Validation of a new procedure to determine plasma fatty acid concentration and isotopic enrichment

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Abstract **Assessment of free fatty acid (FFA) concentration and isotopic enrichment is useful for studies of FFA kinetics in vivo. A new procedure to recover the major FFA from plasma for concentration and isotopic enrichment measurements is described and validated. The procedure involves extraction of plasma lipids with hexane, methylation with iodomethane (CH_3I) to form fatty acid methyl esters (FAME), and subsequent purification of FAME by solid phase extraction (SPE) chromatography. The new method was compared with a traditional method using thin-layer chromatography (TLC) to recover plasma FFA, with subsequent methylation by BF_3/methanol. The TLC method was found to be less reliable than the new CH_3I method because of contamination with extraneous fatty acids, chemical fractionation of FFA species, and incomplete recovery of FFA associated with TLC. In contrast, the CH_3I/SPE method was free of contamination, did not exhibit chemical fractionation, and had higher recovery. The iodomethane reaction was specific for free fatty acids; no FAME were formed when esterified fatty acids (triglycerides, cholesteryl esters, phospholipids) were subjected to the methylation reaction. We conclude that the CH_3I/SPE method provides rapid and convenient recovery of plasma fatty acids for quantification or GC/MS analysis as methyl esters, and is not subject to the problems of contamination, reduced recovery, and chemical fractionation associated with recovery of FFA by TLC.**—Patterson, B. W., G. Zhao, N. Elias, D. L. Hachey, and S. Klein. **Validation of a new procedure to determine plasma fatty acid concentration and isotopic enrichment.** ***J. Lipid Res.*** **1999.** 40: **2118–2124.**

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Accurate assessment of plasma free fatty acid (FFA) concentrations and isotopic enrichment is critical for evaluating fatty acid metabolic kinetics in vivo using stable isotope methodology. Although numerous methods have been reported for the measurement of FFA concentrations and enrichments, these methods are seldom critically cross-validated. Perhaps the most commonly used approach for measuring plasma FFA concentrations and fatty acid enrichment involves extracting the lipid component of plasma, separating FFA by thin-layer chromatography (TLC), and preparing fatty acid methyl ester (FAME) derivatives using boron trifluoride (BF_3) and methanol (1, 2). An internal standard (e.g., heptadecanoic acid, C17:0) is used to quantify FFA concentration by gas chromatography (GC) using a flame ionization detector. The use of TLC requires time-consuming manual manipulations, including spotting samples on TLC plates and recovering TLC scrapings, which limit sample throughput. This approach assumes that recoveries of all fatty acid species are equivalent and that chemical fractionation does not occur.

We developed an alternative approach for preparing FAME by using iodomethane (CH_3I) and solid phase extraction (SPE) cartridges in an effort to decrease sample processing time and effort. In this report, we describe the CH_3I/SPE procedure and identify systematic differences in the concentrations of major plasma FFAs and palmitate isotopic enrichment between this and the traditional TLC/BF_3 procedure. By analysis of appropriate standards and biological samples, we provide evidence that the TLC/BF_3 procedure introduces artifacts which decrease the accuracy of FFA concentration and palmitate enrichment measurements. In contrast, the new CH_3I/SPE approach provides a more reliable analysis and permits a faster sample processing rate.

MATERIALS AND METHODS

Samples and standards

Fatty acid concentration and enrichment were measured in biological plasma samples obtained from human subjects and in FAME and FFA standards. Plasma samples were obtained from subjects participating in two ongoing research protocols that fo-

Abbreviations: FAME, fatty acid methyl esters; FFA, free fatty acids; GC, gas chromatography; GC/MS, gas chromatograph/mass spectrometer; Ra, rate of appearance; SPE, solid phase extraction; TLC, thin layer chromatography; TTR, tracer to tracee ratio.

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cus on the hormonal regulation of whole-body lipolysis. These studies were approved by the Human Studies Committee of the Washington University School of Medicine, and informed consent of the participants was obtained. Samples were specifically chosen to include a broad range of plasma FFA concentration and enrichments. In both study protocols, subjects were infused with 2,2-[$^2H_2$]hexadecanoic acid (98 atom% ^2H, Isotec, Miamisburg, OH) at a rate of 0.04 μmol/kg per min into an antecubital vein and blood samples were taken from a radial artery. Blood samples were collected in chilled tubes containing EDTA as an anticoagulant and placed immediately in ice. Plasma was obtained by refrigerated centrifugation within 20 min of blood drawing and stored at −70°C until subsequent analyses were performed. In one study, blood samples were obtained at regular time intervals during a pancreatic hormonal clamp with 4-stage epinephrine infusion. This study involved infusing somatostatin, insulin, and growth hormone to "clamp" the concentration of pancreatic hormones that affect lipolysis. Epinephrine was infused for 30 min at 0.00125 μg·kg fat free mass (FFM)$^{-1}$·min^{-1}, 0.005 μg·kg FFM^{-1}·min^{-1}, 0.0125 μg·kg FFM^{-1}·min^{-1}, and 0.025 μg·kg FFM^{-1}·min^{-1} in 4 discrete stages, which caused a progressive step-wise increase in plasma FFA concentration and decrease in plasma palmitate enrichment. In the other study, blood samples were taken during an intravenous infusion of propranolol, a nonselective (both β_1 and β_2) β-adrenergic receptor antagonist, which caused a decrease in FFA concentration and an increase in palmitate enrichment. Blood samples from these two studies provided a 3-fold range of plasma FFA concentrations and a 5-fold range in palmitate isotopic enrichments. In addition, certain tests were performed on a larger pool of isotopically enriched plasma that was generated by combining aliquots from multiple plasma samples.

FAME and FFA standards of known fatty acid profile were obtained from Nu-Chek-Prep, Inc. (Elysian, MN). The FAME standard (catalog # GLC-63) consisted of the methyl esters of C12:0 (2.0% by weight), C14:0 (2.0%), C14:1 (1.0%), C16:0 (22.0%), C16:1 (5.0%), C17:0 (10.0%), C18:0 (10.0%), C18:1 (32.0%), C18:2 (8.0%), C18:3 (4.0%), and C20:4 (4.0%). The fatty acid profile in this standard is similar to plasma FFA. The FFA standard (catalog # NIH-D) consisted of C14:0 (11.8%), C16:0 (23.6%), C16:1 (6.9%), C18:0 (13.1%), and C18:1 (44.6%). Sufficient [$^2H_2$]palmitate was added to the FFA standard to provide a palmitate tracer:tracee ratio (TTR) of approximately 12.5%.

CH_3I/SPE method

Aliquots (250 μL) of plasma or water blanks were placed in 13 × 100 mm screw top tubes. Equal volumes (250 μL) of heptadecanoic acid (C17:0) internal standard (0.23 μmol/mL in heptane; stored at −20°C) and water were added. Samples were shaken on a platform vortexer for 3 min before adding 3 mL ice-cold acetone to precipitate plasma proteins. Samples were vortexed again for several seconds and placed at −20°C for 15 min. After centrifugation of precipitated proteins, the supernatant was poured into 16 mm × 125 mm screw-top tubes. Three-mL aliquots of hexane and water were added before securely capping the samples with Teflon-lined caps and shaking them gently in a horizontal platform shaker for 15 min. The samples were then centrifuged to separate the solvent and aqueous phases.

The upper phase (hexane) was transferred into 13 × 100 mm screw-top tubes and dried in a SpeedVac centrifugal concentrator (Savant, Farmingdale, NY) or evaporated under nitrogen. A 0.25-mL aliquot of buffer (0.2 M dibasic potassium phosphate and 0.05 M tetrabutylammonium hydrogen sulfate, pH adjusted to 9.0 with tribasic potassium phosphate) and 0.25 mL iodomethane (Aldrich Chemical Co., Milwaukee, WI) in dichloromethane (1:10 vol:vol) were added, and samples were vortexed for 10 min to form FAME. Three mL of hexane was added and samples were vortexed for 15 min on a platform vortexer to extract FAME. After centrifugation to separate solvent phases, the upper layer (hexane) was transferred to 13 × 100 mm uncapped test tubes and dried in a SpeedVac centrifugal concentrator. Solid phase extraction cartridges (LC-Si, 3 mL size, catalog #505048; Supelco, Bellefonte, PA) were placed on a vacuum manifold and prepared by washing twice with 1.5-mL aliquots of hexane collected into waste tubes without allowing the cartridges to dry. Hexane (1.5 mL) was added to the dried samples and vortexed for 2 min. Samples were transferred to the SPE cartridges and rinsed slowly with 1.5 mL hexane. Waste tubes were replaced with 13 × 100 mm uncapped collection tubes, and FAME were eluted with 2 rinses (1.5 mL each) of 2% ethyl acetate in hexane. When analyzed by TLC, this fraction is free from tri-, di-, and mono-glycerides and phospholipids, but does contain cholesteryl esters. However, these esters do not interfere with subsequent GC/MS analysis of methyl palmitate or quantitative GC analysis of plasma FFA. Samples were dried in a SpeedVac concentrator. Heptane (100 μL) was added to each tube and samples were transferred to autosampler vials for subsequent quantitative GC and GC/MS analyses.

TLC/BF_3 method

This method has been previously described in detail (2). Briefly, C17:0 internal standard was added to plasma or water blanks, lipids were extracted with hexane, dried, and FFA were separated by TLC on silica gel plates using a hexane–ether–formic acid solvent system. FFA were visualized by spraying with 0.01% rhodamine 6G. TLC scrapings containing FFA were extracted with chloroform–methanol 3:1, dried, and FAME were prepared by reaction with BF_3 in methanol.

Additional studies

Variations of the two basic procedures were used to validate the CH_3I/SPE procedure or to delineate artifacts associated with the TLC/BF_3 procedure. One test was used to evaluate the reliability of the solvent extraction procedures; lipids were extracted from plasma with chloroform–methanol (3) rather than with hexane and the CH_3I/SPE and TLC/BF_3 procedures were conducted on the lipid extract. A second test was used to evaluate the effect of varying the size of the FFA band recovered from TLC on FFA concentration and enrichment. Eight identical aliquots of extracted plasma lipids were separated by TLC. After identification of bands by rhodamine, TLC scrapings were recovered which were approximately 7 mm wide ("narrow" cuts, which were entirely within the confines of the visible FFA band) or 12 mm wide ("broad" cuts which extended beyond the visible FFA band) (four lanes per test). A third test evaluated the specificity of the CH_3I methylation procedure by adding approximately 1 mg of triglyceride, containing only C15:0 fatty acid (Nu-Chek-Prep), to 0.25-mL plasma samples before FFA analysis. A fourth test evaluated the importance of the order of FFA extraction and methylation procedures; plasma FFAs were methylated with iodomethane in situ within plasma before hexane extraction, rather than the normal CH_3I/SPE procedure in which lipids were extracted with hexane before methylation. A fifth test evaluated methylation and TLC recovery procedures on FFA standards containing [$^2H_2$]palmitate and C17:0 internal standard. FFA concentrations and palmitate TTR were measured on standards which were methylated with either BF_3/methanol or CH_3I, with or without separation by TLC or SPE. Post-TLC FFA methylation conditions evaluated included methylation with BF_3/methanol or CH_3I, either directly on TLC scrapings or after various solvent conditions which were used to extract FFA (chloroform–methanol

3:1 or 2:1 (vol:vol), single vs. multiple extractions). A final test evaluated the use of TLC rather than SPE for FAME recovery. Identical aliquots (100 μL) of FAME standards were placed directly in autosampler vials or dried in a SpeedVac concentrator, recovered from SPE or TLC, dried in a SpeedVac concentrator again, and reconstituted with 100 μL heptane for quantitative GC analysis. Different solvent extraction conditions (chloroform–methanol at 3:1 or 1:1 (vol:vol), chloroform, or ether) were used for TLC recovery and compared with the normal SPE recovery procedure. FAME concentrations relative to the C17:0 methyl ester internal standard were measured, and recovery of C17:0 methyl ester was determined by comparing GC peak areas in recovered samples.

Quantitative GC and GC/MS analyses

Quantitative GC analysis was determined by using a Hewlett-Packard 5890 GC (Palo Alto, CA) with a 30 m × 0.32 mm Omegawax 250 column (Supelco, Bellefonte, PA) and a flame ionization detector. Instrument response was calibrated by using the GLC-63 FAME standard to relate relative peak areas of FAME peaks to molar ratios of a C17:0 internal standard. For plasma FFAs, only results for major FFAs (C16:0, C16:1, C18:0, C18:1, and C18:2) are reported; fatty acids that contribute less than 2% of the total FFA concentration (C14:0, C18:3, and C20:4) are not reported.

The isotopic enrichment of methyl palmitate was measured by electron impact ionization GC/MS by using a Hewlett-Packard 5971 system with a 30 m × 0.25 mm Omegawax 250 (Supelco) column. Ions with mass to charge ratios of 270 and 272, representing the molecular ion for unlabeled and labeled palmitate methyl ester, were selectively monitored (2, 4). Artifacts resulting from concentration-dependent self chemical ionization of methyl palmitate (4, 5) were minimized by ensuring that similar peak areas were obtained for all samples measured within a single analysis. Tracer:tracee ratios were obtained by measurement of appropriate isotopic enrichment standards.

Materials

Except where noted, reagents and solvents were purchased from Sigma Chemical Company (St. Louis, MO).

Statistics

Values shown are mean ± standard deviation. Values obtained by the TLC/BF_3 and CH_3I/SPE methods were compared by using a 2-tailed Student's *t* test for paired samples. A *P* value of <0.05 was considered statistically significant.

RESULTS

Plasma samples

Figure 1 shows the results from an in vivo tracer infusion study which caused a 3-fold range in plasma FFA concentration. Palmitate concentrations were higher and palmitate isotopic enrichments were lower in samples analyzed using the TLC/BF_3 procedure than the CH_3I/SPE method. The % difference in palmitate concentration observed between methods (11.6 ± 4.1 %) was accompanied by a corresponding difference (−9.5 ± 5.8%) in palmitate enrichment. The differences between methods in both concentration and enrichment values decreased progressively with increasing plasma FFA concentrations.

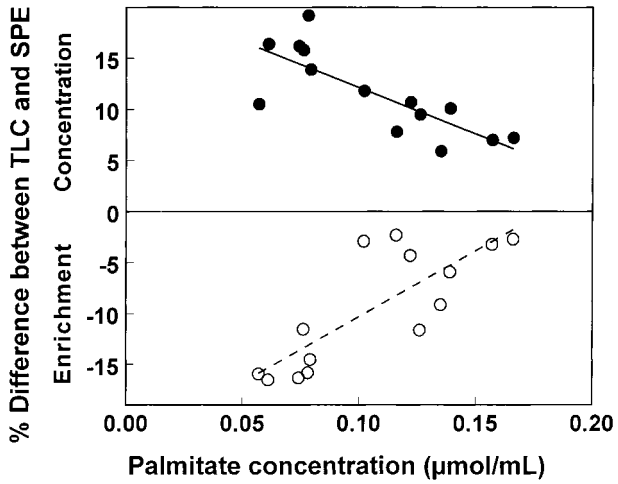

Fig. 1. Differences between CH_3I/SPE and TLC/BF_3 methods for palmitate. Plasma samples from an in vivo tracer infusion study which spanned a 3-fold range of palmitate concentration were processed by the two methods. Plot shows the % difference between the two methods ([(TLC-SPE)/SPE] × 100) for palmitate concentration (●) and isotopic enrichment (○) plotted against the palmitate concentration determined by CH_3I/SPE.

Systematic differences in measured FFA concentration between the CH_3I/SPE and TLC/BF_3 methods were observed for C16:0, C18:0, and C18:2, but not for C16:1 and C18:1 (**Fig. 2**). Saturated FFA concentrations measured by TLC/BF_3 were consistently greater than values measured by CH_3I/SPE. Unsaturated FFA with one double bond were the same measured by both methods, whereas unsaturated FFA with two double bonds had lower concentrations measured by TLC/BF_3 than by CH_3I/SPE. The concentration range measured for each FFA and the mean ± SD difference between the two methods were: C16:0 (range 0.05–0.17 μmol/mL),

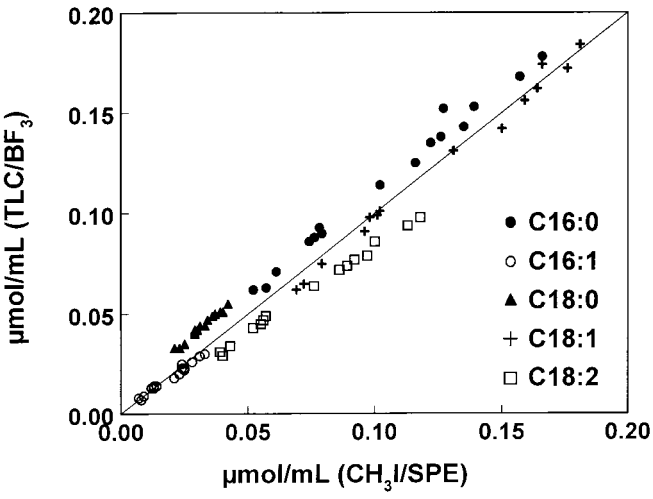

Fig. 2. Comparison of CH_3I/SPE and TLC/BF_3 methods for multiple fatty acids. Concentrations measured by TLC/BF_3 are plotted against concentrations measured by CH_3I/SPE for 6 major plasma FFA. Samples are the same as from Fig. 1. Not shown: C18:3. A line of identity is included for comparison.

13 ± 5%, $P < 0.0001$; C16:1 (range 0.01–0.03 μmol/mL), −3 ± 9%, P = NS; C18:0 (range 0.02–0.04 μmol/mL), 38 ± 7%, $P < 0.0001$; C18:1 (range 0.07–0.22 μmol/mL), −3 ± 4%, P = NS; C18:2 (range 0.04–0.12 μmol/mL), −18 ± 3%, $P < 0.0001$. A regression analysis of the results from Fig. 2 found that the saturated fatty acids (C16:0, C18:0) had significant positive intercepts ($P < 0.005$) and slopes that were not significantly different from unity, whereas the slope for C18:2 was significantly less than unity (0.86 ± 0.01, $P < 0.0001$) with an intercept that was not significantly different than zero. The fraction of total fatty acids represented as palmitate was significantly higher by TLC/BF_3 (30.1 ± 1.5%) compared with CH_3I/SPE (26.9 ± 0.4%; $P < 0.00001$).

Blank samples that contained only the C17:0 internal standard were analyzed by both methods. Blanks evaluated by the TLC/BF_3 procedure demonstrated the presence of significant quantities of C16:0, C18:0, and C18:2 that represented 15%, 45%, and 8%, respectively, of the average sample concentrations that were measured for these three fatty acids; C16:1 and C18:1 were undetectable. In contrast, blanks evaluated by the CH_3I/SPE procedure did not generate detectable quantities of any of these FFAs.

To evaluate the effect of varying the size of the FFA band recovered from TLC, a pool of plasma lipids containing [$^2H_2$]palmitate and C17:0 internal standard was prepared by combining chloroform–methanol 3:1 (vol:vol) extracts from several plasma samples. Identical aliquots from this pool were separated on a TLC plate, and the FFA bands were recovered using "broad" or "narrow" cuts. Identical results were obtained using direct methylation with BF_3/methanol or by extracting FFA with $CHCl_3$–methanol 3:1 (vol:vol) followed by methylation with CH_3I (n = 2 for each treatment); the results for both treatments were thus averaged together. Results for major plasma FFA are shown in **Table 1**. "Broad" cut TLC bands resulted in significantly higher concentrations than "narrow" cut bands for all fatty acids with the exception of C18:0. Although the "broad" and "narrow" cut bands generated significantly different C16:0 concentrations, there was no significant difference in palmitate isotopic enrichment. Qualitative GC/MS with scanning acquisition failed to demonstrate the presence of significant quantities of components other than identifiable FAMEs in samples or blanks recovered from TLC (results not shown), so it is unlikely the effect of TLC band size results from inclusion of contaminants having retention times similar to FAMEs which may affect the quantitative GC results.

TABLE 1. Comparison of "broad" and "narrow" cut bands of plasma FFA recovered from TLC

Fatty Acid	"Broad" Cut n = 4	"Narrow" Cut n = 4	*P* value
C16:0	0.140 ± 0.003	0.111 ± 0.005	<0.001
C16:1	0.021 ± 0.001	0.009 ± 0.001	<0.001
C18:0	0.045 ± 0.006	0.057 ± 0.006	NS
C18:1	0.143 ± 0.005	0.126 ± 0.06	<0.01
C18:2	0.071 ± 0.001	0.025 ± 0.004	<0.001
Palmitate tracer: tracee ratio	0.0295 ± 0.0012	0.0270 ± 0.0011	NS

Values shown are mean ± 1 SD for FFA concentrations (μmol/mL) of identical aliquots of extracted plasma lipids.

TABLE 2. Effect of extraction procedures on plasma fatty acid concentrations determined by the CH_3I/SPE method

Fatty Acid	Hexane Extraction n = 2	Chloroform: Methanol Extraction n = 2	*P* value
C16:0	0.111 ± 0.002	0.465 ± 0.001	<0.001
C16:1	0.020 ± 0.001	0.031 ± 0.001	<0.005
C18:0	0.035 ± 0.001	0.199 ± 0.001	<0.001
C18:1	0.156 ± 0.001	0.217 ± 0.003	<0.001
C18:2	0.065 ± 0.001	0.283 ± 0.004	<0.001
Palmitate tracer: tracee ratio	0.0351 ± 0.0002	0.0083 ± 0.0002	<0.001

Values shown are mean ± 1 SD for FFA concentrations (μmol/mL). Identical aliquots of plasma were processed by the normal CH_3I/SPE method using hexane extraction, or by first extracting plasma lipids with chloroform–methanol 3:1 with subsequent CH_3I methylation and SPE.

The use of hexane for plasma lipid extractions was critical to avoid hydrolysis and/or transmethylation of plasma lipids when using the CH_3I/SPE procedure. The use of chloroform:methanol extraction solvent significantly increased concentrations for all FFA, particularly C16:0, C18:0, and C18:2 (**Table 2**). Palmitate concentration was 4-fold higher and palmitate enrichment was about 4-fold lower when chloroform–methanol extraction was used compared with hexane extraction (Table 2). Chloroform–methanol extraction of plasma spiked with chemically defined triglyceride containing C15:0, followed by CH_3I methylation and SPE, resulted in the formation of a large peak of C15:0 methyl ester identified by electron impact ionization GC/MS. This product was undetectable when

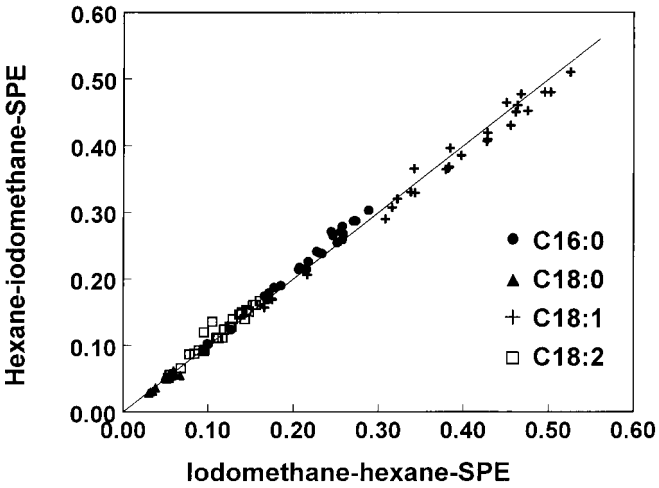

Fig. 3. Variations on the CH_3I/SPE method. Plasma samples that spanned a range of FFA concentrations were analyzed after reversing the order of methylation and hexane extraction steps. Samples were prepared as outlined in Methods (initial hexane extraction followed by CH_3I and SPE; ordinate) or by performing the CH_3I reaction on plasma lipids in situ followed by hexane extraction and SPE (abscissa). Results shown are measured FFA concentrations (μmol/mL). A line of identity is included for comparison.

the normal CH_3I/SPE procedure with hexane extraction was used (results not shown).

Identical results were obtained whether the CH_3I methylation reaction was performed on lipids that were first extracted from plasma by hexane or on lipids in situ within plasma with subsequent hexane extraction (**Fig. 3**); measured FFA concentrations did not differ significantly from the line of unity (Fig. 3).

FFA and FAME standards

A large sample of the NIH-D FFA standard with [$^2H_2$]palmitate and C17:0 internal standard added was prepared and identical aliquots were subjected to seven different treatments that either did or did not involve TLC. Four treatments which included TLC separations gave identical FFA concentrations and TTR values: methylation with BF_3/methanol directly on TLC scrapings, or three different extraction procedures of TLC scrapings (either single or triple extraction with chloroform–methanol 3:1, or single extraction with chloroform–methanol 2:1) followed by methylation with BF_3/methanol. Three treatments that did not involve TLC gave identical FFA concentrations and TTR values: methylation with BF_3/methanol without further separation, methylation with CH_3I without further separation, and methylation with CH_3I followed by separation by SPE. Separation by TLC resulted in significantly ($P \leq 0.0002$) higher concentrations for C16:0 and C18:0 compared with the non-TLC treatments (3.4% and 7.3%, respectively) and significantly ($P = 0.0002$) lower concentrations for C18:1 (4.6%). TLC and non-TLC procedures gave the same concentrations values for C14:0 and C16:1. The higher C16:0 concentration with TLC was associated with a 4.4% lower [$^2H_2$]palmitate isotopic enrichment (0.119 ± 0.003 vs. 0.125 ± 0.001, $P = 0.0003$).

Tests were performed to evaluate the specificity of FFA methylation with CH_3I and subsequent FAME separation by SPE. Methylation with CH_3I was specific for free fatty acids. Fatty acid methyl esters were not generated when large excesses of triglyceride, cholesteryl ester, or phospholipid standards were exposed to the CH_3I reaction conditions (results not shown).

The GLC-63 FAME standard was used to evaluate fractionation and recovery of FAME by SPE compared with TLC. Identical aliquots of the FAME standard in hexane were either placed in autosampler vials or dried, redissolved, recovered from SPE or TLC, and reconstituted to a constant final volume with hexane. Each aliquot was then analyzed by quantitative GC. FAME concentrations of all fatty acids were identical to the starting values when the GLC standard was recovered from SPE (**Fig. 4**). In contrast to the contamination with saturated FFA evident with the TLC method, blanks processed by using the SPE column generated undetectable levels of FAME (data not shown). Recovery of C17:0 methyl ester from SPE averaged $92 \pm 13\%$ as determined by quantitative GC compared to a GLC standard not subjected to SPE. In contrast, TLC resulted in decreased recovery of unsaturated FAME (C16:1, C18:1, C18:2, C18:3, C20:4) compared to the C17:0 internal standard, although saturated FAME recovery was unaltered. The reduced recovery of unsaturated FAME was observed for various extraction solvents including chloroform–methanol 3:1 or 1:1, chloroform, and ether (not shown). Recovery of C17:0 methyl ester determined by quantitative GC from TLC was significantly reduced ($54 \pm 7\%$; $P < 0.0001$) compared with SPE; no major differences were observed between extraction solvents.

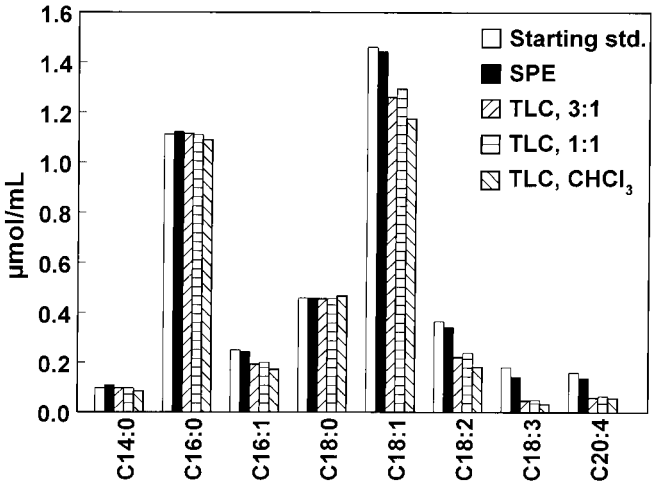

Fig. 4. Effect of SPE and TLC on FAME concentrations. The GLC-63 FAME standard (which contains C17:0 methyl ester internal standard) was analyzed directly (bars), after recovery from SPE (solid bars), or after recovery from TLC using extractions of chloroform–methanol 3:1 or 1:1, or chloroform alone (striped bars).

DISCUSSION

In this report we describe a new procedure for measuring plasma free fatty acid concentrations by quantitative GC and fatty acid isotopic enrichment by GC/MS. This procedure involves the use of iodomethane to methylate plasma FFA and SPE to separate FAME. Each step of the process was validated using chemically defined FFA or FAME standards, and shown to provide more reliable results than a conventional TLC/BF_3 method. The validation procedures are applied to the major components which comprise >95% of the plasma fatty acids; additional tests may be required to extend these studies to minor FFA species.

The new CH_3I/SPE method is specific for free fatty acids without contributions from esterified fatty acids. The derivatization of fatty acids with CH_3I is more convenient than with diazomethane, offering the advantage that CH_3I is commercially available and does not need on-site diazomethane generation (6). However, like diazomethane, CH_3I is a potent carcinogen and must be used in a hood. Furthermore, this approach was not compromised by the two major problems associated with TLC separation of

FFA, contamination of FFA and chemical fractionation of FFA.

We observed contamination with saturated FFA when TLC separation was used. This contamination was manifested as increased FFA concentrations, isotopic dilution of labeled palmitate, and the presence of background FFA when "blanks" were processed. Although subtraction of such background concentrations can improve the accuracy of the TLC/BF_3 method for determining FFA concentration, this contamination will lead to inaccurate enrichment measurements because of isotopic dilution.

Two types of FFA fractionation were observed when using TLC. First, chemical fractionation was identified by decreased recovery of unsaturated FFA relative to the C17:0 internal standard. This fractionation could not be circumvented by varying solvent extraction conditions. This problem was not caused by the use of BF_3/methanol as a methylation reagent, because similar problems were observed when FFA were extracted from TLC and then methylated with CH_3I. The second form of fractionation, spatial fractionation, was based on the observation that measured FFA concentrations (based on the ratio to the C17:0 internal standard) was affected by the size of the TLC band used for FFA recovery. Increasing the TLC band size increased the recovery of FFA relative to the recovery of the C17:0 internal standard. The apparent concentration of palmitate was affected by TLC spot size whereas the isotopic enrichment was not, suggesting that the differences in concentration were due to spatial separation between C16:0 and C17:0 rather than contamination with extraneous C16:0, whereas the deuterated C16:0 co-migrated with unlabeled C16:0. Spatial fractionation was evident for all FFA with the exception of stearate. Spatial fractionation can reduce between-run precision because determining the size of the visualized FFA band for TLC scrapings is subjective. In contrast to these problems of FFA separation by TLC, recovery of FAME by SPE using 2% ethyl acetate in hexane provided reproducible results without chemical fractionation and was not subject to contamination.

Two potential problems which could result in inaccurate FFA measurements by the new procedure were identified and avoided. First, chloroform–methanol should not be used to delipidate plasma because transmethylation of fatty acids may occur under these conditions. This results in exaggerated FFA concentrations (measured as methyl esters) and isotopic dilution of labeled palmitate because these additional methyl esters are formed before FAME are separated from other plasma lipids. Second, once formed, the FAME were separated by SPE rather than TLC because we observed that TLC resulted in decreased FAME recovery and chemical fractionation which was evident as decreased concentrations of unsaturated FAME relative to the C17:0 methyl ester internal standard.

The use of the TLC/BF_3 procedure to process plasma samples may affect the accuracy of assessing lipolytic rates in vivo by using fatty acid tracers. First, contamination with unlabeled FFA during TLC causes a dilution of palmitate isotopic enrichment by approximately 5–15%, depending on the concentration of FFA in the starting sample (Fig. 1). A 10% decrease in isotopic enrichment measured by TLC will result in an approximately 10% overestimation of the true palmitate rate of appearance (Ra), which is determined by dividing the infusion rate of labeled palmitate by the plateau isotopic enrichment (7). Second, accurate FFA concentration measurements are needed to convert palmitate Ra to total FFA Ra, which is determined by dividing palmitate Ra by the percent of total FFA present as palmitate. For example, an increase in the contribution of palmitate to total FFA from 27% (measured by CH_3I/SPE) to 30% (measured by TLC/BF_3) would result in a 10% decrease in calculated total FFA Ra. However, these two errors associated with TLC (isotopic dilution of palmitate combined with overestimation of the percent palmitate) tend to offset each other, so that the effect on total FFA Ra may be negligible. Furthermore, small systematic measurement errors are unlikely to affect the interpretation of data from studies that are designed to assess large differences or changes in FFA Ra, such as during fasting (8, 9) or exercise (10, 11). However, refinement of measuring plasma FFA concentration and enrichment may have a more significant impact in measurement of regional lipolysis by arterio-venous (A-V) difference (12), where systematic errors in methodology may result in significant under- or over-estimation of the true A-V difference.

In summary, the CH_3I/SPE method provides a simple and reliable method for determining plasma FFA concentration and enrichment. The method is not subject to the problems of contamination, reduced recovery, and fractionation associated with recovery of FFA by TLC.

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REFERENCES

1. Morrison, W. R., and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride–methanol. *J. Lipid Res.* **5:** 600–608.
2. Wolfe, R. R. 1992. Radioactive and Stable Isotope Tracers in Biomedicine: Principles and Practice of Kinetic Analysis. Wiley-Liss, New York.
3. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
4. Patterson, B. W., and R. R. Wolfe. 1993. Concentration dependence of methyl palmitate isotope ratios by electron impact ionization gas chromatography/mass spectrometry. *Biol. Mass Spectrom.* **22:** 481–486.
5. Patterson, B. W., G. Zhao, and S. Klein. 1998. Improved accuracy and precision of gas chromatography/mass spectrometry measurements for metabolic tracers. *Metabolism.* **47:** 706–712.
6. Pace-Asciak, C. R. 1989. One-step rapid extractive methylation of plasma nonesterified fatty acids for gas chromatographic analysis. *J. Lipid Res.* **30:** 451–454.
7. Steele, R. 1959. Influences of glucose loading and of injected insulin on hepatic glucose output. *Ann. NY Acad. Sci.* **82:** 420–430.

8. Klein, S., Y. Sakurai, J. A. Romijn, and R. M. Carroll. 1993. Progressive alterations in lipid and glucose metabolism during short-term fasting in young adult men. *Am. J. Physiol.* **265:** E801–E806.
9. Klein, S., V. R. Young, G. L. Blackburn, B. R. Bistrian, and R. R. Wolfe. 1986. Palmitate and glycerol kinetics during brief starvation in normal weight young adult and elderly subjects. *J. Clin. Invest.* **78:** 928–933.
10. Klein, S., E. F. Coyle, and R. R. Wolfe. 1994. Fat metabolism during low-intensity exercise in endurance-trained and untrained men. *Am. J. Physiol.* **267:** E934–E940.
11. Sial, S., A. R. Coggan, R. Carroll, J. Goodwin, and S. Klein. 1996. Fat and carbohydrate metabolism during exercise in elderly and young subjects. *Am. J. Physiol.* **271:** E983–E989.
12. Horowitz, J. F., S. W. Coppack, D. Paramore, P. E. Cryer, G. Zhao, and S. Klein. 1999. Effect of short-term fasting on lipid kinetics in lean and obese women. *Am. J. Physiol.* **276:** E278–E284.