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

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Summary A rapid and precise microfluorometric method for the determination of free fatty acid concentrations in 2–5 μl of plasma is described. The assay is performed directly on plasma, eliminating the need for extraction with organic solvents, and is based on the quantitation of AMP generated from the formation of acyl coenzyme A in the presence of ATP and acyl CoA synthetase. Because of the sensitivity of this assay, reagent quantities, and thus costs, are significantly reduced compared with previously described enzymatic spectrophotometric methods. In addition, the inclusion of fatty acid-free human serum albumin in the standards corrects the previously reported underestimate of plasma free fatty acids with enzymic methods.—**Miles, J., R. Glasscock, J. Aikens, J. Gerich, and M. Haymond.** A microfluorometric method for the determination of free fatty acids in plasma. *J. Lipid Res.* 1983. **24:** 96–99.

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Measurement of free fatty acids (FFA) in plasma has been performed widely for research and, to a lesser extent, for clinical purposes. Numerous modifications of the original method of Dole (1) have been described, all of which require extraction of FFA into an organic solvent (2–8). Although these methods are accurate, they are quite tedious and require considerable care in order to achieve acceptable precision.

An enzymatic spectrophotometric method for the determination of FFA directly on unextracted plasma using a bacterial acyl-CoA synthetase has been recently described (9). The reactions on which this method is based are as follows:

$$\text{FFA} + \text{CoA} + \text{ATP} \xrightarrow{\text{acyl CoA synthetase}} \text{acyl CoA} + \text{AMP} + \text{PPi}$$

$$\text{AMP} + \text{ATP} \xrightarrow{\text{myokinase}} 2\text{ADP}$$

Abbreviation: FFA, free fatty acids.

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$$2ADP + 2 \text{ phosphoenolpyruvate} \xrightarrow{\text{pyruvate kinase}} 2ATP + 2 \text{ pyruvate}$$

$$2 \text{ pyruvate} + 2NADH \xrightarrow{\text{lactate dehydrogenase}} 2 \text{ lactate} + 2NAD^+.$$

Subsequently, this method was modified to a colorimetric one using an enzyme isolated from yeast, acyl-CoA oxidase (10). Both of these novel methods appear to underestimate FFA concentrations in plasma by approximately 10% (10). In addition, reagent costs for both procedures are relatively high for laboratories in which large numbers of samples are processed.

The present report describes a microfluorometric procedure, based on the enzymatic method of Shimizu (9), that is accurate and provides precise determination of FFA concentrations in 2–5 μl of plasma. Because of improved sensitivity, it is less expensive than the original enzymatic procedure; moreover, the problem of incomplete recovery has been solved by the addition of albumin to the standard.

MATERIALS AND METHODS

Materials

Acyl-CoA synthetase, coenzyme A (sodium salt, 90–95% pure), phosphoenol pyruvate (PEP-tricyclohexyl ammonium salt), adenosine 5′-triphosphate (ATP-disodium salt from equine muscle), β-NADH (disodium salt), myokinase (rabbit muscle), L-lactic dehydrogenase (rabbit muscle), pyruvate kinase (rabbit muscle), Triton X-100, $MgCl_2 \cdot 6H_2O$, Trizma base, Trizma hydrochloride, human serum albumin (fatty acid free), sodium oleate, glycerol, 2 mercaptoethanol, ethylenediaminetetraacetic acid (EDTA), and diethyl p-nitrophenyl phosphate (Paroxan), were all obtained from Sigma Chemical Company, St. Louis, MO. Disposable glass tubes (10 × 75 mm) were obtained from Fisher Scientific Co., Pittsburgh, PA. Microconstriction glass pipettes were obtained from H.E. Pederson Co. (Copenhagen) and calibrated colorimetrically to determine their exact volume (11).

Stock solutions

A pH 8.0, 0.08 M Tris buffer containing 0.6 mM EDTA, 10 mM $MgCl_2$, and 0.1% (vol/vol) Triton X-100 was prepared and stored at 4°C. Solutions of ATP (100 mM), PEP (100 mM), NADH (40 mM), and coenzyme A (8 mM) were prepared, aliquoted into 12 × 75 mm polystyrene tubes, and stored at −70°C. NADH was heated for 10 min at 60°C prior to each use (11). Acyl-CoA synthetase was dissolved in pH 8.0, 0.01 1M potassium phosphate buffer containing 5 mM 2-mercaptoethanol and 50% glycerol at a concentration of 0.68 U/ml, and stored at −20°C. All of the reagents were stable for at least 3 months under these storage conditions.

A 4 mM oleate standard was prepared by dissolving sodium oleate in a 10 mM potassium phosphate buffer, pH 7.0, containing 4% human serum albumin. Dissolution was aided by brief sonication in a water bath at 50°C; the standard was then aliquoted into 500-μl microcentrifuge tubes (Scientific Products) and stored at −70°C until use. The standard was calibrated independently by a conventional extraction procedure (6).

Instrumentation

All measurements were made on a ratio fluorometer (Farrand Optical Co. Inc., New York, NY) equipped with a digital display. The instrument was zeroed and calibrated against a quinine standard containing 0.5 μg/ml quinine sulfate in 0.1 N H_2SO_4 (11).

Procedure

Buffer solution is prepared daily by adding NADH (47 nmol/ml), ATP (840 nmol/ml), PEP (970 nmol/ml), myokinase (3.3 U/ml), pyruvate kinase (1.5 U/ml), lactic dehydrogenase (6 U/ml), and acyl-CoA synthetase (1.5 mU/ml) to the stock Tris buffer.

Two-hundred μl of the stock standard is added to 1.6 ml of 50 mM potassium phosphate buffer, pH 7.0, and serially diluted (1:1) in the phosphate buffer to generate six standards ranging from 0.275 to 8.8 nmol. Twenty μl in duplicate of each standard or distilled water is pipetted into 10 × 75 mm disposable glass tubes. The blanks are pipetted in quadruplicate.

Five μl of plasma is pipetted in duplicate into separate tubes (2 or 3 μl if unusually high values are expected). One (1.0) ml of the buffer solution is added to each tube. The tubes are vortexed briefly and allowed to incubate for 10 min. Following a prereading, 80 nmol of coenzyme A is added to each tube using a Hamilton repeating dispenser (Hamilton Co., Reno, NV). The tubes are briefly vortexed and allowed to incubate for 75–90 min at room temperature, after which a final reading is obtained. The decrement in fluorescence due to NADH oxidation is determined by subtracting the final reading from the prereading value.

Validation

In the other enzymatic methods, protein-free standards were used (9, 10). Because of the possibility that underestimation of FFA concentrations in these procedures was due to effects of protein binding, we compared standard curves obtained with standards made up in 4% human serum albumin and protein-free stan-

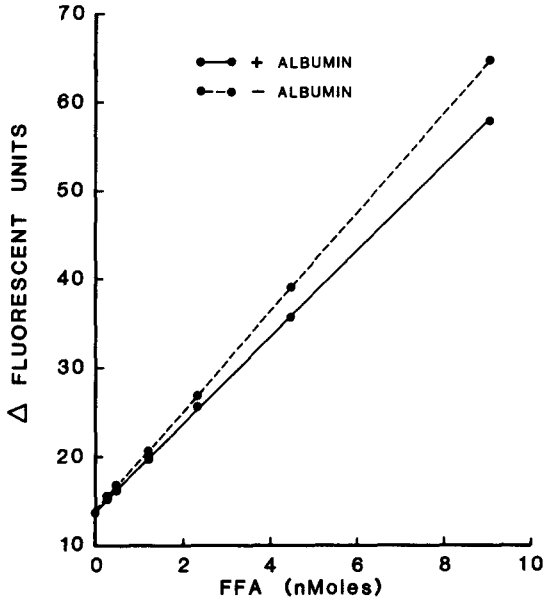

Fig. 1. Standard curves with and without added albumin.

dards. A protein-free 4 mM sodium oleate standard was prepared in a pH 7.0, 0.1 M phosphate buffer containing 0.2% Triton X-100 and 20% ethanol, along with the oleate standard in 4% albumin. The standards were calibrated independently by a gas–liquid chromatographic method (12) as well as by the colorimetric method of Laurell and Tibbling (6) and a modification of the method of Dole (8). In addition, 42 plasma samples from normal (n = 28) and diabetic (n = 14) patients were subjected to parallel analysis by the present method and by the method of Laurell and Tibbling (6). Protein-free standards of octanoic, decanoic, myristic, linolenic, linoleic, and stearic acids were also prepared and assayed by the present method, along with the protein-free oleic acid standard, to determine whether any effect of fatty acid chain length or double-bond content on the reaction could be demonstrated. Finally, a phosphatidylcholine standard and branched-chain α-ketoacid standards (α-ketoisocaproate, α-ketomethyl valerate, and α-ketoisovalerate) were assayed to evaluate the possibility of cross-reactivity.

Tubes containing 0.5 to 2.0 nmol of pyruvate were assayed to determine the duration of preincubation required to completely convert pyruvate to lactate prior to initiation of the FFA-specific reaction with coenzyme A. In addition, blood samples were drawn from a volunteer subject at 10-min intervals prior to (n = 6) and after (n = 6) infusion of heparin and a triglyceride emulsion into tubes containing EDTA both with and without Paroxan (0.04%); these plasmas were assayed to determine whether this inhibitor of lipoprotein lipase-mediated in vitro triglyceride hydrolysis (13) might interfere with the enzymatic reaction.

RESULTS

Fig. 1 depicts standard curves with and without added albumin. The addition of albumin results is a change in the slope of the curve, presumably due to failure of a small fraction of the standard to dissociate from albumin and thus participate in the reaction. The decrement in fluorescence at zero FFA concentration corresponds to the assay blank. **Fig. 2** shows concentrations of free fatty acids in 42 plasmas obtained by the present method as compared with concentrations obtained by the colorimetric method of Laurell and Tibbling. Across a range of physiologic concentrations, results obtained by the present method are 99 ± 1% of the reference method. In the present method, the intraassay coefficient of variation was 2.6% (n = 10), and the interassay coefficient of variation was 3.5% (n = 15). When standard was added to a control plasma, recovery was 97 ± 3% (range, 80–113%) over the physiologic range. Results of assay of protein-free fatty acid standards of chain length from 8 to 18 carbons were essentially identical; furthermore no difference between saturated and unsaturated fatty acids could be demonstrated (data not shown). When read from an albumin-containing standard curve, concentrations of these standards were 12 ± 3% higher than by the Laurell and Tibbling method. Neither phosphatidylcholine nor the branched-chain α-ketoacids cross-reacted in the assay.

Up to 2.0 mmol of pyruvate were completely reacted within 10 min of adding buffer; therefore, 10 min is

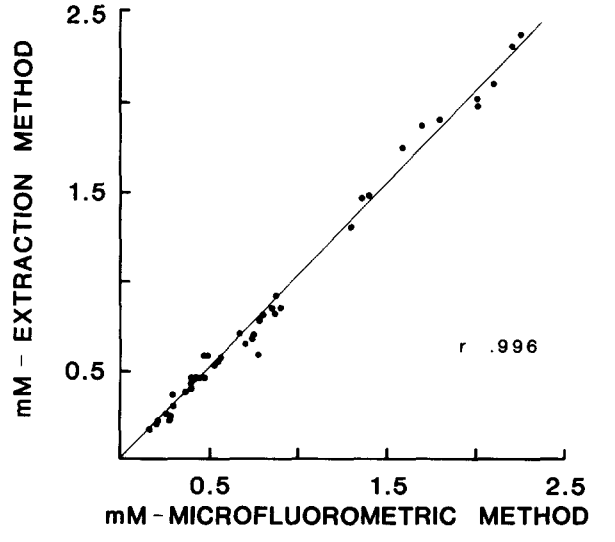

Fig. 2. FFA concentrations in 42 plasmas obtained by the present method compared with results from a conventional extraction procedure.

sufficient for the preincubation for plasma pyruvate concentrations as high as 0.4 mM. Paroxan, a potent lipoprotein lipase inhibitor (13), had no apparent effect on the enzymatic reaction (100 ± 2% compared with no inhibitor in samples obtained prior to heparin administration, n = 6). FFA concentrations from plasmas obtained after heparin administration when no inhibitor was added were 73 ± 5% greater than values obtained in plasma to which Paroxan had been added (n = 6).

DISCUSSION

Previously described enzymatic methods for the determination of plasma FFA (9, 10) are attractive, since they avoid extraction and handling of organic solvents; however, incomplete recovery (10) and high cost of reagents has limited their widespread application. Adaptation of these spectrophotometric methods (9, 10) to a microfluorometric procedure has improved sensitivity, permitting use of reduced amounts of reagents and thus decreasing reagent costs by at least 75%.

The underestimation of plasma FFA in the enzymatic spectrophotometric procedure may be the result of FFA binding to albumin, as previously suggested (10). Addition of human serum albumin to standards in the present method corrects for this incomplete recovery. Since a negative cooperativity model has been proposed for the binding of free fatty acids to albumin (14), it is possible that a small percentage of FFA in plasma cannot be dissociated from protein, and is thus unavailable for participation in the reaction. Finally, Paroxan, an inhibitor of lipoprotein lipase, does not interfere with the reaction in the present method, and is thus suitable for use as an inhibitor in plasmas obtained from patients after heparin administration.

The present data support previous observations that the results obtained from enzymatic procedures are not influenced by fatty acid chain length (C_6–C_{18}) nor by the presence or absence of double bonds (10). If the presence of albumin renders a small percentage of FFA unavailable for the reaction, an overestimate of FFA in protein-free samples might be anticipated, and was, in fact, observed. A number of previous reports (4, 7, 10) suggest that most existing chemical methods for plasma free fatty acid determination actually underestimate plasma free fatty acid content, possibly because extraction from plasma is not quantitative due to protein binding effects (4). Therefore, it is possible that small errors exist in the present method that would make it unsuitable as an absolute reference method, but superior to most methods currently employed for both clinical and research purposes.

In summary, a rapid, accurate, and relatively inexpensive procedure is described for determination of FFA in 2–5 μl of plasma. Because of the elimination of tedious extraction steps and the potential for automation, enzymatic methods such as the one described herein could become the procedures of choice for determination of total free fatty acids in plasma.

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