A Semiautomated Enzymatic Method for Determination of Nonesterified Fatty Acid Concentration in Milk and Plasma

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ABSTRACT: An enzymatic assay for the determination of nonesterified fatty acid concentrations in milk and plasma is described. The procedure is semiautomated for use with a plate luminometer or plate spectrophotometer and enables routine batch processing of large numbers of small samples (≤5 μL). Following the activation of nonesterified fatty acids (NEFA) by acyl-CoA synthetase, the current assay utilizes UDP-glucose pyrophosphorylase to link inorganic pyrophosphate to the production of NADH through the reactions catalyzed by phosphoglucomutase and glucose-6-phosphate 1-dehydrogenase. With this assay sequence the formation of NADH from NEFA is complete within 50 min at 37°C. Enzymatic spectrophotometric techniques were unsuitable for NEFA determination in human milk due to the opacity of the sample. The use of the NADH-luciferase system has overcome this problem, allowing the enzymatic determination of NEFA in human milk. Sample collection and treatment procedures for milk and plasma have been developed to prevent enzymatic lipolysis and to limit interference from enzymes present in milk. The recovery of palmitic acid added to milk and plasma samples was 94.9 ± 2.9 and 100 ± 4.5%, respectively. There was no difference (P = 0.13) in plasma NEFA concentrations determined by the current method and a commercially available enzymatic spectrophotometric technique (Wako NEFA-C kit). Plasma NEFA concentrations determined by gas chromatography were 28% higher compared to both the Wako NEFA-C kit and the current method.

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Determination of the nonesterified fatty acid (NEFA) concentration in biological fluids is of importance in a wide variety of clinical and experimental conditions. As a consequence, considerable effort has been applied to the development of accurate methods for NEFA determination that are sufficiently convenient for routine analysis. Techniques for analysis of NEFA include titrimetry with alkali after extraction (1), colorimetry of NEFA-copper or -cobalt soaps (2), and gas chromatography (GC; [3]). Titrimetric and colorimetric methods enable quantification of NEFA, whereas GC also provides identification of individual NEFA. Nevertheless, these methods involve the use of hazardous chemicals and are complex and labor-intensive.

More recently, enzymatic techniques based on the specificity of acyl-CoA synthetase have been established (4). These methods, although unable to identify individual NEFA, do not require extraction of the sample with organic solvents and hence have enabled the development of relatively simple and convenient procedures. However, current enzymatic methods are costly (Wako NEFA-C kit; Wako Pure Chemical Industries Ltd., Osaka, Japan) and have not previously been reported for the determination of NEFA in milk.

A further limitation of enzymatic techniques is the possibility of interference from biologically active substances in the sample. Reducing agents (ascorbic acid, bilirubin) and alkaline phosphatase inhibit enzymatic colorimetric (5) and luminometric (6) methods, respectively. Alternatively, the action of lipases present in the sample may result in overestimation of NEFA concentration (7). Several methods for collection and treatment of samples for NEFA determination have been described (6–9). However, the appropriate method of choice is dependent on the analytical technique, assay sequence, and nature of the sample. In this context, a sample collection and treatment method suitable for enzymatic determination of NEFA concentration in milk has not previously been described.

Our research on (i) the role of NEFA in the control of human milk synthesis and (ii) NEFA metabolism during exercise involves the collection of large numbers (≥600) of small samples (≤5 μL). To facilitate batch processing of these samples, a suitable, semiautomated method was required. Accordingly, we have developed an enzymatic assay which has been linked to plate luminometry to enable the routine determination of NEFA in milk and plasma. In addition, collection and treatment procedures for milk and plasma, suitable for enzymatic determination of NEFA, have been established.

MATERIALS AND METHODS

Subjects. Human milk samples were obtained from volunteers recruited from the Nursing Mothers' Association of Australia (Western Australian Branch). Human plasma was obtained by finger prick from nonfasting volunteers. Volunteers provided written, informed consent and all procedures were approved by

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Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; GC, gas chromatography; MOPS, 3-[*N*-morpholino]propanesulfonic acid hemisodium salt; NEFA, nonesterified fatty acids; TEA, triethanolamine; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid hemisodium salt.

the Human Rights Committee of The University of Western Australia.

Materials. Bacterial luciferase E.C. 1.14.14.3 (from *Vibrio harveyi*), bovine serum albumin (BSA; essentially fatty acid-free, 0.005%), *n*-caproic acid (6:0, Na salt, 99% pure), *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid hemisodium salt (TES; free acid, 99.5% pure), 3-[*N*-morpholino] propanesulfonic acid hemisodium salt (MOPS, 99% pure), oleic acid (18:1n-9, Na salt, 95% pure), palmitic acid (16:0, Na salt), and tetradecylaldehyde (80% pure) were obtained from Sigma Chemical Company (Castle Hill, Australia). Dipotassium hydrogen orthophosphate 3-hydrate ($K_2HPO_4 \cdot 3H_2O$, 99.0% pure) and potassium dihydrogen orthophosphate (KH_2PO_4, 99% pure) were obtained from BDH Chemicals (Port Fairy, Australia). Ethylenediaminetetraacetic acid (EDTA, tetra-Na salt, 99% pure), hydrochloric acid (HCl, 31.5% w/w), and sodium hydroxide (NaOH, 97% pure) were obtained from Ajax Chemicals (Sydney, Australia). Acyl-CoA synthetase E.C. 6.2.1.3 (from *Pseudomonas fragi*), adenosine triphosphate (ATP; di-Na salt, 99% pure, from yeast), coenzyme A (CoA; lyophilized free acid, grade 1), dithiothreitol, flavin mononucleotide (FMN, 95% pure), glucose-6-phosphate 1-dehydrogenase E.C. 1.1.1.49 (from *Leuconostoc mesenteroides*), nicotinamide adenine dinucleotide (NAD, 98% pure, from yeast), reduced nicotinamide adenine dinucleotide (NADH, di-Na salt, 98% pure), phosphoglucomutase E.C. 5.4.2.2 (from rabbit muscle), triethanolamine (TEA), uridine-5′-diphosphoglucose (UDP-glc, di-Na salt, 98% pure, from yeast), and uridine-5′-diphosphoglucose pyrophosphorylase E.C. 2.7.7.9 (from beef liver) were obtained from Boehringer Mannheim (North Ryde, Australia). Lauric acid (12:0, A grade, 99.5% pure) was obtained from California Corporation for Biochemical Research (Los Angeles, CA). The Wako NEFA-C kit was obtained from Wako Pure Chemical Industries Ltd. Materials used for GC determination have been listed previously (10).

Assay principles. (i) Nonesterified fatty acids. Following the activation of NEFA by acyl-CoA synthetase (4), the current assay sequence utilizes UDP-glucose pyrophosphorylase, phosphoglucomutase and glucose-6-phosphate 1-dehydrogenase to link inorganic pyrophosphate to the reduction of NAD to NADH as outlined below:

$$\text{ATP} + \text{NEFA} + \text{CoA} \xrightarrow{\text{acyl-CoA synthetase}} \text{acyl}-\text{CoA} + \text{AMP} + \text{PPi} \quad [1]$$

$$\text{PPi} + \text{UDP}-\text{glc} \xrightarrow{\text{UDP-glucose pyrophosphorylase}} \text{glucose-1-phosphate} + \text{UTP} \quad [2]$$

$$\text{glucose-1-phosphate} \xrightarrow{\text{phosphoglucomutase}} \text{glucose-6-phosphate} \quad [3]$$

$$\text{glucose-6-phosphate} + \text{NAD}^+ \xrightarrow{\text{glucose-6-phosphate 1-dehydrogenase}} \text{6-phosphoglucono-}\delta\text{-lactone} + \text{NADH} + \text{H}^+ \quad [4]$$

The amount of NADH present is directly proportional to the concentration of NEFA in this reaction sequence.

(ii) Luminescence detection. The principle of the luminescent method, outlined below, has been described previously (11):

$$\text{NADH} + \text{H}^+ + \text{FMN} \xrightarrow{\text{oxidoreductase}} \text{NAD}^+ + \text{FMNH}_2 \quad [5]$$

$$\text{FMNH}_2 + \text{RCHO} + \text{O}_2 \xrightarrow{\text{bacterial luciferase}} \text{FMN} + \text{RCOOH} + \text{H}_2\text{O} + \text{light} \quad [6]$$

This process is metabolite-independent and involves the kinetic measurement of NADH. The amount of light output measured is directly proportional to the NADH present.

The technique of luminescence detection (11) has been modified and semiautomated for the measurement of lactose, galactose, β-hydroxybutyrate, and D-lactate (12) with the ML2250 Microtiter Plate Luminometer (Dynatech Laboratories, Chantilly, VA). In the current study, this technique (12) has been adapted for the determination of NEFA in milk and plasma.

Methods. (i) Sample collection and treatment. Milk samples were collected from mothers at the Department of Biochemistry, The University of Western Australia. Mothers expressed a small amount of milk (<1 mL) before and after each breast feed. Subsamples (250 µL) for NEFA determination were taken immediately and 400 mM EDTA (20 µL) was added according to Chappell *et al.* (13). Samples were frozen at −80°C until required for assay. Prior to assay, milk samples were thawed and then incubated at 80°C in a water bath for 5 min. Following incubation, samples were sonicated (Branson Sonifer B-12; Branson Ultrasonics Corp., Danbury, CT) for 20 s and a subsample (50 µL) was acid-denatured. The method of acid denaturation was modified from Kather and Weiland (6) and involved the addition of 1 M HCl (50 µL) to the 50 µL subsample which was mixed and left to stand at room temperature for 1 min. Samples were neutralized with an equal volume of 1 M NaOH in 100 mM TES.

Finger-prick blood samples were collected in heparinized hematocrit tubes (Chase Instruments, Glen Falls, VT) and centrifuged (H.I. Clements, North Ryde, Australia) for 5 min at $8000 \times g$. Plasma was treated at collection according to a modification of the acid denaturation procedure used for milk. Five microliters of plasma was added to the same volume of 200 mM HCl, mixed, and allowed to stand for 1 min at room temperature. The sample was neutralized by addition of an equal volume of 200 mM NaOH in 100 mM TES and stored at −80°C until required for assay. For GC analysis plasma was obtained from the Blood Transfusion Service of Western Australia.

(ii) Working solutions: reagents. Assay reagent: ATP (4 mM), $MgCl_2$ (10 mM), CoA (30 mM in 25 mM MOPS buffer, pH 5), UDP-glc (3 mM), NAD^+ (8.5 mM), acyl-CoA synthetase (1.2 U/mL), UDP-glucose pyrophosphorylase (0.8 U/mL in 50 mM TEA and 200 µM dithiothreitol), phosphoglucomutase (13.4 U/mL), glucose-6-phosphate 1-dehydro-

genase (6 U/ml), Triton X-100 (0.03%, vol/vol) in 100 mM TEA, pH 8.4. Control reagent: assay reagent excluding acyl-CoA synthetase. Components for the assay/control reagent (except phosphoglucomutase, glucose-6-phosphate 1-dehydrogenase) were stored as stock solutions and were stable for at least 3 mon at −80°C. The complete assay and control reagents were aliquoted and stored at −80°C and were stable for 1 mon. The bioluminescent reagent was prepared according to Thompson *et al.* (12): potassium phosphate (100 mM), BSA (1.6%), EDTA (4 mM), FMN (8 μM), tetradecylaldehyde (0.1%), and bacterial luciferase (0.4 mg/mL) in TES (300 mM, pH 6.8) was aliquoted and stored at −80°C and was stable for at least 4 mon (13). Reagents for the Wako NEFA-C kit were prepared according to manufacturers' instructions. Reagents prepared for modification of the Wako NEFA-C kit have been outlined previously (14).

(ii) Working solutions: standards. A 1 mM NEFA standard was prepared by adding 16:0 to a heated solution of 4% BSA and 0.1% Triton X-100 (50°C). This solution was briefly sonicated and diluted with the BSA/Triton solution to prepare individual standards (range 0–500 μM) which were stored at −20°C. Standards for the Wako NEFA-C kit were prepared according to manufacturers' instructions.

Procedures. Standards were heated to 37°C and vortexed. Two microliters (plasma) and 5 μL (milk) of each standard, sample, blank, and quality control were pipetted in triplicate into a 96-well V-bottom microtiter plate (Flow Laboratories, McLean, VA). Two or 5 μL of either assay or control reagent was added to each well. The plate was mixed (Denley Welltech Wellmixx2, Cytosystems, Castle Hill, Australia), sealed (plate sealer, ICN Biomedicals, Aurora, CA), and incubated at 37°C for 60 min. The procedure for plate luminometry was modified from Thompson *et al.* (12). The reaction was stopped with the addition of 200 μL NaOH (20 mM). Five-microliter aliquots from each well were transferred to a Microlite® 96-well flat-bottom plate (Dynatech Laboratories), diluted with 150 μL NaOH (10 mM), and reacted with 15 μL of bioluminescent reagent in a ML2250 Microtiter Plate Luminometer (Dynatech Laboratories). The luminometer was programmed for a 5-s delay after addition of the bioluminescent reagent which was followed by a 5-s read period. The peak and integrated light response during the read period were automatically calculated. The sample concentration was determined by calculation relative to a standard curve.

The current method for plasma was adapted for use with a plate spectrophotometer (Titertek Multiskan® MCC/340 plate reader; Flow Laboratories) according to the following modifications: 50 μL of each sample, standard, blank, and quality control was plated in duplicate and 50 μL of either assay or control reagent was added to each well. Following incubation, 50 μL of double-deionized water was added to each well and absorbance was measured at 340 nm on the plate spectrophotometer.

Samples were analyzed using the Wako NEFA-C kit according to either manufacturers' instructions or Johnson and Peters (14) using a plate spectrophotometer (Titertek Multiskan®). Assay procedures for NEFA analysis by GC have been previously described (10). Briefly, lipids were extracted from plasma into methanol/chloroform (1:1, vol/vol) with internal standard (heptadecanoic acid). Lipid extracts, evaporated to dryness under N_2 at 40°C, were dissolved in hexane/*tert*-butyl methyl ether (200:3, vol/vol). Nonesterified fatty acids were separated (porasil silica column) and evaporated to dryness under N_2. Dried NEFA samples were methylated by the addition of H_2SO_4 in methanol and incubated at room temperature for 40 min. Methyl esters were extracted into hexane and evaporated to dryness under N_2. Toluene containing internal standard (methyl pentadecanoic acid) was added and methyl esters were separated and quantified by GC at 150°C [(Shimadzu GC-8A (Kyoto, Japan), glass column, 2 m length and 2.6 mm internal diameter packed with 10% SP-2340 on 100/120 Chromosorb (Supelco, Bellefonte, PA)].

Statistics. Paired and unpaired *t*-tests were used to determine the significance of differences between plasma sample collection and treatment procedures and differences in NEFA concentrations determined between methods. Statistical analysis was performed using Statview SE + Graphics® (Abacus Concepts, Inc., Berkeley, CA). All results presented are means ± SE unless stated otherwise.

RESULTS AND DISCUSSION

Our aim was to develop an assay for determination of NEFA concentration in large numbers (≥600) of small (≤5 μL) human milk and plasma samples. An appropriate technique was required to enable routine processing of these samples. In this context the current method has been developed as a semiautomated procedure utilizing either a Microtiter plate luminometer or a Titertek multiscan plate spectrophotometer and 96-well microtiter plates.

Standards. To avoid underestimation of NEFA concentration, standards were prepared with 4% BSA based on the method of Miles *et al.* (15). Light responses increased linearly for 16:0 standards over the range 0–500 μM ($r = 0.998$) and showed close agreement with known concentrations of NADH ($P < 0.001$, $r = 0.999$; $y = -5.06 \times 10^{-3} + 0.978x$, y = NADH, x = 16:0). The plate spectrophotometric procedure showed a linear increase in absorbance for 16:0 standards across the range 0–500 μM ($r = 0.999$). Standards were stable for approximately 1 mon at −20°C.

Sample collection and treatment. (i) Milk. Chemical colorimetric (16,17) and GC (18,19) methods are predominantly used to determine NEFA concentration in milk. These methods are costly, labor-intensive, and require relatively large sample volumes (13). The development of enzymatic spectrophotometric (4) and luminometric procedures (6) for NEFA determination in plasma has overcome many of these problems. Determination of NEFA requires sample treatment to prevent lipolysis during storage and analysis. However, enzymatic determination of NEFA in milk requires additional sample treatment to limit interference from enzymes present in milk.

In vitro enzymatic lipolysis due to lipases in milk can be prevented by the addition of EDTA to milk samples at collection (13). Accordingly, NEFA concentration in samples collected without EDTA increased by 102 ± 28% during 60 min at room temperature (361 ± 17 to 714 ± 64 μM, $n = 5$; $P < 0.001$) whereas samples collected with EDTA increased by 5 ± 5% (361 ± 17 to 375 ± 3 μM, $n = 5$; $P = 0.81$). Production of NADH beyond the incubation period (drift) was observed in milk samples collected with EDTA. This outcome is likely to be a consequence of thioesterase II (20) as well as nondivalent cation-dependent lipases present in milk (13). This drift was abolished by incorporating an incubation step (80°C for 5 min) and acid denaturation after the addition of EDTA during treatment of milk samples.

(ii) Plasma. Heparin is known to activate plasma lipoprotein lipase (21), however, collection of blood samples in heparinized containers is often desirable to prevent clot formation. Despite the potential for *in vitro* enzymatic lipolysis (7), available literature reveals a lack of consistency in the approach to limit this effect (9,22). The efficacy of acid denaturation to prevent enzymatic lipolysis in plasma samples collected with heparinized tubes was examined in the current study. Duplicate plasma samples were either acid-denatured immediately upon collection and stored at −80°C or stored at −80°C and diluted with water prior to assay. Immediate acid denaturation resulted in lower values (17.1 ± 8.5%) for plasma NEFA when compared to samples untreated before storage ($P = 0.059$; $n = 9$). There was no difference when samples were untreated before storage and either acid-denatured or diluted with water prior to assay ($P = 0.979$; $n = 4$). These findings indicate that plasma collected in heparinized containers should be acid-denatured immediately at collection.

The potential for variation in sample final pH following treatment with acid and base during acid denaturation can result in chemical hydrolysis of plasma triacylglycerol and hence overestimation of NEFA concentration. When plasma samples (5 μL) were acid-denatured with HCl (1 M) and NaOH (1 M) according to Kather and Weiland (6), a large range for plasma sample final pH (5–9) was observed. Hydrochloric acid (1 M; 1:1, vol/vol) added to the sample (pH 0–1) and allowed to stand at room temperature for 0–4 min had no effect on NEFA concentration ($P = 0.521$; $n = 4$). However, alkaline conditions are known to cause saponification of triacylglycerol. Duplicate plasma samples were acid-denatured and NaOH was added to the sample/HCl mixture resulting in either an alkaline (range, 9–13) or a neutral pH. Plasma NEFA concentrations were higher in alkaline compared to neutral pH samples immediately following sample treatment ($P = 0.015$; 91 ± 29 μM neutral, 333 ± 53 μM alkaline) and after 60 min at room temperature ($P = 0.001$; 124 ± 33 μM neutral, 539 ± 40 μM alkaline). There was no change in NEFA levels across the 60 min at room temperature in neutral pH samples ($P = 0.203$). In contrast, a 67 ± 14% increase in NEFA was observed during the same period in alkaline samples ($P = 0.009$). Alkaline final pH following plasma sample treatment with acid and base has been avoided by reducing the HCl and NaOH concentrations from 1 M to 200 mM and including a TES buffer (100 mM, pH 7) with the NaOH.

Reagent. All enzymatic methods for determination of NEFA require activation by acyl-CoA synthetase (4). Following this initial reaction, the current assay sequence differs from existing enzymatic methods (4–6,15,23,24) by utilizing UDP-glucose pyrophosphorylase to convert inorganic pyrophosphate to glucose-1-phosphate in the presence of UDP-glc. Glucose-1-phosphate is linked to the production of NADH by way of the reactions catalyzed by phosphoglucomutase and glucose-6-phosphate 1-dehydrogenase. The control reagent (assay reagent excluding acyl-CoA synthetase) was used to account for the presence of glucose-1-phosphate, glucose-6-phosphate, and inorganic pyrophosphate in milk (<5%) and plasma (≤10%).

This assay sequence has enabled a reduction in completion time compared to previous luminometric methods (6). The formation of NADH from NEFA in a 500 μM 16:0 standard and in samples of milk and plasma was complete within 50 min at 37°C and was stable from 60–120 min (Fig. 1). Furthermore, changes in absorbances from a 500 μM 16:0 standard and a human plasma sample showed a similar response for the plate spectrophotometric method (Fig. 1). Accordingly, an incubation period of 60 min at 37°C for both methods was chosen to ensure complete formation of NADH. The concentrations of acyl-CoA synthetase and UDP-glucose pyrophosphorylase, the rate-limiting enzymes for the assay sequence, were optimized for these incubation conditions and to reduce background reagent contamination.

The reactions involved in luminescence detection can be affected by several components present in either the reagent mixture or the sample. Inhibition or stimulation of the light

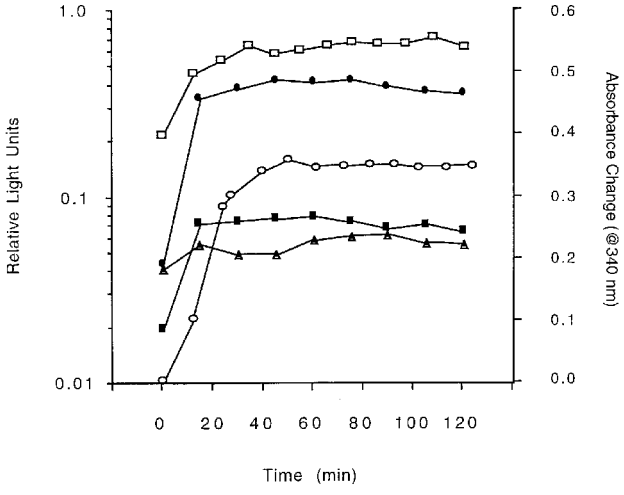

FIG. 1. Time course of nonesterified fatty acid (NEFA) conversion from a 500 μM 16:0 (●) standard and from samples of (▲) human milk and (■) plasma (plate luminometric method, closed symbols; plate spectrophotometric method, open symbols). NADH from NEFA is converted to relative light units as described in the Materials and Methods section of the text. Values are means of duplicate determinations.

TABLE 1
Effect of Reagent and Sample Components on Light Response

Component	Dilution factor[a]	
	10% change[b]	50% change[b]
Milk	65.9	11.5
Plasma	111	4.4
Reagent	520	92.9

[a]Final dilution factor for component in the luminometer well which provided either a 10 or 50% change in relative light units.
[b]Light response normally present in diluted luminescent reagent was taken as 100%.

response by these components was minimized by the high final dilution (1428-fold in the luminometer well) of the reaction mixture. The effects of specific components on the light response are outlined in Table 1.

We found enzymatic spectrophotometric techniques unsuitable for NEFA determination in whole milk and milk whey fractions owing to the opacity of the sample. This problem has been overcome by the use of the NADH-luciferase system. In addition, the combination of the current assay sequence with this system resulted in considerably lower reagent costs when compared to a commercially available spectrophotometric method (Wako NEFA-C kit).

Validation. Recovery, precision, and sensitivity. The recovery of a range of NEFA added to different milk and plasma samples, the interassay variation, and the detection limit (defined as three times the standard deviation of the mean blank) and sensitivity (defined as the detection limit concentration in the luminometer well volume) for the luminometric and spectrophotometric assays are listed in Table 2.

Method comparisons. Human plasma NEFA concentrations determined by the plate luminometric method and the plate spectrophotometric procedure were highly correlated ($r = 0.985$; $P < 0.02$) and showed close agreement ($P = 0.53$; Fig. 2). The plate luminometric method was also compared to a commercially available spectrophotometric method (Wako NEFA-C kit) and a modification of this method for use with 10-µL sample volumes (14). The concentration of NEFA in human plasma samples determined by the Wako NEFA-C kit and the plate luminometric method were highly correlated ($r = 0.998$; $P < 0.001$) and differed by less than 6% ($P = 0.13$; Fig. 2). In contrast, NEFA concentrations determined by the method of Johnson and Peters (14), although highly correlated with the plate luminometric method ($r = 0.989$; $P < 0.001$), were approximately 50% lower ($P = 0.01$; Fig. 2). This difference was found to be due to the effect of sample treatment procedures. Specifically, NEFA concentrations in untreated plasma were lower compared to both acid-denatured ($P = 0.009$) and water-diluted ($P = 0.04$) plasma although all treatments were highly correlated ($P < 0.001$, $r = 0.998$, $y = 11.6 + 1.51x$, y = water-diluted, x = untreated; $P < 0.001$, $r = 0.975$, $y = 112.5 + 1.41x$, y = acid-denatured, x = untreated).

Considerable differences in NEFA concentrations have been reported for blood samples collected under similar conditions, in unrelated studies, when determined by either GC (25) or enzymatic methods (22). When plasma samples were subjected to parallel analysis, we found NEFA concentrations in plasma measured by GC were highly correlated with those determined by the Wako NEFA-C kit and the plate luminometric method ($r = 0.986$, $P < 0.02$; and $r = 0.992$, $P < 0.01$, respectively). However, values derived from GC were 28% higher when compared to those determined by the enzymatic methods ($P = 0.02$, Wako NEFA-C kit; $P = 0.03$, plate luminometry; Fig. 2). These results support the findings of previous direct comparisons between GC and enzymatic methods (26,27). Differences in NEFA–protein interactions between standards and samples have been avoided by the inclusion of albumin with standards (15). This discrepancy is therefore not entirely due to the analytical behavior of the standards. Alternatively, the lower values determined by enzymatic methods compared to GC may be explained by the inability of acyl-CoA synthetase to activate fatty acids tightly bound to albumin (28) or those containing more than 20 carbon atoms (5). As a consequence of the difference in NEFA levels determined by enzymatic procedures when compared to GC, the method of determination should be taken into account when comparing values reported in the literature.

TABLE 2
Recovery of Known Concentrations of NEFA Added to Human Milk and Plasma, the Interassay Variation, and the Detection Limit and the Sensitivity of the Plate Luminometric and Spectrophotometric Methods

NEFA	Recovery (%)	Interassay variation (%)	Detection limit (µM)[a]	Sensitivity (nM)[b]
6:0[c]	97.5 ± 1.52 $n = 8$	7.4 $n = 5$	8.0 ± 0.6 $n = 8$	5.6 ± 0.4 $n = 8$
12:0[c]	96.4 ± 1.64 $n = 8$			
16:0[c]	94.9 ± 0.91 $n = 10$			
18:1n-9[c]	97.4 ± 2.55 $n = 7$			
16:0[d]	100 ± 4.50 $n = 8$	6.8 $n = 7$	9.0 ± 1.7 $n = 10$	2.6 ± 0.5 $n = 10$
16:0[e]	95.8 ± 1.80 $n = 6$	4.9 $n = 5$	13.2 ± 2.7 $n = 5$	4393 ± 916 $n = 5$

[a]Defined as three times the standard deviation of the mean blank.
[b]Defined as the detection limit concentration in the final well volume.
[c]Luminometry for milk.
[d]Luminometry for plasma.
[e]Spectrophotometry for plasma. For abbreviation see Table 1.

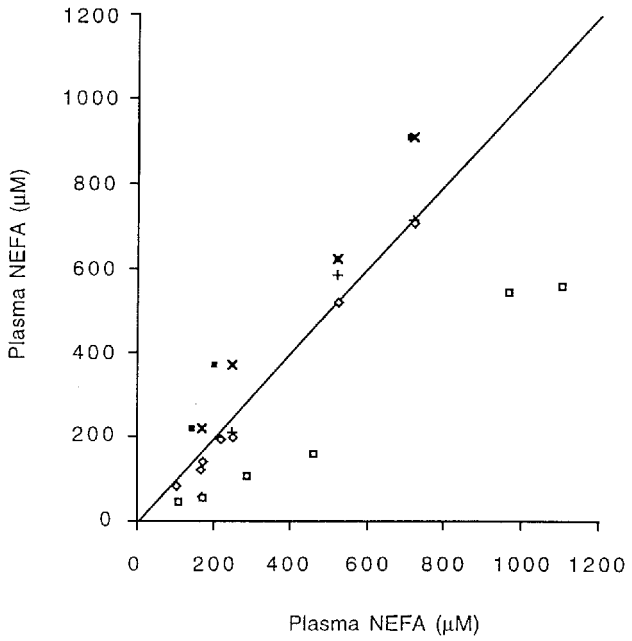

FIG. 2. Comparison of methods for determination of NEFA in human plasma. (+) Plate luminometric method vs. plate spectrophotometric method, $y = -40.67 + 1.09x$; (◇) plate luminometric method vs. Wako NEFA-C kit, $y = -32.23 + 1.04x$; (×) plate luminometric method vs. gas chromatography (10), $y = 37.80 + 1.19x$; (□) plate luminometric method vs. Johnson & Peters (14), $y = -44.23 + 0.57x$; (■) Wako NEFA-C kit vs. gas chromatography (10), $y = 90.73 + 1.12x$; — line of unity, $y = x$ (i.e., slope = 1, intercept = 0). Values are in micromoles and are means of triplicate determinations. For abbreviation see Figure 1.

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