Simultaneous quantitative determination of deuterium- and carbon-13-labeled essential fatty acids in rat plasma

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Abstract This study reports methods for the quantitative determination of stable isotope-labeled essential fatty acids (EFAs) as well as an experiment in which deuterium-labeled linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) were compared with those labeled with carbon-13 in rat plasma in vivo. Standard curves were constructed to compensate for concentration and plasma matrix effects. It was observed that endogenous pools of fatty acids had a greater suppressing effect on the measurements of ^{13}C-U-labeled EFAs relative to those labeled with $^{2}H_5$. Using these methods, the in vivo metabolism of orally administered deuterated-linolenate, ^{13}C-U-labeled linolenate, deuterated-linoleate, and ^{13}C-U-labeled linoleate was compared in adult rats (n = 11). There were no significant differences in the concentrations of the ^{2}H versus ^{13}C isotopomers of 18:2n-6, 18:3n-3, arachidonic acid (20:4n-6), and docosahexaenoic acid (22:6n-3) in rat plasma samples at 24 h after dosing. Thus, there appears to be little isotope effect for $^{2}H_5$- versus ^{13}C-U-labeled EFAs when the data are calculated using the conventional standard curves and corrected for endogenous fatty acid pool size and matrix effects.—Lin, Y. H., R. J. Pawlosky, and N. Salem, Jr. **Simultaneous quantitative determination of deuterium- and carbon-13-labeled essential fatty acids in rat plasma.** ***J. Lipid Res.*** **2005.** 46: **1974–1982.**

Supplementary key words deuterated linolenate • deuterated linoleate • carbon-13-labeled linolenate • carbon-13-labeled linoleate • standard curves • concentration effect curves • gas chromatography-mass spectrometry negative chemical ionization analysis

In essential fatty acid (EFA) metabolic studies, ^{2}H and ^{13}C are the most commonly used stable isotopic atoms in tracer studies (1–6) since their discovery (7, 8) and application to metabolic studies about 70 years ago (9). Compared with ^{2}H, the ^{13}C atom has a relatively small difference in mass with respect to ^{12}C and so is very similar in its physicochemical characteristics. For ^{13}C-labeled fatty acids, a high-precision gas chromatography-combustion isotope ratio mass spectrometric system could detect ^{13}C in fatty acids at very low signal levels, down to 3 pmol per injection (10). ^{2}H-labeled fatty acids were used extensively and measured by GC-MS in electron impact (11), positive chemical ionization (12), and negative chemical ionization (NCI) (13) in biological samples. Other non-mass spectrometry methods, such as conventional GC with a flame ionization detector (FID), could be applied when the deuterated fatty acids had enough mass difference to be chromatographically resolved (13–15) and when sufficient amounts occurred in biological experiments such that they could be adequately detected (15–17). However, for fatty acids, no direct comparisons have been made between the metabolic behavior of ^{2}H and ^{13}C tracers either in vitro or in vivo.

In the course of performing measurements of human (18) and rat (19) plasma samples containing multiple stable isotopes, it became apparent that there were several analytical difficulties. The plasma matrix exerted a suppressing effect on the fatty acid isotopic signals as measured in the NCI mode. This suppression was greater for the ^{13}C signals relative to those of the same fatty acid labeled with ^{2}H. Therefore, standard curves for the various fatty acid isotopes to be used were required. In addition, methods were required for estimating the responses of fatty acid metabolites where isotopic standards were not available. This work describes the standardization and measurement of six stable isotope-labeled EFAs of 18 or 20 carbon length. In addition, the metabolism of ^{2}H- and ^{13}C-labeled linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) ethyl esters are compared to evaluate a possible isotope effect, as this information will also be required to perform valid measurements of EFA metabolism.

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Abbreviations: 18:2n-6, linoleic acid; 18:3n-3, α-linolenic acid; 20:4n-6, arachidonic acid; 20:3n-6, dihomo-γ-linolenic acid; 22:6n-3, docosahexaenoic acid; 20:5n-3, eicosapentaenoic acid; EFA, essential fatty acid; FID, flame ionization detector; ISTD, internal standard; NCI, negative chemical ionization; PFB, pentafluorobenzyl.

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MATERIALS AND METHODS

Stable isotope-labeled fatty acids

Deuterated α-linolenate (17,17,18,18,18-$^2H_5$-18:3n-3 ethyl ester; $^2H > 95\%$), deuterated linoleate (17,17,18,18,18-$^2H_5$-18:2n-6 ethyl ester; $^2H > 95\%$), carbon-13-uniformly labeled linoleate (^{13}C-U-18:2n-6 ethyl ester; $^{13}C > 95\%$), and deuterated dihomo-γ-linolenate (19,19,20,20,20-$^2H_5$-20:3n-6 ethyl ester; $^2H > 95\%$) were obtained from Cambridge Isotope Laboratories (Andover, MA); carbon-13-uniformly labeled eicosapentaenate (^{13}C-U-20:5n-3 ethyl ester; $^{13}C > 95\%$) was obtained from Martek Bioscience Corp. (Columbia, MD). The five isotopes were further purified by HPLC and verified by TLC, GC, NMR and GC-MS. Carbon-13-uniformly labeled linolenate (^{13}C-U-18:3n-3 ethyl ester; $^{13}C > 95\%$) was purchased from Cambridge Isotope Laboratories and used without further purification. Isotope distributions are as follows: 94.4% for $^2H_5$-18:3n-3 (M+5), 43.1% for ^{13}C-U-18:3n-3 (M+18), and 21.8% for (M+17). For isotope distributions in 18:2n-6 isotopomers, the distributions are as follows: 98.9% for $^2H_5$-18:2n-6 (M+5), 58.8% for $^{13}C_{18}$-18:2n-6 (M+18), 23.7% for (M+17), 33.9% for ^{13}C-U-20:5n-3 (M+20), and 89.8% for $^2H_5$-20:3n-6 (M+5). Data were based on three or more determinations. All solvents were of GC or higher grade except acetonitrile, which was HPLC grade.

Preparation of standards

Isotopic standards. Stock solutions at concentrations of 2, 2, 10, 10, 2, and 2 mmol/l of $^2H_5$-18:3n-3, ^{13}C-U-18:3n-3, $^2H_5$-18:2n-6, ^{13}C-U-18:2n-6, ^{13}C-U-20:5n-3, and $^2H_5$-20:3n-6 were prepared for standard curve generation for each isotope. All analyses were conducted by serial dilutions of the stock solution into a series of working solutions.

Unlabeled standards. Unlabeled commercial standards (reference standard 462 with 20:4n-3 added; Nu-Chek Prep, Elysian, MN) were used to construct standard curves of unlabeled fatty acids. Stock solutions at 5~6 mmol/l were made for unlabeled 18:3n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and docosahexaenoic acid (22:6n-3) in the n-3 family and 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, and 22:4n-6 in the n-6 family. The exact concentrations of all standard stock solutions, both the labeled and unlabeled fatty acids, were determined by GC analysis.

Standard curves

Standard curves for stable isotope-labeled fatty acids. Three sets of standard curves were constructed for each C18 isotope in various matrices: *i*) the pure stable isotope; *ii*) isotopes with unlabeled 18:2n-6 (10 nmol) and 18:3n-3 (2 nmol); and *iii*) isotopes with 50 μl of plasma from a rat dosed with olive oil vehicle. For C20 isotopes, the standard curves were made only in the plasma matrix. The concentrations of endogenous fatty acids in the plasma matrix were measured in triplicate by GC-FID analysis and are given as μmol/l of plasma (means ± SD): 70.3 ± 2.4 for 18:3n-3, 1,462 ± 51 for 18:2n-6, 58.7 ± 1.8 for 20:5n-3, and 55.5 ± 1.8 for 20:3n-6.

For each C18 isotopic fatty acid stock solution, a series of serial dilutions was made and a constant amount of internal standard (ISTD) 23:0 ethyl ester (0.13 nmol) was added. The total amounts of isotopic fatty acids in each sample are shown in **Table 1**. However, the amounts injected were much smaller, as one part out of 200 or 2,000 was injected into the GC-MS system. The range of these samples covered the estimated range of the isotope-labeled fatty acids in rat plasma samples. Standard curves were determined using linear regression of the ratio of signal areas between isotopes and ISTD versus the ratio of amounts between isotopes and ISTD.

Standard curves for unlabeled fatty acids. Stock solutions of each unlabeled fatty acid were diluted into a series of working solutions within a range of 2–1,000 pmol per sample with ISTD (0.13 nmol) added to each sample. Both the isotope-labeled and unlabeled samples were processed similarly for GC-MS analysis (see below).

To estimate the slopes of the standard curves for n-3 and n-6 metabolites for which stable isotope-labeled compounds were not available, the ratios of the slopes determined from the standard curves for the various 18:3n-3 isotopomers (18:3n-3, $^2H_5$-18:3n-3, ^{13}C-U-18:3n-3), 18:2n-6 isotopomers (18:2n-6, $^2H_5$-18:2n-6, ^{13}C-U-18:2n-6), 20:5n-3 isotopomers (20:5n-3, ^{13}C-U-20:5n-3), and 20:3n-6 isotopomers (20:3n-6, $^2H_5$-20:3n-6) were used. For example, to determine the standard curve for $^2H_5$-20:5n-3, it was assumed that the relationship of the slopes of 18:3n-3 and $^2H_5$-18:3n-3 was the same as that of unlabeled 20:5n-3 and $^2H_5$-20:5n3. The intercepts were generally very close to the origin and so are not specified.

Concentration dependence curves. The effects of the varying concentrations of endogenous fatty acids on $^2H_5$ and ^{13}C ions were quantified by adding a known amount of a pure stable isotope standard (92 pmol of $^2H_5$-18:3n-3, 69 pmol of ^{13}C-U-18:3n-3, 476 pmol of $^2H_5$-18:2n-6, and 476 pmol of ^{13}C-U-18:2n-6) to various volumes of rat plasma (0, 10, 20, 30, 40, and 50 μl) with normal saline used to bring the amount up to 50 μl. The concentrations of the four isotopes in the sample were chosen to be similar to those of experimental plasma samples. Concentration curves were constructed for the ratio of $^2H_5$ to ^{13}C fatty acid concentration versus the amount of unlabeled 18:3n-3 or 18:2n-6 in the rat plasma.

TABLE 1. Working table for standard curves of isotopic fatty acids

Sample Number	Isotopic Fatty Acids					
	$^2H_5$-18:3n-3	^{13}C-U-18:3n-3	$^2H_5$-18:2n-6	^{13}C-U-18:2n-6	^{13}C-U-20:5n-3	$^2H_5$-20:3n-6
0	0.00	0.00	0.00	0.00	0.00	0.00
1	0.185	0.139	0.952	0.951	0.691	0.839
2	1.85	1.39	9.52	9.51	1.38	1.68
3	18.5	13.9	95.2	95.1	2.77	3.36
4	92.4	69.3	476	476	5.53	6.71
5	185	139	952	951	11.1	13.4
6	369	277	1,903	1,902	22.1	26.8
7	554	416	2,855	2,854	44.2	53.7
8	739	554	3,807	3,805	88.5	107
9	924	693	4,758	4,756	177	215
10		1,386		9,512	354	430

The amounts of the various isotopic fatty acids are expressed as pmol per sample.

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Plasma samples from rats dosed with C18 isotopes

This animal study proposal was approved by the Animal Care and Use Committee of the National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health. Eight week old male and female Sprague-Dawley rats (murine pathogen-free; Taconic, Germantown, NY) were purchased and housed in our animal facility under conventional conditions. A custom diet (Dyets, Bethlehem, PA) based on the AIN-93G standard (20) was used with modifications in the fat sources (10 wt%) to achieve a composition of 40% saturated fat, 42% monounsaturates, 15% 18:2n-6, and 3% 18:3n-3 but with no added C20 and C22 polyunsaturated fatty acids. This diet is described in more detail in the accompanying paper (19). After a 4–5 week equilibration on this diet, the animals were fasted for 5 h before dosing with a mixture of the four stable isotopic tracers (in μmol/kg body weight): $^{2}H_5$-18:3n-3 (45), ^{13}C-U-18:3n-3 (32), $^{2}H_5$-18:2n-6 (210), and ^{13}C-U-18:2n-6 (210) as the ethyl ester in olive oil (n = 11). The olive oil vehicle was given to only one animal of each gender to generate blank plasma with no stable isotopes for background determinations and matrix effect experiments. Body weights at administration were 477 ± 31 g for males (n = 6) and 263 ± 17 g for females (n = 5). Animals had free access to water during the experimental period and to food at 4 h after dosing. Rats were euthanized by decapitation at 24 h after dosing. Blood samples were collected in heparinized tubes. Plasma was separated from whole blood at 1,670 *g* for 15 min, frozen on dry ice, and stored at −80°C until analysis. Standard curves of the isotopic fatty acids in the rat plasma matrix were used to quantify the amounts of the various isotopically labeled compounds in rat plasma samples.

Total lipid extraction

Total lipids of the samples for standard curves and isotopic rat plasma samples were extracted using the method described previously (4, 21). Rat plasma (50 μl) spiked with 13 nmol (for GC analysis) or 0.13 nmol (for GC-MS analysis) of 23:0 ISTD was mixed with 1 ml of methanol containing butylated hydroxytoluene (0.2 mM) and then extracted twice with 2 ml of $CHCl_3$. Total lipid extracts acquired from the chloroform phase were evaporated to dryness and then derivatized for instrumental analysis.

GC-FID analysis of unlabeled fatty acids

The total lipid extract was derivatized to the methyl esters using 14% BF_3-methanol as described by Morrison and Smith (22) and modified by Salem, Reyzer, and Karanian (23). In brief, a fused-silica, narrow-bored DB-FFAP capillary column (30 m × 0.25 mm inner diameter × 0.25 μm; J&W Scientific, Folsom, CA) coupled with a GC 6890 Plus LAN system (Agilent Technologies, Inc., Wilmington, DE) were used for chromatographic separation of the methyl esters with hydrogen carrier gas at 100 Pk_a and a linear velocity of 50 cm/s. The inlet and detector temperatures were set at 250°C. Oven temperature was programmed from 130°C to 175°C at 4°C/min, then to 210°C at 1°C/min, and finally increased at 30°C/min to 245°C, with a final hold for 15 min. A 28 component quantitative methyl ester reference standard (GLC-462) was used to identify the retention times of methyl ester

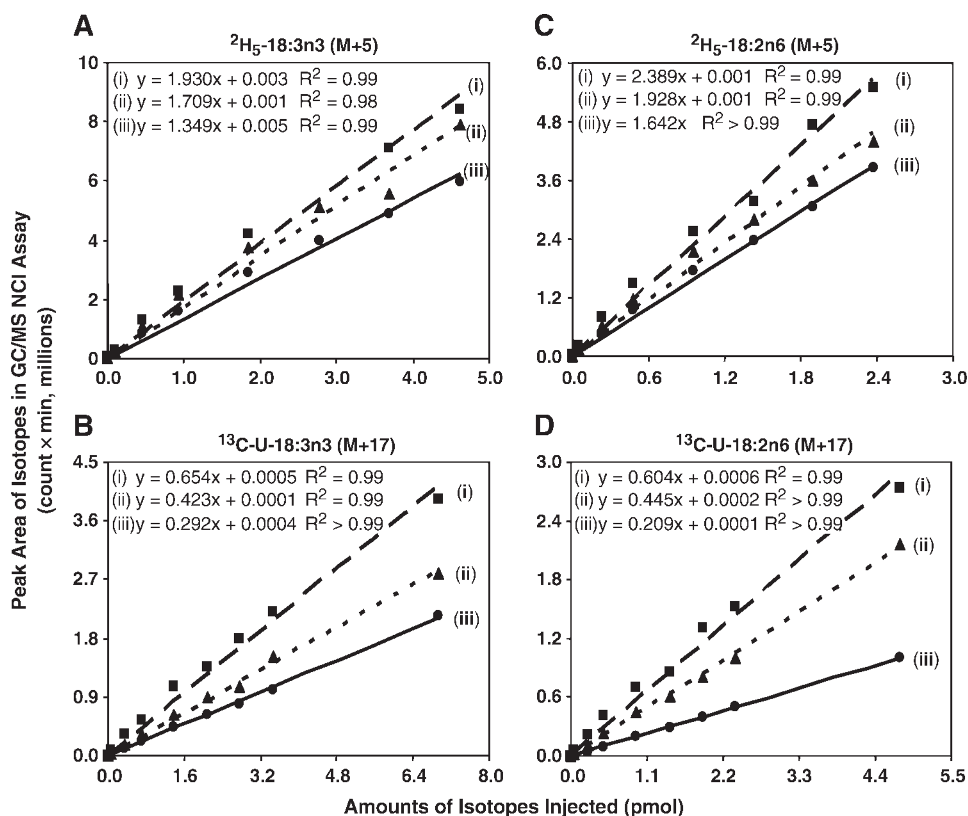

Fig. 1. Plots of MS response (peak areas) of four stable isotope-labeled essential fatty acids versus analyte concentration and effects of unlabeled fatty acid and plasma matrix. Linear ranges were as follows: 0.01–4.6 pmol for $^{2}H_5$-18:3n-3 (A); 0.01–6.9 pmol for ^{13}C-U-18:3n-3 (M+17) (B); 0.005–2.4 pmol for $^{2}H_5$-18:2n-6 (C); and 0.05–4.8 pmol for ^{13}C-U-18:2n-6 (M+17) (D) without any matrix (curve i; squares), with unlabeled fatty acids (curve ii; triangles), and with rat plasma matrix (curve iii; circles).

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peaks and to determine the respective instrumental response factors.

GC-MS analysis of isotope-labeled fatty acids

The total lipid extract was saponified using a 5% KOH-methanol solution, and free fatty acids were derivatized to the pentafluorobenzyl (PFB) esters using the PFB reagent (pentafluorobenzylbromide-diisopropylamine-acetonitrile, 1:100:1,000, v/v/v) followed by heating for 12 min at 60°C for GC-MS analysis in NCI mode as described previously (13, 18). A 1.0 μl portion of the hexane solution was subjected to GC-MS in the splitless mode. The same chromatographic column used in the GC-FID work was used for GC-MS with helium as the carrier gas at a constant column head pressure of 100 kPa. The GC oven temperature was programmed as follows: initially 125°C for 0.5 min, then increased to 245°C at a rate of 8°C/min, followed by an isothermal hold for 39 min. The signal abundances of the anions ($M\text{-}PFB^-$) of the various isotopic fatty acid compounds were detected in the selected ion monitoring mode using an interval of 1.0 amu for each ion. For the deuterated compounds, the ion channels with 5 amu above the ^{12}C fatty acid analog were monitored. For the ^{13}C-labeled fatty acids, ion channels that were 17 amu above the ^{12}C analog were monitored, because this resulted in a better signal-to-noise ratio compared with the 18 amu ion channel. The areas under each chromatographic peak were obtained by integration using the ChemStation integrator. Data analysis was performed using MSD ChemStation software (Agilent G1701DA version D.00.00.38).

Calculations

A least-squares linear fitting procedure was used to determine the standard curves of isotopic fatty acids that were obtained by plotting the ratios of peak areas of the fatty acid to the ISTD versus their molar ratios. The slope of this line was then used to calculate the unknown concentrations of the stable isotope-labeled fatty acids in rat plasma. The percent of dosage was determined by dividing the concentration of the labeled fatty acids and their

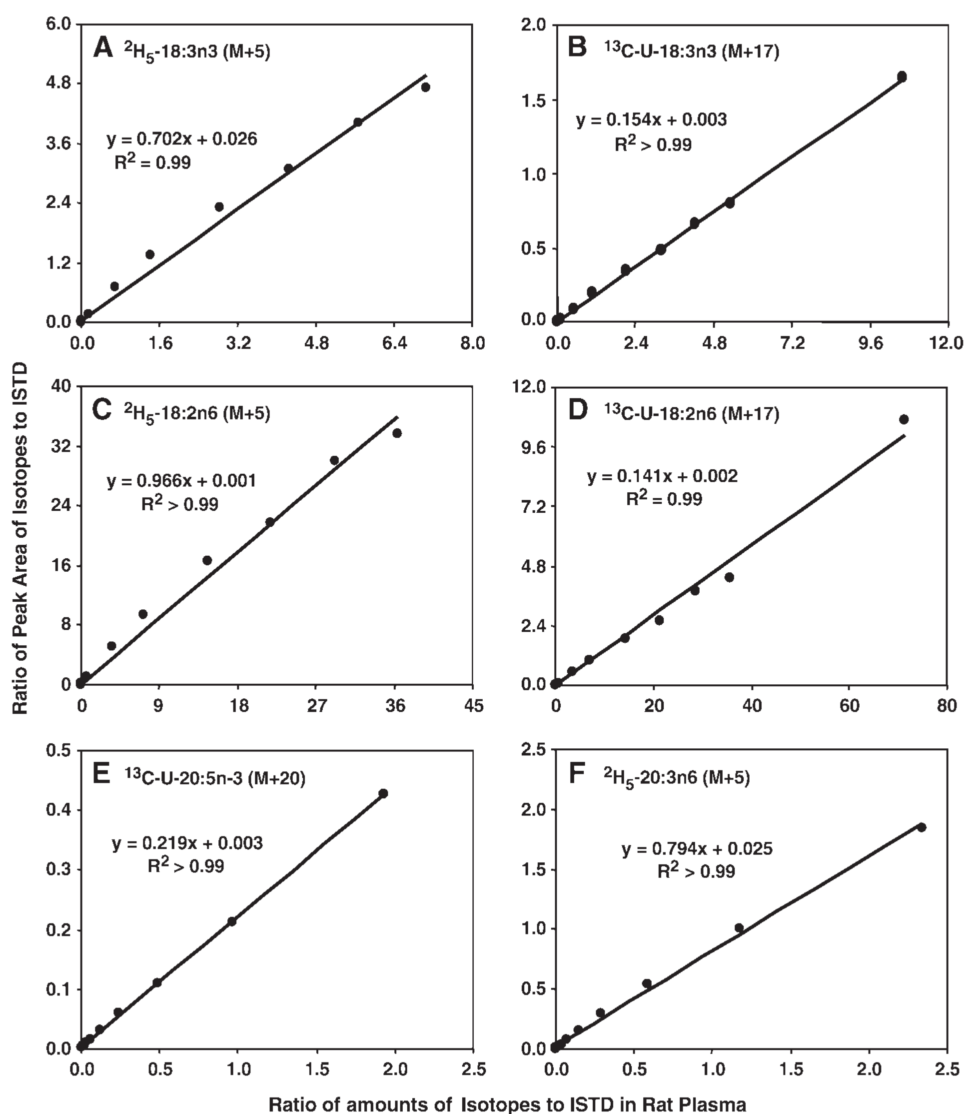

Fig. 2. Standard curves for six stable isotope-labeled fatty acids in rat plasma matrix. The ratio of MS response (peak area) of the isotope to internal standard (ISTD) was plotted against the ratio of the quantity of isotope to ISTD. The regression lines fitted to the experimental data are displayed for $^2H_5$-18:3n-3 (A), ^{13}C-U-18:3n-3 (B), $^2H_5$-18:2n-6 (C), ^{13}C-U-18:2n-6 (D), eicosapentaenoic acid [20:5n-3, EPA (^{13}C-U-20:5n-3)] (E), and dihomo-γ-linolenic acid [20:3n-6, DGLA ($^2H_5$-20:3n-6)] (F).

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metabolites by the amount of isotopes that each rat received. The criteria used to validate the linearity of the standard curves were based on those described by Green (24). When the regression coefficient R^2 was $\geqslant$0.98, it was considered to be an acceptable fit of the data to the regression line. The y intercepts were <5% of the dependent values obtained for the analyte at the target level.

To correct for the effect of the endogenous amount of 18:3n-3 and 18:2n-6 in rat plasma on the concentration of ^{13}C-labeled fatty acids in rat plasma, a nonlinear regression was fitted to the concentration curves. Isotopic enrichment was obtained by calculating the ratio of the amount of isotope-labeled fatty acids to unlabeled endogenous fatty acids and then multiplying by 100. The statistical test used was the two-sided, pairwise Student's *t*-test with $P < 0.05$ considered significant. Both statistical tests and linear regression fitting were conducted using Microsoft® Excel 2002 SP3 for Windows XP Professional (Microsoft, Seattle, WA).

RESULTS AND DISCUSSION

Response curves for $^2H_5$-18:3n-3, ^{13}C-18:3n-3, $^2H_5$-18:2n-6, and ^{13}C-18:2n-6

As the fatty acids occur in a widely different concentration range in the plasma, varying amounts of each C18 isotope were injected into the mass spectrometer over a range from 0.01 to 6.9 pmol after dilution. It was noted that the addition of unlabeled 18:2n-6 (10 nmol) or 18:3n-3 (2 nmol) depressed the response curves of all four of the isotopes (**Fig. 1**). This was also true when using the plasma matrix. However, it is possible to obtain linear responses over a wide range of analyte concentrations. The linear range for each isotope was as follows: 0.01–4.6 pmol for $^2H_5$-18:3n-3, 0.01–6.9 pmol for ^{13}C-U-18:3n-3, 0.005–2.4 pmol for $^2H_5$-18:2n-6, and 0.05–4.8 pmol for ^{13}C-U-18:2n-6.

Most importantly, these data clearly demonstrate that the different response factors are dependent upon the concentration of the endogenous fatty acids, which vary in different samples. In the worst case, the value of the calculated amount of ^{13}C-U-18:2n-6 decreased to ~50% of its actual value. It follows that enrichment is also linear at least up to the range tested in this experiment: at 0–26% for $^2H_5$-18:3n-3, 0–40% for ^{13}C-U-18:3n-3, 0–6.5% for $^2H_5$-18:2n-6, and 0–13% for ^{13}C-U-18:2n-6; the R^2 for the linear regression fit in all four cases was $\geqslant$0.99.

Standard curves for isotopic and unlabeled EFAs

Standard curves of four C18 isotopic fatty acids were examined under three conditions: *i*) no matrix; *ii*) with a constant amount of unlabeled 18:3n-3 and 18:2n-6 added; and *iii*) with the rat plasma matrix added. Also, the C20 isotopes were examined in the plasma matrix. The standard curves were constructed by regression fitting for the ratio of the MS signal abundance of the isotope to that of the ISTD versus the ratio of the actual amount of the isotope to that amount of the ISTD. The regression equations for the standard curves for the six isotopically labeled fatty acids in the plasma matrix are given in **Fig. 2**. It is apparent from this figure that a linear equation provides an excellent fit within the specified ranges, as all had $R^2 \geqslant 0.99$. The slopes of the standard curves alone, when unlabeled fatty acids were added, and in the plasma matrix were as follows: 0.9783, 0.8956, and 0.7015 for $^2H_5$-18:3n-3 and 0.3115, 0.2174, and 0.1535 for ^{13}C-U-18:3n-3 (M+17), respectively.

Standard curves for unlabeled C18-, C20-, and C22 EFAs were constructed in a similar manner as those for the isotopic fatty acids (in the absence of the various matrices). **Table 2** shows the slopes and regression coefficients of the linear regression curves for the unlabeled fatty acids, produced from analyses of a quantitative standard containing all of these fatty acids.

Because the majority of the isotopically labeled n-6 and n-3 fatty acids are not available as standards, it was necessary to estimate their slopes. This was done by assuming that the ratio of the slope of the labeled fatty acid to its unlabeled form is the same as that of its precursor. Thus,

TABLE 2. Slopes and coefficients of regression equations for standard curves of unlabeled fatty acid pentafluorobenzyl esters

	Standard Curve Parameters	
Fatty Acids	Slopes	Coefficient (R^2)
18:3n-3	1.14	0.99
20:3n-3	1.24	0.99
20:4n-3	1.03	0.99
20:5n-3	0.96	0.99
22:5n-3	0.72	0.98
22:6n-3	0.61	0.98
18:2n-6	1.34	0.99
18:3n-6	1.16	0.99
20:2n-6	1.30	0.99
20:3n-6	1.36	0.99
20:4n-6	1.22	0.99
22:4n-6	0.96	0.99

Linear regression of the GC-MS peak area ratio of analytes to internal standard (ISTD) versus the ratio of the amount of the fatty acid to the ISTD over the range of 0.008–4 pmol per injection. Values were based on two determinations with 10 points per curve. The intercepts were all <0.08 (range, 0.006–0.078).

TABLE 3. Calculated slopes of the regression curves for isotopic EFA metabolites in rat plasma

	C18 Isotopes			C20 Isotopes	
Fatty Acids	$^2H_5$	$^{13}C_{18}$	$^{13}C_{17}$	$^2H_5$	$^{13}C_{20}$
18:3n-3	**0.702**	**0.329**	**0.154**	—	—
20:3n-3	0.758	0.348	—	—	—
20:4n-3	0.631	0.290	—	—	—
20:5n-3	0.609	0.279	—	—	**0.219**
22:5n-3	0.460	0.211	—	—	0.098
22:6n-3	0.402	0.184	—	—	0.081
18:2n-6	**0.966**	**0.272**	**0.141**	—	—
18:3n-6	0.726	—	0.170	—	—
20:2n-6	0.907	0.550	—	—	—
20:3n-6	0.849	0.515	—	**0.794**	—
20:4n-6	0.763	0.227	—	0.714	—
22:4n-6	0.598	0.363	—	0.434	—

The slopes for the metabolite of the stable isotope-labeled fatty acids were derived from the slopes of the curves generated with the unlabeled fatty acids (see Table 2). The precursors for which actual standard curves were generated are given in boldface type. The formula used to estimate the unknown slope of the labeled fatty acid metabolites was as follows: unlabeled precursor/labeled precursor = unlabeled metabolites/labeled metabolites.

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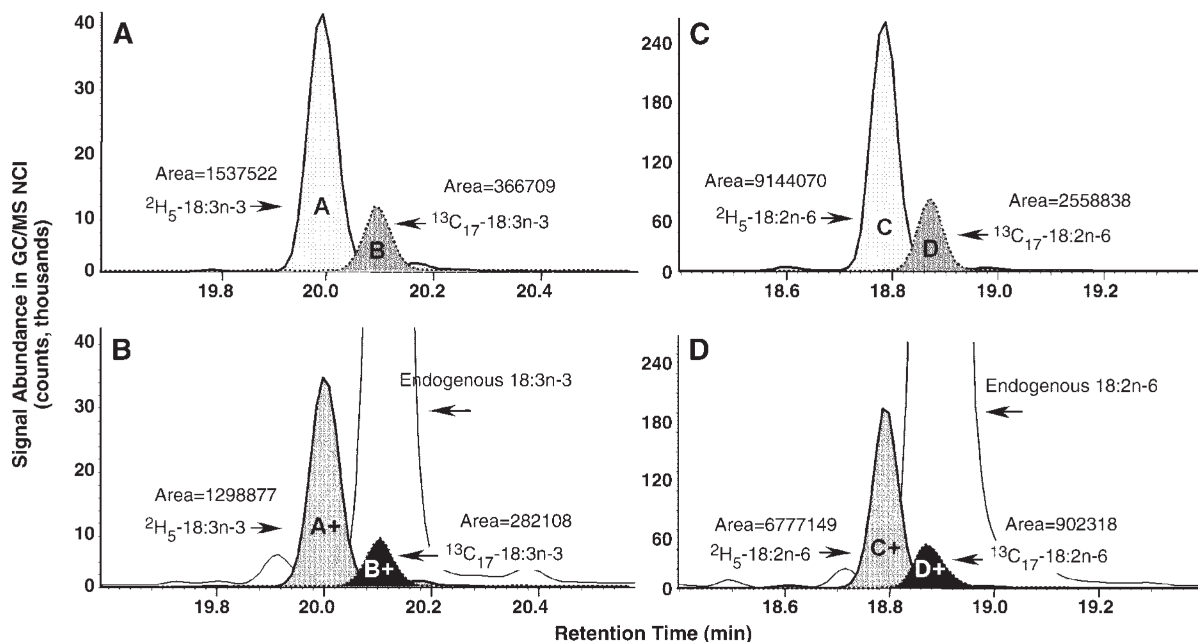

Fig. 3. MS signal suppression attributable to the plasma matrix. Ion chromatograms of $^2H_5$-18:3n-3 (M+5) and ^{13}C-U-18:3n-3 (M+17) without (A) and with (B) endogenous 18:3n-3 and $^2H_5$-18:2n-6 (M+5) and ^{13}C-U-18:2n-6 (M+17) without (C) and with (D) endogenous 18:2n-6 are shown. The peak area was used to compare the signal response of $^2H_5$-labeled ion or $^{13}C_{17}$-labeled ion with the absence (peaks A, B, C, D) or presence (corresponding peaks A+, B+, C+, D+) of endogenous 18:3n-3 and 18:2n-6 from rat plasma.

because three of these four quantities are experimentally determined, the fourth, the labeled metabolite, can be derived. For example, for 22:5n-3, no deuterated or ^{13}C standard was available, so the standard curves for $^2H_5$-22:5n-3 and ^{13}C-22:5n-3 could not be determined experimentally. Therefore, the slopes were determined for the unlabeled 22:5n-3, and the ratio of this to the $^2H_5$-22:5n-3 was calculated by assuming that it was the same as the ratio of 18:3n-3 to $^2H_5$-18:3n-3; similarly, the ratio of the slopes of the 22:5n-3 to ^{13}C-22:5n-3 was assumed to be the same as the ratio of the slopes of 20:5n-3 to ^{13}C-20:5n-3.

For the n-3 fatty acids, the slopes for the deuterated metabolites were calculated similarly using that of $^2H_5$-18:3n-3, and the slopes for the ^{13}C metabolites were calculated using that of ^{13}C-18:3n-3. Similarly, for arachidonic acid (20:4n-6), the isotopically labeled 18:2n-6 was used. In both cases, these determinations were made in the presence of the plasma matrix. However, several minor n-6 fatty acids occur in the plasma at a much lower concentration than 18:2n-6 or 20:4n-6; for these, the slopes were obtained using the labeled 18:2n-6 in the absence of the plasma matrix. In this way, the standard curves for all of the stable isotope metabolites to be measured were estimated (**Table 3**).

The slopes for both ^{13}C-U-20:5n-3 and $^2H_5$-20:3n-6 could be compared with estimated values because these labeled

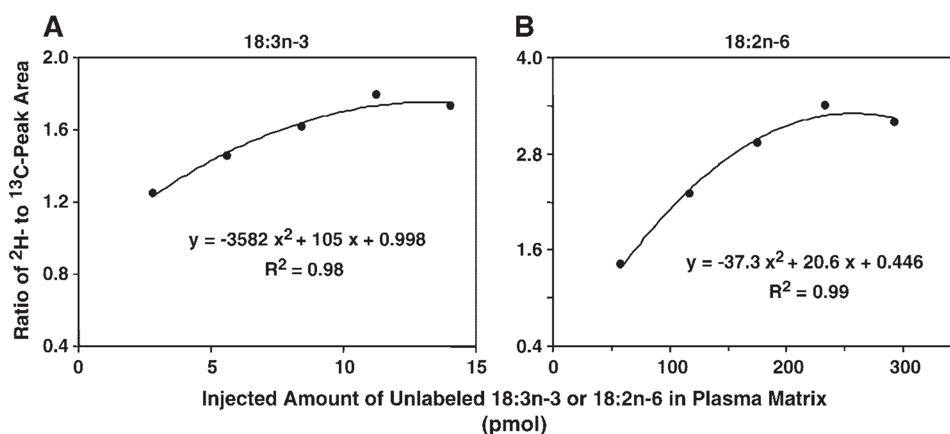

Fig. 4. Concentration dependence curves for ^{2}H- and ^{13}C-labeled 18:3n-3 and 18:2n-6 in which a constant amount of isotopes was added to varying amounts of plasma. The ratios of the concentrations of the two isotopes were plotted as a function of various amounts of endogenous 18:3n-3 (A) and 18:2n-6 (B). A nonlinear, quadratic regression curve was applied for fitting of the experimental data.

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isotopes were available. The measured and estimated slopes, when corrected for the respective isotope distribution of the precursors, for 20:5n-3 were virtually identical (0.6483 vs. 0.6480), and the agreement for the 20:3n-6 slopes was excellent (0.884 vs. 0.858). Slightly greater differences in slopes were noted if different ions were used in place of the parent ions (e.g., M+19 ion in place of the M+20 ion for ^{13}C-U-20:5n-3, and M+17 ion in place of the M+18 ion for ^{13}C-U-20:5n-3). There was significant improvement in precision and accuracy using these standard curves compared with using a response factor calculated from a single concentration over a wide range of concentrations. The latter compromised the accuracy of isotopic fatty acid measurements when the unknown was not close to the amount of the ISTD (25).

Concentration-dependent effects of endogenous fatty acids

Greater signal suppression of ^{13}C- relative to $^{2}H_5$-fatty acid ions by endogenous fatty acids. **Figure 3** depicts the ion chromatograms of pure stable isotope-labeled fatty acids with and without added rat plasma. There was a greater amount of signal suppression on the ^{13}C-labeled fatty acid ions by the endogenous amounts of 18:3n-3 and 18:2n-6 from the plasma compared with the $^{2}H_5$-labeled fatty acid ions. The peak area decreased by 16% for $^{2}H_5$-18:3n-3 (0.5 pmol), 23% for ^{13}C-U-18:3n-3 (0.4 pmol), 26% for $^{2}H_5$-18:2n-6 (3 pmol), and 65% for ^{13}C-U-18:2n-6 (3 pmol) in the presence of rat plasma that contained 4 pmol of 18:3n-3 and 80 pmol of 18:2n-6. A similar suppressive effect of the endogenous pool on stable isotope tracer measurements has been observed previously by Patterson et al. (11, 26) for electron impact mode GC-MS analyses.

Concentration dependence curves. The greater suppressive effect of plasma on ^{13}C-labeled fatty acid ions compared with $^{2}H_5$-labeled fatty acid ions was quantified over a range of plasma fatty acid concentrations by diluting plasma. To demonstrate the relatively greater effect on the ^{13}C-labeled fatty acid ions, the ratios of the $^{2}H_5$ to the ^{13}C signals were plotted on the ordinate versus the injected amount of the fatty acid in the plasma matrix on the abscissa (**Fig. 4**). The curves were ranged from 3 to 14 pmol per injection for 18:3n-3 and 60 to 300 pmol per injection for 18:2n-6, as these were relevant ranges for plasma measurements. The plot follows a nonlinear, quadratic regression equation between the concentration ratio and the amount of endogenous unlabeled fatty acid matrix. A substantially greater suppression was found for the ^{13}C-labeled fatty acid,

TABLE 4. Essential fatty acid composition in rat plasma at 24 h after dosing with four isotopic tracers

Fatty Acids	Fatty Acid Composition
	% of total
18:3n-3	0.67 ± 0.19
20:5n-3	0.68 ± 0.13
22:5n-3	0.54 ± 0.10
22:6n-3	3.84 ± 0.91
24:5n-3	0.23 ± 0.04
24:6n-3	0.19 ± 0.04
18:2n-6	14.11 ± 0.97
18:3n-6	0.16 ± 0.03
20:2n-6	0.13 ± 0.04
20:3n-6	0.54 ± 0.09
20:4n-6	20.16 ± 3.78
22:4n-6	0.22 ± 0.03
22:5n-6	0.14 ± 0.04
24:4n-6	0.11 ± 0.02
24:5n-6	0.12 ± 0.04

Rats were on a controlled diet (40% saturated fat, 42% monounsaturates, 15% 18:2n-6, and 3% 18:3n-3 in 10% of fat in diet) for >4 weeks before beginning this experiment. Data are expressed as means ± SD for 11 rats.

TABLE 5. Comparison of concentrations of $^{2}H_5$-labeled and ^{13}C-labeled fatty acids in rat plasma 24 h after dosing with four isotopic tracers

Fatty Acids	$^{2}H_5$-Labeled	^{13}C-Labeled	Percent Difference
18:3n-3	32.42 ± 7.64	39.92 ± 11.10	−21
20:4n-3	1.16 ± 0.16	0.92 ± 0.09	23
20:5n-3	28.60 ± 3.47	30.71 ± 3.75	−7
22:5n-3	25.18 ± 2.08	29.36 ± 2.30	−15
22:6n-3	40.58 ± 5.08	43.78 ± 8.65	−8
24:5n-3	0.77 ± 0.22	1.00 ± 0.26	−26
24:6n-3	3.96 ± 0.66	4.60 ± 0.78	−15
18:2n-6	192.97 ± 13.35	199.64 ± 20.24	−3
18:3n-6	2.32 ± 0.26	2.05 ± 0.21	12
20:2n-6	1.59 ± 0.14	1.43 ± 0.12	11
20:3n-6	6.68 ± 0.60	5.49 ± 0.50	20
20:4n-6	47.90 ± 6.01	47.19 ± 5.95	1
22:4n-6	0.50 ± 0.05	0.48 ± 0.05	4
22:5n-6	0.18 ± 0.03	0.19 ± 0.03	−7
24:4n-6	0.01 ± 0.00	0.01 ± 0.00	2
24:5n-6	0.02 ± 0.00	0.02 ± 0.00	1

Data are expressed as means ± SEM for 11 rats in units of pmol/ml plasma/μmol oral dose. Percent difference was calculated as the difference of $^{2}H_5$-labeled fatty acids and ^{13}C-labeled fatty acids divided by the average of the two isotopes multiplied by 100.

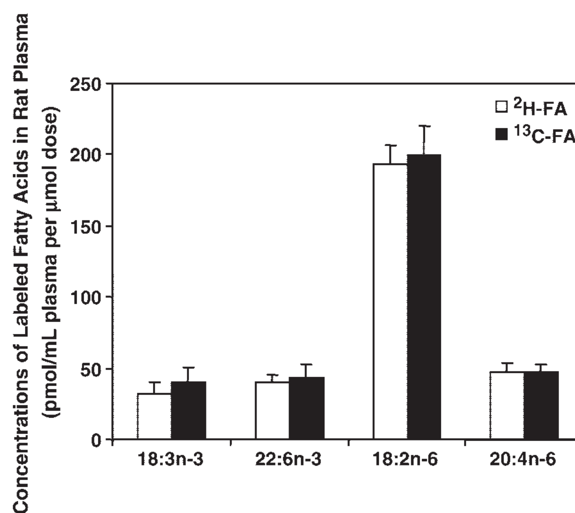

Fig. 5. Comparison of the concentration of isotope-labeled fatty acids per dose (pmol/ml plasma/μmol dose) in rat plasma in vivo. Plotted are the stable isotopic precursors, 18:3n-3 and 18:2n-6, and their major metabolites, 22:6n-3 and 20:4n-6, at 24 h after oral dosing with a mixture of $^{2}H_5$-18:3n-3, ^{13}C-U-18:3n-3, $^{2}H_5$-18:2n-6, and ^{13}C-U-18:2n-6 ethyl esters. No significant difference ($P > 0.05$) was observed in rat plasma between the isotopomers of 18:3n-3, 22:6n-3, 18:2n-6, and 20:4n-6. Values are presented as means ± SEM (n = 11).

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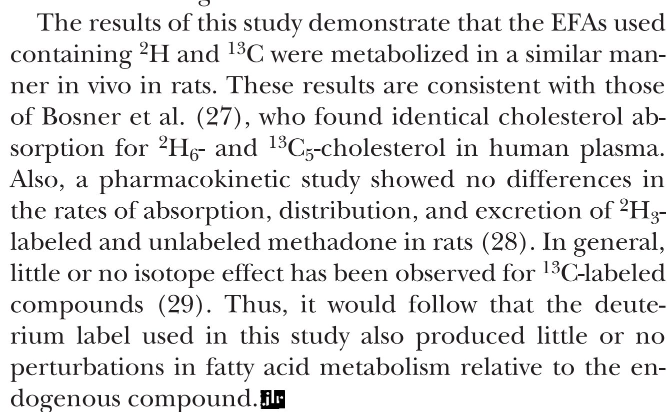
as the ratio was up to 1.8 and 3.4 for 18:3n-3 and 18:2n-6, respectively.

Matrix effects are well known in biological mass spectrometry. In particular, the suppressing effect of higher concentrations of analytes has been reported, for example, for methyl palmitate in the electron impact mode (11). In our NCI analyses, it is assumed that the plasma matrix effect is attributable to suppressed ionization resulting from competition. This is supported by the results presented here demonstrating that the addition of pure fatty acid standards can suppress the signals from the same compound isotopically labeled. Thus, this effect is expected to be an inherent property of this type of analysis and should occur on every instrument. This effect also indicates that the type of ISTD used is a critical aspect of accurate quantitative analysis. It would be optimal to use a ^{13}C-labeled ISTD for the measurement of ^{13}C metabolites and likewise for ^{2}H-labeled compounds. Also, the chemical structures of the ISTDs should be similar to those of the compounds being measured.

Isotope effect in rat plasma in vivo

An attempt was made to directly compare $^{2}H_{5}$- and ^{13}C-labeled 18:2n-6 and 18:3n-3 with respect to the extent of their conversion to longer chain, more unsaturated fatty acids in vivo. Approximately equal doses of each of the forms of each stable isotope-labeled compound were administered orally, and rat plasma was collected 24 h later for analysis. Samples were analyzed by GC-MS, the peak areas were calibrated using the standard curves described above, and the EFA concentration (in nmol/ml plasma) of each isotope was converted to the dosage for each animal, expressed as pmol/ml of plasma over μmol of administered dose. The concentrations of the various endogenous fatty acids in rat plasma were quite different for some of the minor EFA components relative to the more major components 18:2n-6, 20:4n-6, and 22:6n-3 (**Table 4**). Thus, the secondary correction depicted in Fig. 4 was applied to the precursors and to 20:4n-6 and 22:6n-3. This was done by calculating the amount of endogenous fatty acid injected for each of these fatty acids in the rat plasma sample; then, after interpolation from the graphs shown in Fig. 4, the relevant correction was applied between the $^{2}H_{5}$- and ^{13}C-labeled fatty acids. Such corrections were not applied to the fatty acid peaks that occurred at lower concentrations. The accuracy of this approach was verified for the measurement of ^{13}C-18:2n-6 and ^{13}C-20:4n-6. The PFB ester samples was diluted 10-fold before GC-MS analysis and compared with the estimate made at the higher concentration using the secondary correction just described. There were no significant differences between the results with these two approaches in the concentrations (means ± SEM, n = 11) of ^{13}C-18:2n-6 (15.55 ± 0.64 vs. 14.01 ± 1.13 nmol/ml plasma; $P > 0.05$) or ^{13}C-20:4n-6 (3.64 ± 0.26 vs. 3.70 ± 0.36 nmol/ml plasma; $P > 0.05$). Similarly, the values obtained for $^{2}H_{5}$-18:2n-6 (15.58 ± 0.86 nmol/ml plasma) and $^{2}H_{5}$-20:4n-6 (3.71 ± 0.28 nmol/ml plasma) were in close agreement with the corresponding values of the ^{13}C-labeled compound. Thus, sample dilution offers one means to obtain quantitative results for the ^{13}C-labeled fatty acids by decreasing concentration-dependent signal suppression.



Using the correction factors put forward here, there were no significant differences ($P > 0.05$) in the amount of $^{2}H_{5}$-18:3n-3 and ^{13}C-U-18:3n-3 in rat plasma per dosage (**Table 5**). There were also no differences in the amounts of principal n-3 metabolites, $^{2}H_{5}$-20:5n-3 and $^{13}C_{18}$-20:5n-3, or between $^{2}H_{5}$-22:6n-3 and $^{13}C_{18}$-22:6n-3 (**Fig. 5**). Similarly, there were no significant differences in the amounts of $^{2}H_{5}$-18:2n-6 and $^{13}C_{18}$-18:2n-6 or between the isotopomers of the principal n-6 metabolites $^{2}H_{5}$-20:4n-6 and $^{13}C_{18}$-20:4n-6. The more minor n-3 and n-6 metabolites showed very similar accretion values (Table 5). 20:3n-3 could not be measured because of the relatively high baseline of the M+5 ion and its low signal abundance.

The results of this study demonstrate that the EFAs used containing ^{2}H and ^{13}C were metabolized in a similar manner in vivo in rats. These results are consistent with those of Bosner et al. (27), who found identical cholesterol absorption for $^{2}H_{6}$- and $^{13}C_{5}$-cholesterol in human plasma. Also, a pharmacokinetic study showed no differences in the rates of absorption, distribution, and excretion of $^{2}H_{3}$-labeled and unlabeled methadone in rats (28). In general, little or no isotope effect has been observed for ^{13}C-labeled compounds (29). Thus, it would follow that the deuterium label used in this study also produced little or no perturbations in fatty acid metabolism relative to the endogenous compound.

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