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Application of high-performance liquid chromatography of plasma fatty acids as their phenacyl esters to evaluate splanchnic and renal fatty acid balance in vivo

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Abstract

Plasma fatty acids from renal and hepatic veins, and arterialized hand vein obtained in 20 subjects before and after insulin infusion were separated by reversed-phase high-performance liquid chromatography following phenacyl esterification. Separation and quantification over the range 1.0–100 nmol per injection of nine fatty acids was achieved within 60 min using [$^{2}H_{31}$]palmitic acid as internal standard. Analytical recoveries were greater than 90% and the intra- and inter-assay coefficients of variation were less than 2.5 and 4.0%, respectively. Following insulin infusion, net splanchnic uptake of total fatty acids decreased from 3.0±0.3 to 1.0±0.1 μmol/kg min ($p<0.01$), whereas net renal balance remained neutral (−0.04±0.04 vs. −0.06±0.03 μmol/kg min, p=N.S.). Individual fatty acid balance varied from a low of 0.012±0.005 (myristic acid) to a high of 0.95±0.08 (oleic acid) μmol/kg min across the splanchnic tissues and from 0.005±0.002 (stearic acid) to 0.21±0.1 (oleic acid) μmol/kg min across the kidney. There is a substantial diversity in changes in plasma concentration and regional balance of individual fatty acid during short-term fasting and hyperinsulinemia. This method is simple, accurate, and can be applied to assess individual fatty acid metabolism in vivo. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The development of analytical methods for routine quantification of individual fatty acids has enabled significant advancement in our understanding of lipid biochemistry and physiology. The combination of pre-column derivatization and reversed-phase high performance liquid chromatography (HPLC) has been extensively employed for the analysis of fatty acids [1–3]. Although most studies have described accurate and reproducible determinations of fatty acids within a wide range, measurements of small arteriovenous concentration differences to evaluate regional fatty acid metabolism in vivo require very sensitive assays and have not yet been completely investigated. The non-esterified (free) fraction of fatty acids (FFA) in plasma is composed of a mixture of saturated and unsaturated fatty acids with chain-

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lengths varying from 12 to 22 carbon atoms [4]. Routine determinations of plasma FFA often measure the amount of total, not individual, fatty acids [5]. Considering that the use of tracer fatty acids has become the 'gold-standard' technique in the evaluation of systemic and regional lipid kinetics [6,7] the general assumption that one particular fatty acid is representative of the whole plasma FFA is an important limitation of the method which has not been sufficiently emphasized. The current studies were therefore undertaken to examine regional balance of individual fatty acids using an improved method for the determination of circulating FFA levels which could enable measurements of arteriovenous concentration difference across splanchnic tissues and kidney in humans.

2. Experimental

2.1. Reagents and chemicals

Palmitic, palmitoleic, myristic, linoleic, linolenic, arachidonic, elaidic, stearic acids (all >99% pure), [$^2H_{31}$]palmitic acid (98% pure), and 2-bromoacetophenone were purchased from Aldrich (Milwaukee, WI, USA). Sodium oleate (>99% pure), [9,10-$^3H_2$]palmitic acid, glacial acetic acid (99% pure), fatty acid-free bovine serum albumin (>98% pure), triethylamine (>99% pure), heptane, and acetone (HPLC grade) were obtained from Sigma (St. Louis, MO, USA). Methanol, 2-propanol, and acetonitrile (all HPLC grade) were purchased from J.T.Baker (Phillipsburg, NJ, USA). Screw-cap vials with polypropylene caps were supplied by Fisher Scientific (Pittsburgh, PA, USA).

2.2. Standards preparation

The fatty acids external standard solution was prepared by dissolving myristic, arachidonic, linolenic, elaidic, palmitoleic (100 $\mu mol\,l^{-1}$), linoleic, palmitic, oleic (300 $\mu mol\,l^{-1}$), and stearic (600 $\mu mol\,l^{-1}$) acids in 10 m*M* sodium phosphate buffer (pH 7.8) containing 4% fatty acid-free bovine serum albumin. [$^2H_{31}$]Palmitic acid (2 $mmol\,l^{-1}$) in 10 m*M* sodium phosphate buffer (pH 7.8) containing 4% fatty acid-free bovine serum albumin (BSA PBS) was used as internal standard. Stock solutions of both standards were stored at −20°C before use. Serial fatty acids standards were then prepared on the day of the analysis by dilution (1:1×6, v/v) of the fatty acids standard stock solution in BSA PBS. [$^2H_{31}$]Palmitic acid solution (50 μl) was added as internal standard to either 1.0 or 0.5 ml of each standard and to plasma samples before analyses.

2.3. Derivatization procedure

Fatty acids were extracted by the method of Dole and Meinertz [8,9], except that one-half of the original volume of extraction solvents was used. In brief, to release fatty acids from albumin, 5 ml of a mixture containing isopropanol–heptane–0.5 *M* H_2SO_4 in water (40:10:1, v/v) were added to each aliquot (1.0 or 0.5 ml) of fatty acids standard and plasma samples. After 15 min of shaking, 2 ml of heptane and 3 ml of water were added. Next, standards and samples underwent shaking again for 30 min and centrifugation at 800 *g* for 15 min. The supernatant phase was transferred to the screw-cap tubes and reduced to dryness under a flow of nitrogen. Phenacyl esterification was performed using the method of Wood and Lee [10], as modified by Miles at al. [6]. All standards and samples were resuspended in 500 μl of acetonitrile.

2.4. HPLC analysis

Fatty acid analyses were conducted with a Spectra SYSTEM liquid chromatograph (Thermo Separation Products Ink., Riviera Beach, FL, USA). The chromatograph consisted of a solvent delivery isocratic pump (model P1000), autosampler with 100 μl injection loop (model AS3000 Variable Loop), ultraviolet detector (model UV100, variable wavelength), and system controller (model SN4000) linked to a computer running PC1000 software (Version 3.0.1, IBM OS/2 Warp 3.1). The HPLC Spectra SYSTEM was equipped with reversed-phase Kromasil 250× 4.6 mm column packed with 5 μm octadecyl-bonded spherical silica, 100 Angstrom pore diameter (Phase Separations, Franklin, MA, USA) and a fraction collector Foxy Jr. (ISCO, Lincoln, NE, USA). An

aliquot (100 μl) sample was injected and chromatographed at ambient temperature in methanol–water (91:9, v/v) running solvent during 60 min. The flow-rate was 1.15 ml min^{-1} at a pressure of 120 bar, and ultraviolet detection was conducted at 254 nm wavelength.

2.5. Subjects and protocol

After the protocol was approved by the State University of New York Institutional Review Board, informed written consent was obtained from 20 healthy subjects who had normal fasting glucose, blood chemistry and urine analyses, and no personal or family history of diabetes, dyslipidemia, hypertension, hepatic or renal disease. For three days before the study, all had been on a weight-maintaining diet containing at least 200 g of carbohydrate and had abstained from alcohol. After an overnight fast individuals were admitted to the General Clinical Research Center and an antecubital vein was cannulated for continuous infusions of normal saline with either para-aminohippurate (PAH, renal vein studies) or indocyanine green (ICG, hepatic vein studies). Subsequently, a dorsal hand vein was cannulated retrogradely and kept in a thermoregulated Plexiglas box at 65°C for sampling arterialized venous blood throughout the entire experiment. During the 150 min equilibration period subjects had left renal ($n=10$) or right hepatic ($n=10$) veins catheterized. The catheter was then continuously infused with a diluted heparinized saline solution (4.0 U min^{-1}) to maintain patency. During the baseline period (–30 to 0 min) three consecutive blood samples were collected simultaneously from the dorsal arterialized hand vein and from either renal or hepatic vein at 15 min interval for determination of plasma glucose, insulin, fatty acids, PAH or ICG. At 0 min, upon completion of baseline collections, subjects were randomized to receive a 180 min continuous peripheral infusion of either insulin at the rate of 0.250 mU/kg min with a concomitant variable infusion of 10% dextrose to maintain normoglycemia (~90 mg dl^{-1}), or normal saline. Blood samples were again collected from the dorsal hand and either renal or hepatic veins for measurements similar to baseline at 15 min intervals from 150 to 180 min.

2.6. Analyses and calculations

Plasma glucose at the bedside was measured with the Beckman II glucose analyzer (Fullerton, CA, USA). PAH [11] and ICG [12] were determined by a colorimetric method; and insulin by radioimmunoassay [13]. Plasma concentration of total and individual fatty acids was determined using the HPLC method described above. Hepatic and renal plasma flows were calculated by ICG and PAH clearance equations, respectively, as previously described [14]. Net splanchnic and renal balance (Uptake or Output) were calculated for total and each fatty acid (myristic, palmitic, stearic, oleic, linoleic, linolenic, palmitoleic, arachidonic and elaidic) using the formula:

$$\text{Uptake (Output)} = ([FA]_a - [FA]_v) \times PF \quad (1)$$

where [*FA*] is plasma fatty acid concentration in μmol l^{-1} in samples obtained from arterialized hand ($_a$), hepatic or renal vein ($_v$), and *PF* is hepatic or renal plasma flow in ml/kg min.

2.7. Statistics

Values are expressed as mean±SEM, unless otherwise noted, and those obtained in the postabsorptive period were compared to those obtained during the last 30 min of the corresponding experimental infusion period using paired Student's *t*-test. Data obtained during the last 30 min of each insulin infusion period were compared to data obtained during the last 30 min of the saline infusion control period using non-paired Student's *t*-test. *p* values below 0.05 were considered statistically significant.

3. Results and discussion

3.1. Chromatographic conditions

The separation of fatty acid phenacyl derivatives by reversed-phase HPLC procedure is based on the chemical property of decreasing polarity with increasing chain length of fatty acids, and increasing polarity with increasing the number of double bonds for unsaturated fatty acids. Increasing the polarity of the solvent increases the retention times of all fatty

acids, but this is much greater for saturated acids. These effects may in, fact, create problems in the separation of arachidonic, myristic, palmitic and oleic acids within a reasonable (60–90 min) chromatographic time. Using acetonitrile–water or acetonitrile–isopropanol–water solvents as the mobile phase, good resolution is achieved at a flow-rate of 2 ml min^{-1} for 75 min. Acetonitrile and isopropanol have similar effects on chromatographic parameters of saturated and unsaturated fatty acid derivatives. The methanol–water mobile phase enables excellent resolution with a flow-rate of 1.15 ml min^{-1} for 60 min.

3.2. Quantitative analysis of FFA

Recovery tests for the extraction and derivatization procedures were performed by adding a known amount of [9,10-$^{3}H_{2}$]palmitic acid to the fatty acid standard mixture and to human plasma. 20 μl of [9,10-$^{3}H_{2}$]palmitic acid (~1 200 000 DPM = 0.5 μCi) were added to 0.5–1.0 ml of each sample prior to the analyses. Samples were extracted, derivatized and resuspended in 500 μl of acetonitrile, and the mixture (100 μl) was analyzed by HPLC. The eluent was collected in 2 min fractions and counted in a 1219 RackBeta liquid scintillation counter (LKB, Wallac, Finland). The yield for the whole procedure was consistently above 90%.

The inter-assay reproducibility of the extraction and derivatization procedures was evaluated with ten samples (five each of 1 ml and 0.5 ml) of stock external standard solution diluted eight times (one part of standard solution to seven parts of BSA PBS), and results are summarized in Table 1. Data for the extraction and derivatization procedure in either 1 ml or 0.5 ml standard solutions show reproducibility in the range 2–6%.

The chromatogram shown in Fig. 1 illustrates the resolution of the fatty acid derivatives. Calibration curves for each fatty acid were plotted as concentration vs. the area under the peak corrected to the internal standard peak area. Concentrations of fatty acids were calculated using the least-squares method. Linearity was established within the following range of fatty acids: 1.5–100 nmol ml^{-1} for myristic, linolenic, arachidonic, elaidic, and palmitoleic acids; 8–500 nmol ml^{-1} for linoleic, palmitic, oleic acids; and 9–600 nmol ml^{-1} for stearic acid. All correlation coefficients were greater than 0.9990. The intra-assay reproducibility for the HPLC analysis was evaluated with six repeated analysis each of the stock external standard solution diluted four and eight times, and of one undiluted human plasma sample. Results are summarized in Table 2.

Table 1
Reproducibility of fatty acid extraction and derivatization procedures[a]

Standard volume	Area peak for each fatty acid	
	1 ml	0.5 ml
Myristic	1 368 796±66 876	604 038±35 456
Palmitic	6 564 168±133 580	3 036 453±80 731
[$^{2}H_{31}$]palmitic	5 555 343±103 987	5 375 541±212 076
Linoleic	5 830 006±121 266	2 559 616±61 419
Oleic	8 545 486±182 008	3 957 708±98 146
Stearic	6 876 643±180 528	3 160 350±77 754

[a] Stock external standard solution was diluted eight times and each volume analyzed six times. [$^{2}H_{31}$]palmitic acid was added as the internal standard (50 μl of stock solution per sample). Values are presented as mean area under the peak±SD.

3.3. Application

The method was applied to determine fatty acid concentration in plasma sampled from arterialized hand vein, hepatic and renal vein in 20 postabsorptive healthy subjects before and after insulin infusion in an effort to establish the effects of overnight fasting and those of physiological hyperinsulinemia on splanchnic and renal balance of total and individual fatty acids. Plasma glucose was maintained at 4.9±0.3 and 5.2±0.1 μmol ml^{-1}, respectively during insulin and saline infusion. Plasma insulin, however, increased from 42±8 to 92±5 p*M* ($p<0.01$) after insulin infusion, whereas it did not change in the saline controls (30±7 vs. 35±8 p*M*, p = N.S.). Hepatic plasma flow remained constant following either insulin (12.7±0.5 vs. 13.7±1.5 ml/kg min) or saline (13.4±0.9 vs. 13.0±1.0 ml/kg min), and so did renal plasma flow (10.8±0.8 vs. 11.3±0.9 and 9.4±0.9 vs. 9.1±1.0 ml/kg min, respectively during insulin and saline infusion). Table 3 summarizes plasma concentration of individual and total fatty acids in the postabsorptive state and following infusion of either insulin or

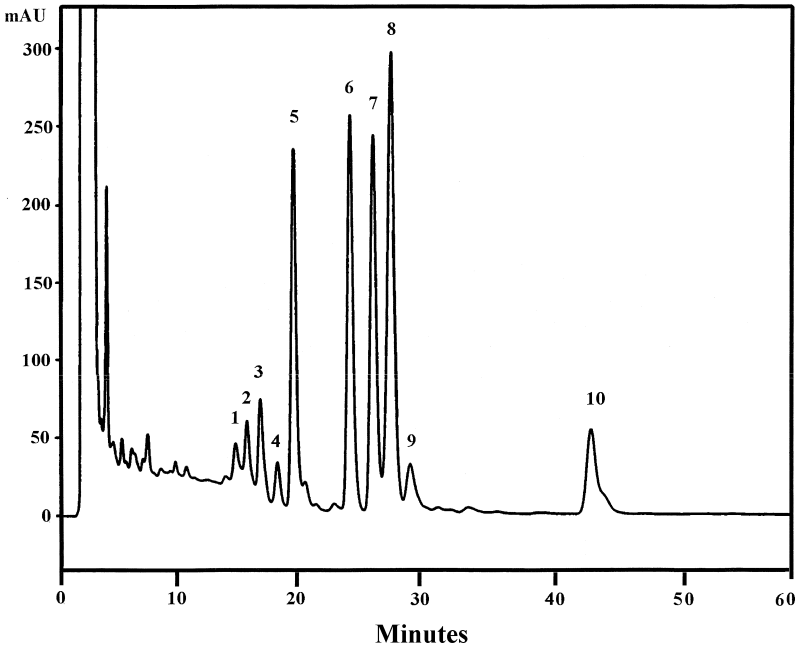

Fig. 1. High-performance liquid chromatogram (HPLC) of phenacyl esters of fatty acids in human plasma. Sample preparation and HPLC conditions are described in Section 2. Peaks: 1, linolenic acid; 2, myristic acid; 3, palmitoleic acid; 4, arachidonic acid; 5, linoleic acid; 6, [$^{2}H_{31}$] palmitic acid (internal standard); 7, palmitic acid; 8, oleic acid; 9, elaidic acid; and 10, stearic acid.

saline. As expected, oleic acid represents the largest fraction (~40%) of circulating fatty acids in post-absorptive subjects, and physiologic hyperinsulinemia induces an approximate 50% reduction in each of the measured fatty acids (all $p<0.001$), with the exception of arachidonic acid, which does not change significantly (4.7 ± 0.5 vs. 4.4 ± 0.6 μmol l^{-1}, $p=0.60$ vs. baseline). In contrast, whereas plasma concentration of each fatty acid increases by ~20% during saline infusion, arachidonic acid decreases by ~25% (4.7 ± 0.5 vs. 3.3 ± 0.4 μmol l^{-1}, $p<0.05$ vs. baseline). These data suggest that, though most circulating fatty acids decrease with plasma elevation of insulin and increase with fasting, opposite changes occur in plasma levels of arachidonic acid; i.e. it increases with insulin and decreases with fasting. Mean data on individual fatty acid balance across splanchnic tissues and kidney in the postabsorptive period and following either insulin or saline infusion are depicted in Figs. 2 and 3. Physiologic hyperin-

Table 2
Reproducibility of the HPLC analyses of fatty acids[a]

Fatty acids	Stock fatty acid standard mixture		Human plasma (nmol ml^{-1})
	Diluted 4 times (nmol ml^{-1})	Diluted 8 times (nmol ml^{-1})	
Myristic ($C_{14:0}$)	25.00±0.51	12.50±0.12	11.19±0.14
Palmitic ($C_{16:0}$)	125.00±0.57	62.50±0.35	104.41±0.21
Linoleic ($C_{18:2}$)	125.01±1.41	62.50±0.54	58.00±0.41
Oleic ($C_{18:1}$)	125.00±0.69	62.5±0.62	137.43±0.21
Stearic ($C_{18:0}$)	150.00±0.82	75.00±0.24	49.22±0.66

[a] The intra-assay reproducibility for the HPLC analysis was evaluated with six repeated analyses each of the stock external standard solution diluted, four and eight times, and of one undiluted human plasma sample.

Table 3
Plasma fatty acid concentrations in arterialized hand vein in the postabsorptive period and during the last 30 min of the insulin and saline infusion period[a]

Fatty acid	Plasma fatty acid concentrations		
	Postabsorptive ($n=20$)	Insulin ($n=12$)	Saline ($n=8$)
Linolenic	5.3±0.7	1.9±0.5[b]	6.9±1.3[b]
Myristic	12.7±1.4	5.5±0.9[b]	16.0±1.7[b]
Palmitoleic	19.9±2.4	6.9±2.1[b]	27.7±3.7[b]
Arachidonic	4.7±0.5	4.4±0.6	3.3±0.4[b]
Linoleic	90.2±8.1	43.6±7.0[b]	108.8±13.0[b]
Palmitic	151.9±15.5	62.3±10.6[b]	195.4±16.2[b]
Oleic	226.1±19.8	94.3±17.2[b]	288.1±28.8[b]
Elaidic	29.5±3.0	9.9±2.2[b]	36.3±5.6[b]
Stearic	58.7±6.1	23.7±3.9[b]	66.5±10.6[b]
Total	597.9±55.2	231.6±44.7[b]	749.5±81.4[b]

[a] Data obtained in 20 healthy volunteers randomized to receive either insulin ($n=12$) or normal saline ($n=8$); individuals in each group are matched for age (mean±SD, 30±5 vs. 33±6 years), gender (6 male/6 female vs. 4 male/4 female), and body mass index (mean±SD, 23±2 vs. 26±4 kg m^{-2}). Values are presented as mean±SE of the mean in nmoles ml^{-1}.

[b] $p<0.05$ vs. postabsorptive.

sulinemia is associated with a decrease in splanchnic uptake of total fatty acid from 2.99±0.30 to 0.99±0.10 μmol/kg min ($p<0.001$ vs. baseline), but it does not change with saline infusion (2.48±0.20 μmol/kg min). Although, splanchnic uptake of total and individual fatty acid correlate closely in all experiments, net splanchnic uptake of oleic acid decreases during saline infusion, despite ~30% elevation in plasma concentration (Table 3). And net splanchnic balance of arachidonic acid remains neutral following either insulin or saline infusion (Fig. 2). These data indicate that, in contrast to most circulating fatty acids, a reduction in oleic acid net uptake across splanchnic tissues during short-term fasting must be secondary to either an increase in splanchnic release, a decrease in splanchnic uptake, or both. The fact that plasma concentration of oleic acid increases, however, strongly suggest that an increase in oleic acid release from splanchnic tissues is most likely responsible for this observation. It is conceivable that arachidonic acid uptake and release by splanchnic tissues in healthy subjects is not responsive to factors which regulate

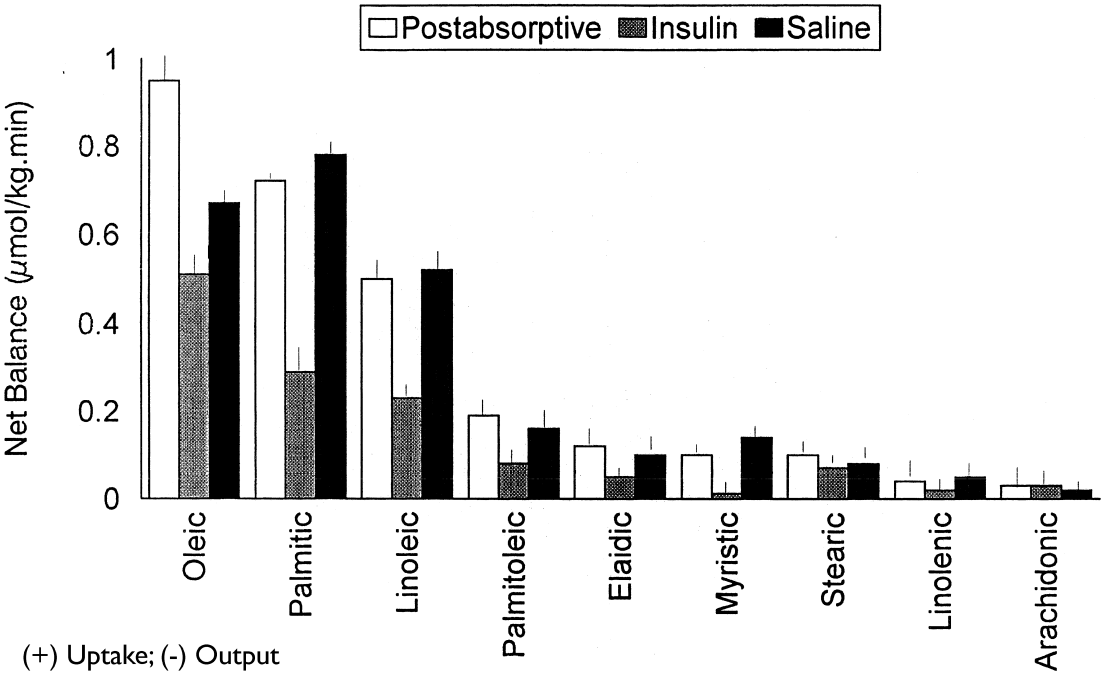

Fig. 2. Mean net splanchnic balance of individual fatty acids in the postabsorptive state and following 180 min of either saline ($n=4$) or insulin infusion ($n=6$) in 10 healthy subjects.

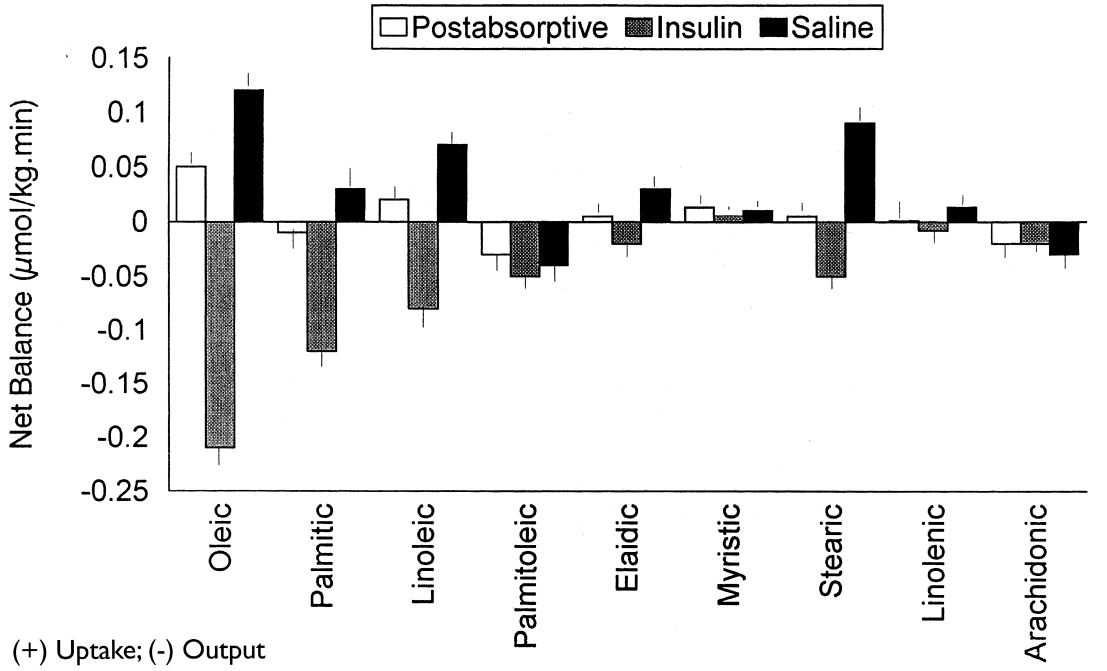

Fig. 3. Mean net renal balance of individual fatty acids in the postabsorptive state and following 180 min of either saline ($n=4$) or insulin infusion ($n=6$) in 10 healthy subjects.

adipose tissue lipolysis and plasma FFA levels, since splanchnic balance of arachidonic acid is unchanged by either fasting or insulin infusion. Data on renal balance of individual fatty acids are depicted in Fig. 3. Physiologic hyperinsulinemia induces a reversal from neutral renal balance (-0.04 ± 0.04 μmol/kg min) in the postabsorptive state to net total fatty acid output of -0.57 ± 0.07 μmol/kg min ($p<0.05$ vs. baseline), whereas saline infusion is accompanied by net uptake of total fatty acid by the kidney (0.30 ± 0.05 μmol/kg min, $p<0.05$ vs. baseline). Analogous to fatty acid balance across splanchnic tissues, renal balance of total and each fatty acid correlate closely, except that renal balance of palmitoleic, myristic and arachidonic acid remain unchanged following either insulin or saline infusion. Although these data are consistent with previous report indicating that splanchnic [15] and renal [16] balance of fatty acids change as a function of circulating levels they underscore the need for caution in extrapolating results obtained with total fatty acids to regional balance of individual fatty acids.

4. Conclusions

Our studies describe an improved method for separation, derivatization and quantification of nine non-esterified fatty acids as phenacyl esters using high-performance liquid chromatography. Application of this method to evaluate individual fatty acid plasma concentration and balance across splanchnic tissues and kidney after an overnight fast and during euglycemic hyperinsulinemic conditions in healthy subjects demonstrates substantial diversity in changes in fatty acid concentration and balance. These observations emphasize the limitations generalization of results obtained with total fatty acids, and indicate the need to assess individual fatty acids in regional balance studies.

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