A sensitive method for the determination of free fatty acids in plasma

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Summary A sensitive micromethod for the colorimetric determination of free fatty acids (FFA) in plasma has been developed. FFA are extracted with chloroform–heptane–methanol with silicic acid being added to eliminate interfering phospholipids. Diphenylcarbazide containing diphenylcarbazone has been chosen as color-developing reagent, since the chromophore formed in the presence of FFA-Cu complex is stable and sensitive. Comparison of the proposed method and a non-routine reference procedure involving, among other steps, isolation of FFA on thin-layer chromatography would indicate that the efficiency of extraction of FFA in the proposed method was 90% that of the reference procedure. The proposed method offers the sensitivity, convenience, and accuracy needed for the determination of FFA in situations where the size of sample available is limiting, as with small rodents.—**Hron, W. T., and L. A. Menahan.** A sensitive method for the determination of free fatty acids in plasma. *J. Lipid Res.* 1981. **22:** 377–381.

Supplementary key words copper soaps · diphenylcarbazide-diphenylcarbazone · phospholipid

Routine determination of free fatty acids (FFA) in plasma usually involves: extraction of the plasma lipids including the FFA into an organic solvent, removal of the co-extracted phospholipids, complexing of the FFA in the organic phase with a divalent metal, usually copper, and finally, assaying for the metal in the organic phase as an index of the quantity of FFA present. Duncombe (1) proposed the use of sodium diethyldithiocarbamate as the color reagent for the determination of FFA-Cu complex in chloroform. A rapid method for determination of FFA in plasma was then proposed (2) which was based on the extraction of FFA into chloroform, formation of FFA-Cu complex, and its detection with diethyldithiocarbamate. Itaya and Ui (3) then added the modification that blood or plasma be extracted with chloroform in the presence of phosphate buffer in order that phospholipids are excluded from the organic phase.

At approximately the same time, Laurell and Tibbling (4) introduced a sensitive method for determination of FFA in plasma in which the interfering phospholipids were eliminated by silicic acid and diphenylcarbazide was chosen for the colorimetric determination of organic phase copper. This method has not been used routinely as the chromophore is unstable and the color must be measured within 5–15 min (4, 5).

More recently, Itaya (5) has reported that a mixture of diphenylcarbazide–diphenylcarbazone gives a color-developing reagent which is sensitive and the color complex is stable. Although linear calibration curves have been reported for the original method of Itaya and Ui (3) based on the color development with diethyldithiocarbamate or its recent modification with diphenylcarbazide containing diphenylcarbazone as color reagent (5), we obtained nonlinear standard curves for both procedures.

By combining the color reagent of diphenylcarbazide containing diphenylcarbazone (5) with the extraction procedure described initially by Laurell and Tibbling (4), we have developed a method that has an efficiency of extraction of FFA from plasma that is comparable to a reference procedure, yet is applicable for the routine micro-determination of FFA in plasma.

MATERIALS AND METHODS

Materials

Diphenylcarbazone, diphenylcarbazide, and sodium diethyldithiocarbamate, all of ACS certified purity, were purchased from Fisher Scientific Co., Chicago, IL. Sodium acetoacetate was prepared as described by Hall (6), which involves the preparation of lithium acetoacetate from ethyl acetoacetate and the conversion of the lithium salt of acetoacetate into the sodium salt by Dowex 50W-X12 ion exchange chromatography. Solutions of acetoacetate were standardized through enzymatic determination with 3-hydroxybutyrate dehydrogenase (7). The concentrations of DL-3-hydroxybutyrate (Sigma Chemical Co., St. Louis, MO) listed in Table 1 are given as the racemic mixture, since the exact proportion of enantiomers in the preparation used was not known.

Bovine Fraction V albumin was obtained from Miles Laboratories, Inc., Elkhart, IN and the free fatty acids were removed by the method of Chen (8) using activated charcoal. The palmitic acid-albumin complex was prepared as described by Oram, Bennetch, and Neely (9) with a final concentration of 4 g/100 ml defatted bovine serum albumin. All other chemicals were of reagent grade and used without further purification.

Abbreviations: FFA, free (nonesterified) fatty acid: TLC, thin-layer chromatography; Cu-TEA, copper-triethanolamine solution.

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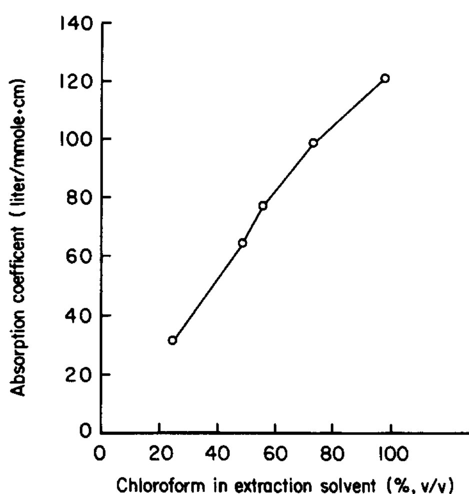

Fig. 1. Influence of extraction solvent composition on the apparent absorption coefficient (550 nm) in the proposed method. Each point shown is the average of duplicate determinations in which 66 nmoles of palmitic acid were extracted in the presence of silicic acid with a solvent mixture of 2% methanol (v/v) in heptane. Heptane in the solvent mixture was replaced by chloroform as indicated.

Animals and collection of blood

Sprague-Dawley rats were obtained from King Animal Laboratories, Oregon, WI and Swiss albino [ARS HA (ICR) f] mice from ARS/Sprague-Dawley, Madison, WI. Mice (25–30 g) and rats (200–250 g) were anesthetized with pentobarbital sodium, 110 or 60 mg/kg intraperitoneally. Lightly heparinized blood was obtained from mice by cardiac puncture after thoracotomy while the abdominal aorta was cannulated when rat blood was obtained. Plasma was separated from lightly heparinized blood by centrifugation for 5 min at 4°C in an Eppendorf Microcentrifuge (Model #543) in the case of mice or, with the larger samples of rat blood, in a Sorvall refrigerated centrifuge (Model RC-5).

Non-routine reference method

Our reference procedure for the non-routine determination of FFA has been modified from a method reported by Duncombe and Rising (10). Lyophilized plasma (4 ml) was extracted with chloroform–methanol 2:1 (v/v) and a high specific activity [1-^{14}C]palmitic acid (5.5×10^5 dpm, 59 mCi/mmol) was added (10). After removal of the extraction solvent on a rotary evaporator and redissolving the lipid extract in 2 ml of chloroform, 100-μl aliquots were applied to 4-cm lanes of 0.25-mm silica gel pre-coated TLC plates (SIL G-25, Brinkman Instruments).

FFA were separated from the other lipid classes using the solvent system by Roch and Grossberg (11). The FFA band was scraped from the plate and the FFA were eluted by extracting the gel three times with 2 ml of $CHCl_3$. The extracts were combined, evaporated to dryness under a stream of nitrogen and redissolved in 1 ml of $CHCl_3$. Duplicate samples were taken both for counting (100 μl) and for FFA assay (250 μl) using the proposed method without the silicic acid step.

Proposed method

The extraction procedure and the complexing of the FFA with copper was adapted from that of Laurell and Tibbling (4). To a 13 × 100-mm screwcap test tube (with a Teflon-lined cap) containing 50 μl of plasma, is added 6 ml of chloroform–heptane–methanol 200:150:7 (v/v/v) and then 330 ± 30 mg of 100-mesh silicic acid (Mallenkrodt), activated at 100°C for at least 16 hr, in that order. The silicic acid is conveniently added with a suitable measuring spoon (⅛ teaspoon). The tubes are shaken 30 times by hand, allowed to stand at room temperature for 15 min and shaken again 6 times. After centrifugation, the solvent containing the extracted FFA is decanted into another tube containing 2 ml of Cu-TEA solution (0.05 M $Cu(NO_3)_2$, 0.1 M triethanolamine, and saturated with NaCl, approximately 33 g/100 ml, and adjusted to pH 8.1). The Cu-TEA solution is made up fresh from stock solutions just prior to use. The tubes are shaken vigorously on a mechanical shaker for 20 min and centrifuged. One ml of the upper organic phase is then pipetted into a clean test tube and 0.5 ml of the color reagent reported by Itaya (5) is added, i.e., a 0.5% solution of

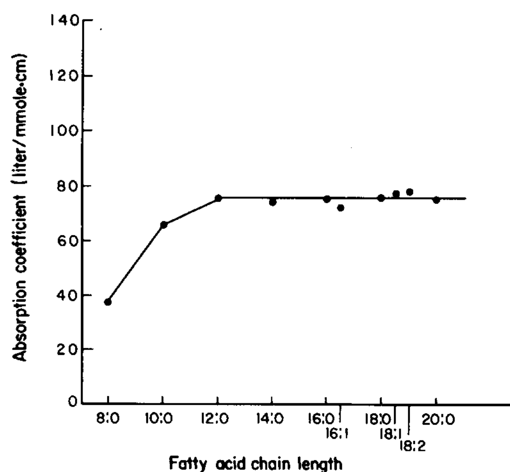

Fig. 2. Dependency of the apparent absorption coefficient (550 nm) in the proposed method with fatty acids of varying chain length and degree of unsaturation. Each point represents the average of triplicate determinations with 117–532 nmoles of indicated fatty acid per assay.

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a mixture of diphenylcarbazone and diphenylcarbazide (5:95) in methanol. The resulting color is read after 10 min but before 2 hr at 550 nm in a spectrophotometer.

RESULTS AND DISCUSSION

The proposed method combines the extraction of plasma FFA with chloroform–heptane–methanol in the presence of silicic acid as described by Laurell and Tibbling (4) with the color-developing reagent of diphenylcarbazide containing diphenylcarbazone reported by Itaya (5).

The effect of solvent composition on the millimolar absorption coefficient in the proposed method was investigated (**Fig. 1**). To assure complete extraction of FFA from plasma, 2% (v/v) methanol was included in each solvent mixture. Increasing the fraction of chloroform in the solvent mixture, at the expense of heptane, augmented the absorption coefficient and thus the sensitivity of the assay. However, when the presence of chloroform exceeded 56% (v/v), the organic phase became denser than the aqueous copper phase. With the organic phase as the lower phase, pipetting of the organic phase became much more difficult and the chance for aqueous copper contamination increased. In the interest of speed and accuracy but at a loss of sensitivity, a solvent mixture of chloroform–heptane–methanol 200:150:7 (v/v/v), that would remain as an organic upper phase after treatment with the copper reagent, was chosen.

When we varied the pH, triethanolamine, and the copper nitrate concentrations of the Cu-TEA solution, results identical to Laurell and Tibbling (4) as to their influence on the determination of FFA were obtained. Thus, the formulation of Laurell and Tibbling (4) was used without modification. This included the addition of 33 g of sodium chloride per 100 ml of Cu-TEA solution in order that aqueous copper phase would be in the lower phase, simplifying and improving the accuracy of pipetting.

TABLE 1. Influence of phospholipids and ketone bodies in the proposed method

Substance	Concentration of Solution Tested	Absorbance
	mmol/l	$A_{550\ nm}$
Palmitic acid	1.3	0.601
Phospholipids		
Phosphatidylcholine	2.9	0.018
Phosphatidylethanolamine	2.7	0.009
Phosphatidylinositol	3.8	0.003
Ketone bodies		
3-Hydroxybutyrate	1.0	0.003 ± 0.003 (6)
	5.0	0.001 ± 0.006 (6)
Acetoacetate	1.1	0.017 ± 0.006 (6)
	5.4	0.023 ± 0.004 (6)

The mean of duplicate determinations is indicated for the phospholipids examined and the $\bar{x}$ ± S.D. with a n = 6 for each concentration of ketone body.

TABLE 2. Recovery of FFA-albumin added to rat plasma in the proposed method

Samples	FFA Found	FFA Expected	Recovery
	nmol/assay		*%*
FFA No. 1	18.2 ± 2.8		
FFA No. 2	10.7 ± 1.3		
Plasma	12.3 ± 1.5		
Plasma + FFA No. 1	26.6 ± 1.4	30.5	87.2
Plasma + FFA No. 2	21.0 ± 3.0	23.0	91.3

The FFA concentration was determined six times on each sample, yet the plasma (25 μl) sample was analyzed ten times. FFA-albumin complex at two levels of FFA (25 μl) combined with 25 μl of defatted albumin solution or rat plasma, was analyzed by the proposed method.

The optimal ratio of diphenylcarbazone to diphenylcarbazide (5:95, w/w) in the color-developing reagent found with the proposed method agrees with the observations of Itaya (5), who showed that the inclusion of 5% diphenylcarbazone in diphenylcarbazide stabilized the chromophore.

All fatty acids with a chain length greater than 12 carbon atoms, both saturated and unsaturated, gave similar absorption coefficients with the proposed method (**Fig. 2**). Thus, variation in chain length and unsaturation of FFA in normal plasma should not influence the values determined with the proposed method.

Interference by phospholipids and ketone bodies in the proposed method was examined (**Table 1**). Phosphatidylcholine, phosphatidylethanolamine, or phosphatidylinositol in amounts equivalent to 2.9, 2.7, and 3.8 mM in plasma, respectively, did not interfere. Likewise, acetoacetate or 3-hydroxybutyrate at concentrations of 1 and 5 mM were without influence in the proposed method.

The proposed method was validated in several ways. The recovery of fatty acid-albumin complex added to rat plasma in the proposed method approached 90% (**Table 2**).

The efficiency of extracting FFA from plasma with the proposed method was also compared with a non-routine reference method consisting of extraction of plasma with chloroform–methanol 2:1 (v/v) and separation of FFA by TLC on silica gel as detailed in the Materials and Methods. With the addition of an internal standard of [1-^{14}C]palmitic

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TABLE 3. Plasma FFA determined in the proposed method and by isolation with thin-layer chromatography

	Thin-layer Chromatography (TLC) Procedure				Proposed Method	
	Apparent	Recovery of Internal Standard	Corrected for Recovery	Plasma FFA (sample-blank)	Plasma FFA	Ratio of Proposed Method/ TLC Procedure
	mmol/l	*percent*	*mmol/l*	*mmol/l*	*mmol/l*	*percent*
Trial 1						
Sample	1.509	81.9	1.842	0.968	0.874	90.3
Blank	0.699	80.0	0.874			
Trial 2						
Sample	1.607	77.1	2.084	1.005	0.909	90.4
Blank	0.818	75.8	1.078			

The mean of duplicate determinations made by both the TLC procedure and the method proposed in this paper on the same pool of plasma is indicated. The comparison was repeated on two separate occasions as indicated by Trials 1 and 2.

acid in the reference method, both the initial extraction of FFA from plasma and any losses through subsequent steps could also be monitored (**Table 3**). Comparison of the proposed method with the non-routine reference procedure in two separate trials would indicate that the efficiency of extraction in the proposed method was 90% that of the reference method. Duncombe and Rising (10) have also recently reported a reference method for determination of FFA which is similar to the one used in the present studies except the FFA fraction was isolated on a KOH-treated silicic acid column. Duncombe and Rising (10) showed that plasma extracted in the presence of chloroform alone gave appreciably lower FFA levels than their reference method. Comparison of FFA values obtained with chloroform–heptane–methanol as extraction solvent in the proposed method with our non-routine reference procedure indicated good agreement with little sacrifice in the extraction efficiency with the proposed method.

To justify further the use of the proposed method, individual mouse plasma samples were analyzed for FFA both by the proposed method and the established routine colorimetric technique of Itaya and Ui (3). At FFA concentrations greater than 0.25 mmol/liter, the correlation between the proposed method and that of Itaya and Ui was excellent (**Fig. 3**). With plasma FFA concentrations below 0.25 mmol/liter, the accord was not good with a correlation coefficient of 0.38. This may be due in part to the loss of sensitivity in the non-linear standard curve obtained with the Itaya and Ui (3) method (**Fig. 4**). Use of diphenylcarbazide containing diphenylcarbazone as color-developing reagent, instead of diethyldithiocarbamate as suggested by Itaya (5), increased sensitivity but still non-linear standard curves were obtained. Yet linear standard curves were obtained with the proposed method (Fig. 4) and a sensitivity was achieved that was at least three-fold greater than that of the procedure with diethyldithiocarbamate

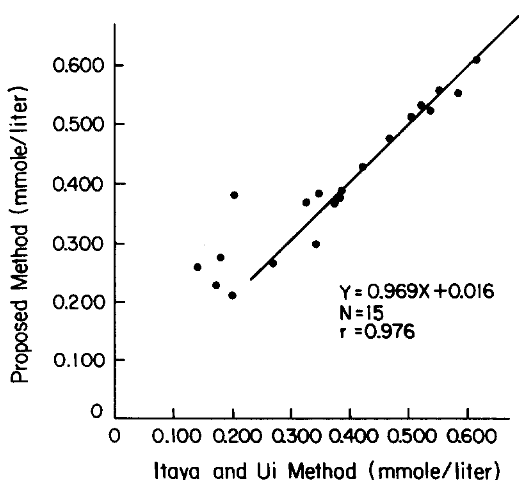

Fig. 3. Comparison between the proposed method, and the procedure of Itaya and Ui (3), for the determination of FFA content in plasma from fed or fasted mice. Regression line was computed by the method of least squares.

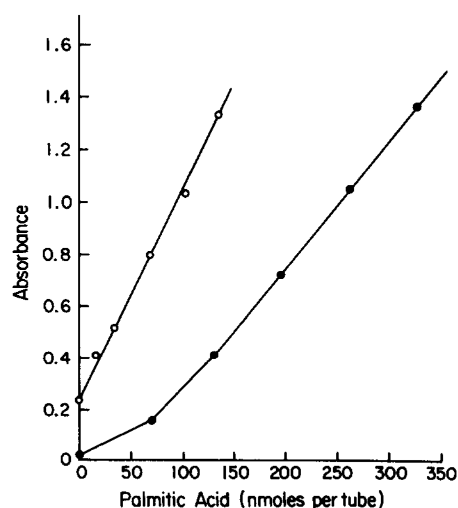

Fig. 4. Palmitic acid standard curves. Each point is the average of triplicate determinations with either the proposed method (○) or the Itaya and Ui (3) procedure (●).

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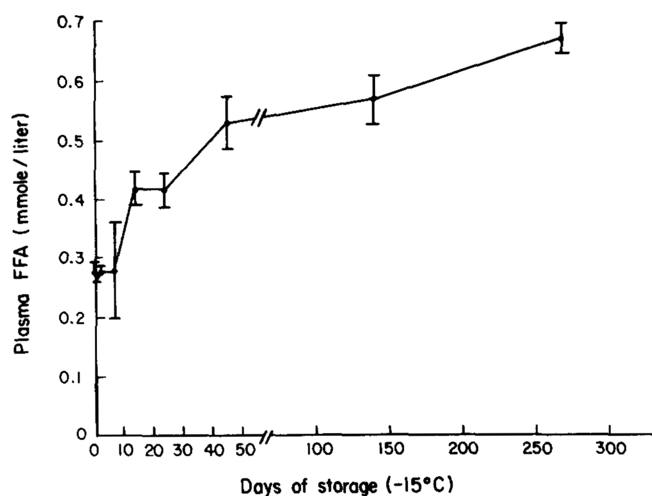

Fig. 5. Effect of storage at −15°C on the FFA content of a plasma pool from fed rats. Determinations were made in triplicate using the proposed method on an individual sample that was thawed only once. Values are given as mean ± SEM (N = 3) for each time point examined.

described by Itaya and Ui (3), when less than 75 nmol of palmitic acid were assayed. The precision of the proposed method was compared to the technique of Itaya and Ui (3) for plasma samples with a FFA concentration greater than 0.25 mmol/liter. Duplicate determinations of mouse plasma samples (separate extractions) in a series indicated a mean value of 0.459 mmol/liter and a standard deviation of the difference of 0.037 for the proposed method (nine determinations). The same nine plasma samples analyzed by the method of Itaya and Ui (3) gave a mean of 0.455 mmol/liter and a standard deviation of 0.030, comparing favorably with the proposed method.

The need for immediate processing of plasma for FFA determinations was demonstrated with the proposed method. Appreciable increases in plasma FFA levels occurred even with storage at −15°C (**Fig. 5**). Increases in both plasma FFA (12, 13) or glycerol (14) upon storage at temperatures as low as −196°C (14) have likewise been demonstrated. Organic extraction of the plasma has been reported to prevent such artifactual increases (13). In the proposed method, organic extracts of plasma samples have been stored routinely following separation from silicic acid before further analysis.

Thus, the combining of a stable and sensitive color-developing reagent of diphenylcarbazide containing diphenylcarbazone (5) with the extraction of FFA with chloroform–heptane–methanol in the presence of silicic acid (4) in the proposed method offers the sensitivity needed for determination of FFA in situations where plasma available for such an assay may be limiting, e.g., using small rodents.

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REFERENCES

1. Duncombe, W. G. 1963. The colorimetric microdetermination of long-chain fatty acids. *Biochem. J.* **88:** 7–10.
2. Duncombe, W. G. 1964. The colorimetric microdetermination of nonesterified fatty acids in plasma. *Clin. Chim. Acta.* **9:** 122–125.
3. Itaya, K., and M. Ui. 1965. Colorimetric determination of free fatty acids in biological fluids. *J. Lipid Res.* **6:** 16–20.
4. Laurell, S., and G. Tibbling. 1967. Colorimetric micro-determination of free fatty acids in plasma. *Clin. Chim. Acta.* **16:** 57–62.
5. Itaya, K. 1977. A more sensitive and stable colorimetric determination of free fatty acids in blood. *J. Lipid Res.* **18:** 663–665.
6. Hall, L. M. 1962. Preparation of crystalline lithium acetoacetate. *Anal. Biochem.* **3:** 75–80.
7. Mellanby, J., and D. H. Williamson. 1974. Acetoacetate. *In* Methods of Enzymatic Analysis. Vol. 4. H. U. Bergmeyer, editor. Academic Press, New York and London. 1840–1843.
8. Chen, R. F. 1967. Removal of free fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* **212:** 173–181.
9. Oram, J. F., S. L. Bennetch, and J. R. Neely. 1973. Regulation of fatty acid utilization in isolated perfused rat hearts. *J. Biol. Chem.* **248:** 5299–5309.
10. Duncombe, W. G., and T. J. Rising. 1973. Quantitative extraction and determination of nonesterified fatty acids in plasma. *J. Lipid Res.* **14:** 258–261.
11. Roch, L. A., and S. E. Grossberg. 1971. Rapid fluorometric micromethod for in situ quantitation of lipids on thin-layer chromatograms. *Anal. Biochem.* **41:** 105–115.
12. Trichopoulou, A., C. Kalaidzidou, and A. Kalandidi. 1976. The relationship between plasma non-esterified fatty acids concentration and conditions of storage. *Clin. Chim. Acta* **69:** 355–356.
13. Bergmann, S. R., E. Carlson, E. Dannen, and B. E. Sobel. 1980. An improved assay with 4-(2-thiazolylazo)-resorcinol for non-esterified fatty acids in biological fluids. *Clin. Chim. Acta.* **104:** 53–63.
14. Steinmetz, J., and E. Panek. 1978. Adaptation sur GSA II Greiner du dosage des triglycérides par voie entièrement enzymatique. Application à l'étude de leur conservation et de certaines interférences analytiques. *J. Clin. Chem. Clin. Biochem.* **16:** 613–619.

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