Automated modification of Duncombe's method for the ultramicro determination of serum free fatty acids

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Summary An automated continuous-flow method is described for estimating free fatty acids in serum using 25-μl samples. The procedure depends on the formation of copper soaps on the surface of a semipermeable membrane, their transfer into chloroform, and subsequent determination of dissolved copper. The membrane separating copper reagent and chloroform is supported between thin-channel dialysis plates. Chloroform extracts of serum free fatty acids are passed through the dialysis unit, and dissolved copper in the outflow is estimated colorimetrically. The procedure gives values that agree with a standard titrimetric method.

Supplementary key words chloroform · copper soaps · semipermeable membrane · thin-channel dialysis · diethylammonium diethyldithiocarbamate

In connection with work on fetal and neonatal lipid metabolism, an automated procedure was developed for estimating free fatty acids (FFA) in very small samples of serum. The method is based on the manual technique of Duncombe (1) and depends on the solubility of copper soaps in chloroform (2) using an aqueous copper nitrate–triethanolamine reagent (3) and diethyldithiocarbamate for the subsequent colorimetric determination of dissolved copper (4). In Antonis' (5) automated adaption of Duncombe's method, a mixing coil is used, to bring together copper reagent and chloroform, followed by a phase separator from which uncontaminated chloroform is sampled. In the present method, greater sensitivity has been achieved by replacing this arrangement with a thin-channel dialysis unit in which fatty acids dissolved in chloroform react with copper reagent that diffuses through a semipermeable membrane. Because the membrane forms a boundary between the two phases, no subsequent separation is required and base-line noise and drift are greatly reduced, enabling detection of fatty acids down to 3×10^{-4} μmoles.

Method. FFA extraction is based on the procedure of Itaya and Ui (6), using 25 μl of serum, 300 μl of phosphate buffer (0.067 M, pH 6.2), and 2 ml of solvent mixture (ethanol-free chloroform–heptane 70:30 [v/v] saturated with water). After shaking for 2 min and centrifuging (in polythene-stoppered 10 cm × 1.2 cm glass tubes), the chloroform phase is loaded onto the sampler. Standards (0.5, 1.0, 1.5, and 2.0 meq/l) are prepared by dissolving purified sodium palmitate in a little hot water and making up to volume with 4% fatty acid-free albumin (bovine albumin, Cohn fraction V, essentially fatty acid-free, Sigma Chemical Co. Ltd., made up in Krebs-Ringer phosphate–saline). Standards and albumin blank are stored at −20°C.

The arrangement of the apparatus for the automated analysis is shown in **Fig. 1.** The sampling device is essentially similar to that described by Antonis (5), but with 1 cm (ID) × 4 cm sample tubes and with a reduced opening to the wash reservoir (3 mm), which is gravity fed with solvent (of the same batch used for the extraction) from a

Abbreviations: FFA, free fatty acids.

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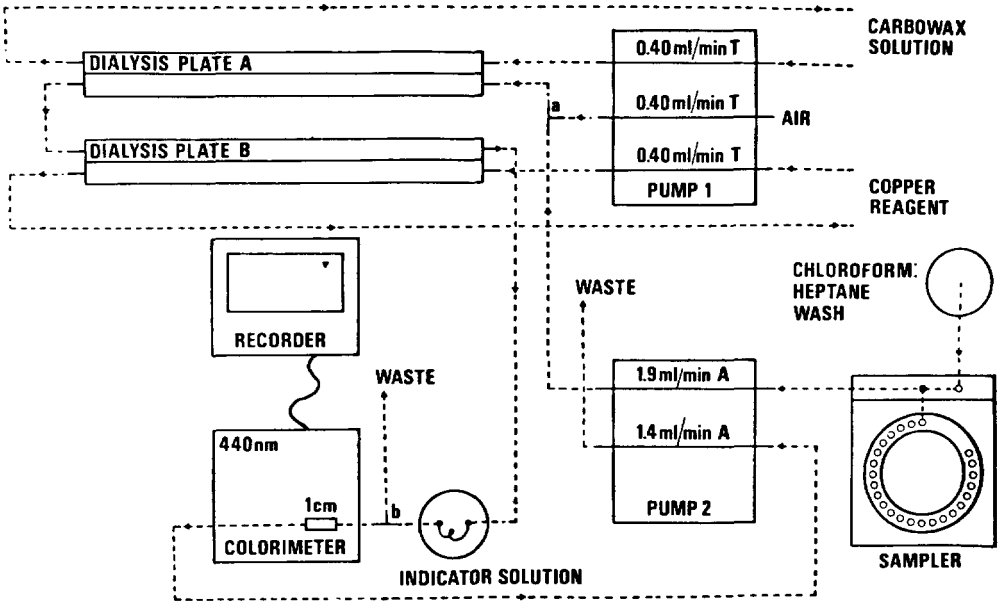

Fig. 1. Flow diagram for ultramicro FFA estimation. Chloroform mixture is fed to the sampler by gravity from a 500-ml reservoir, and the Carbowax and copper reagents are recycled from stoppered flasks. A loop of silicone rubber tube carrying the outflow from the dialysis plates dips into the indicator solution, contained in a stoppered flask. Indicator diffuses into the solvent flow through the tube for color development. Intensity of color is measured in a colorimeter and is displayed on the recorder. Flow rates of the various reagents are indicated in the diagram above the pump tubing lines. A, Acidflex tubing (Technicon); T, Tygon tubing (Technicon); *a*, glass T piece with 1.2-mm-bore side arms; *b*, glass T piece with 1.6-mm-bore side arms.

500-ml stoppered flask (with a small air inlet hole). A sampling period of 50 sec and a wash cycle of 1 min are required. Tubing is also similar to that used by Antonis (5) except that the internal diameter of the chloroform transmission tubing needs to be 1.00 mm and that of the air line (about 15 cm long), 0.50 mm. Pump 1 supplies the air bubbles and circulates the two aqueous reagents from stoppered flasks, each through a set of thin-channel dialysis plates (15.5-cm diameter, 230-cm path) made of polytetrafluoroethylene (*A* and *B*, Fig. 1), fitted with Cuprophan membranes (Technicon Instruments Co. Ltd.).

Pump 2 (Polystaltic, Buchler Instruments Co. Ltd.), fitted with 0.4-mm bore tubing, transmits the chloroform mixture and is adjusted so that about 1.5 ml of extract is sampled. Solvent flow through the colorimeter is regulated by applying tension to the pump tubing until no bubbles pass through the flow cell. Initially, the solvent mixture is dialyzed against the Carbowax reagent (20 g of polyethylene glycol, mol wt 15–20 $\times 10^3$, Carbowax 20M, dissolved in 200 ml of (0.01 N HCl) and then against the copper reagent (30 g of Carbowax 20M dissolved in 200 ml of 0.45 M triethanolamine, 0.133 M cupric nitrate, 0.05 N acetic acid, and 0.027 N *n*-butyric acid). A diffusion device for indicator addition receives the solvent flow emerging from the dialysis plates and consists of a 6-cm loop of silicone rubber tubing (0.13-mm wall, 0.25-mm bore; measurements before solvent swelling), which dips into the indicator solution (200 ml, 3% diethylammonium diethyldithiocarbamate [w/v], Hopkin and Williams Ltd., England, dissolved in solvent mixture and contained in a stoppered flask). 10 cm of tubing at each end is sleeved (when softened in chloroform) over two 12-cm-long stainless steel tubes (19 gauge), which carry the solvent flow. Finally, after color development and removal of air, the chloroform mixture passes through the flow cell of the colorimeter (10-mm light path, 0.08-ml capacity) set at a wavelength of 440 nm. The tube connecting the indicator loop to the debubbler should be at least 100 cm long to allow for color development, whereas the tube connecting the debubbler to the flow cell (0.75-mm bore) should be as short as possible.

In order to conserve solvent mixture between batches of analyses, the speed of pump 2 is reduced. At the end of a run, the solvent lines are flooded with heptane and the indicator loop is removed from the indicator reservoir. Heptane should also be pumped through the dialysis units (with the indicator loop and flow cell disconnected) when new membranes have been fitted in order to dislodge droplets of water that could enter the flow cell.

Results. The calibration graph of albumin-bound palmitic acid standards plotted against extinction is a straight line and passes through the origin (**Fig. 2**). When set up with fresh reagents, the 1.0 meq/l standard gives an extinction of about 0.085, and with 1-min wash intervals carry-over from one sample to the next is negligible (Fig. 2). During the following weeks there may be a slight fall in sensitivity, but standards put on before and after a run do

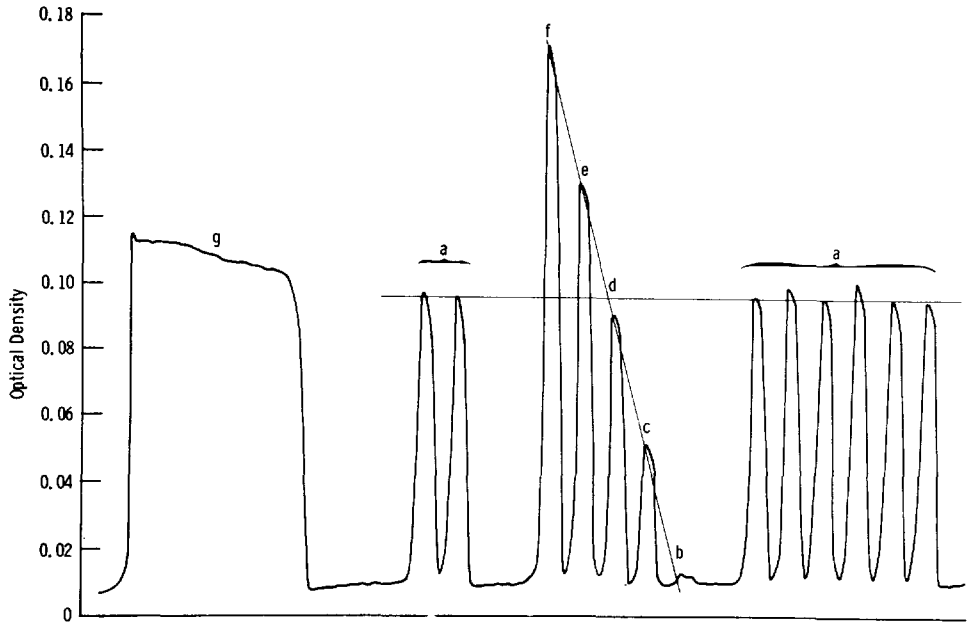

Fig. 2. Colorimeter tracings obtained using the method described in the text for *a*, solution of palmitic acid in extraction mixture, 0.0125 meq/l (equivalent to serum containing 1.00 meq/l); *b, c, d, e,* and *f*, albumin–palmitate standards, blank, 0.5, 1.0, 1.5, and 2.0 meq/l, respectively; and *g*, steady state condition while continuously sampling standard *a*.

not differ significantly. A slight increase in carry-over between samples may also occur with time, but this can be overcome by using a 1.5-min wash cycle. After about 4 wk it is necessary to replace the membranes and reagents. The heights of the peaks are about 85% of the heights of the plateaus produced by sampling for several minutes (Fig. 2) whereas the plateaus are equivalent to those produced by manual techniques. Each peak gently curves towards its apex as it approaches equilibrium, so small variations in sampling time produce a minimal effect on peak height.

Base-line noise is equivalent to an extinction of ±0.001 whereas base-line drift over 2-hr periods varies by an extinction value of ±0.006. This allows detection of fatty acids down to 3×10^{-4} μmoles. At the same time, due to linear response, measurement of up to 0.15 μmole is possible. The precision of the method, shown in **Table 1,** was tested by extracting and analyzing samples of serum and whole blood 20 times consecutively and gave a standard deviation of ±0.02 meq/l.

Table 2 compares the results obtained with different fatty acid standard solutions. The automated procedure gives almost identical responses for fatty acids down to a chain length of 14 carbon atoms; the response falls off sharply after lauric acid (chain length of 12 carbon atoms).

Variations in amount of water dissolved in FFA extracts do not affect the results because initial dialysis against the Carbowax reagent regulates the water content of the solvent mixture. Phospholipids, however, give appreciable interference (**Table** 3), as found by Duncombe (1), but the

TABLE 1. Analytical precision derived from replicate determination on 25-μl samples

Sample (human)	Adult plasma	Fetal plasma	Adult whole blood
No. of estimations	20	20	20
Mean, meq/l	0.65	0.27	0.24
Range, meq/l	0.60–0.69	0.24–0.31	0.21–0.29
Standard deviation	0.02	0.02	0.02
Coefficient of variation	3.7%	7.3%	8.3%

TABLE 2. Comparison of different fatty acids

Fatty Acid		Response as Percentage of Palmitic Acid Reading
Linolenic	18:3[a]	102
Linoleic	18:2	100
Oleic	18:1	100
Stearic	18:0	100
Palmitic	16:0	100
Myristic	14:0	96
Lauric	12:0	88
Capric	10:0	59
Caprylic	8:0	15
Butyric	4:0	4

The various fatty acids (obtained from Sigma or from Koch-Light Laboratories Ltd., >99% pure) were made up in chloroform–heptane 70:30 (v/v) to give solutions containing 0.0125 meq/l. 2 ml of each solution was shaken with 300 μl of buffer and analyzed as described in the text.

[a] Number of carbon atoms:number of double bonds.

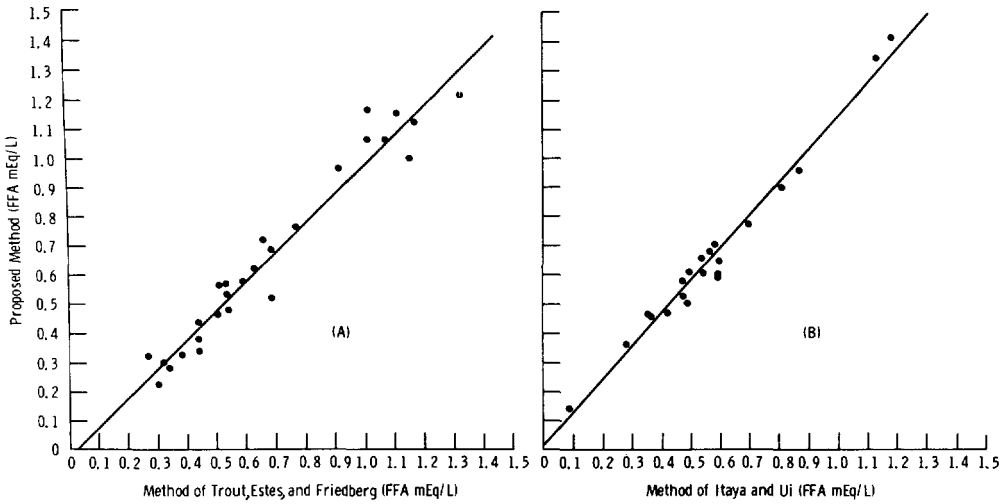

Fig. 3. Comparison between the ultramicro automatic method and two reference procedures for estimating serum FFA. *A*, comparison between values obtained with the method of Dole (7) as modified by Trout, Estes, and Friedberg (8) (x) and with the automated technique (y) ($x = 1.009y - 0.018$; $r = 0.975$; n = 27). *B*, comparison between levels measured with the method of Duncombe (1) using the extraction procedure of Itaya and Ui (6) (x) and with the proposed method (y) ($x = 1.151y + 0.009$; $r = 0.987$; n = 21). Regression lines were calculated using the method of least squares.

extraction technique discriminates against phospholipids. With the original method (6), less than 1.3% is extracted and less than 6% is extracted with the modified procedure used here. At the same time, FFA extraction, estimated by comparing the albumin-bound standards with palmitic acid made up in solvent mixture, is 92% efficient (Fig. 2). This assumes that the fatty acid-free bovine albumin reacts in a manner similar to fresh serum albumin.

Results obtained with the ultramicro method and with a titrimetric method (7, 8) show reasonably good agreement (**Fig. 3**), but when compared with the original method of Duncombe (1), using the extraction procedure of Itaya and Ui (6), the ultramicro method gave consistently higher results. Correlation between methods was good.

Discussion. The use of dialysis plates in place of the mixing coil used by Antonis (5) is a significant advance in the automated analysis of FFA based on the formation of chloroform-soluble copper soaps. Copper reagent cannot contaminate the chloroform phase because inclusion of polyethylene glycol in the aqueous reagents prevents osmotic leak across the membranes. A separator, which requires careful balancing, is therefore not required. FFA are extracted directly into the chloroform mixture, which, because the water content of the solvents is regulated by dialysis, can be sampled immediately without prior evaporation and reconstitution, as would be required with direct extraction in Antonis' method (5). At the same time, the extraction technique discriminates against phospholipid, and interference from this source is negligible. In addition, the extraction procedure is very simple and takes only a few minutes.

An improvement over conventional methods of indicator addition with pulse-free color development and without solvent dilution has been accomplished by allowing indicator to diffuse into the solvent flow through the walls of a fine tube of silicone rubber.

The greater sensitivity of the method over that of Antonis (5) is mainly due to the comparatively noiseless base line resulting from the various modifications, although a 20% better extinction coefficient is also achieved by the use of butyric acid. The latter appears to increase the rate of copper soap formation possibly by functioning as a shuttle, carrying copper from the aqueous phase to FFA in the chloroform mixture.

The method gives values that agree fairly well with a standard titrimetric method (Fig. 3) while giving higher values than the original manual procedures upon which the method is based. The explanation for this discrepancy possibly lies in the extraction process. In our hands, the original method of Itaya and Ui (6) is less efficient than the Dole procedure (7), which gives 96% recovery (of palmitate

TABLE 3. Sensitivity to phospholipids

Phospholipid	Molar Extinction of Phospholipid/ Molar Extinction of Palmitic Acid
Sphingomyelin (bovine brain)	0.084
Dipalmitoyl-DL-α-lecithin (synthetic)	0.236
Phosphatidylethanolamine (egg)	0.520

The phospholipids (obtained from Sigma, >98% pure) were dissolved in chloroform–heptane 70:30 (v/v) to give solutions containing 0.0625 mM phospholipid. 2 ml of each solution was analyzed directly, and the results were compared with a standard solution of palmitic acid.

complexed to fatty acid-free albumin). The method described here appears to extract 92% of FFA, and by standardizing with palmitic acid complexed to albumin possible underestimation of FFA is allowed for (9).

The automated method will analyze 100 to 200 samples daily. Determinations can be made using samples of less than 25 μl by using half the recommended volume of solvent mixture with a 30-sec sampling time. For good results, the dead space of the system needs to be kept to a minimum, especially in the flow cell, and it is essential that glassware and stoppers (which should be handled with forceps) are free from fat and detergents.

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REFERENCES

1. Duncombe, W. G. 1964. The colorimetric micro-determination of non-esterified fatty acids in plasma. *Clin. Chim. Acta.* **9:** 122–125.
2. Ayers, C. W. 1956. Estimation of the higher fatty acids C_7–C_{18}. *Anal. Chim. Acta.* **15:** 77–83.
3. Iwayama, Y. 1959. New colorimetric determination of higher fatty acids. *J. Pharm. Soc. Japan.* **79:** 552–554.
4. Duncombe, W. G. 1963. The colorimetric micro-determination of long-chain fatty acids. *Biochem. J.* **88:** 7–10.
5. Antonis, A. 1965. Semiautomated method for the colorimetric determination of plasma free fatty acids. *J. Lipid Res.* **6:** 307–312.
6. Itaya, K., and M. Ui. 1965. Colorimetric determination of free fatty acids in biological fluids. *J. Lipid Res.* **6:** 16–20.
7. Dole, V. P. 1956. A relation between non-esterified fatty acids in plasma and the metabolism of glucose. *J. Clin. Invest.* **35:** 150–154.
8. Trout, D. L., E. H. Estes, Jr., and S. J. Friedberg. 1960. Titration of free fatty acids of plasma: a study of current methods and a new modification. *J. Lipid Res.* **1:** 199–202.
9. Duncombe, W. G., and T. J. Rising. 1973. Quantitative extraction and determination of nonesterified fatty acids in plasma. *J. Lipid Res.* **14:** 258–261.