Stable isotope dilution method for measurement of palmitate content and labeled palmitate tracer enrichment in microliter plasma samples

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Summary In studies where [1-^{13}C]palmitic acid is employed as isotopic tracer in vivo, we have described a selected ion monitoring gas–liquid chromatography–mass spectrometry micromethod which allows plasma tracer enrichment as well as plasma palmitate content to be determined in the same 100-μl sample through the use of [5,5,6,6-$^{2}H_4$]palmitic acid as assay internal standard. For standard solutions in the range equivalent to plasma palmitic acid concentrations of 10–2500 μM, assay precision was ±5%. For plasma samples in the physiologic range (approximately 30–200 μM palmitate) assay precision averaged better than ±2%. The use of the method is illustrated by measuring palmitate turnover in a 12-hr-old human infant.—**Bougnères, P. F., and D. M. Bier.** Stable isotope dilution method for measurement of palmitate content and labeled palmitate tracer enrichment in microliter plasma samples. *J. Lipid Res.* 1982. **23:** 502–507.

Supplementary key words gas–liquid chromatography–mass spectrometry • palmitate turnover

Extensive evidence suggests that unesterified or free fatty acids (FFA) are mobilized in large amounts and represent the major metabolic fuels for the human newborn soon after birth (1–4). Yet, there are no quantitative measurements for the magnitude of FFA transport in the human neonate. This deficiency is largely due to the lack of a suitable approach to these measurements. Such an approach must satisfy at least two requirements, *1*) it must be non-invasive and ethically acceptable for neonatal investigation, *2*) it must allow both FFA content as well as tracer enrichment to be determined in small microliters plasma samples.

Radiolabeled fatty acids have been used extensively to measure FFA turnover in animals and man (5–8). However, radioactive tracers are unacceptable choices for investigations in newborns. Furthermore, since radiotracer techniques require separate determinations of radioactivity and substrate content, such methods are generally macromethods not suitable for the small sample

Abbreviations: FFA, free fatty acids; GLC-MS, gas-liquid chromatography-mass spectrometry.

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requirements of newborn research. Likewise, while numerous colorimetric and radiochemical microtechniques (9) are available for measuring FFA content, these procedures do not allow tracer isotope enrichments to be determined in the same small plasma samples.

In this manuscript, we present a method that satisfies the above requirements for neonatal research studies. Using non-radioactive carbon-13 labeled palmitic acid as the representative tracer fatty acid and deuterium-labeled palmitic acid as internal standard, both tracer isotope enrichment and palmitate content can be measured in the same 100-μl plasma samples by selected ion monitoring gas–liquid chromatography–mass spectrometry. A similar method using [1-^{13}C]palmitic acid but a larger sample size and separate GLC and GLC–MS measurements of palmitate content and isotope enrichment, respectively, has also been described recently (10).

Materials

Natural palmitic acid was purchased from Sigma Chemical Co. (St. Louis). [5,5,6,6-$^{2}H_4$]Palmitic acid (95.5 atom % $^{2}H_4$, less than 0.3 atom % $^{2}H_1$, and 0.4 atom % $^{2}H_0$) and [1-^{13}C]palmitic acid (84.6 atom % 1-^{13}C) were purchased from Merck Isotopes (Dorval, Quebec). Chemical purity and isotopic purity were confirmed by conventional gas–liquid chromatography and mass spectrometry of the respective methyl ester derivatives.

Stock solutions of natural, [$^{2}H_4$], and [1-^{13}C]palmitic acid were prepared by dissolving 6 mg of accurately weighed fatty acid in 200 ml of heptane. Working calibration standard solutions were prepared by serially diluting various combinations of labeled and natural palmitic acid stock solutions (see Results) which were kept stored at −20°C. For internal standard, the desired amount of [$^{2}H_4$]palmitic acid in heptane was evaporated to dryness under N_2 and redissolved in 7% defatted albumin solution. The latter solution was added to plasma as described.

After verification of non-pyrogenicity by a licensed commercial laboratory, an additional sterile solution of [1-^{13}C]palmitic acid bound to fatty acid free human serum albumin was prepared for newborn infusion as described previously (11).

Analytical methods

In order to separate FFA from neutral lipids prior to methylation, the conventional alkalinization, re-acidification procedure of Hagenfeldt (12) was modified as follows: 0.1 ml of plasma was added to a 1-ml aliquot of 7% albumin solution containing 9.54 nmol of [$^{2}H_4$]palmitic acid. Five ml of Dole's mixture (13) was added and the lipids were extracted by vigorous shaking. Three ml of heptane and 2 ml of distilled water were added, the samples were reshaken, and the upper heptane phase was transferred to another tube containing 2 ml of 0.05% H_2SO_4. The mixture was again shaken vigorously (14), and the upper phase was transferred to another tube to which was added 1 ml of 0.01% thymol blue in 70% methanol. The methanol phase was alkalinized by addition of 1 drop of 1 N NaOH and the neutral lipids were extracted into the heptane phase which was discarded. The methanol phase containing the sodium salts of the fatty acids was washed twice with 3 ml of petroleum ether and then reacidified with 2 drops of 1 N HCl. The regenerated FFA were extracted twice into 2 ml of heptane.

The combined heptane extracts were pooled and evaporated to dryness under N_2 at room temperature. The residues were resuspended in 0.7 ml of 1% H_2SO_4 in methanol, flushed with N_2, and heated at 60°C for 1 hr. After cooling, 0.7 ml of water and 2 ml of heptane were added to each sample and the fatty acid methyl esters were extracted into the heptane. The heptane phase was removed to a 2-ml conical microreaction vial, evaporated to dryness under N_2, redissolved in 40 μl of carbon disulfide, and stored at −20°C until analysis.

Unlabeled and labeled palmitic acid were measured in the methyl ester derivative by electron-impact, selected ion monitoring GLC–MS using a computer-controlled system designed for a Finnigan 3200 Gas Chromatograph-Mass Spectrometer (15). For ion current ratio measurements in the range reported here, this system has a relative precision of ±1% of the measured ratio (15).

Palmitic acid methyl ester separation from other plasma fatty acid methyl esters was accomplished by 160°C isothermal gas–liquid chromatography on a 2-m × 2-mm glass column packed with 3% SP 2300 on Supelcoport 100/200 mesh using helium carrier gas. Mass spectrometer ion source temperature was maintained at 200°C and ionization voltage at 70 eV. Ions at m/z 270, 271 and 274, representing the molecular ions of unlabeled, [1-^{13}C], and [$^{2}H_4$]palmitic acid methyl esters, respectively, were selectively monitored. The corresponding peaks were integrated, and unlabeled palmitate content and [1-^{13}C]palmitate enrichment was calculated as described below. Since each analytical run requires injection of only approximately 1–2 μl of the final CS_2 solution, several replicate measurements can be made on each 0.1 ml plasma sample.

Procedural methods

After obtaining informed consent according to the procedures approved by the Washington University Committee on Human Experimentation, a 2.93-kg, 10-hr-old, unfed newborn was infused through a peripheral vein with albumin-bound [1-^{13}C]palmitic acid at the rate of 0.070 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ for 2 hr. Three hundred-μl capillary "heel-stick" blood samples were obtained prior to the start of the infusion and at 60, 75, 90, 105, and 120 min after the tracer was begun. The plasma was

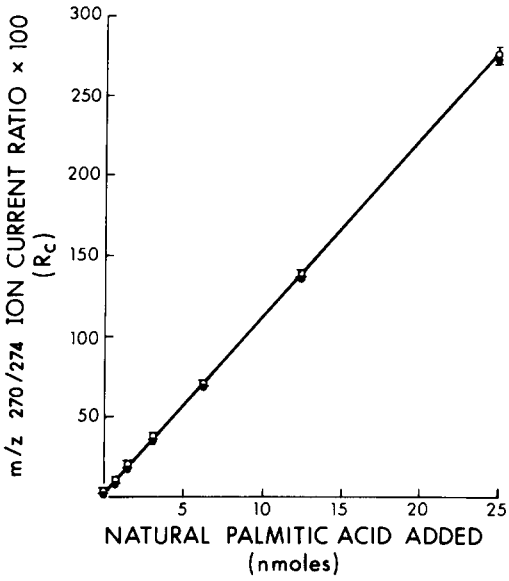

Fig. 1. Calibration curve for determinations of unlabeled (natural) palmitic acid using a [$^2H_4$]palmitic acid internal standard as described. The open circles (±S.D.) represent the observed m/z 270/274 ion current ratio values (R_c) while the closed circles represent the expected values calculated from the known isotopic content of the mixtures. The equation describing the experimental line is $Y = 1.10X + .52$; $r = 0.999$.

immediately separated, added to the albumin-bound [$^2H_4$]palmitic acid internal standard, and extracted into Dole's mixture as described.

Calculations

Plasma palmitate content. Prior to plasma analysis, calibration standards containing the same amount of [$^2H_4$]palmitate, but different amounts of natural palmitate, were analyzed by GLC–MS to establish a standard curve defined by the equation:

$$R_c = R_0 + k(n_0/n_4) \qquad \text{Eq. } 1$$

where R_c is the observed 270/274 ion current ratio of the calibration standard; R_0 is the 270/274 ion current ratio of the pure [$^2H_4$]palmitate alone (this ratio is non-zero due to the presence of some undeuterated palmitic acid in the internal standard); k is the slope of the line; and n_0 and n_4 are the absolute amounts (in moles) of natural and [$^2H_4$]palmitic acid, respectively, in the calibration standards. Since R_c and R_0 are measured and n_0 and n_4 are known, equation *1* can be solved for k.

The absolute amount of endogenous unlabeled palmitic acid in the plasma samples (n_s) was then calculated from the measured 270/274 ion current ratio in the samples (R_s) according to the following rearrangement of equation *1*:

$$n_s = (n_4/k)(R_s - R_0) \qquad \text{Eq. } 2$$

The results were corrected for sample volume and expressed as μmoles of palmitic acid per liter of plasma.

Plasma [1-^{13}C]palmitate enrichment. The same isotope dilution technique is used to calculate [1-^{13}C]palmitate enrichment of the plasma samples, except that a calibration slope is determined from known enrichment standard mixtures of [1-^{13}C]palmitate and natural palmitate in a manner analogous to equation *1* as follows:

$$R'_c = R'_0 + k'(n_1/n_0) + k''(n_4/n_0) \qquad \text{Eq. } 3$$

where R'_c is the observed 271/270 ion current ratio of the calibration standard; R'_0 is the natural 271/270 ion current ratio; n_1 is the absolute amount (in moles) of [1-^{13}C]palmitic acid in the calibration standard; k′ is the slope of the 271/270 ion current ratio as a function of n_1/n_0; and k″ is the slope of the 271/270 ion current ratio as a function of n_4/n_0. This slope is obtained from the internal standard addition data used to establish the standard curve in equation *1* and is necessary to account for the effect of any [$^2H_1$]palmitate in the [$^2H_4$]palmitate internal standard. In the present experiments, k″ (experimentally determined) was equal to zero due to the virtual absence of [$^2H_1$]palmitate. Thus the last term in equation *3* reduced to zero.

From the above determined slope (k′), the atom % [1-^{13}C] enrichment of a sample (E_s) can be calculated from the experimentally observed 271/270 ion current ratios in the tracer-enriched samples (R'_s), and in the pre-infusion (zero time) sample (R'_0), as follows:

$$\Delta R'_s = (1/k')(R'_s - R'_0) \qquad \text{Eq. } 4$$

$$E_s = \Delta R'_s/(1 + \Delta R'_s) \times 100 \qquad \text{Eq. } 5$$

Results

To test the linearity of the assay (**Fig. 1**), a series of standards containing 1–25 nmol of natural palmitic acid in 100 μl of heptane (equivalent to plasma palmitate concentrations of 10–250 μM) was prepared in quadruplicate. Internal standard, 9.54 nmol of [$^2H_4$]palmitic acid, was added to each sample and the sample was analyzed as described. Fig. 1 shows that the assay was linear throughout the measured range and the average relative precision (S.D./mean) of individual estimate was ±4.6%. Except for amounts of palmitic acid corresponding to less than 80 pmol injected into the GLC–MS system, there was no concentration-dependent effect on measurement precision. In the physiological range of plasma palmitate concentrations (approximately 30–200 μM; corresponding to 3–20 nmol on the calibration curve), the average relative precision was ±2.1%.

To examine the effect of the extraction procedure on the results, quadruplicate 1-ml aliquots of the above standard solutions were evaporated to dryness, redissolved in 1 ml of 7% albumin solution containing 95.4 nmol of [$^2H_4$]palmitic acid internal standard, and then taken

through the entire extraction, derivatization, and analytical procedure as described. A comparable set of standards was derivatized directly as in the first experiments above. **Fig. 2** demonstrates that extraction had no significant effect on the determination of natural palmitic acid concentration. It further shows that the assay remains linear over a 10-fold greater range than that shown in Fig. 1.

In order to evaluate the extent of plasma sample size on the assay, five plasma samples were obtained from a single fasting adult. Quadruplicate 0.1- and 1.0-ml aliquots of each sample were prepared and analyzed for palmitic acid content as described. **Fig. 3** shows that sample size in the range tested had no effect on the calculated palmitic acid concentration. Furthermore, the relative precision of the estimate (S.D./mean) was the same for the 0.1- and 1.0-ml samples averaging 1.8% and 1.5%, respectively.

Because of the high m/z 271/270 ion current ratio in natural palmitic acid methyl ester, due principally to natural abundance of ^{13}C from 17 carbon atoms, we assessed the ability of the method to measure increments in this ratio from added [1-^{13}C]palmitic acid tracer (**Fig. 4**). There was a linear response (r = 0.99) with an average relative measurement precision of ±0.14% in the range of 0–4 atom % excess [1-^{13}C]palmitic acid. These data are virtually identical to a similar [1-^{13}C]palmitic acid standard curve obtained previously by Gruenke, Craig, and Bier (16) using a simple voltage sweeping

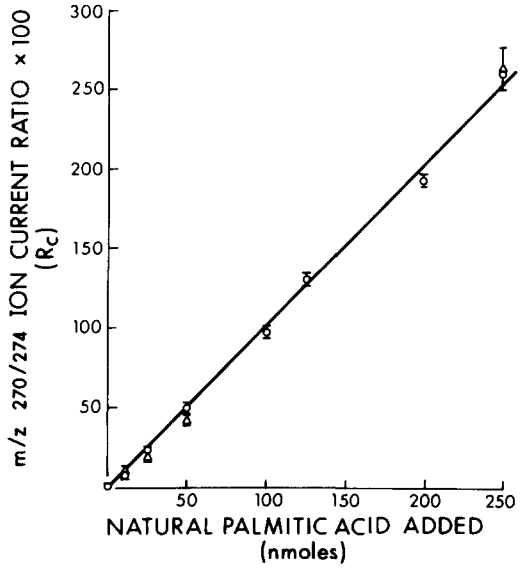

Fig. 2. Calibration curve for determinations of natural palmitic acid using a [$^2H_4$]palmitic acid internal standard as described. The open triangles (±95% confidence limits) show the values obtained from standards derivatized directly; the open circles (±95% confidence limits) show the values for standards taken through the entire extraction procedure (see text). The regression line calculated for all samples is Y = 1.06X + 0.10; r = 0.999.

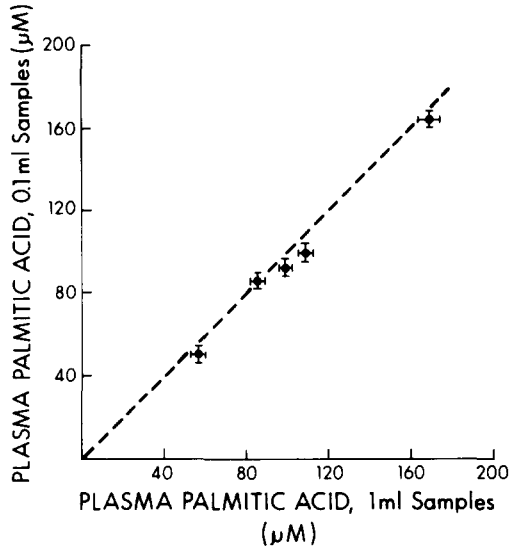

Fig. 3. Plasma palmitic acid values, with associated 95% confidence limits, for 0.1- and 1.0-ml plasma samples analyzed by the current method. The straight line is the line of identity.

circuit designed for magnetic sector mass spectrometers (16, 17).

To compare the presently described method with a conventional GLC assay of plasma FFA (11) and to measure both palmitic acid content and [1-^{13}C] isotopic enrichment in 0.1 ml plasma samples, an additional experiment was performed (**Table 1**). Quadruplicate 0.1-ml aliquots of two plasma samples were analyzed for natural palmitic acid content both by the present method using [$^2H_4$]palmitic acid internal standard and by a conventional GLC method using heptadecanoic acid as internal standard (11). A third plasma sample was treated as the first two, but, in addition, was also spiked with known quantities of [1-^{13}C]palmitic acid. Natural palmitic acid content analyzed by GLC and by reverse isotope dilution were identical in each of the three cases (Table 1). Likewise, measured plasma [1-^{13}C]palmitic acid enrichment was indistinguishable from the calculated expected values (Table 1).

Fig. 5 shows the results of the term newborn infusion study described earlier. From zero to 60 min plasma palmitate increased from 269 to 413 μM corresponding to an increase in total FFA from 640 to 980 μM. The reason for this rise is unclear but may reflect a normal FFA response to fasting. Thereafter, the plasma FFA concentration rise plateaued and, from 60 to 120 min of study, plasma palmitate content and [1-^{13}C] isotopic enrichment were virtually constant, and palmitate flux calculated by standard steady-state tracer dilution (18) was 8.54 $\mu mol \cdot kg^{-1} \cdot min^{-1}$. Since palmitate represented 41.7 ± 1.7% of the total plasma FFA (calculated from area ratios of the remaining fatty acid methyl esters) and since palmitate can be considered a representative tracer

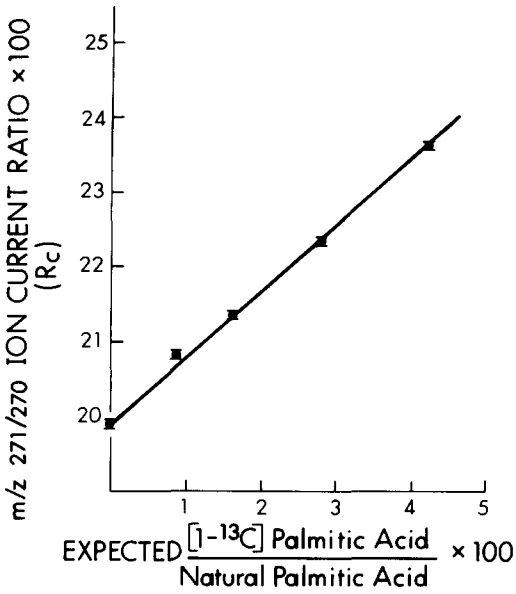

Fig. 4. Calibration curve for mixtures of natural and [1-^{13}C]palmitic acid produced by addition of known amounts of [1-^{13}C]palmitic acid to a natural palmitic acid standard solution. The equation for the line is Y = 0.88X + 19.9; r = 0.998.

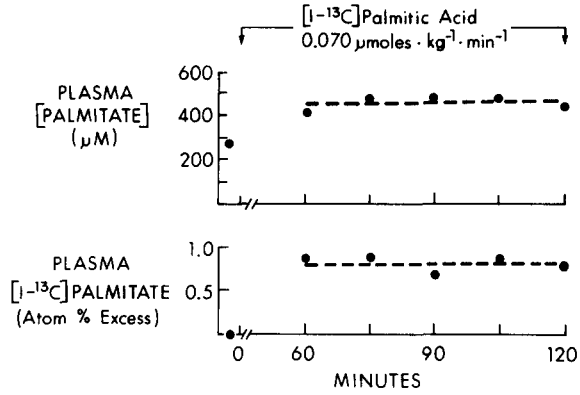

Fig. 5. Plasma palmitic acid content and [1-^{13}C] isotopic enrichment in a term newborn infant (see Text) during the continuous infusion of [1-^{13}C]palmitic acid at the rate of 0.070 μmol·kg^{-1}·min^{-1}. Zero time represents 10 hr of age.

for plasma fatty acids on the whole (19), the measured palmitate flux rate corresponds to a total plasma FFA flux of approximately 20.5 μmol·kg^{-1}·min^{-1}. This value, while higher than basal post-absorptive adult FFA turnover, is in the range of FFA flux found in adults whose plasma FFA levels are elevated to values commonly found in newborns (8), including the infant studied.

Discussion

The use of stable, non-radioactively labeled molecules is a safe and accurate approach to studying substrate fuel transport in newborn infants (20–23). The selected ion monitoring GLC–MS method presented fulfills the requirements for extending the stable isotope approach to measurement of the transport of fatty acids, a major neonatal fuel source. Specifically, it is an ethically acceptable approach that provides accurate and precise replicate determinations of palmitate content and tracer isotopic enrichment in the same microliter blood sample. While described for palmitic acid, similar mixed isotope choices for tracer and internal standard would allow the transport of other fatty acids to be measured as well, since the entire plasma FFA fraction is methylated and GLC separation of individual plasma fatty acid methyl esters is a well established technique. Furthermore, if desired, total plasma fatty acid content can be calculated from summed individual fatty acid methyl ester GLC area ratios referenced to the calculated palmitic acid content.

The practical nature of the method is illustrated by a single newborn example, to our knowledge the first quantitative estimate of fatty acid transport in a human

TABLE 1. Recovery of [1-^{13}C]palmitic acid added to plasma and comparison of plasma palmitic acid concentrations calculated from [$^{2}H_4$]palmitic acid and heptadecanoic acid internal standards

Sample	Palmitic Acid Concentration		Mole Ratio $\frac{[1\text{-}^{13}C]\text{Palmitate}}{\text{Natural Palmitate}} \times 100$	
	Current Method[a]	Hagenfeldt[b]	Observed[a]	Expected
	(μM)			
1	66 ± 8	69		
2	83 ± 3	84		
3[c]	84 ± 5	83	1.85 ± 0.07[c]	
3 + 0.119 nmol [1-^{13}C]palmitate			3.13 ± 0.28	3.12
3 + 0.238 nmol [1-^{13}]palmitate			4.42 ± 0.11	4.38

[a] Mean ± SD (n = 4).
[b] Reference 11.
[c] Sample was obtained from subject infused with [1-^{13}C]palmitic acid (10); thus initial observed value does not equal zero. Subsequent expected values for the "spiked" samples were calculated from this observed ratio.

newborn. Such FFA flux measurements, while contributing needed physiological information in themselves, also have important implications in understanding the interrelationships between substrate transport and metabolic fuel balance in the neonate. Thus, for example, the total FFA transport of 20.5 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ measured in our infant potentially represents the parallel transport of about 7 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ of glycerol from adipose tissue lipolysis. The latter glycerol could contribute up to 12% of neonatal glucose production (21) and thus represents a potentially important gluconeogenic substrate in the unfed newborn.

Finally, while specifically designed for human newborn studies, the sensitivity of the current method may find additional application in small animal studies or in vitro investigations.

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