Quantitative extraction and determination of nonesterified fatty acids in plasma

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Summary A method is described for the determination of nonesterified fatty acids in plasma. Extraction is at least 98% efficient, and losses during subsequent stages are corrected for by the use of an internal radioactive standard. The method is suitable for reference purposes rather than for routine determinations. Higher values are obtained than by other methods of analysis, and it is suggested that some plasma fatty acids remain protein-bound after normal methods of extraction.

In connection with work on methods for the determination of nonesterified fatty acids (free fatty acids) in plasma, we required an absolute method for reference purposes. Careful examination of the published methods showed that none has been rigorously justified, though most are no doubt adequate for clinical or comparative purposes. The recovery of fatty acids added to plasma is frequently taken as a criterion of the quantitative success of a method but, as we have already pointed out (1), the behavior of such added acids may well be different from the endogenous protein-bound ones, and an apparent recovery of 100% of added acids cannot be considered to validate a method.

A further probable cause of error is the use of standards chemically different from the experimental samples. In one commonly used method (2) in which the NEFA are partitioned between aqueous and organic phases, the titration figure for the acids in the organic phase is compared with that for a standard of palmitic acid carried through the same extraction process. However, there is the important difference that the experimental sample contains in addition the plasma proteins, which give a third (solid) phase in the system. This might well result in a significant change in the partition of the NEFA. No evidence is presented as to the efficiency of extraction of NEFA from the plasma protein. Indeed, even in the absence of protein only about 86% of a palmitic acid standard appeared in the organic phase, and it seems probable that plasma free fatty acids which are virtually all protein-bound (3) will be still less efficiently extracted.

Uncertainties of this kind in existing methods made it necessary to devise a quantitatively reliable method, and we present details of such a method in this paper. The initial extraction of NEFA from the plasma is at least 98% efficient, and any losses through all subsequent stages are readily corrected for by using an internal standard of [^{14}C]palmitic acid. The new method has also been compared with a colorimetric method (1), and the results confirm our belief that existing methods do not give a true value for plasma NEFA.

Methods and results

Procedure. The extraction of total lipids from plasma and the subsequent separation of NEFA from other lipids were based, respectively, on methods described by Svanborg and Svennerholm (4) and by McCarthy and Duthie (5).

The combined procedure is described in detail below, and experiments demonstrating the validity of the method are reported later.

Stage 1. To 5 ml of plasma in a round-bottomed flask add 2.5 ml of phosphate buffer (0.2 M, pH 6.0), mix, and freeze-dry. Extract total lipids by refluxing three times for 30 min with 25 ml of methanol–chloroform 2:1 (v/v). Filter extracts through a sintered glass funnel, shake the plasma protein residues with additional solvent mixture, and filter. To the combined extracts add about 0.1 ml of a chloroform solution of high specific activity [1-^{14}C]palmitic acid (ca. 5×10^5 dpm, ca. 50 mCi/mmole) and make up to 100 ml with solvent mixture. Remove duplicate 5-ml portions for liquid scintillation counting (see below) and reduce the remaining 90 ml to dryness by rotary evaporation at about 40°C.

Stage 2. Take up the residue from stage 1 in 50 ml of chloroform and apply 30 ml of the solution to a 1×10 cm column of KOH-treated silicic acid (5), prewashed with 20 ml of chloroform. Elute neutral lipids with 50 ml of chloroform and reject this fraction.

Stage 3. Elute the NEFA fraction with 10 ml of formic acid in chloroform (2% v/v), followed by 40 ml of chloroform. Evaporate this eluate to dryness to remove the formic acid, which interferes with the subsequent colorimetric determination of NEFA. Dissolve the residue in 50 ml of chloroform. Remove replicate 5-ml portions for liquid scintillation counting and for NEFA determination by the method originally described (6) for solutions of pure fatty acids in chloroform. As a blank for the latter determination, run a sample with water instead of plasma through the whole procedure. Correct the radioactivity of the initial lipid extract for the various volume changes involved in the procedure and compare with the radioactivity observed in the final sample. From these figures, deduce the overall efficiency of fatty acid recovery subsequent to the initial extraction stage and correct the observed NEFA concentration in the final sample. From this, calculate the NEFA concentration in the initial plasma sample. The basis for the necessary calculations is given in Table 1.

Abbreviations: NEFA, nonesterified fatty acids.

TABLE 1. Calculation of NEFA concentration

(*a*) Radioactivity calculations	
Stage 1 MeOH–$CHCl_3$ extract; let specific activity	$= n_1$ dpm/5 ml
Then total radioactivity in 100 ml	$= 20\ n_1$ dpm
and activity in 90 ml	$= 18\ n_1$ dpm
This radioactivity is transferred to 50 ml of $CHCl_3$ (stage 2), of which 30 ml is applied to a silicic acid column; the radioactivity, $30/50 \times 18\ n_1$,	$= 10.8\ n_1$ dpm
NEFA fraction from column (stage 3) is dissolved in 50 ml $CHCl_3$. If there are no losses, 5 ml portions should have activity	$= 1.08\ n_1$ dpm/5 ml
Let observed activity of this solution	$= n_2$ dpm/5 ml
Therefore, fractional overall recovery of NEFA after stage 1 extraction	$= n_2/1.08\ n_1$
(*b*) NEFA calculations	
Let observed NEFA concentration in stage 3 $CHCl_3$ solution	$= c$ mmoles/l
Concentration corrected for recovery (see under [*a*], above)	$= 1.08\ cn_1/n_2$ mmoles/l
Total corrected NEFA in 50 ml of stage 3 $CHCl_3$ solution	$= \frac{50}{1000} \times 1.08\ cn_1/n_2$
	$= 0.054\ cn_1/n_2$ mmoles
This was derived from 30 ml of the 50 ml of the stage 2 $CHCl_3$ solution, which therefore contained total NEFA	$= \frac{50}{30} \times 0.054\ cn_1/n_2$
	$= 0.09\ cn_1/n_2$ mmoles
Stage 2 $CHCl_3$ solution was derived from 90 ml of 100 ml of the initial MeOH–$CHCl_3$ extract, which therefore contained total NEFA	$= \frac{100}{90} \times 0.09\ cn_1/n_2$
	$= 0.1\ cn_1/n_2$ mmoles
This extract was derived from 5 ml plasma, which therefore had NEFA concentration	$= \frac{1000}{5} \times 0.1\ cn_1/n_2$
	$= 20\ cn_1/n_2$ mmoles/l

TABLE 2. NEFA concentrations of various samples

Sample	NEFA[a]	Residual NEFA after Extraction[b]
	mmoles/l	*mmoles/l*
Horse plasma	1.020 ± 0.040 (4)	0.02
Lipid mixture[c]	0.075 ± 0.028 (4)	
Phosphate buffer (0.2 M, pH 6.0)	0.01, 0.01 (2)	

[a] Determined by the present method. Results are means ± SEM or individual determinations for number of determinations indicated in parentheses.

[b] Determined in hydrolysate of plasma residue by visual estimation of thin-layer chromatograms. Expressed as equivalent concentration in plasma.

[c] Composition as given in Methods and Results.

Radioactivity measurements. Samples in solvents were evaporated just to dryness in a liquid scintillation counting vial, and dissolved in a xylene-based scintillator solution (Koch-Light, type KL 352). Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer and internally standardized using [1-^{14}C]-hexadecane.

Validation of the method. The efficiency of the initial extraction was investigated by measuring the NEFA in the solid plasma residue after extraction. The latter was heated in an evacuated, sealed tube with 50% concentrated HCl at 100°C for 24 hr, thus hydrolyzing the protein and releasing any residual NEFA bound to protein. The hydrolysate was evaporated to dryness under vacuum and extracted by refluxing successively with chloroform and light petroleum (bp 40–60°C). The NEFA content of the extract was too low to determine by the colorimetric method (6). The extract was therefore concentrated and then analyzed by thin-layer chromatography with graded fatty acid standards of known concentration (silica gel G, hexane–ether–acetic acid 60:40:2 [v/v/v]; the thin-layer plates were sprayed with 0.01% [w/v] dichlorofluorescein in ethanol). Visual comparison of the spots showed that a small quantity of NEFA, corresponding to about 2% of the total, could be found in the acid hydrolysate of the extracted plasma residue.

To check that no stage of the standard method caused hydrolysis of lipids, leading to a falsely high value for the final observed NEFA level, the standard method was applied to a preparation of typical plasma lipids other than NEFA. This contained the following components (concentrations in mg/100 ml): cholesteryl palmitate, 60; cholesterol, 12; triglyceride, 48; diglyceride, 6; monoglyceride, 6; sphingomyelin, 18; cephalin, 10; and lecithin, 60; all dispersed by ultrasonication in aqueous 4% (w/v) bovine serum albumin free from fatty acids (7). A small NEFA level was found (Table 2), probably originating from free fatty acids in some components of the artificial lipid mixture that are difficult to obtain free from such contamination. However, the possibility of a small contribution by hydrolysis of lipids cannot be excluded.

The silicic acid column fractionation leaves phospholipids firmly bound on the column after elution of free fatty acids, and we have also shown by thin-layer chromatography that the latter fraction is uncontaminated. Interference by other lipids does not, therefore, occur.

A blank phosphate buffer run through the whole standard method gave a very low NEFA value (Table 2).

The above results demonstrate that the initial extraction is nearly quantitative and that no chemical interference is produced during the entire procedure. Once the plasma fatty acids have been extracted into solvents and mixed with the internal standard of radioactive fatty acid (of negligible weight relative to the sample),

TABLE 3. Example of analytical observations and calculation of NEFA levels

	1st Aliquot	2nd Aliquot
Specific radioactivity of stage 1 extract (dpm/5 ml) = n_1	19,930	19,778
Specific radioactivity of final chloroform solution (dpm/5 ml) = n_2	14,425	18,994
NEFA concentration in final chloroform solution (mmoles/l) = c	0.038	0.052
Plasma NEFA concentration (mmoles/l) (see Table 1) = 20 n_1c/n_2	1.05	1.08

Duplicate aliquots of a sample of horse plasma were analyzed by the present method. Symbols as in Table 1.

TABLE 4. Comparison of two methods for the determination of plasma NEFA levels[a]

Sample	NEFA Concentration: Present Method	NEFA Concentration: Colorimetric Method
Citrated horse plasma	0.61	0.33
	0.60	0.33
Citrated human plasma	0.53	0.16
	0.51	0.18
	0.51	0.18
Dog serum	2.53	2.32
Citrated rabbit plasma	1.33	0.75
Recalcified horse plasma[b]	2.32	1.64
	1.17	0.93
	2.01	1.17
	1.53	0.93
	1.83	1.24
	1.42	0.57
Heparinized cat plasma	0.80	0.34
	1.69	1.04

[a] Determinations were made (*a*) by the method described in this paper and (*b*) by the colorimetric method described in Ref. 1. Values, expressed as mmoles/l, are the means of duplicate determinations on three separate aliquots from each sample (present method) or triplicate determinations on each sample (colorimetric method).

[b] Wellcome Reagents Ltd., no. 2. Six different batches were sampled.

any small losses at any stage of the procedure are quantitatively corrected for by measuring the recovery of radioactivity. The final chloroform solution of NEFA used for the colorimetric determination is free from other lipids, and the determination is based on standard solutions in chloroform; the results are therefore quantitative.

Table 3 shows the results for duplicate plasma samples run separately through the entire procedure; the observed values for radioactive measurements and NEFA values are substituted in the final expression derived in Table 1. It will be noted that although the recoveries for the duplicates are different, as indicated by the different observed NEFA levels in the final chloroform solution, the corrected NEFA levels for the original plasma are nevertheless in good agreement.

Comparison with a colorimetric method. Table 4 shows NEFA levels in a number of plasma samples determined in parallel by the method described in this paper and by a colorimetric method (1). In all cases the latter method gave lower apparent NEFA levels.

Discussion

The method described in this paper provides a nearly absolute determination of plasma NEFA. A small amount of NEFA, up to about 2% of the total, can be found in an acid hydrolysate of the extracted plasma residue. This may represent NEFA either not extracted or possibly arising by hydrolysis of traces of unextracted lipids. The initial extraction of NEFA is thus at least 98% and possibly 100% efficient, and since all other losses throughout the procedure are corrected for by a radioactive internal standard, the overall method measures at least 98% of the plasma NEFA. The procedure is comparatively lengthy and we do not claim that it is suitable for routine determinations, but we believe it to be valuable as a standard when investigating other methods and for use in particular cases when absolute determinations are necessary.

The higher NEFA levels found by the present method, as compared with the colorimetric method, are of significance in that the colorimetric method has been found (1, 8) to give results in reasonable agreement with established titration methods (9, 10). The basic colorimetric method for solutions of pure fatty acids (6) has also been used with a number of modified procedures for the extraction of plasma NEFA and the results have been compared (11–13) with other well-known titrimetric methods (2, 14); again reasonable agreements were obtained. All these results suggest that the various extraction procedures used in the titrimetric methods and in the colorimetric method and its modifications result in incomplete recovery of NEFA from plasma.

This is probably caused by part of the NEFA fraction remaining protein-bound. Bovine serum albumin has been shown (15) to contain two distinct classes of high-energy binding sites for fatty acids and numerous weaker binding sites, and it seems likely that in albumin of most species the fatty acids bound to some of the higher energy sites will not be released by normal extraction methods for liquid plasma. In the present method, on the other hand, extraction of the lyophilized plasma with a polar solvent mixture is sufficient to release fatty acids from all binding sites.

In many cases a knowledge of the absolute level of plasma NEFA is of little importance, interest being centered on relative changes after some experimental or therapeutic treatment. In such cases the colorimetric

method (1) or other analytical methods will be adequate. If, on the other hand, absolute values are required, it will be necessary to use a method that has been quantitatively evaluated throughout. The one described in this paper appears to be the first to fulfill this requirement.

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