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Rapid quantitation of free fatty acids in human plasma by high-performance liquid chromatography

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Abstract

We report a rapid and sensitive method for separation and quantitation of free fatty acids (FFAs) in human plasma using high-performance liquid chromatography (HPLC). Two established techniques of lipid extraction were investigated and modified to achieve maximal FFA recovery in a reasonably short time period. A modified Dole extraction method exhibited greater recovery (~90%) and short processing times (30 min) compared to the method of Miles et al. Reversed-phase HPLC using UV detection was used for plasma FFA separation and quantitation. Two phenacyl ester derivatives, phenacyl bromide and *p*-bromophenacyl bromide, were investigated in order to achieve optimal separation of individual plasma FFAs (saturated and unsaturated) with desirable detection limits. Different chromatographic parameters including column temperature, column type and elution profiles (isocratic and gradient) were tested to achieve optimal separation and recovery of fatty acids. Phenacyl bromide esters of plasma fatty acids were best resolved using an octadecylsilyl column with endcapped silanol groups. An isocratic elution method using acetonitrile–water (83:17) at 2 ml/min with UV detection at 242 nm and a column temperature of 45°C was found to optimally resolve the six major free fatty acids present in human plasma (myristic [14:0], palmitic [16:0], palmitoleic [16:1], stearic [18:0], oleic [18:1] and linoleic [18:2]), with a run time of less than 35 min and detection limits in the nmol range. The entire process including plasma extraction, pre-column derivatization, and HPLC quantitation can be completed in ~90 min with plasma samples as small as 50 μl. Over a wide physiological range, plasma FFA concentrations determined using our HPLC method agree closely with measurements using established TLC–GC methods ($r^2 \geq 0.95$). In addition, by measuring [^{14}C] or [^{3}H] radioactivity in eluent fractions following HPLC separation of plasma FFA, this method can also quantitate rates of FFA turnover in vivo in human metabolic studies employing isotopic tracers of one or more fatty acids.

Keywords: Free fatty acids; Phenacyl esters

1. Introduction

Long-chain fatty acids are an important source of oxidative fuel [1,2] and also act as mediators of signal transduction [3,4]. Rapid and non destructive measurement of individual free (unesterified) fatty acid concentrations in plasma is an important step in studying lipid metabolism. Although free fatty acids (FFAs) represent a relatively small fraction of total fatty acids present in the circulation, they are the most actively metabolized lipid substrates and are responsible for a significant amount of energy release through oxidation. The predominant source of FFAs in plasma are the triacylglycerol stores in adipose

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tissue. Because long chain fatty acids are hydrophobic and have low solubility at physiological pH, FFAs are transported in the circulation bound to serum albumin. The circulating concentrations of FFAs can vary significantly under metabolic conditions characterized by low lipolytic activity (e.g., hyperinsulinemia [5,6]) or high lipolytic activity (e.g., fasting [7] or exercise [8,9]). A fast and accurate method for determination of concentrations of individual fatty acids present in plasma is essential for studies of in vivo FFA metabolism.

Analysis of individual FFAs in plasma is complicated by the fact that FFAs do not contain chromophores to facilitate detection in the ultraviolet–visible (UV–Vis) or fluorescent domains. Traditionally gas chromatography (GC) or thin-layer chromatography (TLC) coupled with GC has been used for fatty acid quantitation [10,11]. GC methods require high temperatures that may not be appropriate for accurately quantifying thermally labile moieties like polyunsaturated fatty acids. High-performance liquid chromatography (HPLC) offers the advantages of speed, resolution, high sensitivity and specificity. A major advantage of HPLC over GC is the lower temperature during analysis, which reduces the risk of isomerization of unsaturated fatty acids [12]. HPLC also facilitates the collection of desired fractions of separated components for further analysis, which is essential in isotope dilution studies using radiolabeled fatty acids.

Advances in derivatization of fatty acids to provide easier and sensitive detection groups has led to the development of many methods employing HPLC. Toyo'oka [13] presents an excellent review of the different techniques and tagging agents available for derivatization of fatty acids for UV–Vis, fluorescent, chemiluminescent and electrochemical detection. A number of ultraviolet sensitive derivatives have been used for HPLC analysis of fatty acids from a variety of sources. These derivatives include benzyl [14], *p*-nitrobenzyl [15,16], phenacyl [17–20], *p*-bromophenacyl [21,22], *p*-methylthiobenzyl [23], *p*-phenylazophenacyl [24] and 1-napthylamine [25] esters. Use of such UV sensitive derivatives has made detection and quantitation of various FFAs possible with a high degree of sensitivity.

However determination of individual fatty acid concentrations in plasma by HPLC is still a time consuming process. Prior to derivatization and analysis, FFAs must be extracted from the aqueous phase for better chromatographic separation. The extraction of lipids and fatty acids from plasma has been studied by many researchers previously [26,21,27]. We investigated and modified established techniques of fatty acid extraction to optimize recovery of FFAs in a reasonable amount of time. We also modified existing techniques of derivatization and HPLC separation [27,28] to achieve optimized chromatographic separation of the six major FFAs present in plasma in a relatively short time (35 min) with a detection limit in the nmol range. These six fatty acids, myristic (14:0), palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), and linoleic (18:2) represent approximately 99% of the total FFAs present in plasma [5,9]. The overall process from extraction to quantitation took approximately 90 min. Plasma FFA measurements using our optimized HPLC method correlated well with established TLC–GC analytical methods.

2. Experimental

2.1. Reagents

Individual fatty acids (myristic, palmitic, palmitoleic, stearic, oleic and linoleic acids) and α-bromoacetophenone (phenacyl bromide) were purchased from Sigma (St. Louis, MO, USA). Triethylamine (spectra grade), acetone, acetic acid were obtained from Fisher Scientific (Pittsburgh, PA, USA). Acetonitrile, *n*-heptane and ''Omni-solve'' water (all HPLC grade) were purchased from EM Science (Gibbstown, NJ, USA). 2-Propanol, 18-Crown-6 ''gold label'' and phosphoric acid were purchased from Aldrich (Milwaukee, WI, USA). α,*p*-Dibromoacetophenone (*p*-bromophenacyl bromide) was from Fluka (Ronkonkoma, NY, USA) and [$^{2}H_{31}$]-palmitic acid was obtained from MSD isotopes (Montreal, Canada). Ultima Gold LSC cocktail was purchased from Packard Instrument (Meriden, CT, USA). [1-^{14}C]- Palmitic acid, [9,10-^{3}H]-palmitic acid and [3-^{3}H]-glucose were purchased from New England Nuclear (Boston, MA, USA).

2.2. Extraction

Plasma used for FFA determination was stored at −70°C and thawed to room temperature prior to extraction. All glassware was rinsed with acetone and air dried prior to use to prevent contamination of samples from exogenous sources of fatty acids like soaps. To quantify the efficiency of extraction and derivatization, plasma samples were spiked with a known amount of [1-^{14}C]- or [9,10-^{3}H]-palmitate (approximately 200 000 dpm). Recovery of [^{14}C] or [^{3}H]-palmitate was measured by determining the palmitate radioactivity (dpm) after the extraction, derivatization and HPLC separation steps. Separation of the organic from the aqueous components of human plasma by the extraction procedure was assessed by spiking human plasma samples with [9,10-^{3}H]-palmitate, [3-^{3}H]-glucose, or both. Radioactivity (dpm) in the organic extracts of the spiked samples was measured by liquid scintillation counting and compared to extracts of unspiked plasma and to heptane blanks. Two extraction methods were tested as described below.

2.2.1. Modified Dole extraction

This method was modified from the method suggested by Puttmann et al. [28] and is based on the Dole and Meinertz extraction procedure [26]. The extraction solvent was prepared by mixing isopropanol–heptane–phosphoric acid (2 *M*) (40:10:1, v/v) and was thoroughly stirred before use. Human plasma (0.5 ml) was mixed with 50 μl [$^{2}H_{31}$]-palmitic acid in *n*-heptane (500 μ*M*), used as an internal standard, in an 8-ml Pyrex glass tube (Corning, NY, USA) with PTFE lined screw caps. Extraction solvent (2.5 ml) was added and the tubes were thoroughly vortexed. The tubes were then immersed in a sonicator water bath (Branson Ultrasonic, Danbury, CT, USA) and the samples were sonicated in 30 s intervals for 2 min. Care was taken to avoid heating the samples during sonication. The samples were then vortexed rigorously and allowed to incubate at room temperature for 10 min. Heptane (1 ml) and water (1.5 ml) were added and the tubes were thoroughly vortexed and sonicated again for one min. Tubes were centrifuged at 1000 *g* for 10 min at 4°C. After centrifugation the top organic layer, which was yellowish in color, was seen to separate cleanly from the milky aqueous layer. A 1.5-ml aliquot (88% of the total organic layer of 1.7 ml) of the top layer was transferred carefully using a pipettor to 2.0 ml reactivials (Supelco, Bellefonte, PA, USA) and dried under a stream of nitrogen (N-Evap; Organomation, Berlin, MA, USA). These dried samples were then used for derivatization.

2.2.2. Miles extraction

This method was used by Miles et al. [27] to extract FFAs from dog plasma. Briefly the extraction solvent was prepared by mixing chloroform–heptane–methanol (56:42:2, v/v). To 0.5 ml of plasma, 50 μl of [$^{2}H_{31}$]-palmitic acid in *n*-heptane (500 μ*M*), used as internal standard, was added. The plasma samples were extracted by adding 10 ml of the extraction solvent and 200 μl of HCl (2 *M*) and mixing them thoroughly on a rotary mixer (Glass-Col, Terre Haute, IN, USA) for 1 h. The samples were then centrifuged in an inverted position at 800 *g* for 15 min at 4°C. The tubes were carefully removed and frozen inverted at −70°C overnight. The organic phase was transferred to polypropylene tubes, dried under a stream of nitrogen to a volume of 1–1.5 ml, and then transferred to 2-ml reactivials and dried completely.

2.3. Derivatization

Phenacyl ester derivatives of fatty acids formed by using phenacyl bromide [20] and *p*-bromophenacyl bromide [21,28] were investigated to determine which reaction yielded better derivatization efficiency. The phenacyl ester derivatives were also tested to determine which derivative exhibited better chromatographic separation of the individual fatty acids with desirable detection limits. The reaction for preparation of phenacyl esters using α-bromoacetophenone (phenacyl bromide) can be written as:

$$C_6H_4COCH_2Br + RCOOH + R'_3N \rightarrow C_6H_4COCH_2OCOR + R'_3NH^+Br^-$$

A tertiary amine, triethylamine, was used to catalyze the reaction and scavenge HBr from the

medium. The reaction for *p*-bromophenacyl bromide derivatization proceeds similarly, except 18-Crown-6 was used as a phase transfer catalyst with $KHCO_3$.

2.3.1. Phenacyl bromide derivatives

This method was modified from the procedure used by Wood and Lee for analyzing fatty acids in seed oils [20]. α-Bromoacetophenone (20 mg/ml), triethylamine (25 mg/ml) and acetic acid (10 mg/ml) were all dissolved in acetone and stored at 4°C. Fifty μl of α-bromoacetophenone solution and 50 μl of triethylamine solution were added to the dried lipid extract in the reactivials which were then capped tightly and vortexed. These vials were placed in a heater block (Fisher Scientific) and heated at 100°C for 15 min. The vials were allowed to cool and 75 μl of acetic acid was added. The vials were then re-capped and vortexed, heated for an additional 5 min at 100°C, and allowed to cool. The product at this stage has a golden yellowish color and was dried under a stream of nitrogen to remove all traces of acetone and then reconstituted in the mobile phase. The reconstituted sample was vortexed and centrifuged at room temperature to pellet any particulate matter prior to injecting over the HPLC column.

2.3.2. p-Bromophenacyl bromide derivatives

The procedure described by Puttmann et al. [28] was used in preparing these derivatives. Briefly, the chromophore-catalyst reagent was prepared by mixing *p*-bromophenacyl bromide (50 mmol/l) and 18-crown-6 (5 mol/l) in acetonitrile. To the dried lipid extract, 500 μl of acetonitrile, 6 μl of the chromophore-catalyst reagent and ~1 mg of $KHCO_3$ were added and the vials were tightly capped. These were then vortexed vigorously and heated at 85°C for 45 min. The vials were cooled and then centrifuged to pellet the $KHCO_3$. The supernatant was transferred to another vial to be dried, reconstituted in the mobile phase and used for HPLC injections.

2.4. FFA standard preparation

Individual fatty acids were dissolved in *n*-heptane to prepare stock solutions of 1 m*M* concentration. Purity of the individual fatty acid stock solutions was confirmed by HPLC (data not shown). These solutions were stored at 4°C. Mixed fatty acid standards used for generating calibration curves were prepared by mixing 1 ml each of the individual fatty acid stock solutions and followed by serial 1:1 dilutions in *n*-heptane to obtain concentrations of 250, 125, 62.5, 31.25, 15.6 and 7.8 μ*M*. A 0.5-ml aliquot of each mixed FFA standard was transferred to the reactivials and 50 μl of [$^2H_{31}$]-palmitic acid (0.5 m*M*) was added as internal standard. The vials were dried under a stream of nitrogen and derivatization was accomplished in the same manner as for the plasma samples. A new standard curve was generated for each HPLC run and the calibration was checked again at the end of each run by running one mixed standard of known composition.

2.5. HPLC apparatus and conditions

HPLC was carried out using an apparatus consisting of a pair of isocratic pumps (Model 510), a pump control module, an autosampler (Model 717), a programmable multi-wavelength UV detector (Model 490) and a column heater (Model 1122), all from Waters Chromatography (Milford, MA, USA). An Apex C_{18} 25 cm×4.6 mm; 5 μm (Jones Chromatography, Lakewood, CO, USA), a Resolve C_{18} 30 cm×3.9 mm; 5 μm (Waters Chromatography, Milford, MA, USA) and a Spherisorb C_6 15 cm×4.6 mm; 3 μm (Phase Separation, Norwalk, CT, USA) reversed-phase columns were used to test the chromatographic separations. A Resolve C_{18} Guard-Pak cartridge (Waters Chromatography) was used as a guard column. The column temperature was maintained at 22±1, 35±1 or 45±1°C. The mobile phase was degassed thoroughly prior to start of the run by sonicating it under vacuum and then sparged with nitrogen. The dried phenacyl esters were first dissolved in 166 μl acetonitrile and vortexed thoroughly, then 34 μl water was added to bring the acetonitrile concentration to 83% (that of the mobile phase) and 100 μl was injected over the column. Since the fraction corresponding to the palmitate peak was collected and analyzed for [^{3}H]- palmitate or [^{14}C]-palmitate radioactivity, the injection volume was kept large so as to allow for reasonable counts of radiolabeled palmitate to be collected without overloading the column. For most analyses, the solvent flow-rate was 2 ml/min (back pressure ~2100 p.s.i.; 1 p.s.i. = 6894.76 Pa). For some gradient runs differ-

ent flow-rates were tested. The mobile phase was acetonitrile–water. Different mobile phase compositions were tested for both isocratic and gradient elution schemes. For gradient runs different gradient profiles were tested using the pump control module. A back pressure regulator (Upchurch Scientific, Oak Harbor, WA, USA) was used at the exit port of the UV detector to prevent air bubble formation in the flow cell and to produce a smoother baseline. Data acquisition and analysis were performed on a x486 personal computer using the Millennium chromatography manager (Waters Chromatography), which communicated with the HPLC equipment by a Bus LAC/E card using IEEE 488 connections. A fraction collector (Foxy 2000; ISCO, Lincoln, NE, USA) was used to collect fractions of interest for counting and further processing. Absorbance was measured at 242 and 254 nm and the peaks were identified via their retention times in relation to the internal standard ([$^2H_{31}$]-palmitic acid). The concentrations of individual fatty acids were computed by the internal standard method from peak area ratios using the standard curves of mixtures of free fatty acids. Column life during routine use averaged over 750 injections. Flushing the column overnight with 100% acetonitrile after a set of injections (20 or more) extended the column life to over 1200 injections. The guard column required replacement after approximately 150 injections. Palmitate radioactivity was determined by drying the relevant eluent fractions, reconstituting them in 1 ml acetonitrile, adding 10 ml Ultima Gold liquid scintillation cocktail and counting them using a Packard CA2200 Tri Car liquid scintillation analyzer (Packard Instruments, Downers Grove, IL, USA).

2.6. TLC–GC analysis of FFAs

Total and individual free fatty acid quantitation by TLC–GC was performed using the method of McDonald-Gibson and Young [10].

2.7. Human studies

Blood samples were collected from healthy non-obese human volunteers ($n=15$) in the basal, resting state following a 12 h overnight fast and during 2 h of continuous cycle ergometer exercise and 90 min of post-exercise recovery. Resulting plasma total FFA concentrations ranged from 400 to 800 μmol/l. All blood samples were collected in chilled EDTA tubes to prevent in vitro hydrolysis of plasma triacylglycerols. The plasma was promptly separated by centrifugation at 800 *g* for 20 min at 4°C and then frozen at −70°C until analysis. Multiple cycles of thawing and re-freezing did not affect plasma FFA concentrations (data not shown).

2.8. Calculations and statistics

Extraction efficiency was calculated by measuring the recovery of [^{3}H]-palmitic acid and [$^2H_{31}$]-palmitic acid (the internal standard). For recovery of radiolabeled palmitic acid, a known amount of [^{3}H]-palmitic acid was added to the plasma samples prior to extraction. The recovery of [^{3}H]-palmitic acid was calculated as the ratio of [^{3}H] counts in the organic layer and the radioactivity added to the plasma samples. For recovery of the internal standard, 50 μl of [$^2H_{31}$]-palmitic acid (0.5 m*M*) was added to plasma samples and to a mixture of pure fatty acid standards prepared in heptane. The plasma samples were extracted and the extracted plasma and the pure fatty acid mixtures were then derivatized and injected over the HPLC column. The recovery for the internal standard was calculated as the ratio of [$^2H_{31}$]-palmitate peak areas from the extracted plasma sample relative to the mixed fatty acid standard in heptane. Chromatographic separation was quantified by computing the capacity factor k', selectivity α, and the resolution R_s [29]. The capacity factor (k') is defined as $(V_R - V_0)/V_0$, where V_R and V_0 are the retention volumes for the peak of interest and the unretained components, respectively. V_0 was measured by injecting 10 μl of acetone. The selectivity α is defined as k'_1/k'_2, where k'_1 and k'_2 are the capacity factors of the two peaks of interest. The resolution R_s is defined as $2(t_{R1} - t_{R2})/(w_1 + w_2)$, where t_{R1}, t_{R2} are the retention times and w_1, w_2 are the peak widths of the two peaks of interest. The statistical significance of comparisons between different methods was tested using paired and unpaired *t*-tests. Values of $P<0.05$ were considered statistically significant. All statistics were computed using SigmaStat (version 2.0; Jandel Scientific, San Rafael, CA, USA).

2.9. Validation of the analytical method

Calibration curves were constructed from the peak area ratios of FFAs to internal standard ([$^2H_{31}$] palmitic acid) against the expected FFA concentration. Linear regression analysis was used to calculate the slope, intercept and correlation coefficient (r^2) of each calibration curve. The assay precision (C.V., %) was assessed by expressing the standard deviation of repeated measurements as a percentage of the mean value. The accuracy of the method was expressed as the ratio of concentration determined by the assay to the known concentration of the quality control samples. Intra-day precision was estimated from six replicates of quality control samples (concentrations ranging from 31.25 μM to 250 μM). Inter-day precision was estimated from the analysis of freshly prepared control samples (concentrations ranging from 8.9 μM to 285 μM) on six separate days.

3. Results

3.1. Plasma FFA extraction

The modified Dole and the Miles extraction procedures were investigated for extraction of FFAs from human plasma. Extraction efficiency (i.e., recovery), reproducibility and time required for extraction were superior with the modified Dole extraction method (Table 1). The recovery was approximately 90% for [^{3}H]-palmitate and [$^2H_{31}$]-palmitate using the modified Dole procedure whereas the recovery rates for the Miles procedure were approximately 75% ($P<0.001$). The intra-assay coefficients of variance (C.V.s) for the modified Dole and Miles procedures averaged 3.6 and 4.8%, respectively ($P<0.001$).

The degree of separation of the organic from the aqueous fractions in human plasma using the modified Dole extraction procedure was investigated by spiking human plasma samples ($n=4$) with [^{3}H]-palmitate (17 841±363 dpm), [^{3}H]-glucose (14 984±294 dpm), or both. The measured radioactivity in the organic extracts of spiked plasma samples was compared to extracts of unspiked plasma and to heptane blanks. Average counts in the organic extracts of samples spiked with both [^{3}H]-palmitate and [^{3}H]- glucose (16 021±667 dpm) were similar to the samples spiked with [^{3}H]-palmitate only (15 560±414 dpm, $P=$NS). Counts in the organic extracts of samples spiked only with [^{3}H]-glucose (17±3 dpm) were indistinguishable from the background counts present in unspiked samples (16±1 dpm) or the heptane blanks (14±2 dpm).

3.2. Plasma FFA derivatization

Experiments were performed to determine the optimal conditions for derivatization of FFAs in human plasma using α-bromoacetophenone. Since unreacted α-bromoacetophenone produces broad interfering peaks in HPLC chromatograms of plasma FFAs [20], experiments were conducted to optimize the concentration of α-bromoacetophenone in the reaction mixture. Fig. 1 shows the effect of increas-

Table 1
Comparison of modified Dole and Miles extraction procedures for FFA extraction from human plasma

	Modified Dole	Miles
[^{3}H]-Palmitate recovery (%)	89.1±0.36[a]	76.8±0.68
[$^2H_{31}$]-Palmitate recovery (%)	89.9±2.2[b]	73.3±3.1
Intra-assay C.V. for [$^2H_{31}$]-palmitate recovery (%)	3.6[b]	4.8
Organic solvents volume (ml)	5	5
Volume to evaporate (ml)	1.5	4
Total time required	30 min	>6 h

Values are expressed as mean (±S.E.M.). Human plasma samples (0.5 ml) were spiked with known quantities of [9,10-^{3}H] or [$^2H_{31}$]-palmitate and FFA extraction was performed as described in Section 2.
[a] $P<0.0001$.
[b] $P<0.001$ vs. extraction method of Miles et al.

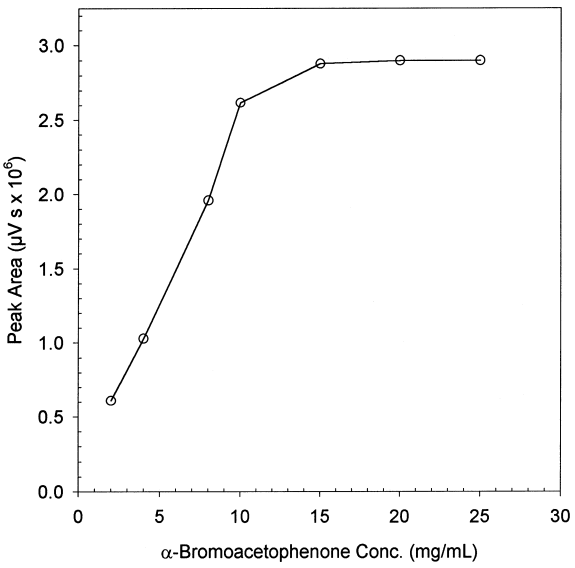

Fig. 1. Effect of derivatization reagent (α-bromoacetophenone) concentration on peak area for 100 nmol [$^{2}H_{31}$]-palmitic acid. α-Bromoacetophenone was dissolved in acetone and free fatty acids were derivatized as described in Section 2. Peak areas were determined by measuring absorbance at 242 nm.

ing concentrations of α-bromoacetophenone on the peak areas of a sample fatty acid ([$^{2}H_{31}$]-palmitic acid). The peak areas increased with increasing concentrations of α-bromoacetophenone up to a concentration of 15 mg/ml beyond which the peak areas did not change. To minimize excess derivatization agent and maximize detection limits and sensitivity the concentration of α-bromoacetophenone was subsequently kept at 15 mg/ml. Concentration of triethylamine was kept at 25 mg/ml as varying its concentration did not have any appreciable effect on the peak areas (data not shown). α-Bromoacetophenone dissolved in acetone and stored at −4°C was found to be stable for up to four weeks.

The efficiency of the derivatization reaction using α-bromoacetophenone was studied by adding known amounts of [^{14}C]-palmitate to a mixture of pure fatty acid standards, and measuring the [^{14}C] counts in eluent fractions following HPLC separation. The fraction corresponding to the palmitate peak (eluting at 18.2 min) had over 85% of the [^{14}C]-palmitate counts added. In contrast the [^{14}C] radioactivity of the earliest eluting fraction (0–4 min) was less than 5% of the total [^{14}C]-palmitate radioactivity added. Since all unreacted FFAs would be expected to elute with the void volume fraction, this indicated that over 95% of the plasma FFAs were derivatized to form their corresponding phenacyl esters (i.e., the reaction efficiency was over 95%).

In order to determine which derivatization procedure yielded better HPLC separation and quantitation, mixed FFA standards in heptane were derivatized to their respective phenacyl esters using either α-bromoacetophenone or *p*-bromophenacyl bromide. To achieve derivatization efficiencies comparable to that of the α-bromoacetophenone derivatives, it was necessary to carry out the reaction using *p*-bromophenacyl bromide at elevated temperatures (85°C or higher) for a much longer time (45 versus 15 min). Although the *p*-bromophenacyl bromide derivatives gave slightly bigger peaks compared to the phenacyl bromide derivatives, the chromatographic separation of the *p*-bromophenacyl bromide derivatives was inferior, especially for the [$^{2}H_{31}$]-palmitate (internal standard), palmitate and oleate peaks (data not shown). Two different columns (C_{18} and C_{6}) were tested for the separation of *p*-bromophenacyl bromide derivatives. The separation of critical pairs like palmitate/oleate using both types of columns was inferior compared to that for phenacyl bromide derivatives. The selectivity α was used to quantify the level of chromatographic resolution achieved for the *p*-bromophenacyl and phenacyl derivatives. α for the palmitate/oleate peaks averaged 1.02 for *p*-bromophenacyl derivatives as compared to 1.23 for phenacyl derivatives ($P<0.01$). Therefore, because of the shorter reaction time and superior chromatographic resolution, we chose to utilize phenacyl bromide derivatives for all subsequent chromatographic analyses.

3.3. Separation of individual FFAs in human plasma by HPLC

A series of experiments were performed to identify the optimal HPLC method for separation of the six major long chain fatty acids in human plasma (myristate [14:0], palmitate [16:0], palmitoleate [16:1], stearate [18:0], oleate [18:1] and linoleate [18:2]). The effects of column type, column temperature and isocratic and gradient elution profiles (ace-

tonitrile–water mobile phase) were examined to optimize separation of individual FFAs with reasonable elution times (<60 min). Representative chromatograms of a mixed FFA standard in heptane and an extracted human plasma sample are shown in Fig. 2.

Firstly, two different reverse phase, octadecylsilyl (ODS) columns were evaluated to compare their chromatographic separation of the phenacyl derivatives of the six major plasma free fatty acids. An

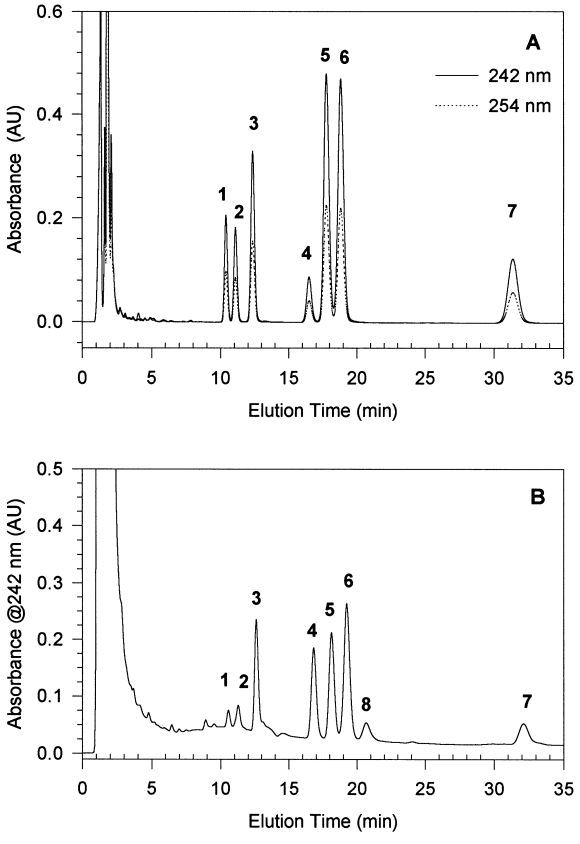

Fig. 2. Representative HPLC chromatograms of free fatty acids: (A) mixed fatty acid standard (125 μmol/l); (B) human plasma sample. Both samples contain [$^2H_{31}$] palmitic acid added as internal standard: 100 nmol for A and 25 nmol for B. Peak identification: 1=myristic (14:0), 2=palmitoleic (16:1), 3=linoleic (18:2), 4=[$^2H_{31}$]-palmitic (internal standard), 5=palmitic (16:0), 6=oleic (18:1), 7=stearic (18:0), 8=elaidic (*trans* 18:1ω9). Absorbance was monitored at 242 nm (solid line) and 254 nm (dotted line) for (A). See Section 2 for details of plasma extraction and derivatization. HPLC conditions: isocratic elution with acetonitrile–water (83:17) at 2 ml/min and column temperature=45°C.

ODS column with end-capped silanol groups (Apex I, Jones Chromatography; 25 cm×4.6 mm, 5 μm particle size) was compared with an ODS column with free silanol groups (Resolve, Waters Chromatography; 30 cm×3.9 mm, 5 μm particle size). Under identical chromatographic conditions [isocratic elution with acetonitrile–water (83:17) at 2 ml/min and 22°C], the six major FFAs in human plasma eluted in 61 and 69 min using the Apex I and Resolve ODS columns, respectively. Moreover, chromatographic separation for the palmitate/oleate peaks was better with the Apex column. The selectivity for the palmitate/oleate peak pair (α_{PO}) averaged 1.23 for the Apex column compared with α_{PO} = 1.14 for the Resolve column. Because of the shorter run times and better chromatographic separation, we chose the Apex column for all subsequent analyses.

Next, isocratic elution schemes were evaluated to determine the optimal acetonitrile–water mobile phase for HPLC separation of individual FFAs in plasma. The flow-rate was maintained at 2 ml/min and column temperature at 22°C for all isocratic elutions tested. Absorbance was monitored at a UV wavelength of 242 nm, since UV absorbance for phenacyl esters at 242 nm was significantly greater than at 254 nm, as shown in Fig. 2A. Acetonitrile concentration in the mobile phase was varied between 75% and 90%. As the proportion of acetonitrile increased, FFA elution times and capacity factors (k') progressively decreased (Fig. 3A). For example, stearate (18:0) elution time decreased from 86.2 min to 18.9 min by increasing the acetonitrile concentration in the mobile phase from 75% to 90%. Fig. 3A presents the capacity factors (k') for the four main FFAs as a function of acetonitrile concentration. The close overlap of the capacity factors for palmitate (16:0) and oleate (18:1) illustrates the difficulty in resolving these two peaks. As shown in Fig. 3A, the difference in their respective capacity factors was greatest at acetonitrile–water (83:17), indicating optimal isocratic chromatographic resolution of these peaks at this mobile phase composition.

Thirdly, the effect of column temperature (22, 35 and 45°C) was assessed on the run time and chromatographic resolution of the [$^2H_{31}$]-palmitate/palmitate/oleate peaks. As column temperature increased, retention times decreased. For example, stearate retention time decreased from over 60 min at

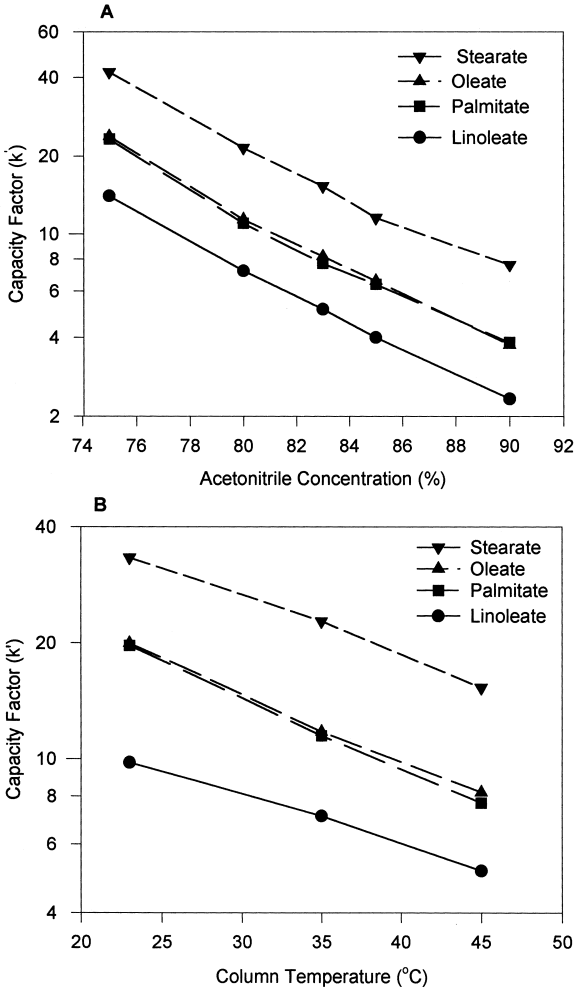

Fig. 3. Capacity factors (k') for individual free fatty acids as a function of: (A) acetonitrile concentration in mobile phase and (B) column temperature. Mobile phase acetonitrile concentration was increased from 75% to 90% and column temperature increased from 22 to 45°C. HPLC separations of mixed FFA standards were performed using isocratic elutions with acetonitrile–water at 2 ml/min.

22°C to ~50 min at 35°C and 32 min at 45°C. Fig. 3B shows the effect of column temperature on the capacity factor (k') for the four major FFAs in plasma. As shown by the diverging capacity factor plots for palmitate and oleate, the reduced elution times at 45°C were achieved along with improved resolution of these FFA peaks. Thus, a column temperature of 45°C was selected for all subsequent analyses.

Lastly, gradient elution schemes were tested for optimal separation of fatty acid derivatives using the Apex ODS column at 45°C. Fig. 4 shows a few sample chromatograms for different gradient elution profiles. Linear (Fig. 4A), exponential (Fig. 4B), step

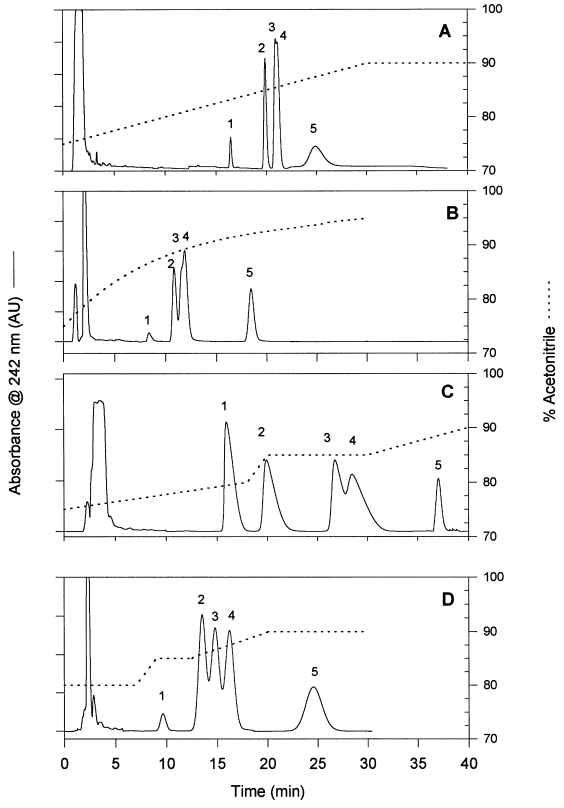

Fig. 4. Representative HPLC chromatograms of mixed fatty acid standards using different gradient elution profiles: (A) linear gradient: 0–30 min, 75–90% acetonitrile; 30–35 min, 90% acetonitrile; (B) convex exponential gradient: 0–30 min, 70–95% acetonitrile; (C) step-linear gradient: 0–18 min, 75–80% acetonitrile; 18–20 min 80–85% acetonitrile; 20–30 min, 85% acetonitrile; 30–40 min, 85–90% acetonitrile; and (D) combination of linear and convex exponential gradients: 0–7.5 min, 80% acetonitrile; 7.5–9 min, 80–85% acetonitrile (linear gradient); 9–12 min, 85% acetonitrile; 12–20 min, 85–90% acetonitrile (convex gradient); 20–35 min, 90% acetonitrile. Mobile phase: acetonitrile–water. Column: ODS 25 cm×4.6 mm, 5 μm particle size (Apex I column, Jones Chromatography). Column temperature=45°C. Flow-rate: 2 ml/min for A, B, D; 1.5 ml/min for C. UV absorbance at 242 nm (solid lines) is plotted on the left-hand y-axes; mobile phase acetonitrile content (dashed lines) is plotted on the right-hand y-axes. Peaks: 1=linoleic (18:2), 2=[$^2H_{31}$]-palmitic (internal standard), 3=palmitic (16:0), 4=oleic (18:1), 5=stearic (18:0).

(Fig. 4C) and combination (Fig. 4D) gradients were investigated. The linear gradient shown in Fig. 4A reduced the retention time for stearate to 25 min, yet the retention times for the other peaks of interest did not change. Although the [$^2H_{31}$]-palmitate and palmitate peaks were adequately resolved, the resolution of palmitate and oleate declined markedly (the selectivity for the palmitate/oleate peaks, $\alpha_{PO} < 1.01$). Similarly, the convex exponential gradient as shown in Fig. 4B produced shorter retention times for all peaks, but the resolution of the palmitate/oleate peaks was unacceptable ($\alpha_{PO} < 1.005$). Fig. 4C shows a step linear gradient elution profile tested to optimize the separation of the three closely eluting [$^2H_{31}$]-palmitate, palmitate and oleate peaks. The separation achieved between the [$^2H_{31}$]-palmitate and palmitate peaks was excellent, but again the palmitate/oleate peaks were not resolved ($\alpha_{PO} < 1.02$). Finally a combination of linear and concave exponential gradients were evaluated (Fig. 4D). Although the [$^2H_{31}$]- palmitate/palmitate/oleate peaks were better resolved compared to the previous gradient elution schemes, the resolution of the palmitate/oleate peaks was not significantly enhanced when compared to isocratic elution using acetonitrile–water (83:17, v/v) (Fig. 2). For other gradient elution schemes examined improved resolution of the palmitate-oleate pair was achieved at the expense of reduced resolution of the internal standard and palmitate peak pair (data not shown).

3.4. Quantitation of individual FFAs by HPLC and comparison with GC

Phenacyl esters of mixed fatty acid standards of known concentration were prepared and analyzed by HPLC using the optimized conditions (Fig. 2A). The resulting standard curves were linear up to a concentration of 400 μmol/l. The limit of detection was approximately 1 nmol. Calibration curves for all FFAs of interest had r^2 values consistently >0.98.

Plasma FFA quantitation using the optimized HPLC method described above was compared with gas chromatography (Table 2 Fig. 5). Plasma total and individual FFA concentrations were determined in the basal resting state in healthy human volunteers ($n=15$) and every 15–30 min during cycle ergometer exercise and post-exercise recovery. Basal FFA concentrations measured by the HPLC and GC methods were identical (Table 2). Likewise, FFA concentrations during exercise and recovery were similar as measured by HPLC and GC over a wide concentration range (Fig. 5).

Table 2
Comparison of concentrations of four major plasma FFA in healthy human subjects measured by HPLC vs. TLC–GC

Free fatty acids	HPLC (μmol/l)	TLC–GC (μmol/l)	P[a]
Palmitic (16:0)	86±10	81±6	0.65
Stearic (18:0)	40±4	36±2	0.31
Oleic (18:1)	135±15	137±12	0.91
Linoleic (18:2)	65±8	60±5	0.59
Total FFAs	326±39	315±26	0.81

Values are expressed as mean±S.E.M. Blood samples were collected from healthy, non-obese human subjects ($n=15$) following a 12 h overnight fast in the basal resting state.
[a] Differences in FFA concentrations by the two methods are not statistically significant.

3.5. Precision and accuracy of the analytical method

Intra-day variations in the measured concentrations of the four major FFAs are shown in Table 3. The intra-day precision was consistently between 1.9% to 8% for all FFAs. The data of the inter- day variation are shown in Table 4. The inter-day coefficients of variation were 1.3% to 12% for concentrations from 8.9 μ*M* to 285 μ*M*. The accuracy of FFA quantitation using this HPLC method was within ±7% of the expected concentration for all six major fatty acids in human plasma (Tables 3 and 4).

4. Discussion

We report an optimized method for quantitation of FFAs in human plasma by HPLC. Established techniques for lipid extraction, fatty acid derivatization and HPLC separation were modified to yield a rapid, sensitive and highly reproducible method suitable for use in human studies. Our optimized method compares favorably with traditional GC methodology for quantitation of individual long-chain FFAs in human plasma. Moreover, our method possesses a number

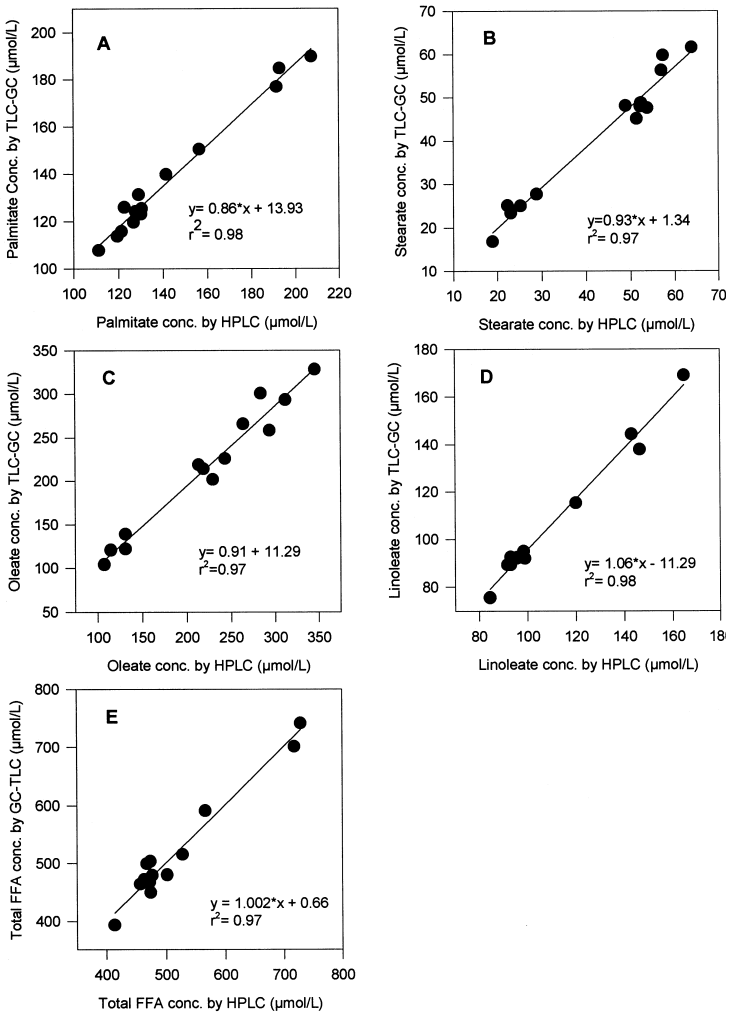

Fig. 5. Comparison of plasma FFA concentrations (µmol/l) measured using HPLC and TLC–GC methods in a healthy human subject. (A) Palmitate; (B) stearate; (C) oleate; (D) linoleate, and (E) total FFAs. Linear regression lines (solid lines), regression equations, and r^2 values for the comparisons between HPLC and TLC–GC measurements are shown. Blood samples were collected in the basal, resting state after a 12 h fast and every 15–30 min during continuous cycle ergometer exercise (120 min) and post-exercise recovery (90 min).

of advantages over a previously reported method for FFA quantitation in human plasma.

Extraction of FFAs from a proteinaceous aqueous medium is critical to prepare the fatty acids for further analysis by derivatization and liquid chromatography. The extraction step concentrates the FFAs in the organic layer and removes water and aqueous constituents present in plasma. Presence of water has been shown to lower the reaction extent for the derivatization step [28], thus removing all traces of water from the extracted FFAs and dissolving them in an organic phase allows the derivatization reaction to proceed to near completion. As shown in Table 1, extraction efficiency and reproducibility were superior with the modified Dole extraction method compared to the Miles procedure. Moreover, extraction using the modified Dole procedure was significantly more rapid compared to the Miles procedure (30 min vs. >6 h). Importantly the extraction solvent used in the Miles method contains chloroform which can release fatty acids from phospholipids in biological materials [30], thus artificially

Table 3
Intra-day precision and accuracy of the HPLC method at different FFA concentrations ($n=6$)

FFA standard	Concentration (μmol/l)	HPLC method (μmol/l)	Precision (C.V., %)	Accuracy (% of expected value)
Myristate	250	253.4±5.6	2.2	101
(14:0)	125	129.6±4.7	3.6	104
	62.5	64.1±4.3	6.7	102
	31.3	33.3±2.2	6.7	107
Linoleate	250	255.8±4.9	1.9	102
(18:2)	125	131.2±3.6	2.8	105
	62.5	61.45±4.6	7.4	98
	31.3	33.1±2.5	7.7	106
Palmitate	250	252.6±6.0	2.4	101
(16:0)	125	127.2±2.6	2.1	102
	62.5	65.1±2.6	3.9	102
	31.3	30.3±2.2	7.3	97
Oleate	250	257.4±6.0	2.3	103
(18:1)	125	129.8±2.6	2.0	104
	62.5	62.7±1.7	2.8	101
	31.3	31.2±2.5	8.1	100

Values are expressed as mean (±S.D.).

raising the measured concentration of certain fatty acids. The use of isopropanol and heptane in the modified Dole procedure avoids this artifact during extraction. Thus, for the reasons of superior extraction efficiency and reproducibility, shorter processing time, and the absence of chloroform in the extraction solvent, the modified Dole extraction procedure was chosen for all further plasma FFA extractions.

Since metabolic studies in human subjects may involve simultaneous infusion of radioisotopes of palmitate and glucose [7,9], it was important to confirm that radiolabeled glucose was not ''contaminating'' the organic layer and, therefore, precluding accurate determination of palmitate radioactivity in the plasma extracts. Along these lines, the [^{3}H] counts in the organic layer for plasma samples spiked with [^{3}H]-glucose were indistinguishable from the background counts in unspiked samples and heptane blanks. Moreover, organic extracts of plasma samples spiked with both [^{3}H]-glucose and [^{3}H]-palmitate contained [^{3}H] counts indistinguishable from samples spiked with [^{3}H]-palmitate only. Thus, the modified Dole extraction procedure produces complete separation of the organic and aqueous constituents present in human plasma, allowing simultaneous administration of [^{3}H]-glucose and [^{3}H]-palmitate in metabolic studies in vivo.

Because fatty acids do not contain chromophores, detection in the UV-range requires fatty acid derivatization prior to HPLC separation. Two phenacyl esters, phenacyl bromide and *p*-bromophenacyl bromide, with high molar extinction coefficients in the UV range were tested to determine the optimal derivative for chromatographic separation with an acceptable detection limit (<5 nmol). For both derivatization procedures examined, the extent of reaction exceeded 95% and detection of these ester derivatives in the UV range (242 nm) was possible at levels lower than 1 nmol. However, phenacyl bromide derivatives were chosen over *p*-bromophenacyl bromide derivatives because of superior chromatographic separation. Although both derivatization reactions required elevated temperatures (85–100°C) to accelerate the rate of reaction, heating the reaction mixture to these temperatures produced no detectable changes in a variety of fatty acids when compared to phenacyl derivatives produced overnight at room temperature [20]. Thus the extent of isomerization of polyunsaturated fatty acids during reaction at tem-

Table 4
Inter-day precision and accuracy of the HPLC method at different concentrations for the six major FFAs in human plasma ($n=6$)

FFA standard	Concentration (μmol/l)	Assay result (μmol/l)	Precision (C.V., %)	Accuracy (% of expected value)
Myristate	71.4	71.1±3.7	5.2	99
(14:0)	35.7	38.4±3.7	9.7	107
	17.9	19.9±1.2	6.3	111
	8.9	9.4±1.1	11.7	105
Palmitoleate	71.4	70.7±2.9	4.2	99
(16:1)	35.7	36.9±2.4	6.6	103
	17.9	19.2±1.7	9.0	107
	8.9	9.4±1.1	11.7	106
Linoleate	142.9	142.1±9.8	6.9	99
(18:2)	71.4	71.3±1.0	1.3	100
	35.7	35.5±1.0	2.7	99
	17.9	18.5±1.1	6.1	103
Palmitate	285.7	290.6±12.0	4.1	102
(16:0)	142.9	151.0±6.5	4.3	106
	71.4	74.8±2.2	3.0	105
	35.7	35.6±1.7	4.7	100
Oleate	285.7	280.9±13.9	5.0	98
(18:1)	142.9	145.7±3.2	2.2	102
	71.4	73.7±1.4	1.9	103
	35.7	35.7±2.8	8.0	100
Stearate	142.9	140.9±6.1	4.3	99
(18:0)	71.4	73.0±1.9	2.6	102
	35.7	37.2±2.0	5.2	104
	17.9	16.8±1.5	9.1	94

Values are expressed as mean (±S.D.).

peratures of 85–100°C appears to be insignificant. Moreover, we evaluated the effect of oxygen on FFA derivatization at high temperature. During paired analyses, some reaction vials were topped with a few milliliters of nitrogen gas and sealed tightly prior to heating to minimize oxygen availability. Peak areas of samples derivatized by excluding oxygen in this way were not significantly different compared to samples derivatized in the presence of oxygen (data not shown).

Next, we evaluated several factors in an attempt to optimize the synthesis of phenacyl bromide derivatives of plasma FFAs. Because unreacted α-bromoacetophenone produced broad interfering peaks in FFA chromatograms [20], the concentration of α-bromoacetophenone was carefully controlled to minimize the amount of unreacted derivatization agent (Fig. 1). For amounts of FFAs present in human plasma, a concentration of 15 mg/ml gave maximal peak heights with detection limits <1 nmol and no broad interfering peaks in the FFA chromatograms due to unreacted α-bromoacetophenone. Although acetic acid was added at the end of the derivatization step to convert the unreacted α-bromoacetophenone to phenacyl acetate, minimizing the amount of phenacyl acetate produced by this step was observed to improve the column life. Also, it has been previously reported that unreacted derivatization reagent affected the stability of the phenacyl derivatives [20]. Thus, for all the above reasons, the concentration of α-bromoacetophenone was kept at 15 mg/ml.

We performed a series of experiments to identify the optimal HPLC method for separation and quanti-

tation of the major FFAs present in human plasma. An isocratic method using acetonitrile–water (83:17, v/v) was observed to give the best overall chromatographic separation with a reasonable run time (Fig. 2A Fig. 3A). The saturated fatty acids eluted according to carbon chain length. Thus stearic acid (18:0) exhibited the longest retention time and myristic acid (14:0) the shortest among the fatty acids studied. The presence of one double bond in the hydrocarbon chain reduced the retention time by the equivalent of two carbon atoms in chain length. Thus oleic acid (18:1) and palmitic acid (16:0) exhibited similar retention times, as did myristic (14:0) and palmitoleic (16:1). The internal standard, [$^2H_{31}$]-palmitic acid, eluted slightly before palmitic acid. For some plasma samples a small peak representing elaidic acid (*trans* 18:1ω9) was seen following the palmitate/oleate pair (Fig. 2B). Peak areas were found to be approximately 40% larger at a UV wavelength of 242 nm as compared to 254 nm, giving significantly better detection limits for small volume samples (Fig. 2A). For purposes of quantitation, the four major FFA present in human plasma (palmitic, stearic, oleic and linoleic acids) account for over 95% of the total circulating FFA. If the minor fatty acids, myristic (14:0) and palmitoleic (16:1) acids, are included, over 99% of the total FFAs present in plasma can be quantified using our HPLC method [5,9]. Since palmitic and oleic acids constitute over 60% of the FFAs present in human plasma, and radiolabeled palmitate or oleate are often used to trace FFAs in metabolic studies in vivo, it was essential to achieve good chromatographic separation between these two constituents.

To optimize chromatographic resolution of the internal standard ([$^2H_{31}$]-palmitate), palmitate and oleate peaks, the effects of mobile phase composition were assessed (Fig. 3A). A mobile phase of acetonitrile–water (70:30) gave good chromatographic separation, but the run times were excessively long (stearate retention time >80 min). Capacity factors (k') for the four major FFAs increased logarithmically with decreasing acetonitrile concentration, although the slopes of the capacity factor plots differ (Fig. 3A). The capacity factors for palmitate and oleate were very similar leading to incomplete resolution of the peaks with most mobile phase compositions. The difference between the two capacity factors was maximal at 83% acetonitrile, indicating that this isocratic mobile phase composition gave the best separation of palmitate and oleate. Moreover, the increase in retention times with decreasing acetonitrile concentration is greatest for longer chain fatty acids. Conversely, as the solvent strength increases, the retention times for less hydrophobic compounds (shorter chain lengths or higher degree of unsaturation) will decrease faster. Induced dipole interactions between double bonds in the fatty acids and the triple bonds in the nitrile group of the mobile phase may play a role in this phenomena [31,32]. Changes in solvent strength also lead to changes in elution order of some fatty acids. For example at a mobile phase concentration of $\geq$95% acetonitrile, oleic acid elutes ahead of palmitic acid ($k'_{\text{oleic}}=3.86$, $k'_{\text{palmitic}}=4.1$).

Next, we sought to examine the effects of column temperature on chromatographic resolution. Similar to the effects of increasing solvent strength, increasing the column temperature reduced the retention time and the capacity factor for each fatty acid (Fig. 3B). The decrease in retention time was largest for stearate, the most hydrophobic component (from 60 min at 22°C to 32 min at 45°C). Another advantage of increasing the column temperature from 23°C to 45°C was the improved selectivity (α) for palmitic and oleic acid. ($\alpha_{23}=1.09$ vs. $\alpha_{45}=1.23$, $P<0.001$). Thus, retention times were reduced and the resolution of the palmitate/oleate peaks was improved at higher column temperatures.

The separation and quantitation of phenacyl esters of FFAs by HPLC are not unique to this paper [20,27]. However, our extraction, derivatization and HPLC method reported here has a number of advantages over existing methods and is suitable for analysis of large numbers of plasma samples from in vivo metabolic studies. Compared to the method of Miles et al. [27], our method is equally sensitive (limit of detection <1 nmol) and reproducible (C.V. <10%) but significantly more rapid (90 min vs. ≥ 6 h per sample). The greatly reduced time for analysis makes our method more suitable for analysis of large sample numbers. Moreover, our method quantifies all six major fatty acids in human plasma, compared to only palmitate and oleate with the method of Miles et al. [27]. Furthermore, our isocratic HPLC method resolved critical pairs of fatty acids (palmitate and

oleate) without resorting to time and solvent consuming gradient elution techniques. Importantly, our HPLC method compares favorably with TLC–GC [11] over a wide range of FFA concentrations (Table 2 and Fig. 5). Lastly, in addition to measurements of plasma FFA concentrations our method was developed to quantify rates of FFA turnover, when combined with infusion of radiolabeled fatty acids, by measuring the fatty acid radioactivity in collected eluent fractions following HPLC separation [8,9]. Plasma samples of 0.5 ml were used when measuring fatty acid radioactivity in eluent fractions to maximize [^{14}C]- or [^{3}H]-palmitate counts. However, if quantitation of plasma FFA concentrations is the sole objective, then plasma volumes as small as 50 μl yielded equivalent sensitivity and reproducibility (data not shown).

In summary, we report an optimized method for the HPLC quantitation of individual FFAs in human plasma. Our HPLC method separates and quantitates the six major fatty acids present in human plasma with a high degree of sensitivity ($\leq$1 nmol) and reproducibility (C.V. $<$10%) and compares favorably with traditional GC methodology. This optimized HPLC method will be useful in future studies designed to investigate the regulation of in vivo FFA metabolism in humans.

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