Colorimetric determination of free fatty acids in biological fluids

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SUMMARY Following the introduction by Duncombe of a colorimetric micromethod for the determination of free fatty acids (FFA) dissolved in chloroform, we studied the optimum conditions for chloroform extraction of FFA from blood or other biological fluids. A degree and rate of extraction similar to those with Dole's extraction mixture were obtained.

Phospholipids are not extracted by the procedure described. As little as 0.2 ml of whole blood, instead of serum or plasma, can serve as a FFA sample in the subsequent colorimetric determination, which has been modified to render it simpler and more sensitive.

KEY WORDS free fatty acids · extraction · colorimetric determination · serum · blood · calcium · albumin · incubation media

THE PROCEDURES currently available for the analysis of free fatty acids (FFA) in body fluids or other biological materials such as the incubation media of fat bodies are mostly based on the titration by dilute alkali of fatty acids after their extraction with organic solvents (1, 2). In 1962, Duncombe (3) suggested in a preliminary communication that the selective transfer of the copper salts of fatty acids into chloroform, the principle of which was first introduced by Ayers (4), may be applicable to colorimetric determination of fatty acids. Recently, a full report (5) was published by Duncombe, who succeeded in measuring FFA colorimetrically in chloroform at concentrations as low as 0.01 μeq/ml with satisfactory accuracy and simplicity.

Duncombe's procedure, which was intended for the determination of FFA in chloroform eluates from paper chromatograms, does not discuss any process for the extraction of FFA in an aqueous phase into organic solvents. The purpose of the present study was to determine the best conditions for the transfer of FFA from an aqueous phase into chloroform, in which the Cu complex of FFA can be formed and measured colorimetrically. Since phospholipids such as lecithin, according to Duncombe (5), form a chloroform-soluble complex with Cu ions, the extraction rate of serum phospholipids by chloroform was also studied.

MATERIALS AND METHODS

Colorimetric Procedures

The procedure described by Duncombe (5) was applied either to a standard solution of recrystallized palmitic acid in chloroform or to the chloroform extract of aqueous samples containing FFA (final concentration of FFA, 0.02–0.1 μeq/5 ml of chloroform). The chloroform was distilled once before use. Six milliliters of the chloroform solution were shaken with 3 ml of copper–triethanolamine solution [1 M triethanolamine–1 N acetic acid–6.45% $Cu(NO_3)_2 \cdot 3H_2O$ 9:1:10 (v/v/v)] (6). To the chloroform layer, after separation and filtration, was added a small amount of sodium diethyldithiocarbamate solution dissolved in *n*-butanol. The color developed was measured at 440 mμ. Details of the colorimetric procedure standardized by the present authors, along with some experimental findings leading to the standardization of the method, will be briefly mentioned in the later part of Results.

Preparation of FFA–Albumin Solution

As the test solution containing FFA, besides rat serum and whole blood, we employed Krebs-Ringer bicarbonate solution containing palmitic acid and serum albumin, because most of the in vitro studies concerning FFA metabolism in animal adipose tissues so far reported

from many laboratories have been done by incubating fat pads in Krebs-Ringer solution containing albumin as an acceptor of released FFA.

Recrystallized palmitic acid was dissolved in dilute NaCl with bovine serum albumin (Fraction No. V, Armour Pharmaceutical Co., Kankakee, Ill.) as described by Evans and Mueller (7) and further diluted with appropriate salt solution to make Krebs-Ringer bicarbonate solution. The concentration of albumin was 2% unless otherwise specified. The resultant Krebs-Ringer bicarbonate solution containing FFA and albumin will be referred to as "FFA–albumin solution" in the present paper.

RESULTS

Chloroform Extraction of FFA from Biological Fluids

In the preliminary experiments, serum or FFA–albumin solution diluted with water was shaken with chloroform in the presence or absence of one drop of N HCl and the amount of FFA transferred to chloroform layer was measured colorimetrically. Exposure of the bicarbonate-buffered serum or FFA–albumin solutions to air (which has a lower partial pressure of CO_2) resulted in a rise of the pH to 8.0 or higher. The effect of pH of the aqueous phase in the extraction process on the ability of chloroform to extract FFA from serum or FFA–albumin solution was examined, the results being presented in Fig. 1. The maximal extraction rate was obtainable from either serum or FFA–albumin solution when the pH was maintained between 6 and 7 (Fig. 1*A*); a marked discrepancy between serum and FFA–albumin solution was observed at lower pH levels. The discrepancy can be attributed, at least in part, to the lower Ca and higher albumin content of the serum: Fig. 1*B* illustrates the effect of omitting Ca from the buffered FFA–albumin, and Fig. 1*C* that of increasing the albumin content to 8%. These results indicate that Ca ions somehow promote the extraction of unionized FFA into chloroform.

If the pH of the medium was kept between 6 and 7, any buffer so far examined provided a similar favorable condition for the transfer of FFA into chloroform. Tris–maleate buffer was used in the experiment shown in Fig. 1 because of its indifference to Ca ions, and phosphate buffer at pH 6.2 (five volumes or more of the test solution) was employed in the remaining experiments of the present study.

Figure 2 shows that any period of shaking longer than 60 sec was satisfactory for maximal extraction of FFA.

Comparison with Dole Extraction Method

Although maximal transfer of FFA from serum to chloroform was achieved in phosphate buffer at pH 6–7, it remained to be decided whether the FFA were quantitatively transferred. A comparison was made, therefore, with the extraction process of Dole (2). Rat serum or FFA–albumin solution (0.2 ml) was added to 5 ml of isopropanol–heptane–N H_2SO_4 40:10:1 as described by Dole (2). The heptane layer was separated by further addition of 2 ml of water, and 3 ml of it was evaporated to dryness at 40° in an atmosphere of nitrogen. The residue was dissolved in 5 ml of chloroform. Table 1 shows that FFA survive such an evaporation process. Another portion of the same serum was directly extracted with chloroform in the presence of

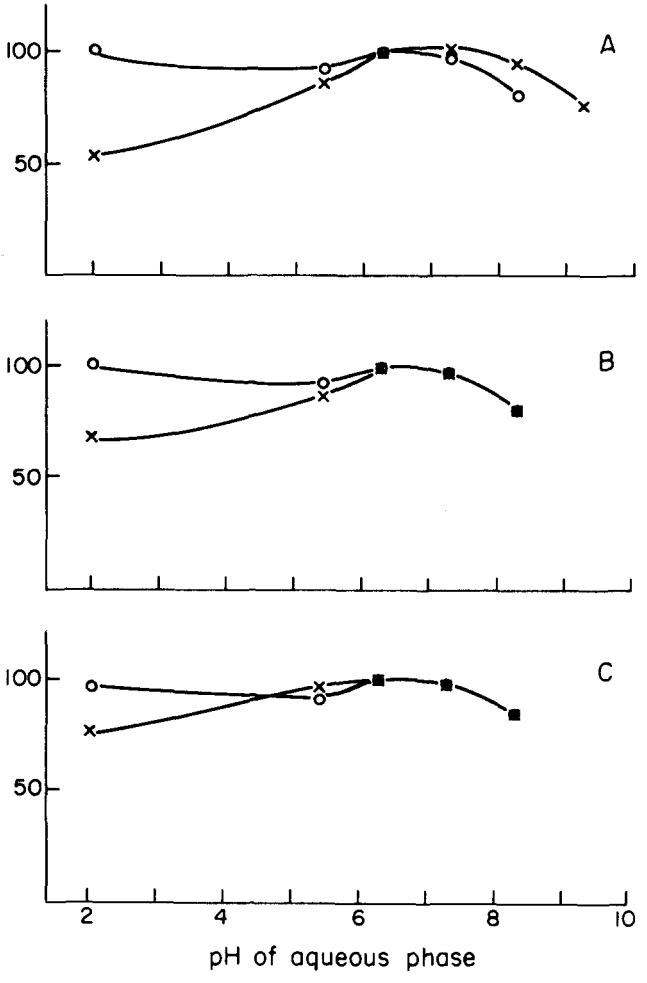

FIG. 1. FFA extraction by chloroform from aqueous solution at different pH levels. The relative amount of FFA transferred to chloroform was plotted as a function of pH in the aqueous phase (the value obtained at pH 6.2 is 100 in each case). One milliliter of Tris–maleate buffer was employed for 0.1 ml of serum or FFA–albumin solution and the medium at pH 2.0 was obtained by adding N HCl. *A*, ○———○ FFA–albumin solution (Krebs-Ringer bicarbonate solution); ×———× serum. *B*, ○———○ FFA–albumin solution (Krebs-Ringer bicarbonate solution); ×———× FFA–albumin solution in Ca-omitted Krebs-Ringer bicarbonate solution. *C*, ○———○ FFA–albumin solution (Krebs-Ringer bicarbonate solution); ×———× FFA–albumin solution fortified with higher (8%) concentration of albumin.

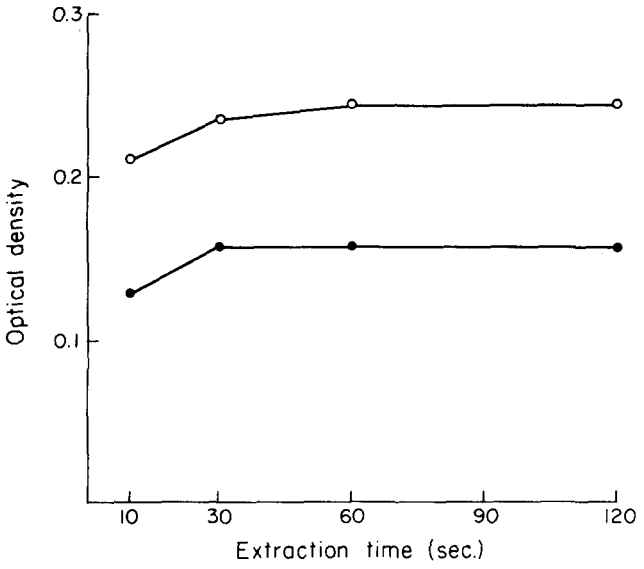

FIG. 2. Rate of FFA extraction by method described. Two milliliters of phosphate buffer (pH 6.2) and a 0.2 ml portion of a sample were shaken with 6.0 ml of chloroform for a period indicated on abscissa. The amount of FFA transferred to chloroform layer and colorimetrically determined is expressed in optical density (ordinate). ○———○, rat serum used as a sample; ●———●, FFA–albumin solution used as a sample.

phosphate buffer (pH 6.2). The two chloroform layers thus obtained were subjected to the colorimetric estimation of FFA. Table 2 shows that extraction with chloroform in the presence of phosphate buffer, pH 6.2, was as effective as with Dole's extraction mixture. Quantitative recoveries of FFA–albumin added to rat serum are shown in Table 3.

Analysis of FFA in Whole Blood

The titration technique now widely adopted for the determination of blood FFA (1, 2) is applied to the serum but not to whole blood, because the pigment extracted from blood cells seriously interferes with the detection of the end point during microtitration. Chloroform in the presence of phosphate buffer extracts no colored substances, so that whole blood can serve as a test solution for the present colorimetric method (Table 2). Relatively lower values of FFA obtained for whole blood may be ascribed to the much lower FFA concentration of blood cells.

No comparison could be made between the amounts of FFA extractable from whole blood by the present extraction technique and those by the extraction method of Dole, because the colored substances having absorption near 440 mμ, which are transferred to Dole's extraction mixture, dissolved in chloroform after removing the heptane by evaporation. However, as Table 4 shows, fatty acid added to rat whole blood was recovered as quantitatively as in the case of rat serum.

Extractability of Phospholipids by Chloroform

As already reported by Duncombe, lecithin is the only lipid which interferes with the colorimetric determination of FFA, because the molar extinction produced by lecithin is comparable to that produced by FFA. Triglycerides, cholesterol, or cholesterol esters produced only very low optical densities when subjected to the colorimetric procedure. Chloroform–phosphate and warm methanol–chloroform (8) extracts of the same serum were subjected to thin-layer chromatography and the phosphorus content of the phospholipid areas was determined by the method of Bartlett (9). In the

TABLE 1 THE EFFECT OF EVAPORATION OF THE SOLVENT ON FFA VALUE

FFA Standard Solution	Optical Density	
	Solvent Evaporated Once*	Solvent Not Evaporated
μeq/ml		
0.02	0.173	0.171
0.03	0.260	0.259
0.04	0.354	0.355
0.05	0.443	0.442

* Chloroform of the standard solution was evaporated off at 40° in an atmosphere of N_2. The dried residue was redissolved in the original volume of chloroform and subjected to the colorimetric procedure together with the samples stored without evaporation of the solvent.

TABLE 2 FFA EXTRACTED BY CHLOROFORM IN THE PRESENCE OF PHOSPHATE BUFFER COMPARED WITH THOSE EXTRACTED BY DOLE'S EXTRACTION MIXTURE

Samples		FFA Extracted		Ratio (*a*/*b*)
		Chloroform + Phosphate Buffer (*a*)	Dole's Extraction Mixture (*b*)	
		μeq/liter		
FFA – albumin solution	1	1660	1621	1.02
	2	801	768	1.04
	3	599	591	1.01
	4	501	486	1.03
	5	429	417	1.03
	6	285	279	1.02
	7	122	117	1.04
Serum from fasted rat	1	777	750	1.04
	2	618	594	1.04
	3	603	586	1.03
	4	498	507	0.98
Serum from nonfasted rat	1	330	339	0.97
	2	295	303	0.97
Whole blood from nonfasted rat	1	246		
	2	194		
	3	153		
	4	123		

The values in this table were obtained by comparing optical densities with the standard curve.

three samples of serum and one of rat liver mitochondria examined, the lipid phosphorus extracted by chloroform–phosphate was less than 1.3% of the total extractable by methanol–chloroform.

Standardized Procedure

The following procedure was adopted for the routine colorimetric analysis of FFA in biological preparations, as being the simplest and most efficient.

To a glass-stoppered pyrex test tube (16 x 150 mm) containing 6.0 ml of chloroform and 1.0–2.0 ml of phosphate buffer (pH 6–7), 0.2 or 0.3 ml of a sample is added, and the mixture is shaken for 90 sec. After a settling period of 15 min or more, the upper layer is aspirated with a fine-tipped pipette. Protein precipitate formed can be aspirated together with the upper layer. The chloroform phase is then decanted into a second glass-stoppered tube and to it is added 3.0 ml of Cu–triethanolamine solution. The tube is shaken 30 times. After 15 min or more, the Cu–triethanolamine solution is aspirated with a fine-tipped pipette. The residual chloroform layer is filtered, and 2 drops of sodium diethyldithiocarbamate solution are added. The yellowish brown color developed immediately is measured at 440 mμ against a reagent blank.

Some comments follow. Although the reduction in the amount of the chloroform after several decanting steps may occur to a somewhat different extent from one tube to another, the concentration of FFA in chloroform is maintained constant throughout the transfer steps. One drop (about 0.02 ml) of the color-developing reagent was found to be sufficient for determining FFA in a concentration up to 0.08 μeq/ml. When the concentration of FFA exceeds this level, 2 drops of the reagent must be added.

Water must be removed from the chloroform layer after shaking with Cu–triethanolamine and prior to the addition of sodium diethyldithiocarbamate because traces of Cu ions in aqueous solution develop color. To avoid the contamination of chloroform layer with droplets of water, Duncombe used centrifugation for a few minutes. We found, however, that filtering the chloroform layer once was simpler and just as effective for removing water. Any filter paper may be satisfactorily used except those that have been pretreated with either HF or HCl in the manufacturing process. This destroys the linear relationship of the optical density to the concentration of FFA.

When serum or other biological fluids containing no erythrocytes serve as a test solution, one decanting step may be omitted, i.e., the extraction of FFA with chloroform and the formation of the Cu complex can be carried out in the same test tube. In this case, 3 ml of Cu–triethanolamine reagent is added to the chloroform extract after pipetting off most of the upper layer. When whole blood is analyzed, however, the chloroform extract must be completely freed from droplets of hemolyzed blood, because the latter produces a coloration during the formation of the copper complex.

TABLE 3 RECOVERY OF FFA–ALBUMIN ADDED TO SERUM

Expt. No.	Samples	Optical Density		Recovery
		Found	Expected	
				%
1	FFA No. 1 + H_2O	0.079		
	FFA No. 2 + H_2O	0.152		
	FFA No. 3 + H_2O	0.251		
	Serum + H_2O	0.148		
	Serum + FFA No. 1	0.220	0.227	96.9
	Serum + FFA No. 2	0.308	0.300	102.6
	Serum + FFA No. 3	0.404	0.399	101.3
2	FFA No. 4 + H_2O	0.108		
	FFA No. 5 + H_2O	0.215		
	Serum + H_2O	0.181		
	Serum + FFA No. 4	0.285	0.289	98.6
	Serum + FFA No. 5	0.387	0.396	97.7

FFA–albumin solution at various FFA levels, 0.1 ml, combined with 0.1 ml of H_2O or rat serum, was subjected to the colorimetric analysis.

DISCUSSION

The procedure established here for the colorimetric determination of the FFA in aqueous phase was developed in order to obtain a simple and sensitive analytical method. Some techniques such as transfer of the chloroform layer by decantation instead of pipetting,

TABLE 4 RECOVERY OF FFA–ALBUMIN ADDED TO WHOLE BLOOD

Expt. No.	Samples	Optical Density		Recovery
		Found	Expected	
				%
1	FFA No. 1 + H_2O	0.046		
	FFA No. 2 + H_2O	0.174		
	FFA No. 3 + H_2O	0.287		
	Whole blood + H_2O	0.041		
	Whole blood + FFA No. 1	0.096	0.087	110.3
	Whole blood + FFA No. 2	0.203	0.215	94.4
	Whole blood + FFA No. 3	0.329	0.328	100.3
2	FFA No. 4 + H_2O	0.036		
	FFA No. 5 + H_2O	0.140		
	FFA No. 6 + H_2O	0.242		
	Whole blood + H_2O	0.046		
	Whole blood + FFA No. 4	0.083	0.082	101.2
	Whole blood + FFA No. 5	0.191	0.186	102.5
	Whole blood + FFA No. 6	0.283	0.288	98.2

FFA–albumin solution at various FFA levels, 0.1 ml, combined with 0.1 ml of H_2O or of rat whole blood, was submitted to the colorimetric analysis.

combination of filtration and dehydration steps, and the use of whole blood as a sample without centrifuging off the blood cells or deproteinization were adopted for the purpose of both raising sensitivity and saving time.

The sensitivity of the present method is based on the ability of diethyldithiocarbamate to react with a trace amount of Cu complex in chloroform layer, and merits no further discussion. As to specificity, it was found by Duncombe that none of the lipids examined form a measurable amount of chloroform-soluble complex with Cu ions except phospholipids such as lecithin. No significant amount of phospholipids was transferred to the chloroform layer when aqueous samples were shaken with chloroform for 90 sec in the presence of phosphate buffer. Success in extracting FFA completely without extracting interfering substances is indicated by the good agreement between analytical values obtained by the present method and those provided by the combined method of Dole's extraction and colorimetric determination (Table 2). Analysis of variance on the colorimetric determination of standard fatty acid in chloroform was already carried out by Duncombe (5) to give an acceptable degree of precision. When the extraction step was superimposed on the colorimetric procedure, the standard deviation obtained was 9.1 for a mean value of 521 μeq FFA per liter of serum and 10.2 for a mean value of 403 μeq FFA per liter of FFA–albumin solution. The number of observations was 6 in each case.

One of the advantages of the present method over the titrimetric methods is that whole blood can be directly analyzed for FFA content without separation of serum or plasma. As little as 0.2 ml of whole blood may serve as a sample for FFA analysis. Thus, periodical analysis of the blood FFA level of a rat has been rendered possible by the application of the present method.

Quite recently, during the course of this study, Duncombe published (10) the results of a tentative application of his analytical method to human sera, the FFA content of which had been measured by another investigator according to Gordon's method (1). He found that when 5 ml of chloroform, 0.5 ml of serum, and 3 ml of Cu–triethanolamine solution were vigorously shaken and the chloroform layer separated by centrifugation was analyzed colorimetrically, the FFA values were at variance with those obtained by Gordon's microtitration method. The higher pH (presumably 8.5–9.0), as well as the formation of a disk of protein precipitate which we have observed when chloroform is shaken with these larger volumes of serum in the presence of Cu–triethanolamine reagent, might have resulted in the more variable and less reliable analytical values.

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