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PAPER

Validation of deuterium labeled fatty acids for the measurement of dietary fat oxidation: a method for measuring fat-oxidation in free-living subjects

SB Votruba[1], SM Zeddun[1] and DA Schoeller[1]*

[1]*Department of Nutritional Sciences, University of Wisconsin, Madison, Wisconsin, USA*

BACKGROUND: Fatty acid oxidation has frequently been measured using ^{13}C or ^{14}C labeled fatty acids. While providing valuable data, the breath test method is hindered by the need for a controlled environment to measure VCO_2 and collect frequent breath samples. Additionally, the CO_2 breath tests require the use of ^{13}C- or ^{14}C-acetate to correct for isotope exchange in the TCA cycle. We validated d_{31}-palmitic acid for measuring dietary fat oxidation. When oxidized, the deuterium appears as water and mixes with the body water pool providing a cumulative record of fat oxidation.
METHODS: The recovery of deuterium from d_{31}-palmitic acid at 10 h post-dose was compared to that of $^{13}CO_2$ from [1-^{13}C]-palmitic acid in nine subjects (body mass index (BMI) = 23.6 ± 2.8; percentage body fat (%BF) = 22.6 ± 5.3; mean ± s.d.). Subjects were studied at rest. [1-^{13}C]-acetate (2 mg/kg) was dosed in a liquid breakfast. On a second day, [1-^{13}C]-palmitic acid (10 mg/kg) and d_{31}-palmitic acid (15 mg/kg) were dosed with the same liquid breakfast.
RESULTS: Recovery of $^{13}CO_2$ from [1-^{13}C]-acetate at 10 h post-dose was 53.7 ± 10.4%. Recovery of d_{31}-palmitic acid was 13.2 ± 7.7% (mean ± s.d.) and [1-^{13}C]-palmitic acid recovery was 6.4 ± 3.6%. When the ^{13}C data was corrected for [1-^{13}C]-acetate (Na salt) recovery, the mean difference in percentage recovery between the two tracers was 0.5 ± 2.8% and cumulative recoveries through 10 h post-dose were highly correlated ($y = 1.045x - 0.47$; $r^2 = 0.88$, $P < 0.0002$). Our data shows both labels to be equivalent in their ability to measure dietary fat oxidation in resting subjects.
CONCLUSIONS: The use of deuterium labeled palmitic acid eliminates the need for rigid control over the subjects' environment. Frequent sampling and measurement of VCO_2 are not needed for accurate calculation of percentage recovery of the deuterium label. In addition, the deuterium label has a decreased potential for isotopic exchange compared to ^{13}C or ^{14}C, so a recovery correction factor is probably not required.
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Keywords: fatty acid oxidation; deuterium; ^{13}C breath tests

Introduction

The stable isotope ^{13}C has been used for measuring fat oxidation for the past few decades.[1–4] It has been given by constant infusion to measure plasma fat oxidation and orally to measure dietary fat oxidation. While the latter makes use of tracer technology to differentiate between exogenous and endogenous fats, both types of applications are aimed at quantification of some aspect of fat oxidation. In each setting, the ^{13}C fatty acid oxidation is measured based on the ratio of $^{13}CO_2$ above baseline in breath.

Despite the advantages and ease of the technique, some limitations still exist with the use of ^{13}C-labeled fatty acids. A controlled environment is needed for frequent collection of breath samples. For accurate analysis, respiratory gas exchange measurements are necessary because the CO_2 flux is needed to calculate ^{13}C recovery, which requires access to either a metabolic cart or a human respiratory chamber. Methods of obtaining VCO_2 are not only time consuming but also result in greater subject involvement and restriction of daily activity. A second limitation of the ^{13}C method is the need for frequent breath sampling, again interfering with free-living conditions. Lastly, the use of

*Correspondence: DA Schoeller, Department of Nutritional Sciences, University of Wisconsin, Madison, WI 53706, USA.
E-mail: dschoell@nutrisci.wisc.edu
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^{13}C-labeled fatty acids may be limited due to the potential for isotopic exchange of ^{13}C at several steps along the TCA cycle.[5,6] The exchange of label results in a decrease in $^{13}CO_2$ excretion and, therefore, an underestimation of fatty acid oxidation. A solution to this problem has been to correct fatty acid recovery with the recovery of ^{13}C-acetate.[5–8]

Given the limitations of $^{13}CO_2$ breath tests, the development of an alternative method would be useful. The use of deuterium labeling may serve as such an alternative. Tritium has been used by researchers in the past, but the use of radioactivity is not always feasible.[9,10] The loss of label in the TCA cycle is virtually eliminated when deuterium is used rather than ^{13}C and therefore should not require a correction for exchange reactions. Deuterium eliminates the need for frequent sampling as well as the measurement of VCO_2. When oxidized, ^{2}H appears as H_2O and mixes with the body water pool. Other than minimal losses (7%/day) as urine and insensible water,[11] the remainder of the label should provide a cumulative record of fat oxidation measurable in urine.

The aim of this study was to validate the use of deuterated labeling of fatty acids to measure dietary fat oxidation. The null hypothesis to be tested is that recovery of $^{13}CO_2$ from [1-^{13}C]-palmitic acid, corrected for isotopic exchange, and deuterium recovery from d_{31}-palmitic acid are equal.

Methods

Materials

Labeled fatty acids were obtained from Cambridge Isotope, Andover, MA. [1-^{13}C]-palmitic acid (CLM-150) was 99 atom % ^{13}C, d_{31}-palmitic acid (DLM-215) was 98 atom%. ^{2}H, and [1-^{13}C]-acetate, Na salt (CLM-156) was 99 atom% ^{13}C. The ^{18}O was obtained as water (Isotec, Inc, Miamisburg, OH) and was 10.8 atom%.

Subjects

A group of nine healthy subjects (six females, three males) volunteered for this study and their characteristics are summarized in Table 1. Three additional subjects (two female, one male) were recruited for measurement of natural background variations in the isotopes (age = 26.3 ± 2.5 y, body mass index (BMI) = 22.6 ± 1.5 kg/m^2; mean ± s.d.). The subjects were thoroughly briefed on the experimental protocol and a signed consent form was obtained. The protocol was approved by the IRB of the College of Agriculture and Life Sciences at the University of WI, Madison. No medication, smoking, alcohol, or caffeinated beverages were allowed during the two test days.

Table 1 Subject characteristics; mean ± s.d.

	Female	*Male*	*All*
n	6	3	9
Age (y)	26.5 ± 5.3	29.0 ± 5.3	27.3 ± 5.1
Weight (kg)	60.7 ± 5.2	76.9 ± 14.9	66.1 ± 11.8
Percentage body fat	24.2 ± 3.9	19.6 ± 7.5	22.6 ± 5.3
BMI	23.4 ± 1.8	24.2 ± 4.9	23.6 ± 2.8

Protocol

This study took place over two days, separated by 2–7 days. On the first day, subjects ingested 2 mg/kg of [1-^{13}C]-acetate in a liquid replacement meal (Boost Plus, Mead Johnson). On day 2, subjects were given [1-^{13}C]-palmitic acid at 10 mg/kg and d_{31}-palmitic acid at 15 mg/kg in the breakfast drink, heated to 50°C to insure mixing. On day 2, subjects were also given a dose of 10 atom% ^{18}O as water at a dose of 0.2 g/kg.

Both test days were similar except for the specified tracers. Subjects reported to the laboratory at 07:30 after an overnight fast. Baseline breath and urine samples were collected, and drinks containing the labeled materials were given orally as part of a liquid replacement meal at 08:00. Subjects remained in the laboratory until 16:00. Meals were provided and consisted of replacement meals and nutrient bars given at the subjects' estimated energy expenditure using BEE calculated from the WHO equation[12] multiplied by an activity factor of 1.5. Meals were provided at 08:00 and 12:00, and were 50% carbohydrate, 35% fat and 15% protein. The 08:00 liquid meal provided 20% and the 12:00 meal 30% of each subject's estimated energy requirement for the day. Respiratory gas exchange was measured while the subjects were quietly sitting upright for 20 min out of each hour using the Delta Trac metabolic cart (Sensor Medics, Yorba Linda, CA). The O_2 and CO_2 analyzers were calibrated with a standard gas containing 96% O_2 and 4% CO_2. A mouthpiece was attached to a two way non-rebreathing t-shaped valve and a noseclip to ensure that all gas exchange was through the mouth (Hans Rudolph, Kansas City, MO). The valve was exhausted into the Delta Trac ventilated canopy system for measurement of respiratory gases. VCO_2 values were extrapolated to represent an average CO_2 production for the hour corresponding to the breath sample time.

Sample collection and analysis

On both days, breath samples for the collection of $^{13}CO_2$ were taken hourly by having subjects blow through a straw into a 15 ml Vacutainer™, which was then capped (Becton Dickinson, Franklin Lakes, NJ). Breath CO_2 was sampled directly from the Vacutainer™ with a syringe and $^{13}CO_2/^{12}CO_2$ was measured with continuous flow IRMS (Delta S, Finnigan MAT, Bremen, Germany).[13] CO_2 was introduced into a helium stream onto choromosorb-Q to separate CO_2 from the less polar gases and directed into the source of the isotope ratio mass spectrometer (IRMS).[13] Each sample was injected twice for isotope ratio analyses. The average standard deviation for all injections was 0.10‰. Isotopic enrichment was determined relative to a tank calibrated CO_2 standard. Excess ^{13}C was calculated relative to

baseline breath CO_2 prior to label administration and was corrected for natural fluctuations in ^{13}C-breath content that occurs as a function of meals.[11]

^{2}H and ^{18}O were measured in urine samples collected every 4 h after dosing. Urine (5 ml) was mixed with 200 mg carbon black to remove impurities and filtered through a 0.45 μm filter. One milliliter of urine was placed into auto-sampler vials and deuterium was measured with IRMS. Urine samples were analyzed for deuterium content as a ratio of $^{2}H/^{1}H$ using the Delta Plus mass spectrometer (Finnigan MAT, Bremen, Germany). From each 1 ml of sample, 0.8 μl were injected into a quartz tube packed with chromium powder held at 850°C to reduce the water to hydrogen gas. Each run included three injections of the sample with independent analysis. Data were corrected for both H^{3+} and memory. Results were corrected to the Standard Mean Ocean Water (SMOW) scale. A second 1 ml of clean urine was allowed to equilibrate with CO_2 at 25°C for 48 h. The ^{18}O concentration was measured by continuous flow IRMS and the body water was calculated by dilution.[11,13]

Label calculations

Recovery of the stable isotopes was calculated at 2, 6, and 10 h post-dose. For the labeled subjects, one baseline breath/urine sample was taken each day before ingesting the dose. This value was subtracted from the subsequent dose values for each subject, so that each time point is expressed as a per mille* increase above the subjects' own baseline ($\Delta\delta$). The control subjects' averaged values were subtracted from those of the labeled subjects to correct for background variation. This resulted in values that were adjusted for each individual's baseline as well as meal related natural background variation.

Total CO_2 production was obtained from the respiratory gas exchange measurements and total excess ^{13}C expressed as amount of dose recovered during each hour.

$$\%\text{recovery} = 100(VCO_2 \cdot \Delta\delta \cdot R_{STD}/1000)/(D \cdot P \cdot n/MW \cdot 100)$$

where $R_{STD} = {}^{13}C/{}^{12}C$ of standard CO_2; $P = {}^{13}C$ isotope atom%; n = number of labeled atoms per molecule;[1] MW = molecular weight ([1-^{13}C]-acetate, Na salt = 83; [1-^{13}C]-palmitic acid = 257); D = dose (g); and VCO_2 = carbon carbon dioxide production ratio in M/h. Cumulative recovery was calculated using the trapezoid rule.

Because the $^{13}CO_2$ excretion following ^{13}C-acetate at 8 h post-dose had not quite returned to baseline as had been expected, the terminal elimination rate was extrapolated from 8 to 10 h to allow for calculation of the 10 h ^{13}C-palmitate value. The average cumulative percentage dose recovery between 8 and 10 h from acetate was small (1.8 ± 0.9%).

Recovery of deuterium from palmitic acid oxidation was calculated as excess ^{2}H times TBW divided by the dose of ^{2}H administered:

$$\%\text{recovery} = 100((TBW \cdot 2 \cdot \Delta\delta \cdot R_{STD}/1000)/(D \cdot P \cdot n/MW \cdot 100))$$

where $R_{STD} = {}^{2}H/{}^{1}H$ of SMOW; $P = {}^{2}H$ isotope atom% n = number of labeled atoms per molecule,[31] MW = molecular ecular weight (d_{31}-palmitic acid = 287); TBW (moles); and D = dose (g). The hours post-dose for ^{2}H recovery in urine were assumed to equal the midpoint between sequential voids.

Statistical analysis

An analysis of agreement was done for [1-^{13}C]-palmitic acid and d_{31}-palmitic acid.[14] The difference between ^{2}H and ^{13}C percentage recovery was plotted against the average recovery of the two isotopes. Bias and precision were evaluated using the mean and s.d. of the difference between the labeled fatty acid oxidation. The limits of agreement were defined as the mean difference ± 2 s.d. A regression analysis was completed followed by a paired Student's *t*-test quantifying the strength of the relationship between the two methods. All analyses were performed with Statview 5.0.1 (SAS Institute, Cary, NC) and significance was set at a $P < 0.05$.

Results

Peak recovery of $^{13}CO_2$ following acetate administration occurred at 1.5 h post-dose (Figure 1). After [1-^{13}C]-palmitic acid administration, peak $^{13}CO_2$ recovery occurred at 5 h. Comparison of ^{13}C with deuterium required that the ^{13}C results be expressed as the cumulative recovery. The total recoveries for [1-^{13}C]-acetate at 2, 6, and 10 h post-dose were 22.0 ± 5.4%, 48.4 ± 8.3% and 53.7 ± 10.4%. This indicates that most of the label was recovered by 6 h post dose in our resting subjects. The 2, 6 and 10 h $^{13}CO_2$ percentage recoveries of [1-^{13}C]-palmitic acid (without the [1-^{13}C]-acetate correction) were 0.4 ± 0.4%, 3.5 ± 2.6% and 6.4 ± 3.6%. D_{31}-palmitatic acid recoveries at 2, 6 and 10 h post-dose were 4.1 ± 3.8%, 9.8 ± 7.1% and 13.2 ± 7.7%. The [1-^{13}C]-palmitic acid recovery values are considerably lower than those of d_{31}-palmitatic acid, but also represent loss of tracer in the TCA. To correct for this, the [1-^{13}C]-palmitic acid recovery at each time point was divided by the [1-^{13}C]-acetate recovery at the corresponding time point. The correction results in a [1-^{13}C]-palmitic acid recovery at 2, 6 and 10 h of 1.8 ± 1.9%, 8.0 ± 6.4% and 12.9 ± 8.5%. These values are not statistically different from the d_{31}-palmitatic acid recovery when a Student's *t*-test was performed. Correction with [1-^{13}C]-acetate recovery at 10 h was not affected by whether the group

*Del per mille (δ)= ($R_U/R_{STD} - 1$)1000

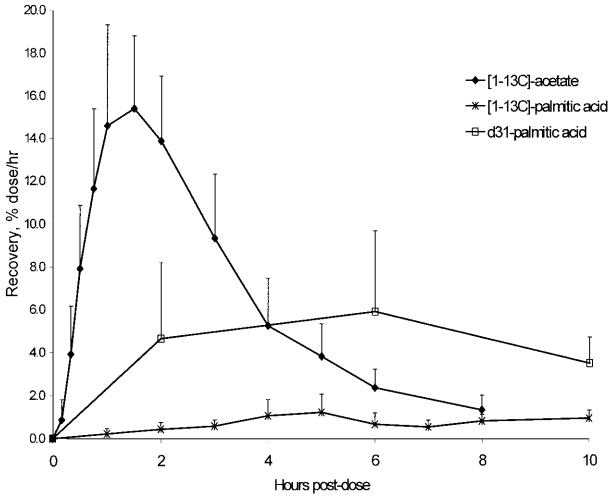

Figure 1 Recoveries of labels expressed as percentage dose/h for acetate and palmitic acid. [1-^{13}C]-palmitic acid recovery has not been corrected for isotopic exchange. d_{31}-Palmitic acid recovery has been calculated as the instantaneous recovery based on change of enrichment between successive voids. All data are mean ± s.d.

average recovery or individual recovery values were used (12.4 ± 10.4 *vs* 12.9 ± 8.5%, respectively).

When d_{31}-palmitatic acid recovery is plotted against [1-^{13}C]-palmitic acid recovery corrected with the group mean [1-^{13}C]-acetate recovery, the correlation coefficient $r^2 = 0.88$ ($y = 1.045x - 0.47$), is significant at $P < 0.0002$ (Figure 2). The *y*-intercept of the regression line is not different from zero ($P = 0.83$). If each subject's [1-^{13}C]-palmitic acid recovery is corrected with his/her own [1-^{13}C]-acetate recovery, the correlation $r^2 = 0.74$ ($y = 0.801x + 2.08$). A Bland–Altman plot was made with the group corrected

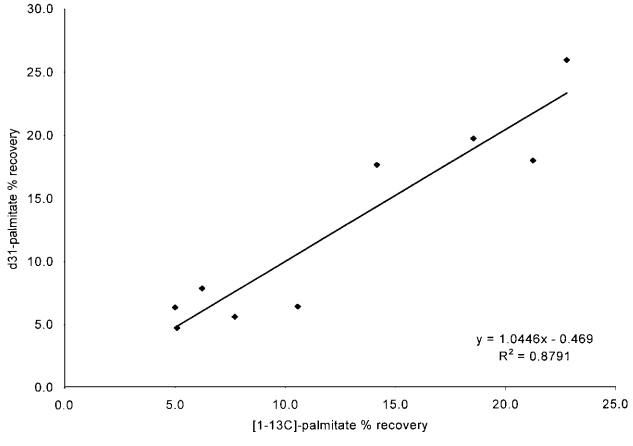

Figure 2 d_{31}-Palmitic acid recovery *vs* corrected [1-^{13}C]-palmitic acid recovery at 10 h post-dose. Group mean acetate recoveries were used for label fixation.

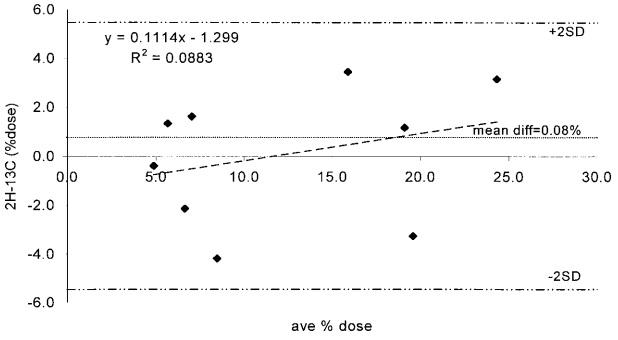

Figure 3 Comparison of the average and difference between percentage recovery of both d_{31}- and [1-^{13}C]-palmitic acid at 10 h post-dose. Group means have been used to correct for label fixation.

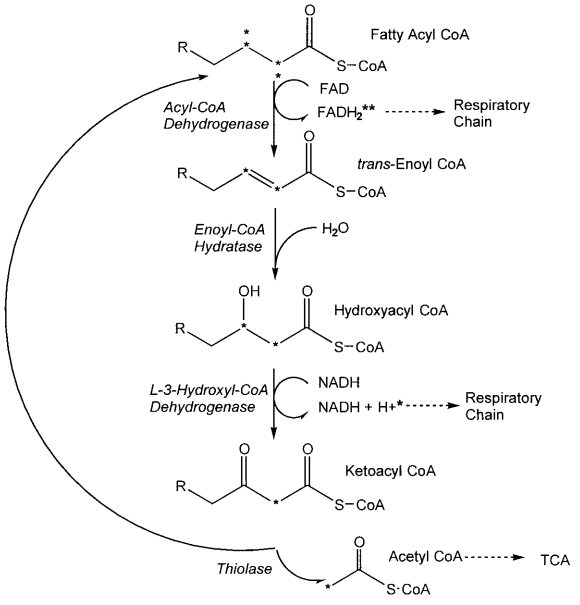

Figure 4 Diagram of fatty acid β-oxidation. Asterisks represent places of potential hydrogen exchange.

[1-^{13}C]-palmitic acid values to more closely examine the agreement of the two labels (Figure 3). The mean difference between the methods is 0.08 ± 2.8%, implying that both measurements are accurate. All points on the plot fall within ± 2 s.d., which suggests that random error was minimal.

Discussion

Our study showed that the deuterium labeled fatty acid is a valid method of measuring dietary fat oxidation when compared to the traditional ^{13}C label in subjects at rest. Deuterium labeling offers several advantages over the use of ^{13}C. When deuterium is used, a large acetate correction factor for the calculation of recovery is no longer needed. In addition,

deuterium can be used in free-living conditions with decreased sampling frequency and no VCO_2 measurements.

The use of deuterium labeling minimizes the opportunity for loss of tracer. Inspection of the reactions occurring during β-oxidation and acetate metabolism demonstrate this (Figure 4). The ^{13}C has a greater potential for isotopic exchange. The ^{13}C-free fatty acid (FFA) goes through β-oxidation, with acetyl-CoA units being sent to the TCA cycle. From the TCA cycle, one would expect that the ^{13}C label leaves the system as $^{13}CO_2$ in the breath. Unfortunately, there are several sites in the TCA cycle where isotopic exchange may occur. As suggested by Sidossis *et al*,[5,6] label may be temporarily lost through oxaloacetate to pyruvate/lactate and possibly glucose. Label may also be lost through conversion of α-ketoglutarate to glutamate/glutamine, resulting in temporary label fixation in the amino acid or protein pool. This problem can be corrected with the use of the ^{13}C-acetate.[5,6] When entering the cell, acetate is directly converted to acetyl-CoA and directly enters the TCA cycle. The fraction of acetate retained in the body is equal to the amount of label fixed through exchange in the TCA cycle.

In contrast, the ^2H label is removed from the fatty acids during β-oxidation and the TCA cycle as flavoproteins, $NADH + H^+$ and $FADH_2$, which enter the respiratory chain and are oxidized to body water (Figure 4). The first step of β-oxidation is dehydration catalyzed by acyl-CoA dehydrogenase. In this step, two ^2H are eliminated and oxidize to water. In the next step (hydration with enoyl-CoA hydratase), H_2O adds an unlabeled H bond to the fatty acid. Two more hydrogens are eliminated during the next dehydration step. These hydrogens may be either labeled or unlabeled. If one of the deuteriums remains on the acetyl-CoA that enters the TCA cycle, then the ^2H may be lost when carbon isotopic exchange occurs. The ^2H may still be incorporated into $NADH + H^+$ and $FADH_2$ in several reactions of the TCA cycle. Since at least 75% of the hydrogens for each acetyl-CoA are already shuttled to the respiratory chain in β-oxidation, only one-fourth of the labeled H has the potential for exchange during transit through the TCA cycle. The [1-^{13}C]-acetate recovery in our study suggests that $\sim$50% of the label remains sequestered at 10 h post-dose. We thus predict that only 12% (50% of 25%) of the labeled H from fatty acid oxidation could be lost in the TCA cycle.

Though the acetate correction factor was initially done with ^{14}C-acetate given on the same day as the ^{13}C-FFA,[6] later evidence suggests that ^{13}C-acetate may be used on a separate day to make the correction if test conditions are reproduced.[7] Schrauwen *et al* infused [1,2-^{13}C]-acetate in six subjects on two different days (2–4 weeks apart) to determine intrasubject reproducibility of the measurements. In those subjects, no differences in ^{13}C recovery and a high correlation between the two days were seen at all time points. They reported the mean percentage difference in acetate recovery in each subject between the two tests to be 5.4%, with an intrasubject coefficient of variation over the infusion time of $4.0 \pm 1.5\%$. This implies that, even when measured on a different test day, the within subject recovery variability in ^{13}C from acetate is smaller than the between individual variation and it is valid to use a separate test day to measure the ^{13}C-acetate recovery and eliminate the use of radiolabels.

Schrauwen *et al* caution that a separate correction factor needs to be determined for each subject in a study due to the high inter-individual variability in the recovery of ^{13}C-acetate (coefficient of variance $= 8.3 \pm 0.6\%$).[7] In our data, however, there was no statistical difference between using a group mean correction and an individual correction. Indeed, the fit of the regression line between the ^{13}C and ^2H measures was better using the group correction. This may, however, be specific to our small sample of individuals. Our methods also differ from previous studies employing the acetate correction factor. We give all of our labels orally and measure recovery over longer time periods, while most studies use short infusion periods. The short-term kinetics of acetate may be more variable than the long-term recovery.

The use of ^2H-labeled FFA is highly advantageous when compared to ^{13}C-FFA because they have the potential to be used in free-living conditions. A controlled environment is no longer necessary and samples do not have to be taken as frequently. We only required a urine sample from our subjects once every 4 h. If only a 24 h dietary fat oxidation value is desired, one could potentially only require two samples from each subject: baseline and enriched. A potential disadvantage does exist for the recovery of the deuterium label in the urine. If a protocol requires frequent measurements, it may be difficult to obtain a large number of urine samples from some subjects. In that case, plasma or saliva samples may be used as an alternative. Saliva, however, requires that care be taken to reduce fractionation secondary to evaporation prior to collection.

Another major advantage with the deuterium label is that measurement of VCO_2 is not needed to calculate recovery. It is, however, necessary to measure the water pool size of a subject. This can be done without tracer interference through the use of ^{18}O dilution.[11,13] The ^{18}O enrichment can be measured from the same urine sample used to analyze deuterium. Again, plasma or saliva samples may be used. If, as may be the case, ^{18}O is not readily available, several other methods of measuring TBW exist. Any one of these methods may be used, as long as error inherent in that particular measurement is kept in mind.

When using deuterium tracers, however, it is important to be aware of potential label sequestration into ketone bodies. During periods of low glycogen availability, the liver converts acetyl-CoA resulting from β-oxidation of fatty acids into acetoacetic acid and β-hydroxybutric acid. About $\sim$75% of the deuterium will be released to water during β-oxidation, but the carbon and remaining hydrogen will be converted into ketone bodies. Formation of ketone bodies can result in discrepancies between ^{13}C and deuterium when measuring fatty acid oxidation, when the ketone body pool size increases, or when ketones are spilled into the urine. In normal healthy individuals, the average plasma levels of

acetoacetate and β-hydroxybutyrate are $\sim$100 and 300 μmol/l, respectively, and urinary excretion is effectively zero.[15] Over a 48 h fast, plasma ketone concentrations can increase by $\sim$2.5 mM/l.[16] If we assume average ketones to have four carbons, then this corresponds to about 1.2 g/day of long chain FA being sequestered into ketones in a reference male (ECF = 14 l). When a FA tracer dose is given in a 500 kcal meal (35% energy as fat), $\sim$9.7 g/day of fat is being administered. Ignoring the contribution of endogenous FA, this would lead to, at most, a 6% loss of dose per day into the enlarging ketone pool. In situations of severe ketoacidosis, plasma ketone levels may increase to 14 mM/l.[16] If urine concentration is assumed to equal plasma concentration, and urine output is assumed to be 2 l/day, then $\sim$1.8 g/day of FA may be lost. In a situation where tracer is infused at 10% of endogenous FFA production, at most 0.1% per day loss can occur in urine. These situations are rare, however, and are unlikely to be problematic for metabolic studies in normal individuals. Of course since the fate of most ketone bodies is oxidation, the carbon and remaining hydrogen labels will be released as $^{13}CO_2$ and $^{2}H_2O$ and represent substrate oxidation despite the different route of oxidation.[6]

In conclusion, we found that the deuterium label eliminates many of the problems encountered when using ^{13}C to measure dietary fat oxidation. Deuterium can be used in free-living, resting subjects with minimal sampling times, no VCO_2 measurement is required, cost is minimal, and no correction factor is needed. Further studies that validate the use of deuterium labeled fatty acids during non-resting conditions are necessary. Incidentally, the use of deuterium in this study also confirms the need for the acetate correction factor when using the ^{13}C label. The relatively minimal chance for exchange of deuterium suggests that the oxidation of the label represents an accurate estimation of dietary fat oxidation.

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