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Errors in measuring plasma free fatty acid concentrations with a popular enzymatic colorimetric kit

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ABSTRACT

Objectives: Our goal was to test whether an enzymatic, colorimetric assay, the WAKO NEFA kit, provides information equivalent to liquid chromatography (LC) LC-based measures of free fatty acid (FFA).
Design & methods: We reanalyzed nadir FFA samples from 109 volunteers from a previous study where we demonstrated that maximal suppression of FFA concentrations predicts metabolic abnormalities in humans; the results from the WAKO NEFA kit, which has been widely used for over three decades, could not replicate our findings. We conducted additional studies to directly compare results from this kit to our LC-mass spectrometry (LC/MS) method that was validated by our LC-UV detection method.
Results: Plasma samples with FFA concentrations ranging from 0.015 to 1.813 mmol/L were measured both by LC-mass spectrometry (LC/MS) and by the WAKO NEFA kit. Despite good overall agreement ($R^2 = 0.86$), the slope was significantly different from 1.0 and the intercept was significantly different from zero. The results from the kit were especially discrepant with FFA concentrations < 0.200 and > 1.000 mmol/L. Some of the discrepancy was related to the use of oleate as the standard solution for the kit and the substrate specificity of the kit enzymes for different fatty acids. Despite attempts to improve the kit by modifying the reaction time, sample volume and the types of standard solutions, we could not obtain a satisfactory agreement between the WAKO NEFA results and LC/MS.
Conclusions: The WAKO NEFA kit should not be used when high precision and accuracy of FFA concentrations over a wide range is required.

1. Introduction

Free fatty acids (FFA), also referred to as non-esterified fatty acids (NEFA), are the major circulating lipid fuel, even though FFA concentrations are considerably less than triglyceride concentrations. When present in excess, FFA can contribute to insulin resistance [1,2]. Elevated FFA concentrations are commonly found in obesity/type 2 diabetes [3–6], and are implicated in the pathogenesis of non-alcoholic fatty liver disease [7–10]. The failure to normally suppress FFA concentrations is associated with a greater risk of type 2 diabetes in women with gestational diabetes [11]. Plasma FFA concentrations can be quite variable, ranging from over 1.5 mmol/L in fasting [12] or diabetic ketoacidosis [13] to less 0.01 mmol/L in the insulin suppressed state [12,14–16]. We found that the degree of post-meal suppression of plasma FFA concentrations was an independent predictor of insulin sensitivity and serum triglyceride concentrations, even within the lower range of FFA [14,15]. Thus, the accuracy of FFA concentration measurements level over a wide range is important both for clinical and research purposes.

The techniques used to measure FFA concentrations has evolved over the years, ranging from titrimetric determination, thin-layer chromatography (TLC) to gas chromatography (GC) and liquid chromatography (LC)-based methods [17–20]. Microfluorometric and colorimetric approaches that include chemical and enzymatic methods are also available to determine total FFA concentrations [21–23]. The WAKO NEFA kit (FUJIFILM WAKO Diagnostics U.S.A. Corporation) employs enzymatic-colorimetric approaches to measure total FFA concentrations [24] and has been very widely used since the 1980s [25–27].

If the concentrations of both total FFA and each species of fatty

Abbreviations: BCA, bicinchonionic acid; BSA, bovine serum albumin; FFA, free fatty acid; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; NEFA, non-esterified fatty acid; OD, optical density; TLC, thin-layer chromatography; UV, ultraviolet-visible

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acids are needed, GC or LC approaches are required. However, these methods are more laborious and time consuming than approaches such as the WAKO NEFA kit if only total FFA concentrations are needed. Although a few previous studies reportedly compared the WAKO NEFA kit to other methods [28,29], the reported testing wasn't sufficiently rigorous to convince us to change. We therefore assessed whether FFA concentrations measured by the WAKO NEFA kit could replicate our findings that nadir suppression of FFA (measured using an LC/UV detection approach) is predictive of fasting triglycerides and insulin sensitivity. Because our analysis indicated that the WAKO NEFA kit could not meet that standard, we undertook a series of experiments to understand the reason for the differences in order to attempts to improve the accuracy of the kit.

2. Materials and methods

2.1. Subjects and sample collection

To test whether we could obtain the same predictive value for plasma FFA concentrations using the WAKO NEFA kit as we have observed for LC-based FFA measures we analyzed plasma samples from 109 volunteers from our previous study that identified the predictive value of suppressed FFA concentrations with respect to metabolic abnormalities [14]. Body composition was measured using dual-energy x-ray absorptiometry and single-slice abdominal computed tomography scan [30]. Insulin sensitivity was measured with both intravenous (IV) glucose tolerance test (SI_{IVGTT}) and mixed meal tolerance test (SI_{Meal}) [14]. The plasma FFA concentrations in this study were measured by an LC-UV detection method [31] that we have used to calibrate and validate our newer LC/MS method [32]. In that study we were primarily interested in palmitate kinetics as measured using tracer methodology; we therefore did not measure the full complement of FFA species in all samples. In previous studies we found that plasma palmitate concentrations averaged between 22% [33] and 25% [34] of total FFA. For this analysis we used the palmitate concentrations for each participant and calculated total FFA concentrations assuming that palmitate was 24% of total FFA based upon these [33,34] and a large number of other studies.

We collected blood samples from 17 volunteers under overnight, postabsorptive conditions and/or during an intravenous insulin and glucose infusion – a so-called insulin clamp - or during an infusion of somatostatin to suppress insulin secretion and allow FFA concentrations to rise above fasting levels.

These studies were approved by the Mayo Clinic Institutional Review Board. Blood samples were immediately centrifuged at 3000 RPM for 15 min at 4 °C in the centrifuge of J-20 XPI from Beckman Coulter (Pasadena, CA) to separate the plasma, which was then stored at −80 °C.

2.2. Total FFA concentrations measured by the WAKO kit

The principle of the WAKO NEFA kit (WAKO Chemicals Inc. Richmond, VA, USA) assay is a two-step enzymatic conversion of non-esterified fatty acids to 2,3-trans-Enoyl-CoA and H_2O_2, which results in the H_2O_2 catalyzing a chemical reaction that results in a color change that is detected as a change in optical density (OD). The kit includes NEFA-HR2 Color Reagent A and B, NEFA-HR2 Color Solvent A and B, NEFA linearity solution, NEFA standard solution, control serum I and II. The flat bottom 96-well plates used in these experiments were purchased from Thermo Fisher Scientific (Waltham, MA). We used a SpectraMax M2 spectrophotometer (Molecular Devices, San Jose, CA) to read the OD values. The WAKO NEFA standard solution was diluted to create concentration standards so as to generate the formulas needed to calculate the concentration of each sample and the WAKO NEFA Linearity Solution was to test the suggested test range. The Control Serum samples I and II provide the reference samples for abnormal and normal plasma; these are measured for each assay to assure proper setup.

The assay requires 5 μL of plasma sample in each well and plasma sample was measured in triplicate, with appropriate blanks included. After the three steps outlined in the kit instructions are completed the absorbance of the mixture is read and the final absorbance was calculated per instructions. The concentration of each sample is calculated with the formula generated from the absorbance of WAKO standard solution.

2.3. Total FFA concentrations measured by LC/MS

We used separate aliquots of the same, unthawed plasma samples described above. The procedures for measure total FFA using LC/MS have been described [32]. We used our LC/UV detection method of measuring plasma FFA concentrations [31] to validate and calibrate the LC/MS method, and have converted all analyses to the LC/MS method because it provides the same precision and accuracy while using less plasma. Briefly, we use serial dilutions of a mixed FFA standard solution with 12 fatty acid species during each assay to create a standard curve and we include two different quality control samples with each assay to assure inter-assay variability is minimal. The mixed FFA concentration standard solution and a newly created (for the purpose of this study) oleate standard solution are prepared in our laboratory using pure (as measured by LC/MS), gravimetrically measured fatty acids (Sigma-Aldrich, St. Louis, MO) suspended in fatty acid free bovine serum albumin (BSA) (Sigma-Aldrich). The amounts of each fatty acid we include are proportional to their usual presence in human plasma. To each standard and sample we add an aliquot of heptadecanoate in fatty acid free bovine serum albumin to serve as an internal standard. The FFA are extracted using a Dole solution and analyzed on the LC/MS system. The concentration of each fatty acid was calculated by the standard curve generated with the mixed FFA standard solution made in our laboratory with a range of 10 different concentrations.

To measure the concentration of WAKO standard solution (that contains only oleate) by LC/MS we diluted the solution, assuming the manufacturer's stated concentration was correct, to create a series of samples with the highest and lowest concentrations calculated to be 1.0 and 0.1 mmol/L. An aliquot of a “blank” containing only the internal standard served as the zero concentration sample. The WAKO control serums were also diluted using a similar approach. All samples were analyzed in duplicate.

2.4. Protein concentrations measured by bicinchoninic acid kit

Oleate is not soluble in an aqueous solution; we used fatty acid free BSA to create our FFA concentration standards. The compound used to maintain oleate in solution for the WAKO standard solution is proprietary; to test whether it might be albumin we tested the WAKO standard solution for protein content using a bicinchoninic acid (BCA) kit (Prod 23225, Thermo Fisher Scientific, Waltham, MA).

2.5. Statistical analysis

To analyze the difference between paired data such as concentrations or ratios we used *t*-tests if the data was normally distributed. If the data was not normally distributed we performed a log transformation to achieve a normal distribution. If the data could not be transformed to achieve a normal distribution we used the Wilcoxon signed-rank test. Correlations between the concentrations and ratios were done using Pearson test if the data was normally distributed or could be transformed to achieve a normal distribution. The Spearman rank test was applied if otherwise. To determine the predictors of fasting plasma triglyceride concentrations and insulin sensitivity, multiple linear regression analysis and type II squared partial correlations were performed to identify the combined predictors or the predictive value of

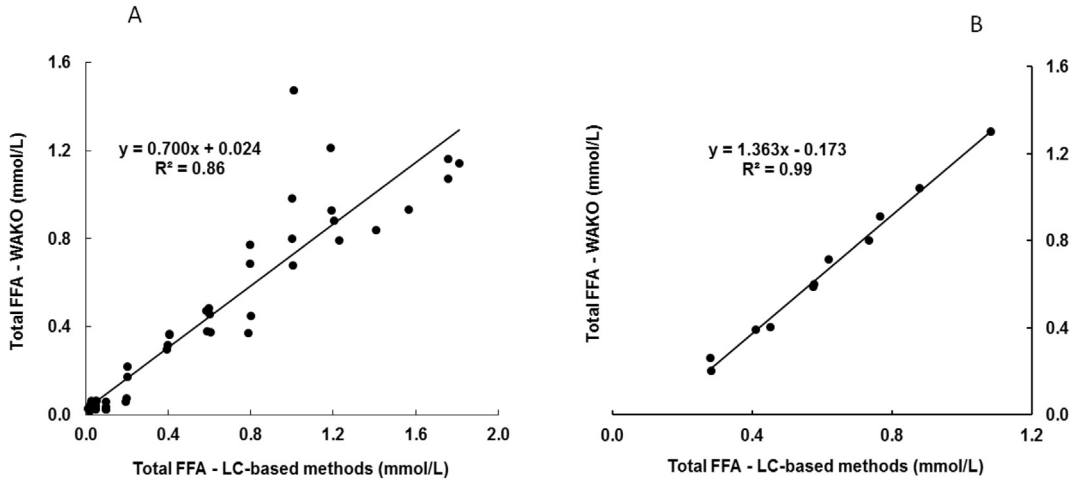

Fig. 1. Relationship between FFA concentrations measured by LC/MS and the WAKO NEFA kit. Panel A depicts total FFA concentrations in 47 separate samples measured using both LC/MS and the WAKO NEFA kit. Panel B depicts total FFA concentrations of the WAKO control serum samples, including several dilutions to achieve a wider range of values.

each factor by measuring model R^2 and partial R^2. Differences were considered statistically significantly if the *P*-value was < 0.05. Statistical analysis was performed using SPSS version 20.0 (SPSS Inc. Chicago, IL, USA).

3. Results

We first assayed in triplicate the linearity solution and the two control serum samples provided with the WAKO NEFA kit to assure that our experimental procedures provided the expected results. A formula was generated using the WAKO standard solution (stated to be oleate) following the instructions and the concentrations of different samples were calculated using the OD values and the formula. The OD values were read immediately after Reagent B was added and mixed well as per the manufacturer instructions. The concentrations and standard deviations of the two control serum samples were consistent with those described in the instructions with the kit; the mean ± SD FFA concentrations of control serum I and control serum II were 1.21 ± 0.064 and 0.81 ± 0.009 mmol/L, respectively. This indicates that the kit was performing according to the manufacturer's metrics in our hands.

3.1. Comparison of WAKO NEFA kit measurement vs. LC-based methods of FFA

To test whether the WAKO NEFA kit provides FFA concentration values that are as predictive of metabolic abnormalities as our LC-based method, we assayed total FFA concentrations in samples from 109 volunteers that we had stored the nadir FFA concentration time point. In that study we found the nadir suppressed FFA concentrations were predictive of fasting plasma triglyceride concentrations and parameters of insulin resistance [14]. For this set of samples, the LC-based measure of FFA concentrations was correlated with fasting plasma triglyceride concentrations ($R^2 = 0.369$, $p < .001$) and SI_{Meal} ($R^2 = -0.236$, $p = .007$). The FFA concentrations on the same samples measured using the WAKO kit were somewhat less predictive of SI_{Meal} ($R^2 = -0.207$, $p = .034$) and were not correlated with fasting plasma triglyceride concentrations.

Another finding of our previous study was that the strongest combined predictors of plasma triglyceride concentrations were visceral fat, post-meal nadir FFA concentrations, sex and age while the best predictors of SI_{Meal} were meal nadir FFA concentrations, upper body subcutaneous fat mass, VO_2 max and sex. To test if the FFA concentrations measured by the WAKO NEFA kit could serve equally as well as LC-based measures of FFA in predicting plasma triglyceride concentrations and SI_{Meal}, we calculated the model R^2 and partial R^2 using multiple linear regression analysis and type II squared partial correlations as described in the previous study [14]. We re-ran the model of predictors of plasma triglyceride concentrations using meal nadir FFA concentrations (LC-based), visceral fat, sex and age; this combination continued to predict plasma triglyceride concentrations ($p = .001$). For the model of predictors of $SI_{Meal,}$ the combination of meal nadir FFA concentrations (LC-based), upper body subcutaneous fat, VO_2 max and sex continued to explain 21% of the total variance ($p < .001$). The LC-based methods of measuring plasma FFA concentrations contributed significantly to the models for both plasma triglyceride concentrations and SI_{Meal} (partial R^2 of 12.3% and 13.3%, respectively, $p < .001$). In contrast, FFA concentrations measured by the WAKO NEFA kit were not significant predictors of plasma triglyceride concentrations. In regression model predicting SI_{Meal}, using the WAKO kit measures of nadir meal FFA, the combination of factors could predict only 14.7% of the variance ($p = .003$) and the partial R^2 of WAKO measured FFA concentrations was reduced to 6.5% ($p = .01$). These results indicated to us that the WAKO kit measures of plasma FFA, at least under suppressed conditions, lacked the robustness of LC-based methods. In order to understand why, and to determine if we could improve the kit performance, additional studies were conducted.

3.2. Results of WAKO vs. plasma and control serum

Plasma FFA concentrations were measured on 46 samples from obtained from the studies being conducted by our laboratory using the WAKO kit and LC/MS. The range of total FFA concentrations as measured by LC/MS was from 0.015 to1.813 mmol/L. The concentrations as measured by the WAKO kit vs. LC/MS are depicted in Fig. 1. The overall agreement was good, ($R^2 = 0.86$), however, the slope was significantly different than 1.0 and the intercept was significantly different from zero (Fig. 1A).

We then measured the concentrations of the WAKO kit control serums by diluting them to create samples with a range of concentrations that would fall within the linear range of LC/MS; all samples were run in triplicate. The WAKO control serum samples contained the full range of fatty acid species. The FFA concentrations of the two WAKO control serum samples measured by LC/MS were greater (up to 1.4 times) than those indicated by the manufacturer and those measured using the WAKO kit assay. Fig. 1B plots the concentrations created by serial dilutions of the two WAKO control serum samples vs. the concentrations measured by LC/MS. Although the correlation was excellent, the slope was > 1.0 ($p < .0001$) and the intercept was < 0.0

Table 1
Concentrations of 12 different free fatty acids measured by WAKO.

Sample	WAKO value (mmol/L)
Oleate	0.362
Linolenic	0.363
Linoleic	0.465
Palmitoleic	0.423
Palmitelaidic	0.478
Palmitic	0.442
Elaidic	0.493
Arachidonic	0.564
Stearic	0.460
Myristic	0.541
EPA	0.093
DHA	0.122

All fatty acid concentrations were created to be 0.500 mmol/L. EPA –eicosapentaenoic acid; DHA – docosapentanoic acid.

(p = .0002).

From these results we concluded that there are systematic differences between LC/MS measures of total FFA and the WAKO kit. In order to understand the reasons for the differences we conducted additional experiments.

3.3. Effects of FFA species on FFA concentrations

To investigate the systematic differences between the two measurements, we examined the possibility that differences in individual FFA species might explain our findings. We measured the concentrations of the components of the kit using LC/MS. The standard solution from the WAKO kit contained only oleate.

It is not well-known whether enzymes used for the WAKO kit assay exhibit substantial substrate specificity. If it did, this might explain both the discrepancy between LC/MS measure of the control serum and the varying time courses of OD change we observed at different concentrations. To test for fatty acid species specificity with the WAKO kit we measured the concentrations of 12 different, individual FFA solutions (in fatty acid free BSA) that were created to have concentrations of 0.500 mmol/L. As shown in Table 1, the WAKO kit-measured concentrations for oleate and linolenic were ~30% less than the true concentration, EPA and DHA were ~80% less than the true concentrations, and the remainder were within 15% (usually less than) of the known concentration.

Because the WAKO kit measured the FFA oleate concentration as 30% less than that which we created, and because the WAKO standard solution is made of only oleate, we investigated the WAKO standard solution more thoroughly. To do this we created a dilution series from 1.0 to 0.025 mmol/L using the WAKO standard solution according to the manufacturer's directions and measured the oleate concentrations using LC/MS. The oleate concentration of the WAKO standard solution measured by LC/MS was more discrepant (up to 2.5 times greater) at low than at high oleate concentrations. Fig. 2 depicts the calculated oleate concentration from diluting the standard solution vs. the concentration measured by LC/MS. Although the agreement was excellent (R^2 = 0.97), the slope was significantly > 1.0 and the intercept was significantly > zero.

This did not seem possible unless the oleate concentrations we measured by LC/MS were in error due to problems with our mixed FFA standard solution. To test this possibility we created a new oleate solution in fatty acid free BSA with concentrations ranging from 0.1 mmol/L to 1 mmol/L and measured the concentrations against our mixed FFA standard solution. The LC/MS measured oleate concentrations agreed extremely well (R^2 = 0.9997, p < .0001, slope = 1.002 – p = NS vs. 1.0, intercept = 0.004, P – NS vs. 0). This indicated to us that the discrepancy between the WAKO standard solution and LC/MS was not due to problems with our standard curve or our instrument.

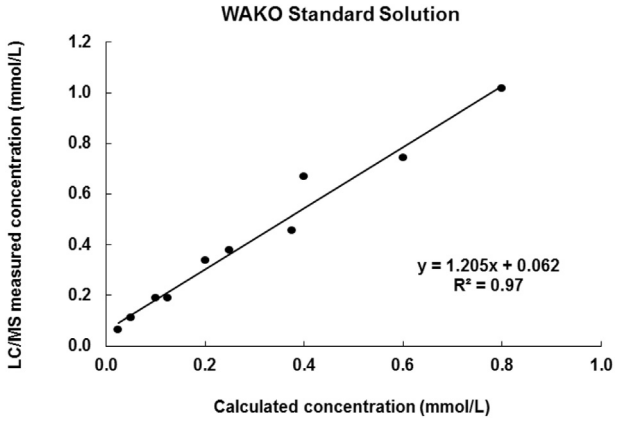

Fig. 2. Relationship between the concentrations of oleate in dilutions of the WAKO standard solution as calculated from the manufacturer's guidelines vs. as measured by LC/MS.

Having excluded problems with our LC/MS standard curve and instruments, we returned to the issue of why the WAKO standard solution (oleate) did not give the expected concentrations by LC/MS. Our laboratory oleate solution is in fatty acid free BSA, which provides a stable solution at different FFA concentrations. The means by which oleate is solubilized in the WAKO standard solution is proprietary, but to understand whether it might be albumin also, we tested the solution for protein (BCA) and found none. Thus, the method used to keep oleate in solution is not albumin or another protein.

This caused us to consider whether, in the process of diluting the WAKO standard solution to create a standard curve we were creating/exaggerating an issue of oleate miscibility, e.g., causing it to concentrate more in the upper layer of the tube. To test this hypothesis we created two, 10 mL samples of the WAKO standard solution with concentrations of 1.000 and 0.025 mmol/L. The two solutions were vortexed for 5 s and we then serially removed 1 mL aliquots from the top of each tube, saving a portion from each aliquot for assay in duplicate by LC/MS. In this way we could measure the concentration in each of ten different layers. We found that the LC/MS measured oleate concentration in the tube containing 0.025 mmol/L oleate declined significantly from the topmost to bottommost layer (p < .05, Fig. 3). In contrast, the WAKO standard solution with an oleate concentration of 1.0 mmol/L was not different between layers.

From these experiments we concluded that the WAKO oleate standard solution reacts with the enzymes used for the measurement less robustly that many other FFA species and that, in the process of making

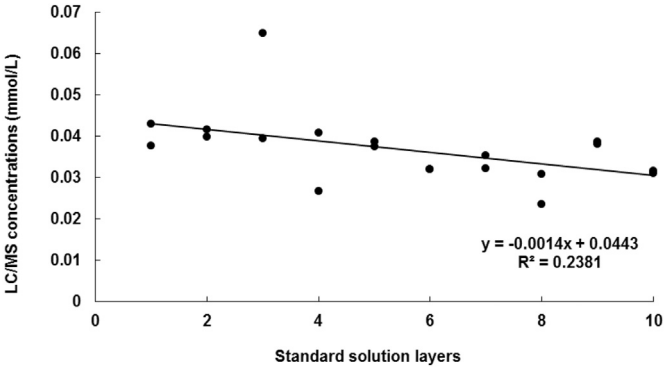

Fig. 3. Oleate concentrations measured in duplicate by LC/MS in layers 1–10 (1 being the top layer) of a 10 mL sample of the WAKO standard solution diluted to a calculated concentration of 0.025 mmol/L. The relationship is significant (r = −0.49, p < .05).

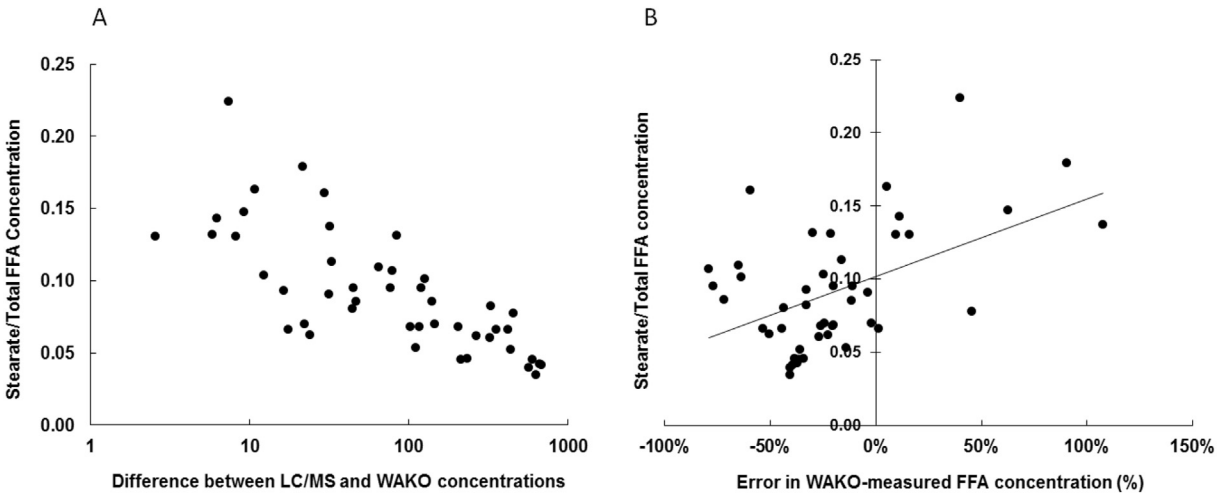

Fig. 4. Relationship between the proportion of FFA that was stearate and the difference between total FFA concentrations measured using the WAKO NEFA kit and LC/MS. Panel A is the absolute difference (plotted on a log scale) between the WAKO NEFA kit and LC/MS vs. the proportion of total FFA that was stearate. Panel B depicts the percent difference between the WAKO NEFA kit and LC/MS vs. the proportion of total FFA that was stearate.

dilutions for the standard curve according to the manufacturer's directions, it is possible to create inhomogeneous concentrations that can affect the standard curve if aliquots are taken from the top of a test tube.

Another implication of these findings is that natural variations in the FFA species as they contribute to total FFA concentrations may influence the WAKO results. We examined whether some of the errors in WAKO-measured total FFA concentrations is related to the variation in the proportions of total FFA from different species. To do this we examined the relationships between the percentages of four most abundant (palmitate, oleate, linoleate and stearate) FFA and the disagreement between the two methods. The relationships between the fraction of total FFA that was stearate and absolute difference between WAKO and LC/MS total FFA concentrations is shown in Fig. 4A (x-axis is log transformed to better display the data). The relationship between the fraction of total FFA that was stearate and percent error between WAKO and LC/MS total FFA concentrations was highly significant ($r = 0.50$, $p = .0004$, Fig. 4B). The concentrations measured using WAKO kit tended to be less than LC/MS when stearate was < 10% of total FFA and greater than LC/MS when stearate was > 10% of FFA, however, this observation was confounded by the fact that the percent of FFA that is stearate was consistently less when total FFA were lower and greater when total FFA are higher. The percent of FFA that were oleate, palmitate or linoleate didn't correlate with the error between the two methods; we saw no consistent pattern between total FFA concentrations and the percent of oleate, palmitate and linoleate.

3.4. Approaches to improve the WAKO kit results

3.4.1. Effects of larger sample plasma volume on WAKO-measured FFA concentrations

Plasma samples with the lowest total FFA concentrations by LC/MS had the greatest error when measured by the WAKO kit. Because the signal from the WAKO assay is related to the generation of H_2O_2 in direct proportion to the non-esterified fatty acid content of the sample, we considered that there may be an insufficient quantity of fatty acids in 5 μL to reliably generate a detectable signal at low FFA concentrations. If this were true, then increasing the volume of plasma used to assay samples with low concentrations should increase the signal and improve the accuracy/sensitivity. To test this hypothesis we selected two samples with FFA concentrations by LC/MS of ~0.025 mmol/L to assay by WAKO using 5, 10 and 20 μL of plasma. The calculated final concentrations were corrected for the volume of plasma used. Table 2 provides the measured concentrations of these 2 samples using the WAKO formula read at 15 and 60 min. Although the concentration of one sample was read correctly by WAKO with the 5 and 10 μL aliquot at 15 min, the concentration measured by WAKO in the other sample was twice as high with the 3 different volumes and 2 different incubation times. Thus, for plasma samples with low FFA concentrations, neither a greater sample volume nor extending the time of the assay uniformly mirrored concentrations measured by LC/MS.

3.4.2. Use of different standards to create concentration calculation formulas

Because of the problems we identified using pure oleate for the WAKO concentration curve, we used the mixed FFA solution from our LC/MS concentration standard curve to generate the curve for the WAKO kit. We also used a plasma sample that was diluted to different concentrations in attempts to achieve better consistency between the WAKO kit and LC/MS. The OD value of each standard and each sample were read at 15 min and the concentrations of our plasma samples with low concentrations (Table 2) and with a range of concentrations (Fig. 5) were calculated with these three formulas. For low concentration samples neither of the alternative standard curves were uniformly accurate (Table 3). For the comparison with a range of FFA concentrations (Fig. 5), the standard curve created using plasma samples was the closest to a slope of 1.0 and an intercept of zero.

3.4.3. Optimal FFA concentration range for the WAKO kit

The plasma FFA concentrations measured by the WAKO kit were at least 30% different than concentrations measured by LC/MS for samples with FFA < 0.2 mmol/L and the WAKO concentrations were 20–40% less for samples with FFA > 1.2 mmol/L. To assess whether there is a range where the WAKO kit agrees best with LC/MS, we divided the 46 sample concentrations (by LC/MS) into those with concentrations < 0.2 (n = 18), 0.2–1.0 (n = 15) and > 1.0 (n = 13) mmol/L. The concentrations measured by WAKO with 3 different curves (WAKO, the mixed FFA standard and the plasma FFA standard) for the 3 different groups were compared by ANOVA and, if significant differences were found, the 3 formulas were compared with the LC/MS results using paired *t*-tests (Table 3). For samples with total FFA < 0.2 mmol/L by LC/MS, the WAKO concentrations from both the mixed standard formula and plasma sample formulas were significantly different from LC/MS. For samples with total FFA 0.2–1.0 mmol/L by LC/MS, the WAKO concentrations calculated from all the formulas were not statistically different from LC/MS. For samples with FFA > 1.0 mmol/

Table 2
Influence of sample volume on WAKO concentrations.

Sample	Sample volume	LC/MS result (mmol/L)	WAKO formula (mmol/L)		Mixed standard formula (mmol/L)		Plasma sample formula (mmol/L)	
			15 min	60 min	15 min	60 min	15 min	60 min
1	5	0.027	0.029	0.068	0.020	0.063	0.020	0.076
	10		0.022	0.044	0.018	0.043	0.021	0.053
	20		0.014	0.028	0.013	0.029	0.015	0.035
2	5	0.024	0.046	0.112	0.038	0.113	0.044	0.139
	10		0.056	0.102	0.056	0.108	0.069	0.135
	20		0.047	0.082	0.050	0.089	0.063	0.113

LC/MS – liquid chromatography/mass spectrometry.

L by LC/MS, the WAKO formula results differed significantly from LC/MS (Table 3).

We tested the agreement between the LC/MS and the WAKO kit for each of the ranges and each of the formulas using linear regression analysis. For samples with FFA < 0.2 mmol/L by LC/MS, none of the WAKO kit formulas had an r > 0.42 and none of the *P* values were significant. For samples with FFA from 0.2–1.0 mmol/L by LC/MS, all of the WAKO kit formulas had an r > 0.87 and all of the p values < .0001. For samples with FFA > 1.0 mmol/L by LC/MS, the WAKO kit formulas had r values ranging from 0.27 (p = .37, WAKO kit) to 0.39 (p = .19, plasma sample formula).

4. Discussion

Our goal was to determine whether we could use the WAKO NEFA assay to measure plasma total FFA concentrations in place of the more laborious LC/MS method. Our initial assessment of the WAKO results vs. LC-based methods suggested the kit underperformed at least in some circumstances. To further evaluate why this occurred, we performed additional comparisons of the kit vs. LC/MS measures of plasma FFA; the result indicated a more significant, systematic disagreement between the two methods. These findings lead us to conduct experiments designed to determine the cause of the discrepancy in order to understand if we could modify the assay conditions to improve the results. Despite varying the reaction time, the sample volume and employing more physiological (multiple FFA species) concentration solutions for the standard curve, we could not find an approach that improved the agreement between WAKO NEFA kit and LC/MS to the point where we can rely on it to measure suppressed and elevated FFA concentrations.

We found a number of factors that likely contribute to the discrepancies between the WAKO NEFA assay and total FFA measured by LC/MS. The WAKO NEFA standard solution consists only of oleate that is suspended in an aqueous medium by a proprietary compound. This compound appears to have reduced ability to maintain oleate miscibility when diluted, allowing oleate to concentrate in the upper layer of test tubes used to create low concentration standards. Furthermore, different fatty acids react differently with the enzymes used in the kit and oleate was one of the less reactive fatty acids. The agreement between LC/MS measures of total FFA and different dilutions of the WAKO control serum samples was better than the agreement using our 46 plasma samples; this may reflect the greater heterogeneity of FFA species in the latter. The poor agreement between the WAKO NEFA kit and LC/MS when plasma FFA concentrations are < 0.200 mmol/L is most probably a sensitivity issue that we could not correct with greater volumes of plasma or longer assay times. This phenomenon would affected the accuracy of the WAKO NEFA kit in studies aimed to investigate the correlation between suppressed lipolysis and FFA level with insulin sensitivity or glucose metabolism and the predictive value of nadir FFA concentrations towards insulin sensitivity and triglyceride concentrations. [14,33,34] Finally, the consistent underestimation of plasma FFA by the WAKO NEFA assay when plasma FFA exceed 1.000 mmol/L is almost certainly due to the use of pure oleate as a standard solution because the mixed FFA standard curve and plasma sample standard curve did not underestimate these sample (Table 3).

The enzymes acyl-CoA synthase and acyl-CoA oxidase (ACOD) are used in WAKO NEFA kit to generate hydrogen peroxide. The varying reaction velocities of these enzymes with the different fatty acids might explain why the WAKO results vary as a function of time (Table 2). A description of the specific acyl-CoA synthase that is used in the WAKO kit has been published [23]. The Pseudomonas sp. ACS used for the WAKO kit is widely used in laboratory research. If WAKO uses the ACS enzyme homolog FadD1, it would be more specific for long-chain fatty acids (18 carbons) [35] that are common in plasma FFA. It is unclear whether the fatty acid species specificity we observed can be accounted for by the properties of this enzyme. The ACOD in the WAKO kit is from Arthrobacter sp., which has different activity towards each acyl-CoA substrate. The ACOD enzyme activity is such that stearoyl-CoA had the

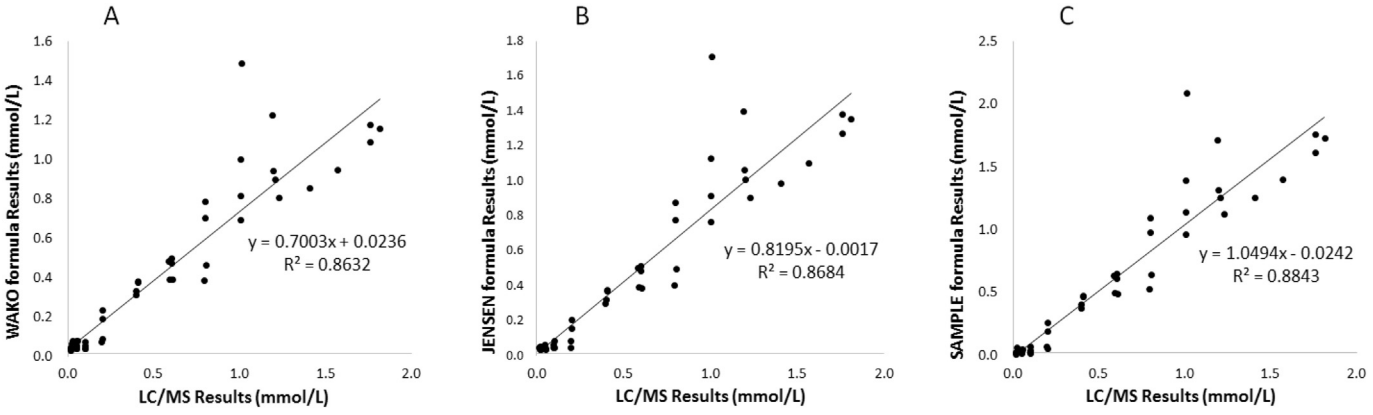

Fig. 5. Total FFA concentrations measured by LC/MS vs. the WAKO NEFA kit using formulas developed from WAKO NEFA standard solution (panel A), a laboratory-made mixed FFA standard curve (panel B) and a plasma sample diluted to create a range of concentrations (panel C).

Table 3
Comparison between concentrations from different measurements.

	Concentration < 0.2 mmol/L (n = 18)	Concentration between 0.2 and 1.0 mmol/L (n = 15)	Concentration > 1.0 mmol/L (n = 13)
LC/MS	0.051 (0.026, 0.101)	0.590 (0.399, 0.793)	1.206 (1.009, 1.663)
WAKO formula	0.035 (0.024, 0.058)	0.373 (0.315, 0.468)	0.929 (0.818, 1.149)[a]
Mixed standard formula	0.032 (0.022, 0.043)[b]	0.375 (0.303, 0.484)	1.082 (0.933, 1.351)
Plasma formula	0.019 (0.003, 0.025)[a]	0.474 (0.382, 0.621)	1.374 (1.175, 1.701)

LC/MS – liquid chromatography/mass spectrometry. Data is in median and quartile.
[a] $p < .05$ compared to LC/MS results.
[b] $p < .01$ compared to LC/MS results.

lowest Vmax among the FFA tested in our study while oleoyl-CoA had the greatest Vmax [36]. Although the ACOD tested in this study [36] was from *Candida tropicalis*, the activity for various long-chain acyl-CoA's is very similar between ACODs from *Candida tropicalis* and Arthrobacter sp. [37]. Because the Arthrobacter sp. ACOD is the one used in the WAKO kit, the difference of Vmax might account for the phenomenon that oleate provided a lower signal than the other FFA with the same carbon chain length.

Our results are somewhat at odds with those who have compared the WAKO kit and other FFA measurements, such as HPLC-MS and thin-layer chromatography (TLC). Shmeeda et al. [38] concluded that the WAKO kit results were in excellent agreement with other approaches, but didn't compare the FFA concentration results with a gold standard, independent measure of FFA. Other publications that comment on the WAKO kit include one where it was compared with TLC for the lipids [39] and one where the WAKO kit was also compared with TLC to measure liposome lipid [28]. The only other study we found noted that NEFA measured by the WAKO kit changed in the same direction as serum cholesterol and α-tocopherol measured by HPLC [29]. In short, there seem to have been no robust comparisons of the WAKO NEFA assay with a gold-standard plasma FFA assay.

On the positive side, the WAKO NEFA kit results agreed with LC/MS with an R^2 of ~0.8 with plasma FFA concentrations between 0.2 and 1.0 mmol/L. Given this finding, if samples from groups of humans are within this range and a very high level of accuracy is not required, the WAKO NEFA kit is a reasonable choice for measuring FFA concentrations due to its simplicity, speed and reduced cost. The WAKO kit can measure a large number of samples and, although it may miss small differences, it can distinguish differences of 20% with reasonable group sizes. For samples with concentrations of < 0.200 or > 1.000 mmol/L, however, other approaches should be considered.

In summary, the WAKO NEFA kit is a fast, easy way to measure plasma FFA concentrations, but has a somewhat limited range of accuracy, especially if it is important to measure suppression of FFA. We could not overcome the limitations at low concentrations by increasing the reaction time, the sample volume or using a more physiological standard solution. There are several reasons this kit underperforms at low and high FFA concentrations and no single approach is likely to correct the deficiencies we identified. We suggest that for those investigators who need to be able to accurately measure low and high FFA concentrations, or if there is a need to know the individual species of FFA, chromatography based approaches will be necessary.

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