380–E391.
18. Hellerstein, M. K., Benowitz, N. L., Neese, R. A., Schwartz, J.-M., Hoh, R., Jacob, P., III, Hsieh, J., and Faix, D. (1994) Effects of cigarette smoking and its cessation on lipid metabolism and energy expenditure in heavy smokers. *J. Clin. Invest.* **93**, 265–272.
19. Sial, S., Coggan, A. R., Hickner, R. C., and Klein, S. (1998) Training-induced alterations in fat and carbohydrate metabolism during exercise in elderly subjects. *Am. J. Physiol. Endocrinol. Metab.* **274**, E785–E790.
20. Gastaldelli, A., Coggan, A. R., and Wolfe, R. R. (1999) Assessment of methods for improving tracer estimation of non-steady-state rate of appearance. *J. Appl. Physiol.* **87**, 1813–1822.
21. Matthews, D. E., Pesola, G. R., and Kvetan, V. (1991) Glycerol metabolism in humans: Validation of ^2H and ^{13}C -labeled tracers. *Acta Diabetol.* **28**, 179–184.