Measurement of plasma glycerol specific activity by high performance liquid chromatography to determine glycerol flux

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Abstract **Previous methods for measuring plasma glycerol specific activity (SA) are suboptimal, making the determination of glycerol kinetics in vivo with radiotracers difficult. A new high performance liquid chromatography (HPLC) method is described that permits the accurate and specific measurement of glycerol SA. The method involves isolation of glycerol from plasma and the formation of a tribenzoyl derivative. Glycerol rate of appearance was measured in five human volunteers using both [2-^{3}H]glycerol and [$^2H_5$] glycerol. There was close agreement between the glycerol appearance rates measured using the two approaches (1.66 ± 0.14 vs. 1.70 ± 0.10 μmol·kg^{-1}· min^{-1}, respectively, *P* = NS). This HPLC method offers improved specificity over existing methods of measuring glycerol turnover using radiotracers.—**Judd, R. L., R. Nelson, S. Klein, M. D. Jensen, and J. M. Miles. **Measurement of plasma glycerol specific activity by high performance liquid chromatography to determine glycerol flux. *J. Lipid Res.* 1998.** 39: **1106–1110.**

Supplementary key words lipolysis • HPLC • stable isotopes • radioisotopes

Glycerol release is an important marker of adipose tissue lipolysis because it is produced from the hydrolysis of intracellular triglycerides by hormone-sensitive lipase, but cannot be locally re-esterified by adipocytes (1). It also serves as an oxidative substrate for certain tissues (2), provides the backbone for lipids, and functions as a critical gluconeogenic substrate (1). Glycerol appearance has been measured isotopically in numerous human and animal studies to determine rates of adipose tissue lipolysis and provide an index of fat mobilization in vivo (3–11).

Although excellent methods using stable isotopes of glycerol are available (9, 12, 13), not all investigators have access to gas chromatography/mass spectrometry (GC/MS) facilities. The techniques that use glycerol radiotracers are limited by nonspecificity and imprecision (2–4, 6, 14). Winkler et al. (15) used a tribenzoyl derivative of glycerol to measure glycerol specific activity; however, the techniques described to separate glycerol from other radiolabeled compounds present in plasma are tedious and time consuming. In addition, this approach (15) does not take advantage of the UV absorbency of tribenzoyl glycerol, instead relying on less accurate gravimetric determinations of tribenzoyl glycerol mass to calculate glycerol specific activity (SA). We describe herein a new method for measurement of glycerol SA using high performance liquid chromatography (HPLC) to fully separate glycerol from other compounds and UV detection to more precisely and accurately measure glycerol mass. This approach allows the measurement of glycerol flux with radioisotopes in vivo with much greater ease and accuracy than has been previously available.

MATERIALS AND METHODS

Supplies

Analytical grade or better glycerol, glucose, zinc sulfate ($ZnSO_4$, 0.3 N), barium hydroxide ($BaOH_2$, 0.3 N), pyridine, butyl acetate, benzoyl chloride, glycerol kinase, and 4-dimethylaminopyridine (DMAP) were obtained from Sigma-Aldrich Corporation (St. Louis, MO and Milwaukee, WI). Tribenzoyl glycerol was kindly donated by Unitex Chemical Corporation (Greensboro, NC). Hydrochloric acid and HPLC grade chloroform were supplied by Baxter (Muskegon, MI). HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Analytical grade anion (AG1-X8, 200–400 mesh, hydroxide form) and cation (AG-50WX8, 100–200 mesh, hydrogen form) exchange resins were obtained from Bio-Rad (Hercules, CA). [2-^{14}C] and [2-^{3}H]glycerol were supplied by Amersham Corporation (Arlington Heights, IL) and counted in OptiFluor liquid scintillation cocktail (Packard Instruments, Meriden, CT).

[$^2H_5$]glycerol was purchased from Merck Isotopes (Montreal,

Abbreviations: GC/MS, Gas chromatography–mass spectrometry; FFA, free fatty acids; SA, specific activity; HPLC, high performance liquid chromatography; R_a, rate of appearance; MPE, moles percent excess; DMAP, dimethylaminopyridine.

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Canada). Four dram (8.0 ml) derivatization vials were obtained from Kimball (Toledo, OH) and their caps were fitted with Teflon liners (Arthur H. Thomas, Philadelphia, PA). Disposable 1 × 8 cm plastic Quik-Sep columns were supplied by IsoLab, Inc. (Akron, OH). Microfuge tubes (250 μl) were obtained from Chromtech (Minneapolis, MN).

Stock solutions and standards

An 800 μmol/L aqueous solution of glycerol (250 ml) containing [^{14}C]glycerol (~50,000 dpm/ml) was prepared and stored in a glass reagent bottle. DMAP (30 mg/ml) was dissolved in 250 ml pyridine and stored in a reagent bottle. A 25% (vol/vol) solution of benzoyl chloride in butyl acetate (50 ml) was stored in a screw-cap extraction tube. All standards and reagents were prepared fresh each day to assure optimum results, and all container caps were fitted with Teflon liners. Anion and cation exchange resins were washed and stored in H_2O (1:1, vol/vol) at 4°C prior to their addition to disposable plastic chromatography columns.

Procedure

One ml H_2O was added to each of seven derivatization vials. One ml of the glycerol standard was added to the first vial and serially diluted (1:1), discarding 1.0 ml from the last vial, thus generating 7 standards of 400, 200, 100, 50, 25, 12.5, and 6.25 nmol each.

Columns were prepared by pipetting 2.0 ml of the resin suspension into each column, producing a bed volume of 1.0 ml. The columns were stacked in custom-made racks so that the anion column was on top and the cation column was on the bottom.

One ml plasma was aliquoted into 13 × 100 mm borosilicate tubes. The sample was briefly vortexed and deproteinized by the sequential addition of 1.0 ml BaOH and 1.1 ml $ZnSO_4$. After vortexing again, the sample was centrifuged at 2000 rpm for 10 min. The supernatant was transferred to the top (anion) column, collecting the eluate in a 2-dram derivatization vial. The pellet was washed twice with 1.0 ml H_2O, vortexed, and centrifuged at 2000 rpm and the supernatant from these washes was also placed over the columns. The columns were rinsed twice with 1.0 ml H_2O, collecting all of the eluate (total volume, ~6 ml). One hundred microliters of sucrose (100 mm) was added to all samples and standards and vortexed. Samples were then frozen at −20°C and lyophilized overnight (15 h) (Savant SpeedVac, Holbrook, NY).

After lyophilization, 5 drops of 2,2-dimethoxypropane was added to each sample, mixed, and dried under air. It is critical that the samples are completely dry. One ml DMAP (30 mg/ml) was added to each vial, vortexed, and sonicated for 5 min at 45°C. It is important to limit this sonication–solubilization step to no more than 5 min, because several interfering chromatographic peaks appear when the sonication step is continued for a longer period. Benzoyl chloride (100 μL) was then added and the samples were vortexed until a white precipitate appeared. The vials were transferred to a 45°C water bath for 15 min, removed, and allowed to cool to room temperature. Methanol (50 μL) was added and the samples were again vortexed and heated for 5 min at 45°C. The samples were then dried under a stream of air (Multivap, South Berlin, MA) at 45°C for 60 min and resuspended in 4.0 ml chloroform by vortexing and sonicating. Pyridinium salts and other polar byproducts of the reaction were extracted into 2.0 ml 1 N HCL, the samples were vortexed for 15 sec, centrifuged (2000 rpm for 5 min), and the aqueous layer (supernatant) was aspirated and discarded. The HCL extraction was repeated once, the supernatant was again discarded, and the infranatant (chloroform layer) was evaporated under a stream of air.

The samples were resuspended in 72% acetonitrile by first adding 172.8 μL of 100% acetonitrile to the vials and then 67.2 μL of water. Samples were vortexed well, centrifuged for 2 min on a table top microcentrifuge, and loaded on the deck of an HPLC autosampler (710B WISP, Waters Corporation, Milford, MA). Two hundred (200) μL was injected onto a 5μ Apex C18 250 mm ODS column (Jones Chromatography, Lakewood, CO) and eluted with acetonitrile in H_2O (56:44, solvent A) at 1.3 ml/min. The run time was 60 min, with the column being flushed with 95% acetonitrile between samples, beginning 30 min into the run and switching back to the original buffer 40 min into the run for column re-equilibration. Detection was accomplished at 254 nm with a SpectraPhysics FP-8450 UV detector. Data were collected and stored by a SpectraPhysics plotter/integrator using WINner 386 software on an IBM PS-2 computer.

Under these conditions, tribenzoyl glycerol eluted at approximately 26 min. The column effluent was directed to a waste container except when glycerol eluted from the column; a 5 min fraction beginning 2 min before the anticipated peak was collected in a liquid scintillation vial on an automated fraction collector (ISCO, Inc., Lincoln, NE) with the aid of an automated diverting valve (General Valve Corp, Fairfield, NJ). The fraction was partially dried (to ~1 ml) under a stream of air prior to addition of scintillation cocktail (7.0 ml) and then counted on a dual channel liquid scintillation spectrometer (Wallac Model 1410, Gaithersburg, MD).

Glycerol enrichment was determined by GC/MS using an MSD 5971 system (Hewlett-Packard, Palo Alto, CA) with an HP-1 12-m × 0.2-mm fused silica capillary column (Hewlett-Packard) as described previously (12). Plasma proteins were precipitated with barium hydroxide and zinc sulfate, and after centrifugation the supernatant was passed through a mixed cation and anion exchange column. A trimethylsilyl derivative of glycerol was formed and injected into the GC/MS where ions were produced by electron impact ionization. Glycerol tracer:tracee ratio was determined by selectively monitoring ions at mass-to-charge ratios 205.1 and 208.1. Column temperature was set at 105°C for 4 min and programmed to increase at 70°C/min to a final temperature of 280°C. Helium carrier gas flow was set at 20 ml/min.

Glycerol concentration was determined enzymatically on a centrifugal analyzer (Cobas, Roche Diagnostics).

In vivo experiments

These studies were approved by the Mayo Clinic Institutional Review Board. Informed written consent was obtained from six healthy volunteers, ages 18–35 years and body mass index <24 kg/m^2. All studies were conducted after an overnight fast in the Mayo Clinic General Clinical Research Center while the volunteers rested in bed. A controlled (30 ml/h) infusion of 0.9% NaCl was administered through a forearm vein, which was also used for tracer infusions. A dorsal hand vein was cannulated in a retrograde fashion and used for sampling of arterialized venous blood (16). For five of the volunteers, infusions of [2-^{3}H]glycerol and [$^2H_5$]glycerol were started at 0700 h at rates of ~0.3 μCi/min and ~0.08 μmol·kg^{-1}·min^{-1}, respectively. Thirty minutes was allowed to achieve isotopic steady state after which blood samples were taken every 10 min for 30 min. Blood was collected in chilled EDTA tubes, centrifuged, and the plasma was stored at −70°C until analyzed. The studies were repeated on three or four consecutive days to test the intra- and inter-subject variability and agreement between the two methods. One additional volunteer received a 3-h infusion of both [2-^{3}H]glycerol and [2-^{14}C]glycerol in order to allow labeling of other, non-glycerol components of plasma. This allowed us to collect all fractions from the sample injected onto the HPLC to assess the separation of the glycerol peak from other ^{3}H and ^{14}C-containing peaks. As part of

this study, duplicate samples of the volunteer's plasma were treated with glycerol kinase under appropriate conditions to convert glycerol to glycerol phosphate. This should result in the retention of glycerol phosphate on the exchange columns, therefore eliminating most or all of glycerol from the sample prior to derivitization. If the chromatographic peak also were eliminated, or if the peak decreased in size but the specific activity remained the same, this would provide evidence that the chromatographic peak is specific for glycerol.

Calculations

The rate of appearance (R_a) of glycerol ($\mu mol \cdot kg^{-1} \cdot min^{-1}$) was calculated using Steele's equation (17) as it applies to isotopic steady-state conditions. Using [2-^{3}H]glycerol, the systemic glycerol R_a was calculated using the steady-state formula:

$$\text{glycerol } R_a = F \div [^3H]\text{glycerol SA},$$

where F is the [2-^{3}H]glycerol infusion rate (dpm/min). Using deuterated glycerol, systemic glycerol R_a was calculated using the steady state formula:

$$\text{glycerol } R_a = \frac{F}{TTR}$$

where F is the [$^2H_5$]glycerol infusion rate ($\mu mol \cdot kg^{-1} \cdot min^{-1}$), and TTR is the tracer:tracee ratio of plasma glycerol at isotopic equilibrium.

All values are expressed as mean ± SEM. The Student's *t* test for paired comparison was used to determine whether glycerol flux determined by the two methods was significantly different. All *P* values <0.05 were considered statistically significant.

RESULTS

Maximum absorbency of tribenzoyl glycerol was observed at 230 nm, but this wavelength provided excessive sensitivity for the relatively large amount (usually 5–50 nmol) of glycerol recovered from 1.0 ml of plasma. For this reason, detection was accomplished at a less sensitive wavelength (254 nm).

During development of this method, difficulty was encountered separating the glycerol peak from multiple benzoyl glucose derivative peaks. To solve this problem, the plasma was passed over an AG1-X8 hydroxide column to adsorb glucose, but this increased the volatile losses of glycerol during the dry-down procedures. Therefore, sucrose was added in small quantities to decrease the evaporative losses of glycerol and improve recovery. This improved the chromatography because the sucrose peak elutes much later than the glycerol peak in the chromatogram. Excessive evaporative losses of glycerol occurred when the lyophilization step was extended for many hours after the sample was effectively dry. The duration of the lyophilization we report was chosen to ensure complete removal of water (necessary for a satisfactory derivatization) without volatilization of glycerol. 2,2-Dimethoxypropane was added to assure complete evaporation of the water from the glycerol.

Recovery of glycerol radioactivity added to the samples of the external standard curve averaged 92% (corrected for incomplete sample injection), indicating incomplete derivatization. This made it necessary to add a radiolabeled internal standard to the external standard curve samples in order to correct for incomplete recovery of standards. Recovery of labeled glycerol from plasma was somewhat lower (~76%) because of losses during deproteinization, etc. The coefficient of variation for plasma glycerol specific activity was 3%.

A representative chromatogram of plasma glycerol is provided in **Fig. 1**. The derivatized glycerol standard peak and both radiolabeled derivatized glycerol tracers were found to coelute with tribenzoyl glycerol. The elution time of glycerol was stable provided that the mobile phase reservoir was stirred to prevent demixing, allowing complete recovery of glycerol radioactivity from a narrow (5 min) fraction. A standard curve for glycerol content (used for the SA calculation) is depicted in **Fig. 2**; as can be seen, the curve is linear and intersects the x-axis at zero. The plasma of the subject receiving the 3-h radiotracer glycerol infusion was subjected to a complete radiochromatogram to assess the separation of the glycerol peak from other ^{3}H- and ^{14}C-containing peaks. The glycerol peak was ~2 min wide and separated by 7 min from the peak containing ^{14}C that eluted when the column was flushed with 95% acetonitrile. Treatment of the plasma sample with glycerol kinase virtually eliminated the HPLC tribenzoyl glycerol peak together with the ^{3}H and ^{14}C counts in the fraction normally containing the tribenzoyl glycerol. In one of the samples the glycerol peak was incompletely (84%) eliminated; however, the [^{3}H]- and [^{14}C] glycerol SA were the same in the treated and untreated samples (1.38 vs. 1.44 dpm/nmol and 0.74

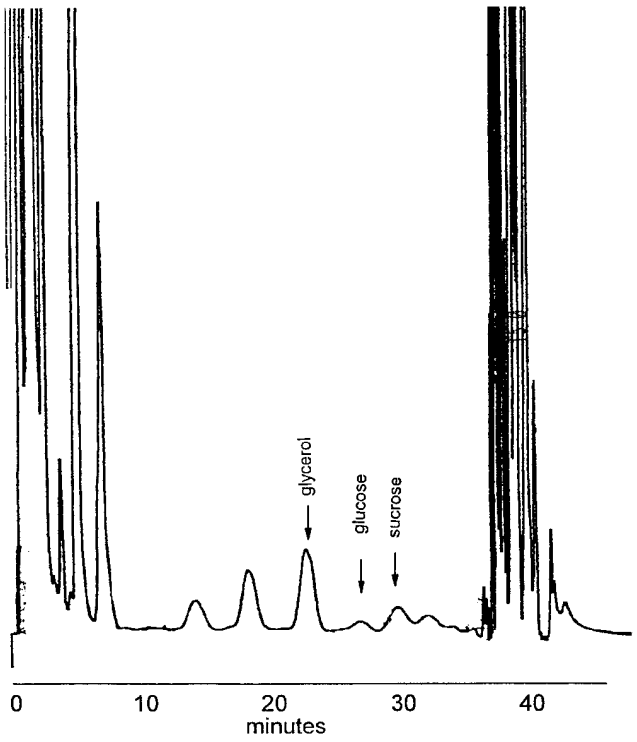

Fig. 1. An HPLC chromatogram of plasma showing the tribenzoyl glycerol peak.

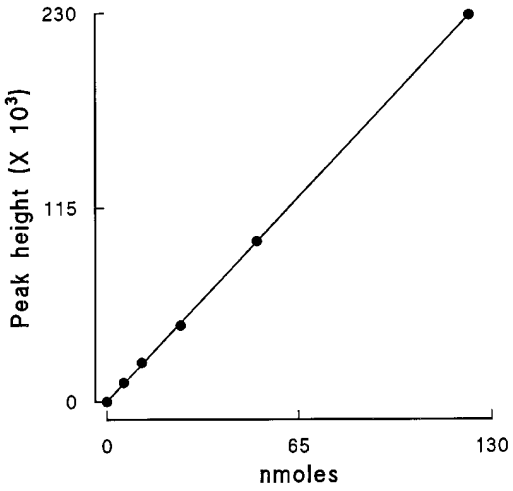

Fig. 2. External standard curve for glycerol content.

vs. 0.75 dpm/nmol, respectively). Thus, there are no non-glycerol contaminants of the HPLC tribenzoyl glycerol fraction.

As shown in **Table 1**, plasma glycerol flux was similar using [2-^{3}H]glycerol and [$^2H_5$]glycerol. The mean values for [2-^{3}H]glycerol and [$^2H_5$]glycerol flux for all five volunteers were almost identical (1.66 ± 0.14 and 1.70 ± 0.10 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, respectively, P = NS). More importantly, between-day differences in glycerol flux within the same individual as measured by [$^2H_5$]glycerol were generally in agreement with the flux assessed by the radiotracer method.

To examine whether the total rate of glycerol flux influenced the relationship between the measured values with the two methods, the between-method differences in glycerol flux were plotted against the flux measured using [$^2H_5$]glycerol (**Fig. 3**). The differences are scattered evenly about the zero line without a consistent distribution pattern, suggesting that the variances in glycerol flux measured by the two methods were random and independent of glycerol kinetics.

DISCUSSION

Isotopic tracer studies are important tools to study glycerol metabolism in vivo and excellent stable isotope methods are available (8, 9, 11–13). Radiotracer studies have also been conducted, but the results have been somewhat variable, suggesting analytical problems. For example, investigators measuring both glycerol and free fatty acid (FFA) appearance have reported marked differences in the FFA:glycerol appearance ratio. Both measurements are thought to reflect adipose tissue lipolysis, therefore, an appearance ratio close to the theoretical 3:1 would be expected, especially since more recent studies (18) suggest that previous evidence for significant in situ FFA re-esterification (19) in adipose tissue may have been due to analytical artifacts. In fact, when specific and precise GC/MS methods are used to measure FFA and glycerol kinetics, the FFA:glycerol appearance ratio varies only between 2.6:1 and 3.0:1 in infants (8) and adult humans (11). On the other hand, when radioactive tracers were used, ratios as high as 5.4:1 (6) and as low as 1.4:1 (14) have been reported. These discrepant results most likely reflect either contamination of the glycerol fraction with glucose (synthesized in vivo from radiolabled glycerol) or loss of the glycerol fraction during sample preparation. It should be acknowledged that the measurement of glycerol SA represents a significant analytical challenge because of its volatility, as we found in our investigation. The method described in this report for measuring glycerol SA uses HPLC to assure separation of glycerol from potentially interfering substances, and is particularly suited to the determination of glycerol flux in vivo.

Free glycerol is difficult to analyze by HPLC because of its high polarity and low UV absorbency. We previously reported that glycerol can be isolated from other carbohydrates using ion exclusion/partition chromatography (20). However, whether glycerol radioactivity is isolated by this type of HPLC or on borate columns (2), it is necessary to measure glycerol in a separate enzymatic procedure, introducing additional tedium and imprecision. The current technique uses the tribenzoyl derivative of glycerol prepared by Winkler et al. over 30 years ago (15), but uses more straightforward purification and derivatization techniques combined with HPLC and UV detection to specifically determine glycerol SA. If a different radiolabeled internal standard is added to plasma (e.g., [^{14}C]glycerol in the context of a [^{3}H]glycerol turnover study), both concentration and SA could be measured in a single analytical step. Considering the precision of the SA measure-

TABLE 1. Plasma glycerol flux and concentration

	Subject 1 72.6 kg			Subject 2 92.1 kg			Subject 3 86.1 kg			Subject 4 77.2 kg			Subject 5 63.0 kg		
	$^2H_5$ Flux	^{3}H Flux	Conc	$^2H_5$ Flux	^{3}H Flux	Conc	$^2H_5$ Flux	^{3}H Flux	Conc	$^2H_5$ Flux	^{3}H Flux	Conc	$^2H_5$ Flux	^{3}H Flux	Conc
Day 1				1.63	1.62	68	1.26	1.24	81				1.55	1.75	51
Day 2	1.92	2.02	74	1.60	1.59	77	1.93	1.46	93	2.01	1.98	81	1.94	1.79	59
Day 3	1.78	1.94	71	1.12	0.96	58	1.66	1.77	68	2.00	1.81	93	1.90	1.59	56
Day 4	1.66	1.80	69	1.06	1.11	43	1.52	1.98	59	1.55	1.44	59	1.66	2.02	59
Mean	1.79	1.92	70	1.35	1.32	62	1.59	1.61	75	1.85	1.74	78	1.76	1.79	56

Plasma glycerol flux values measured by [$^2H_5$]glycerol and [^{3}H]glycerol ($\mu mol \cdot kg^{-1} \cdot min^{-1}$) are provided together with plasma glycerol concentrations (μmol/L) for the five subjects participating in the comparison study.

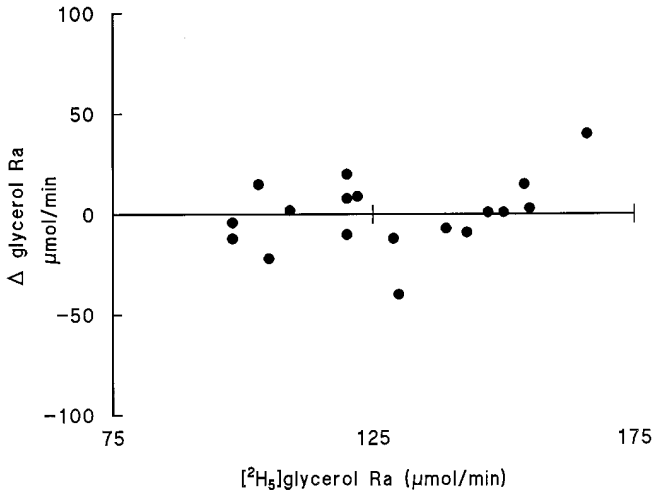

Fig. 3. The difference between glycerol rate of appearance (R_a) measured using [2-^{3}H]glycerol and [$^2H_5$]glycerol is plotted versus glycerol flux measured using [$^2H_5$]glycerol.

ment, the concentration measurement using this approach should be equally good over a wide range of values. The precision of the method is similar to GC/MS, which is not surprising as both HPLC and GC/MS measure tracer:tracee ratios and are thus relatively immune to effects of recovery on precision. The measures of glycerol appearance rate provided by this HPLC method to determine [^{3}H]glycerol SA were similar to estimates from an established GC/MS method.

It should be noted that the recovery of glycerol from plasma was ~75%, which also results in incomplete recovery of the tracer. If low tracer infusion rates are used for glycerol flux studies, this could result in few dpm in the HPLC fraction, requiring longer counting times or resulting in reduced precision of the SA measurement. This problem can be addressed by processing larger plasma samples or increasing the tracer dose. The former option would clearly be preferable for those wishing to limit exposure of their volunteers to radioactivity.

In summary, we have developed a new method for determining glycerol SA that uses HPLC to separate a glycerol derivative that is easily detectable by UV absorbency. This technique is uniquely suited to the measurement of in vivo glycerol kinetics and could be readily modified to allow simultaneous determination of glycerol concentration. The flux values obtained with the new method agree well with those obtained using an accepted stable isotopic technique. Investigators who do not have access to GC/MS facilities should be able to apply this technique to the study of adipose tissue lipolysis to more accurately measure glycerol flux.

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