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Determination of plasma non-esterified fatty acids and triglyceride fatty acids by gas chromatography of their methyl esters after isolation by column chromatography on silica gel

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Abstract

Non-esterified fatty acids (NEFA) and triglycerides were isolated from human plasma by column chromatography on silica gel. Eight principal fatty acids of each of these lipid classes were determined by gas chromatography of their methyl ester derivatives and quantified relative to multipoint standard curves. Within-day relative standard deviations for plasma non-esterified fatty acid and triglyceride fatty acid determinations were 2.4 and 3.2%, respectively. Day-to-day relative standard deviations for plasma non-esterified fatty acid and triglyceride fatty acid determinations were 1.4 and 1.1%, respectively. The total plasma concentration and the relative proportions of the eight non-esterified fatty acids determined by this method were significantly different from results obtained according to two generally accepted methods for direct plasma non-esterified fatty acid determination without a specific isolation step. These comparisons suggested that considerable fatty acid ester lipid hydrolysis occurred during these direct determination procedures, and that this hydrolysis resulted in 3-fold overestimation of plasma NEFA content by those methods. Measured levels of arachidonic acid are substantially overestimated by these direct determination methods in which non-esterified fatty acids are not isolated before derivatization.

1. Introduction

Many methods have been proposed for gas chromatographic determination of non-esterified fatty acids in biological samples. These methods generally involve solvent extraction of total sample lipids [1–5] and derivatization of NEFA [6] before gas chromatography. The total NEFA concentration in plasma of human subjects of normal health and nutritional status is about 3–10% of the total concentration of fatty acids (FA) contained by either the plasma triglycerides (TG) or the plasma total phospholipids [7]. Release of only 3% of fatty acids from any major plasma FA ester lipid class by any mechanism during sample preparation would therefore result in overestimation by 30–100% in the determined plasma NEFA concentration. The relative pro-

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portions of FA contained by the plasma cholesteryl ester and phospholipid fractions differ substantially from those found in the plasma NEFA [7]. Non-selective release of fatty acids from either of these ester lipid classes during sample preparation would distort the relative proportions of fatty acids determined as NEFA, as well as the total determined NEFA concentration.

Both NEFA and the constituent fatty acids of fatty acid ester lipids usually are determined by gas chromatography of their methyl ester derivatives. These derivatives usually are prepared by acid-catalyzed methanol acylation [6,8,9]. Reaction conditions for carboxylate methyl ester preparation by this reaction mechanism [6] and for acid-catalyzed methanolysis of fatty acid ester lipids [6,10] are similar. Some workers have described direct derivatization of NEFA in plasma extracts under selective methanol acylation conditions claimed to result in negligible rates of methanolysis of sample fatty acid ester lipids [11–15]. However, the sensitivity of these methods to small variation in reaction conditions [12,13,16] leaves some question concerning the accuracy of results obtained by these techniques. Preliminary isolation of NEFA could eliminate any question concerning the actual lipid class origin of fatty acids determined as NEFA. Consequently, others have chosen to separate sample NEFA from ester lipids [17–24] before derivatization and chromatographic determination. Fatty acids also can be derivatized by other carboxylate-specific reaction mechanisms under conditions expected to minimize the rate of competing fatty acid ester lipid hydrolysis. Diazomethane [25] and substituted diazomethanes [6] have been used for purported selective derivatization of NEFA in the presence of coextracted fatty acid ester lipids [6,11,26]. Recently, two groups have published comparisons [27,28] of plasma NEFA determination methods that involve preliminary chromatographic isolation of NEFA with methods for direct methanol acylation [13] or direct derivatization by reaction with diazomethane [15]. Authors who described selective isolation of NEFA before determination [24,29,30] generally reported lower total plasma NEFA concentrations and different relative proportions of plasma NEFA than those obtained by direct derivatization methods [13,15].

Large procedural blank values also can complicate plasma NEFA determination. Fatty acids are ubiquitous in nature, and they are constituents of commercial lubricants, surfactants, and cosmetics [31]. The cumulative blank contribution of palmitic and stearic acids during sample preparation can exceed the quantity of these compounds present as NEFA in small plasma specimens. This problem has been described by some authors whose sample isolation conditions and method validation experiments actually would disclose it [17,24,32,33]. Great technical care is required to minimize these blank values.

We developed a method for isolation of plasma NEFA and six other lipid classes by chromatography on small silica-gel columns [33]. The method produced no quantitatively significant NEFA procedural blank value in relation to the quantities of NEFA in 0.2 ml of human plasma. We report here the application of that plasma lipid separation technique to accurate determination of plasma NEFA and triglyceride fatty acids (TGFA) by gas chromatography of their methyl ester derivatives.

2. Experimental

2.1. Equipment

Gas chromatography of fatty acid methyl esters was accomplished with a Hewlett-Packard (Avondale, PA, USA) Model 5890 instrument equipped with a splitless capillary injection port, two flame ionization detectors, and a Model 7673 automated liquid sampler. A Hewlett-Packard Model 3365 chromatographic data system was used for collection and reduction of data from the gas chromatograph. A DuPont-Sorvall Model GLC-4 tabletop centrifuge (DuPont Laboratory Instruments, Newtown, CT, USA), a Big Vortex unit (Glas-Col Apparatus, Terre Haute, IN, USA), an Evapo-Rac sample evaporation unit (Cole-Parmer Instruments, Chicago, IL, USA), and a Multi-Blok heater (Labline Instruments, Melrose Park, IL, USA) were used for preparation of samples. All transfer of solvents

and samples during sample preparation was carried out with apparatus constructed entirely of glass. All glassware used in this procedure was rinsed thoroughly with HPLC-grade methanol or absolute ethanol and dried before use.

2.2. Materials

Merck silica gel type 60 (230–400 mesh, EM Science catalog number 9385-3) was obtained from Curtin-Matheson Scientific (Cleveland, OH, USA), and prepared for use in sample isolation columns as described previously [33]. Isooctane (HPLC grade), ethyl acetate, chloroform, methanol, diethyl ether, potassium hydroxide, hydrochloric acid and glacial acetic acid were obtained from Fisher Scientific (Cleveland, OH, USA). Ethanol was obtained from the hospital pharmacy. Acetyl chloride, 2,2-dimethoxypropane, and *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide were obtained from Aldrich (Milwaukee, WI, USA). Acetyl chloride was distilled before use. A standard mixture of tetradecanoic acid (myristic acid, $C_{14:0}$, 2% w/w), hexadecanoic acid (palmitic acid, $C_{16:0}$, 28% w/w), *cis*-9-hexadecenoic acid (palmitoleic acid, $C_{16:1}$, 7% w/w), octadecanoic acid (stearic acid, $C_{18:0}$, 3% w/w), *cis*-9-octadecenoic acid (oleic acid, $C_{18:1}$, 39% w/w), *cis*-9-*cis*-12-octadecadienoic acid (linoleic acid, $C_{18:2}$, 16% w/w), *cis*-9-, *cis*-12-, *cis*-15-octadecatrienoic acid (linolenic acid, $C_{18:3}$, 3% w/w), and *cis*-5-, *cis*-8-, *cis*-11-, *cis*-14-eicosatetraenoic acid (arachidonic acid, $C_{20:4}$, 2% w/w) in the approximate relative proportions of NEFA found in normal human plasma [7] was prepared and shipped in sealed ampoules by Nu-Check Prep (Elysian, MN, USA). Heptadecanoic acid, pentadecanoic acid, tripentadecanoin, and all other synthetic and natural lipid compounds used in preliminary phases of this work were purchased from Sigma (St. Louis, MO, USA). They were used without further purification. Solutions of diazomethane were prepared in diethyl ether [34] and used immediately.

Water was purified by passage through a MilliQ apparatus (Millipore, Bedford, MA, USA). Chloroform–methanol solution (3:1, v/v) and the phosphate buffer solution (0.67 *M*, pH 6.2) used for lipid extraction from plasma samples were prepared daily [33]. Air used for sample concentration was passed through a refrigerated gas drier at the compressor outlet and through a silica trap at the point of use in the laboratory.

Human plasma was obtained from healthy adults after an overnight fast. Blood samples were collected in evacuated blood collection tubes containing potassium EDTA, chilled immediately by immersion in an ice-water bath, and centrifuged under refrigeration (1°C) at 1200 *g*. The plasma was transferred to clean, stoppered glass tubes. Plasma not used immediately was stored at −80°C until needed.

2.3. Standard solutions

A solution of 800 μM tripentadecanoin in isooctane–ethyl acetate (75:25, v/v) was prepared for use as a recovery standard in the triglyceride fatty acid determination procedure. About 0.025 g of the commercial fatty acid standard mixture was weighed precisely by subtraction from sealed shipping ampoules and dissolved in 20.0 ml of isooctane–ethyl acetate (75:25, v/v). To this was added 10.0 ml of the 800 μM tripentadecanoin recovery standard solution to produce a stock solution that was used for TGFA standard curve establishment. About 0.025 g of the fatty acid standard mixture was weighed precisely and dissolved in 150.0 ml of isooctane–ethyl acetate (75:25, v/v) to give a stock solution for preparation of calibration standards for plasma NEFA determination. Two solutions of heptadecanoic acid were prepared at 800 μM and 100 μM in isooctane–ethyl acetate (75:25, v/v) for use in triglyceride fatty acid determination and non-esterified fatty acid determination, respectively.

2.4. Extraction and chromatographic isolation of plasma TG and NEFA

A 200-μl volume of the tripentadecanoin triglyceride recovery standard solution and 200 μl of the 100 μM heptadecanoic acid NEFA internal standard solution were added to 13 × 100 mm test tubes. The solvent was evaporated under a

stream of compressed air. Plasma sample aliquots of 200 μl, 0.3 ml of 0.67 *M* phosphate buffer solution, and 2.0 ml of chloroform–methanol (3:1, v/v) were added to these tubes. The tubes were vortex-mixed for 2 min, and then centrifuged at 1200 *g* to effect phase separation. The top aqueous phase was removed with a glass Pasteur pipette and discarded. The bottom organic phase was transferred with glass Pasteur pipettes to clean 13 × 100 mm glass test tubes and evaporated to dryness under a stream of compressed air. The dried extracts were reconstituted in mobile phase and subjected to column chromatographic isolation of TG and NEFA exactly as described previously [33]. The TG and NEFA fractions were collected in 13 × 100 mm glass tubes and dried under compressed air. Very large groups of liquid fractions occasionally were sealed and stored overnight at −80°C before concentration and subsequent procedural steps.

2.5. Derivatization of NEFA

The NEFA fraction residues were treated with 0.5 ml of methanol, 0.5 ml of 2,2-dimethoxypropane, 0.05 ml of water, and 0.02 ml of 12 *M* HCl. The tubes were vortex-mixed, covered with clean glass marbles, and left to stand for 30 min at room temperature [33]. The samples were concentrated to about 0.2–0.3 ml total volume, diluted with 0.2 ml of water, vortex-mixed with 0.2 ml of isooctane, centrifuged at 1200 *g* for 5 min to effect phase separation, and 0.05 ml of the organic phase was transferred to glass autosampler vial inserts. The vials were sealed with PTFE-lined rubber septa. Aliquots of 1 μl were injected onto the gas chromatographic system.

2.6. Saponification and derivatization of TGFA

The TG fraction residues were reconstituted in 0.2 ml of the 800 μM heptadecanoic acid internal standard solution and evaporated to dryness. These residues were treated in closed tubes with 0.3 ml of 0.5 *M* KOH in 95% ethanol at 85°C for 30 min. Samples were vortex-mixed once after half of this reaction time had elapsed. The samples were cooled, acidified by addition of 0.02 ml of concentrated hydrochloric acid, diluted with 0.2 ml of water, and extracted with 1.0 ml of isooctane by vortex-mixing for 2 min. The organic phase was transferred with a glass Pasteur pipette to clean 13 × 100 mm test tubes and evaporated to dryness under a stream of compressed air. These residues were derivatized and prepared for gas chromatography as described above for NEFA. Aliquots of 1 μl were injected onto the gas chromatographic system.

2.7. Calibration sample preparation

Varied volumes of the plasma NEFA calibration standard solution were transferred to 13 × 100 mm glass test tubes to give a set of five calibration samples which represented sample total fatty acid concentrations of 140–1670 μM. These samples were evaporated to dryness. To these residues were added 0.2 ml of water, 0.2 ml of the 100 μM heptadecanoic acid internal standard solution, and 0.3 ml of 0.67 *M* phosphate buffer solution. These solutions were extracted, evaporated to dryness, reconstituted, subjected to silica column chromatography [33], and derivatized exactly as described above for plasma specimens. All NEFA calibration samples were prepared and analyzed in duplicate.

Varied volumes of the TGFA calibration standard solution were transferred to 13 × 100 mm glass test tubes to give a set of five calibration samples which represented sample total triglyceride fatty acid concentrations of 1255–6275 μM. These samples were evaporated to dryness. These residues were reconstituted in 0.2 ml of 800 μM heptadecanoic acid standard solution, dried, and extracted as described above. The extracts were dried, saponified, derivatized, and prepared for gas chromatographic analysis as described for plasma TGFA. All TGFA calibration samples were prepared and analyzed in duplicate.

2.8. Gas chromatography

Gas chromatography of carboxylate methyl esters was accomplished simultaneously on two 15-m fused-silica capillary columns coupled to

one injection port through a 2-hole polyimide/graphite ferrule (Supelco, Bellefonte, PA, USA). One of these columns contained SP-2330 cyanopropyl polysiloxane (0.25 mm I.D., 0.20 μm film thickness; Supelco). The second column contained HP-1 dimethylpolysiloxane (0.20 mm I.D., 0.33 μm film thickness; Hewlett-Packard, Kennett Square, PA, USA). Helium was used as the carrier and flame-ionization detector make-up gas at flow-rates of 1.5 ml/min and 36 ml/min, respectively. The injection port and detector temperatures were 230°C and 250°C, respectively. The initial column oven temperature was 60°C. Sample injection initiated a programmed column oven temperature increase by 30°C/min to a final temperature of 240°C. The column oven was maintained at 240°C for 5 min to complete the separation of fatty acid methyl esters.

2.9. Quantitation

Peak heights of the fatty acid methyl esters and the internal standard heptadecanoic acid methyl ester in the calibration sample chromatograms were measured by the data system. Fatty acid:heptadecanoic acid methyl ester peak-height ratios were calculated, and standard curves of these peak-height ratios vs. calibration sample fatty acid concentration were constructed. The sample concentration of each non-esterified or triglyceride fatty acid in experimental samples was calculated by interpolation from the peak-height ratio found for that acid in relation to the algebraic least squares regression line equation found for the same fatty acid in the corresponding set of calibration standard results. Pentadecanoic acid methyl ester derived from the tripentadecanoin TGFA recovery standard was determined in each sample prepared for TGFA determination. The quantity of pentadecanoic acid recovered in each sample was compared with the quantity of pentadecanoic acid added initially to the sample as tripentadecanoin. This ratio was used to correct the determined triglyceride fatty acid concentrations in each sample for loss during extraction and column chromatography. All calibration standard and experimental samples were prepared and analyzed in duplicate.

2.10. Statistical calculations

Statistical calculations and group comparisons were carried out with the microcomputer program SigmaStat (Jandel Scientific, San Rafael, CA, USA). Arithmetic means and population standard deviation estimates were calculated for each determined fatty acid and for sums of determined fatty acids in each group of sample replicates. Sets of fatty acid concentration measurements intended for group treatment comparisons were subjected to the Komolgorov/Smirnov normality and Levene median equal variance tests. Pairs of data sets which did not satisfy normality and equal variance tests were compared by the Mann–Whitney rank sum test. Kruskal–Wallis ANOVA on ranks and subsequent Student–Newman–Keuls all-pairwise comparison of groups were applied to data sets obtained during experimental comparisons of three treatments.

2.11. Comparison of NEFA determination procedures

Plasma was prepared from blood of one adult male volunteer as described above, transferred to separate storage tubes, and frozen. On three successive days, one of these tubes was thawed, and 10 replicate aliquots of 200 μl of this plasma were transferred to glass test tubes. The first group of 10 replicate samples then was subjected to NEFA determination by a published direct-derivatization procedure with methanol–acetyl chloride [13]. The second group of aliquots was prepared and analyzed according to a published procedure for direct NEFA determination in plasma extracts by reaction with diazomethane [15]. The remaining group was prepared for NEFA determination by extraction, column chromatography on silica gel, and reaction with methanol–HCl as described in this report. NEFA methyl esters were determined by gas chromatography as described above in each of these experiments (see Table 1).

2.12. Comparison of derivatization methods

One plasma specimen prepared from blood of one human male volunteer was thawed. Thirty aliquots of 200 μl were transferred to glass test tubes. NEFA were isolated from all of these samples by extraction and column chromatography as described above. The isolated NEFA fractions were separated into 3 groups of 10 replicates. One of these groups was derivatized by reaction with methanol–acetyl chloride [13]. The second group was subjected to reaction with diazomethane [15]. The remaining group was derivatized by reaction with methanol–HCl as described above. NEFA methyl ester derivatives were concentrated, transferred to sealed vials, and determined by gas chromatography in each of these treatment groups as described above (see Table 2).

2.13. Comparison of evaporation procedures

Standard NEFA (2304 μ*M*) were added to each of 20 replicate plasma aliquots. These were divided into 2 groups of 10 replicates. TG and NEFA were extracted and isolated by column chromatography. TGFA and NEFA were derivatized and determined in both groups by gas chromatography as described above. All evaporation steps applied to one of these groups were carried out with dry nitrogen. All evaporation steps applied to the remaining group were carried out with compressed air (see Table 3).

2.14. Lipid class origin of excess directly determined NEFA

Standard solutions of commercial cholesteryl pentadecanoate, tripentadecanoin, and dipentadecanoyl phosphatidyl choline were examined for contamination by non-esterified pentadecanoic acid. In these experiments 6 replicate aliquots of one ester lipid standard solution were applied to silica-gel columns. The NEFA fraction was eluted, concentrated, derivatized, and analyzed by gas chromatography as described above. In subsequent experiments, about 60 nmol of a single synthetic pentadecanoate ester lipid compound was added to each of 18 replicate 200-μl aliquots of one plasma specimen. Six of these samples were subjected to NEFA determination as described above. Six samples were subjected to direct NEFA determination by reaction with methanol–acetyl chloride [13]. The remaining group of 6 replicates was subjected to direct NEFA determination by reaction with diazomethane [15]. Pentadecanoic acid was determined in each of these treatment groups. The quantity of pentadecanoic acid determined in each sample was compared with the quantity of pentadecanoic acid added as pentadecanoate ester at the outset of the experiment. This provided an estimate of the extent of release of fatty acids from the lipid class represented by the added synthetic ester lipid.

2.15. Method precision study

One frozen plasma sample was thawed, and 6 aliquots of 200 μl of this sample were transferred to glass test tubes. These sample replicates were subjected to TGFA and NEFA determination as described above. The experiment was repeated twice on consecutive days with separately frozen samples of the same plasma specimen.

3. Results and discussion

Some time ago, we planned a series of stable-isotope-labeled fatty acid turnover experiments. For this purpose a method was needed for the specific determination of NEFA in plasma without dilution by fatty acids originating either from fatty acid ester lipid solvolysis during sample preparation [28] or through large procedural blank contamination by palmitic and stearic acids [17,19,32]. Thin layer chromatography for sample lipid separation was rejected after considering a report of oxidative losses of certain fatty acids from some lipid classes during isolation by TLC [32] and the difficulty of location and efficient recovery of separated lipid classes from TLC plates.

We developed and described a method for extraction and chromatographic isolation of

cholesteryl esters, triglycerides, cholesterol and diglycerides, NEFA, monoglycerides, ethanolamine phospholipids, and choline phospholipids from human plasma specimens [33]. This sample preparation method provides highly reproducible overall recoveries of the named lipid classes. The procedural blank concentration values for palmitic and stearic acids in the NEFA fraction isolated by this procedure are quantitatively insignificant. This isolation procedure is reliable when it is performed with carefully cleaned glass apparatus and with the materials specified. Evaporation steps carried out to dryness should be stopped immediately when sample tubes contain no visible liquid.

We used a commercial mixture of the eight principal non-esterified fatty acids of human plasma in their expected approximate relative proportions [7] for preparation of calibration standard solutions. We used heptadecanoic acid as a procedural internal standard for fatty acid determination. Line equations for the NEFA calibration results were linear with near-zero y-intercept values for the saturated n-carboxylates. Line equations obtained for linolenic and arachidonic acids were linear, but they gave negative y-intercept values proportional to sample concentrations of about 3 μM for each of these acids. This result suggested that some small but finite procedural loss of these fatty acids occurs before detection. We performed a series of experiments in which varied amounts of linolenic and arachidonic acids were added to plasma sample aliquots and then determined according to this procedure. Plots of determined concentrations of these fatty acids vs. concentration added were linear, with y-intercepts equal to the determined concentration of each respective fatty acid in the untreated plasma specimen. These losses of linolenic and arachidonic acids were negligible in relation to TGFA standard curves and the comparatively large quantities of TGFA in plasma.

We used synthetic tripentadecanoin as a recovery standard for determination of plasma TGFA. We made a standard addition of tripentadecanoin to every plasma specimen before extraction and isolation of sample triglycerides. The TGFA calibration standards were subjected to the saponification reaction to control for any loss during that procedure. Pentadecanoic acid derived from the tripentadecanoin added to plasma specimens then was determined with other TGFA. The determined quantity of pentadecanoic acid in each sample was compared with the quantity added initially to the plasma specimens as tripentadecanoin. This ratio was used to correct for triglyceride loss from each sample during the extraction and column chromatographic sample preparation steps. The overall recovery of pentadecanoic acid derived from tripentadecanoin in TGFA determination experiments typically was 60–70%.

We compared our NEFA determination method with two popular methods for plasma NEFA determination without preliminary isolation [13,15]. We carefully applied all three determination methods to replicate groups of 10 aliquots of one plasma specimen. The results of this experiment are represented in Table 1. Direct derivatization of plasma NEFA by either derivatization procedure gave total NEFA concentration values of eight principal plasma fatty acids which were about 3-fold greater than those obtained after isolation of NEFA from the same plasma by silica-gel column chromatography. The molar fraction of arachidonic acid determined as NEFA by the direct procedures was about 4-fold greater than that found by our method. We then isolated NEFA from three replicate groups of 10 plasma aliquots and applied one derivatization procedure to each group. The results obtained using our methanol–HCl acylation procedure, the acetyl chloride–methanol acylation procedure [13], and derivatization by reaction with diazomethane [15] are presented in Table 2. The derivatization method-dependent concentration differences shown in Table 2 are statistically significant by ANOVA on ranks ($p < 0.05$) except for linolenic acid ($C_{18:3}$). However, these apparent reaction condition-dependent variations in methyl ester derivative yield or selective loss of polyunsaturated fatty acids during the derivatization reactions are far smaller than the 3-fold concentration differences shown in Table 1. Thus, the data pre-

Table 1
Comparison of plasma NEFA concentrations determined after chromatographic isolation and by two previously published methods for direct determination

Fatty acid	Present method		Previously published direct methods			
			Acetyl chloride–methanol [13]		Diazomethane [15]	
	μM	mol%	μM	mol%	μM	mol%
$C_{14:0}$	6 ± 3	2	17 ± 6	<2	7 ± 3	1
$C_{16:0}$	82 ± 1	25	301 ± 2	27	354 ± 120	30
$C_{16:1}$	15 ± 2	5	43 ± 3	4	33 ± 10	3
$C_{18:0}$	30 ± 1	9	91 ± 11	8	160 ± 60	14
$C_{18:1}$	134 ± 1	40	307 ± 2	27	275 ± 85	23
$C_{18:2}$	57 ± 1	17	289 ± 2	26	237 ± 95	20
$C_{18:3}$	3 ± 2	<1	6 ± 1	1	4 ± 1	<1
$C_{20:4}$	6 ± 4	2	64 ± 9	6	101 ± 42	9
Sum	333 ± 3	100	1118 ± 5	100	1171 ± 30	100

Replicate groups of 10 aliquots of plasma from one healthy, fed human adult male were subjected to NEFA determination according to the method described in this report and also according to two recently published methods for direct determination of NEFA by reaction with acetyl chloride–methanol [13] or by reaction with diazomethane [15]. Concentration data are expressed as mean ± standard deviation ($n = 10$). Molar fractions (mol%) are given as percent of the total concentration of the eight named fatty acids. Individual and sums of plasma NEFA concentrations obtained by the method described here differed significantly from those obtained by either direct determination method ($p < 0.0005$).

Table 2
Effect of derivatization reaction conditions on fatty acid concentrations determined after isolation by column chromatography on silica gel

Fatty acid	Present method		Derivatization method			
			Acetyl chloride–methanol [13]		Diazomethane [15]	
	μM	mol%	μM	mol%	μM	mol%
$C_{14:0}$	4 ± 1	1	7 ± 1	2	n.d.	
$C_{16:0}$	64 ± 1	24	72 ± 4	24	42 ± 9	16
$C_{16:1}$	6 ± 1	2	8 ± 1	3	1 ± 2	<1
$C_{18:0}$	22 ± 1	8	24 ± 2	8	29 ± 6	11
$C_{18:1}$	90 ± 3	33	95 ± 3	32	101 ± 11	38
$C_{18:2}$	75 ± 2	28	81 ± 3	27	81 ± 9	31
$C_{18:3}$	3 ± 0	1	4 ± 1	1	2 ± 3	1
$C_{20:4}$	6 ± 0	<3	5 ± 0	<3	7 ± 1	3
Sum	270 ± 5	100	296 ± 12	100	263 ± 24	100

NEFA were isolated from 3 groups of 10 aliquots of human plasma by extraction and column chromatography on silica gel according to the procedure described in this report. Each group was derivatized as indicated, and sample fatty acid concentrations were determined by gas chromatography of their methyl ester derivatives. Data are given as mean μM concentrations ± standard deviation ($n = 10$, n.d.: not detected). Molar fractions (mol%) are given as percent of the total concentration of the eight named fatty acids. Concentration differences are significant ($p < 0.05$) by ANOVA on ranks except for linolenic acid ($C_{18:3}$).

sented in Table 2 show clearly that the method-dependent differences in determined plasma NEFA concentrations displayed in Table 1 do not result primarily from differences among the three derivatization procedures.

We performed experiments in which either cholesteryl pentadecanoate, tripentadecanoin, or dipentadecanoyl phosphatidylcholine was added to each of six replicate plasma specimens before determination of NEFA by the direct determination procedures (data not shown). We determined the pentadecanoic acid concentration in each sample and compared the determined quantity with that added initially as synthetic lipid. This calculation provided an estimate of the extent of release of fatty acids by all operative mechanisms during the determination procedure from the lipid class represented by the added synthetic lipid. In the direct acetyl chloride–methanol acylation procedure, we observed quantities of pentadecanoic acid proportional to release and derivatization of 5% of plasma cholesteryl ester fatty acids, 3% of plasma triglyceride fatty acids, and 15% of plasma phosphatidylcholine fatty acids. This result is similar to that reported by Hallaq et al. [28], who described extensive phospholipid hydrolysis during direct determination of plasma NEFA by the acetyl chloride–methanol acylation procedure [13]. Similarly, we applied the direct diazomethane procedure [15] to plasma specimens containing added synthetic lipids. We observed even greater release of pentadecanoic acid from added dipentadecanoyl phosphatidylcholine. This result corresponds with reports by Borra et al. [17] and by Lin et al. [27] of significant release of fatty acids from plasma phospholipids during direct determination of plasma NEFA by reaction with diazomethane. This evidence demonstrates that fatty acids can be released from plasma phospholipids during some procedures for direct plasma NEFA determination. The quantities of fatty acids so released are at least similar to those present initially as NEFA in the plasma specimen. We conclude that plasma NEFA concentrations determined by either of these direct derivatization methods without preliminary isolation of NEFA should be regarded as inaccurate in the absence of definite contrary evidence in each analyzed sample. We believe that isolation of NEFA from plasma fatty acid ester lipids is fundamental to accurate determination of NEFA.

Early in this work we were surprised by an incidental finding of no practical difference in determined plasma NEFA concentrations when air was substituted for nitrogen during sample evaporation steps. We confirmed this finding in experiments in which about 2300 μM of NEFA were added to twenty replicate aliquots of one plasma specimen. This addition was intended to reveal small losses of polyunsaturated NEFA which are present in plasma at very low concentrations. The samples were divided into two equal groups. NEFA and TGFA were determined in both groups. All concentration steps applied to one group of samples were carried out with compressed air. All sample concentration steps applied to the remaining treatment group were carried out with dry nitrogen. The results of this experiment are summarized in Table 3. Concentration differences observed for NEFA were significant by Mann–Whitney rank-sum comparison ($p < 0.05$) only for palmitoleic acid ($C_{16:1}$), stearic acid ($C_{18:0}$), and oleic acid ($C_{18:1}$). Concentration differences observed for triglyceride fatty acids were not significant ($p >= 0.05$). We also experimented with nitrogen for evaporation of the calibration samples during concentration steps. This did not improve the slightly negative y-intercept values obtained for some of the unsaturated fatty acid calibration standard curves.

These results were surprising in relation to many published reports of selective loss of polyunsaturated fatty acids or general loss of fatty acids from samples prepared without exclusion of oxygen. The column chromatographic lipid separation procedure employed in our work permits isolation of NEFA and ester lipids in discrete liquid fractions of volatile organic solvents. Evaporation of these fractions proceeds rapidly, and we halt the evaporation immediately when no visible liquid remains in the test tubes used for fraction collection. Fatty acids and ester lipids isolated in this way are distributed over

Table 3
Effect of use of air or nitrogen for sample concentration on determined plasma NEFA and TGFA concentrations

Fatty acid	Concentration (mean ± S.D.) (μM)					
	NEFA			TGFA		
	Nitrogen	Air	p	Nitrogen	Air	p
$C_{14:0}$	57 ± 6	57 ± 1	n.s.	64 ± 2	65 ± 1	n.s.
$C_{16:0}$	757 ± 8	762 ± 8	n.s.	976 ± 24	989 ± 21	n.s.
$C_{16:1}$	183 ± 2	180 ± 2	0.026	105 ± 13	100 ± 12	n.s.
$C_{18:0}$	96 ± 1	102 ± 2	0.0001	175 ± 4	179 ± 5	n.s.
$C_{18:1}$	990 ± 10	975 ± 10	0.021	1638 ± 46	1631 ± 35	n.s.
$C_{18:2}$	463 ± 5	458 ± 5	n.s.	1223 ± 29	1210 ± 29	n.s.
$C_{18:3}$	80 ± 1	78 ± 2	n.s.	58 ± 2	57 ± 2	n.s.
$C_{20:4}$	60 ± 3	61 ± 2	n.s.	42 ± 4	44 ± 4	n.s.
Sum	2686 ± 27	2673 ± 27	n.s.	4281 ± 111	4275 ± 97	n.s.

Fatty acids (2304 μM) were added to each of 2 groups of 10 replicate plasma specimens. NEFA and TGFA were extracted, isolated by column chromatography on silica gel, derivatized, and determined by gas chromatography as described in this report. All evaporation steps during sample preparation were carried out with nitrogen or with air as indicated. Mean concentrations are given as μM ± standard deviation ($n = 10$). Error probabilities returned by Mann–Whitney rank-sum comparison are given where $p < 0.05$; n.s.: not significantly different, $p > = 0.05$.

about half of the bottom surface of the test tubes used for fraction collection. TLC has been used most often for isolation of NEFA and ester lipids. TLC separation ultimately distributes isolated FA and ester lipids over the effectively far larger surface area of particulate chromatographic adsorbents. We did not attempt to identify specific factors in our procedure which provide apparent effective protection against oxidative losses of fatty acids suggested by the data shown in Table 3. We chose to use compressed air for the method developmental work described in this report after confirming the results shown in Table 3. However, we use nitrogen for sample evaporation in experimental applications of this determination method.

The precision of the method was studied by six replicate determinations of NEFA and TGFA in one plasma specimen on three consecutive days. Relative standard deviations for NEFA and TGFA determinations were 2.4% and 3.2%, respectively, for one assay event. This within-day precision of the method also is implied by the data shown in Tables 1–3. Episodic relative standard deviations were 1.4% and 1.1%, respectively, for three assay repetitions.

We applied the NEFA and TGFA determination method to plasma specimens obtained from 28 healthy adults after an overnight fast. These results are presented in Table 4. The average total plasma concentration of the non-esterified fatty acids measured in this experiment is about one-half of that obtained by a direct NEFA determination method used in a similar study of normal subjects [13]. The average molar fraction of non-esterified arachidonic acid determined by our method is approximately one-third of that reported in the same study.

The procedure reported here combines an efficient small-scale column chromatographic procedure for isolation of plasma NEFA with familiar techniques for methyl ester derivatization and gas chromatographic analysis. We have extended our method for TGFA determination to measurement of plasma cholesteryl ester fatty acid concentrations by using cholesteryl pentadecanoate as a procedural recovery standard for the isolation steps. We have applied these de-

Table 4
Plasma NEFA and TGFA concentrations in 28 healthy adult human subjects after an overnight fast

Fatty acid	NEFA concentration			TGFA concentration		
	Mean ± S.D. (μM)	Range (μM)	mol%	Mean ± S.D. (μM)	Range (μM)	mol%
$C_{14:0}$	8 ± 3	4–16	<3	44 ± 21	12–87	2
$C_{16:0}$	79 ± 37	27–146	24	492 ± 202	187–940	24
$C_{16:1}$	17 ± 10	5–46	5	97 ± 50	29–207	5
$C_{18:0}$	29 ± 10	14–52	9	55 ± 26	25–127	3
$C_{18:1}$	121 ± 55	44–233	37	801 ± 32	365–1419	39
$C_{18:2}$	63 ± 30	20–120	19	470 ± 205	221–921	23
$C_{18:3}$	7 ± 4	1–5	2	33 ± 19	10–82	<2
$C_{20:4}$	3 ± 2	0–7	1	59 ± 29	22–139	3
Sum	327 ± 145	122–576	100	2051 ± 785	897–3523	100

Blood samples were obtained from 28 healthy male and female adult human volunteers after an overnight fast. The samples were processed immediately for determination of plasma NEFA and TGFA as described in the text. Mean concentrations of NEFA are given as μM ± standard deviation ($n = 28$). Concentration ranges are given as the minimum and maximum μM values observed in this population sample. Molar fractions (mol%) are given as percent of the total concentration of the eight named fatty acids.

termination methods to several hundred samples obtained in the course of a fatty acid turnover study in human subjects. The results of that study will be reported elsewhere.

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