Rapid colorimetric micromethod for free fatty acids

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ABSTRACT Free fatty acids (FFA), the uranyl ion, and the basic dye Rhodamine B form colored complexes, which are extractable into toluene or benzene. Fatty acids of different chain lengths above C_{10} and different degrees of unsaturation gave constant molar yield. Complexes in toluene alone are unstable, especially in the light, but a small amount of aqueous uranyl acetate stabilizes them sufficiently for determination.

At constant uranyl and Rhodamine B concentrations, a plot of optical density vs. FFA concentration yields two straight lines of different slope, i.e., a biphasic standard curve. Phospholipids interfere, and must be removed with zeolite during FFA extraction. Recovery of FFA added to rat plasma was very similar to that with titration. Assay of rat and dog plasma samples under fasting and fed conditions gave good agreement with the titration method. Values of human plasma samples tended to be higher by the colorimetric procedure; a few samples gave significant disagreement.

The method compares well with previous methods in sensitivity and accuracy, and offers advantages in speed, simplicity, and possibly specificity.

KEY WORDS free fatty acids · colorimetric assay · Rhodamine B · uranyl ion

THERE IS an increasing need for methods that measure microquantities of free fatty acids in biological systems and food products. Especially needed is a method that will permit simple and rapid analysis of large numbers of samples. Dole and Meinertz (1) have described a method for extraction of free fatty acids from plasma and other biological fluids (using a heptane–isopropyl alcohol–sulfuric acid system), and their determination by microtitration. Baker (2) reported a colorimetric procedure for determining free fatty acids in grain by the formation of copper soaps in benzene. Iwayama (3) reported the formation of copper soaps in chloroform, and Duncombe (4) increased the sensitivity of this method by using sodium diethyl dithiocarbamate for the detection of copper. Recently, Novak (5) reported a colorimetric method in which cobalt is used instead of copper, and the cobalt is determined with α-nitroso-β-naphthol.

Most investigators have used the Dole extraction procedure (1) or its modification as reported by Trout, Estes, and Friedberg (6) for the isolation of free fatty acids. Mendelsohn (7) reported a modification of an extraction procedure described by Delsal (8, 9) which employs methylal–methanol and petroleum ether for the extraction of lipids from biological materials. Mendelsohn used *p*-rosaniline for colorimetric determination of the extracted fatty acids. Feigl (10) suggested the use of Rhodamine B for fatty acid detection as a spot test, but did not discuss its suitability for microquantitative analysis.

In the procedure described below, Rhodamine B and uranyl ion are used to form a colored complex with free fatty acids. This complex is extractable into benzene or toluene and can be measured colorimetrically in these solvents. The sensitivity of the method can be adjusted, by variation of reagent proportions to provide convenient ranges for different applications. The procedure described is that used in this laboratory for assay of lipoprotein lipase in rat plasma (measurement of FFA liberated from an Ediol emulsion).

MATERIALS AND METHODS

Apparatus and Reagents

The spectrophotometers used were the Bausch & Lomb Spectronic 20 and the Perkin-Elmer model 350. The

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Abbreviation: FFA, free fatty acid(s).

mechanical shaker was an Eberbach reciprocating type.

The glassware used in this procedure consisted of test tubes with Teflon-lined screw caps, glass-stoppered test tubes (10 ml capacity), and colorimeter tubes 13 × 100 mm matched to a tolerance of ± 0.5% transmittance. All glassware used was rinsed in alcoholic HCl and distilled water, and dried in the air before use.

Reagents were: toluene, analytical reagent grade; Rhodamine B hydrochloride (British Drug Houses,[1] Poole, Dorset, England, distributed in the United States by Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, L.I., N.Y.), an aqueous solution 1 mg/ml; 1% aqueous uranyl acetate (Fisher Certified Reagent, Fair Lawn, N.Y.). Fatty acids were: caproic (Matheson, Coleman, and Bell, Cincinnati, Ohio), lauric, myristic, palmitic, stearic, oleic, and linolenic (Applied Science Laboratories Inc., State College, Pa.), purity greater than 99% by gas–liquid chromatography; petroleum ether, boiling point 40–60°C, redistilled; heptane, redistilled; methylal (Matheson, Coleman, and Bell); methanol, reagent grade; isopropanol, reagent grade, redistilled; zeolite (W. A. Taylor & Company, Baltimore, Md.), ground to 100 mesh and dried in an oven at 110°C overnight.

Procedure

Most of the data in this work were obtained by the following procedure, which was developed specifically for the assay of lipoprotein lipase in rat plasma. Appropriate modifications for other purposes are given, or are suggested, in later parts of this paper.

The extraction method of Mendelsohn (7) is preferred to others for the isolation of free fatty acids from plasma systems. 1 ml of plasma or aqueous plasma mixture is placed in an extraction tube. Two drops of 85% phosphoric acid and 3 ml of methylal–methanol (4:1) are added with mixing. 4 ml of methanol, 1 ml of distilled water, and 3 ml of petroleum ether are added. The tube is then closed and shaken on a mechanical shaker for 15 min. The extraction tube is centrifuged for 5 min at 2000 rpm. An aliquot of the extract is placed in a glass-stoppered test tube and the solvent is removed by evaporation on a warm water bath under a stream of nitrogen. The residue is dissolved in 4 ml of toluene (benzene can also be used, but is more toxic). 3 ml of a mixture of the Rhodamine B hydrochloride and uranyl acetate solutions (1:1) are added. The mixture is shaken (1 min vigorous shaking by hand is sufficient, although 15 min on a mechanical shaker was used in most of this work.) The two phases are separated by centrifugation for about 1 min. A sample of the upper phase is transferred to a colorimeter tube and read in a spectrophotometer at 545 mμ. The colorimeter tube should contain a drop of uranyl acetate solution to prevent fading, and should be kept from strong light. A reagent blank is made up of the same volume of solvent and reagent mixture and used to adjust the instrument to 100% transmittance. Palmitic acid standards are run with each experiment. When the method is used to assay physiological levels of FFA in plasma, sensitivity should be increased by increasing the Rhodamine B concentration from 1.0 to 3.0 mg/ml, and phospholipids must be removed as described below.

[1] Rhodamine B from one other source was not satisfactory for this use.

RESULTS

Absorption Spectrum of Rhodamine B–Uranyl Ion–Fatty Acid Complex

The absorption spectrum of the Rhodamine B–uranyl ion–fatty acid complex was determined for caproic, stearic, oleic, and linolenic acids in the Bausch & Lomb Spectronic 20 spectrophotometer. Fig. 1 shows that all had spectra with a maximum absorption at 545 mμ. The spectrum of palmitic acid was determined at various concentrations with a Perkin-Elmer model 350 recording spectrophotometer. The spectrum at each concentration displayed a single maximum at 545 mμ. Since palmitic

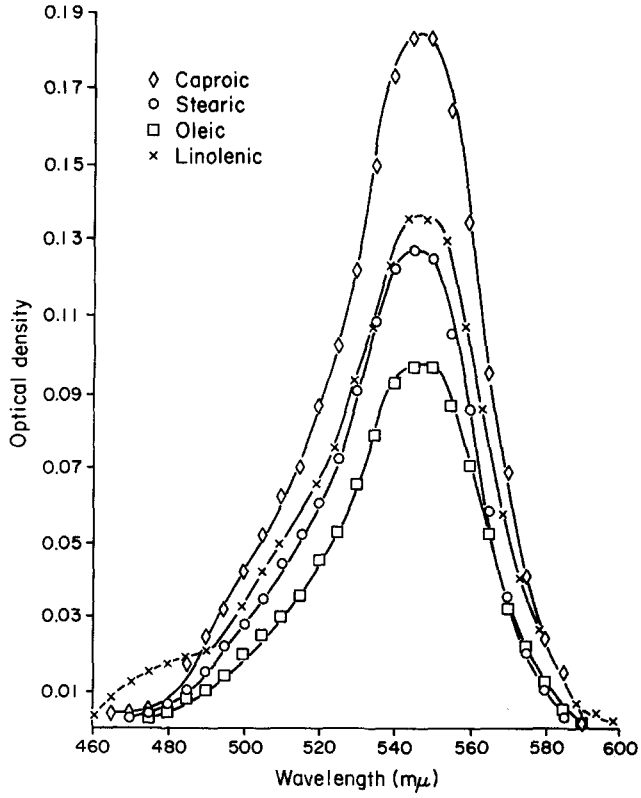

FIG. 1. Absorption spectra of fatty acid–uranyl–Rhodamine B complexes in toluene. Concentrations of fatty acids: caproic, 0.3; stearic, 0.08; oleic, 0.07; and linolenic, 0.09 μmole/ml.

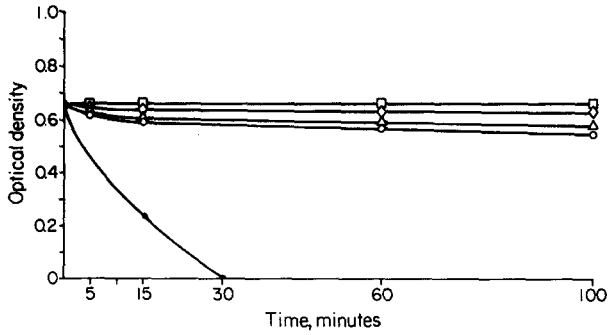

FIG. 2. Effects of time and exposure to light on the palmitic acid complex. All tubes contained the complex formed with 0.3 μeq of palmitic acid per ml of toluene. △, Complex, kept in dark, no uranyl acetate present; □, complex, in dark, one drop of 1% aqueous uranyl acetate in tube; ◇, complex, in laboratory light, one drop of 1% aqueous uranyl acetate in tube; ○, complex, in laboratory light, no uranyl acetate present; ●, and complex, in direct sunlight, no uranyl acetate present.

acid was determined on a different instrument, it was not included in Fig. 1. At 0.125 μmole/ml toluene, palmitic acid gave a maximum absorption of 0.2 absorbance. These data indicate that there was no difference in spectra between short-chain, long-chain, saturated, or unsaturated fatty acids, when complexed with Rhodamine B and uranyl ion.

Color Stability

The color complex in the toluene phase will fade when it is removed from the aqueous uranyl acetate phase. Fig. 2 shows the effect of ordinary laboratory light on color intensity in the absence or presence of one drop of 1% uranyl acetate solution in the bottom of the colorimeter tube. This figure shows that the colored complex fades slowly in the absence of uranyl acetate solution, even in the dark. Fading is accelerated by light, but for many purposes ordinary laboratory light can be tolerated if uranyl acetate solution is present. No fading occurred with uranyl acetate in the dark during the interval studied (100 min). Samples left in the color development tube in contact with the original aqueous phase were stable for 24 hr under laboratory light (not shown in figure).

Color Yield at Different Concentrations of Palmitic Acid

Seven separate standard curves for palmitic acid were run over a 6 wk period in a Spectronic 20 spectrophotometer. The average of these seven determinations was plotted and the 95% confidence limits for a single run were determined.[2] The results are shown in Fig. 3.

Beer's law is not obeyed over the full range, but rather the optical density versus concentration curve is made up

[2] 95% confidence limits of y for a single value of x.

$$y \pm t_{0.025}\, S_{y.x} \sqrt{1 + \frac{1}{N} + [(X - \bar{X})^2/(N-1)S_x^2]}$$

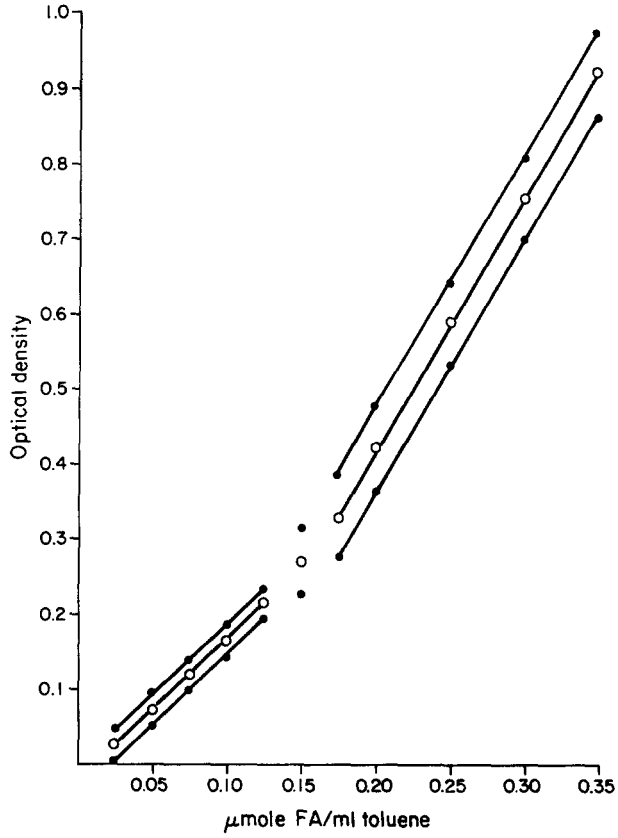

FIG. 3. Standard curve for palmitic acid, with 95% confidence limits for a single run (based on seven determinations, over a 6 wk period). ○, Average; ●, 95% confidence limits. FA, fatty acid.

of two straight-line portions with different slopes. The upper slope is twice the lower. Since no shift in absorption maximum occurs with concentration, the ratio of dye to fatty acid in the complex probably changes.

If the color intensity obtained is greater than the instrument can accurately measure, the colored solution can be diluted with reagent blank. A palmitic acid complex of 1.0 μmole/ml of toluene was made and diluted stepwise with reagent blank; the resulting curve was well within the confidence limits in Fig. 3. Alternatively, optical densities approaching 3.0, measured on the Gilford spectrophotometer, model 2000, have demonstrated that the upper straight line extends into this range.

Variation of Color Yield at Different Concentrations of Reagents

The concentration of Rhodamine B was varied at constant concentrations of fatty acid and uranyl acetate, and color yield was determined. The color yields for palmitic acid at 0.1 μmole/ml and uranyl acetate at 1% showed that Beer's law is followed, from 0.5 mg/ml to 5 mg/ml of Rhodamine B (optical density at 5 mg/ml, 0.68). The blanks used to adjust the instrument contained 1%

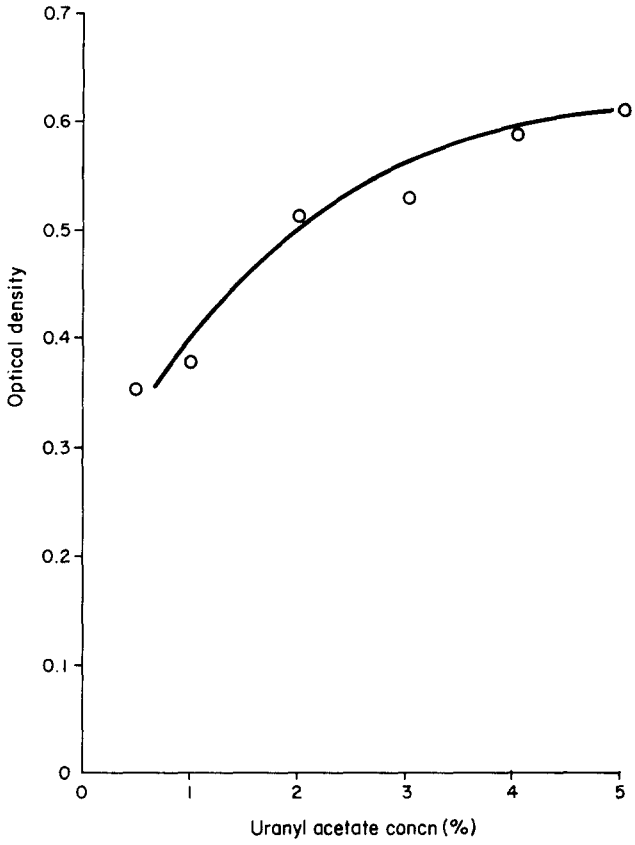

FIG. 4. Color yield as a function of uranyl acetate concentration. Palmitic acid at 0.2 μmole/ml toluene and Rhodamine B at 1 mg/ml of aqueous reagent.

uranyl acetate solution and Rhodamine B of the experimental concentration.

Fig. 4 records the results of varying the uranyl acetate concentration while the concentration of Rhodamine B was maintained at 1 mg/ml and that of palmitic acid at 0.2 μmole/ml. Although the color yield increases with increasing uranyl concentration, the rate of increase is not linear and Beer's law is not obeyed; the amount of color tends to reach a plateau at higher concentrations.

Standard curves for palmitic acid with variation of uranyl ion (Fig. 5) or Rhodamine B (Fig. 6) show that both these factors give increasing color yield with increasing amounts. Uranyl acetate concentrations of 2% and higher caused cloudiness in the toluene phase of some tubes, and therefore are not of practical use.

Color Yield of Various Fatty Acids

Caproic, myristic, palmitic, stearic, oleic, and linolenic acids were dissolved in petroleum ether in concentrations of 1.0 μmole/ml. Various volumes were pipetted into glass-stoppered test tubes and the solvent was evaporated. The residue was dissolved in toluene and the Rhodamine B–uranyl acetate mixture was added. Color intensities were measured in the Spectronic 20 at 545 mμ; the resulting curves for each fatty acid are shown in Fig. 7. The data for fatty acids above C_{10} lie within the 95% confidence limits for palmitic acid as shown in Fig. 3. Caproic acid, however, produced a lower color yield. This is probably due to the greater solubility of its complex in the aqueous phase. This type of partition is similar to that reported by Duncombe (4) for copper soaps.

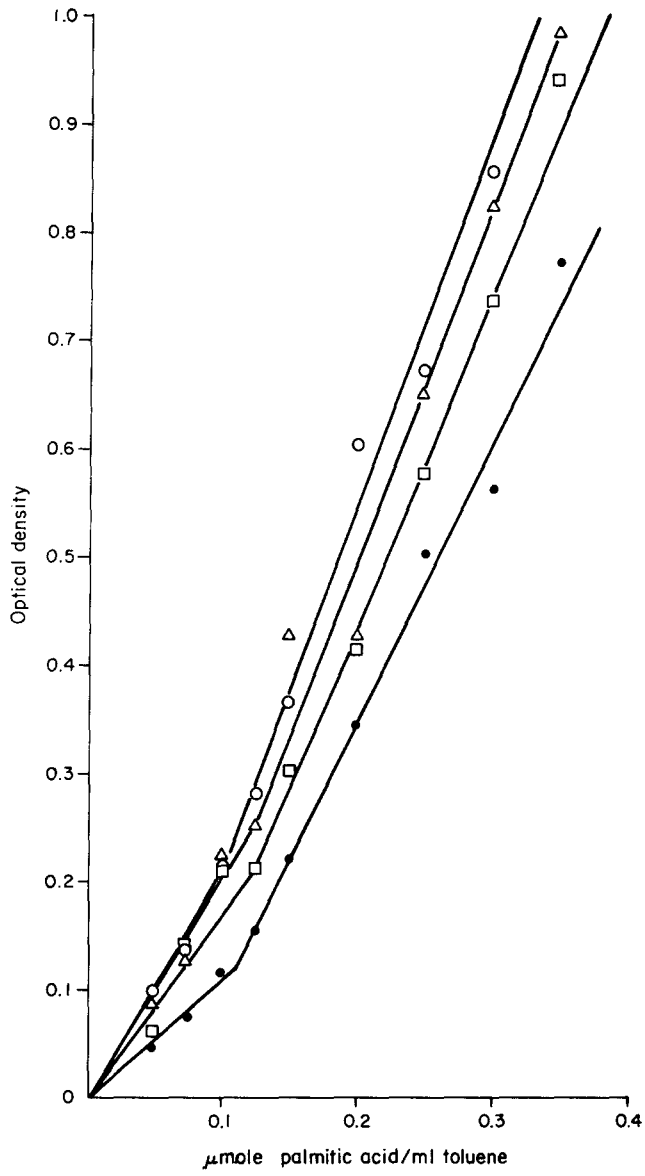

FIG. 5. Standard curves for palmitic acid at various concentrations of uranyl acetate. Rhodamine B concentration, 1 mg/ml of aqueous reagent. Uranyl acetate, ●, 0.5% in aqueous reagent; □, 1.0%; △, 2.0%; ○, 4.0%.

Interfering Materials

Many detergents, especially anionic ones such as arylalkyl sulfonates, give intense color with Rhodamine B in benzene and toluene. All glassware should therefore be rinsed well before use. Alcoholic hydrochloric acid will

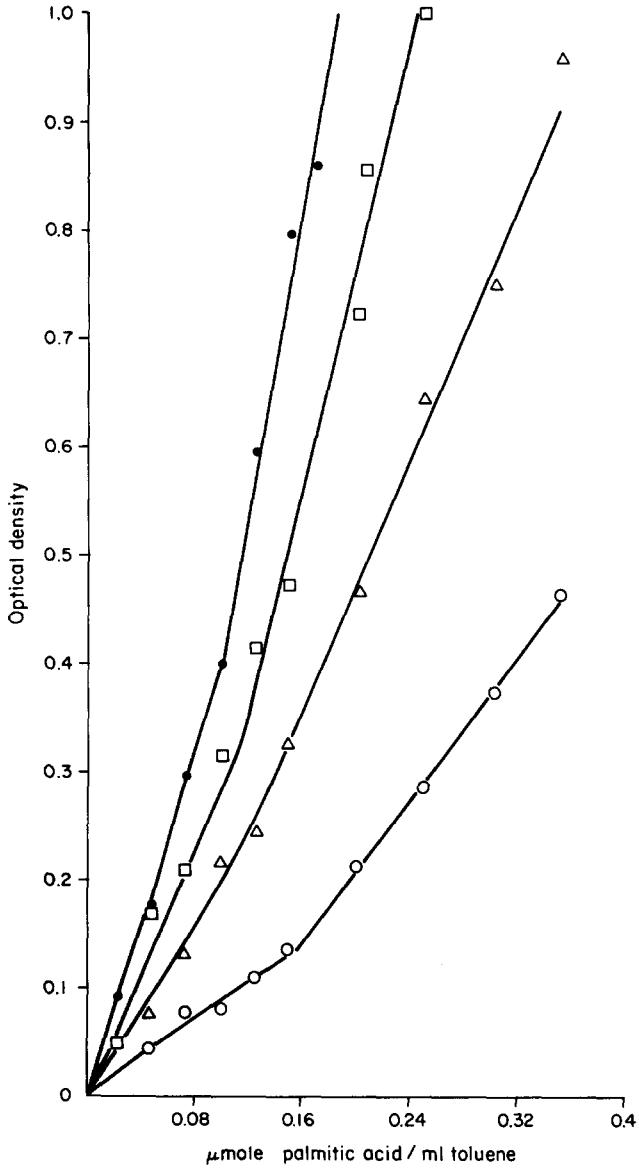

FIG. 6. Standard curves for palmitic acid at various concentrations of Rhodamine B. Uranyl acetate, 1% in aqueous reagent. Rhodamine B, ○, 0.5 mg/ml of aqueous reagent; △, 1.0 mg/ml; □, 2.0 mg/ml; ●, 3.0 mg/ml.

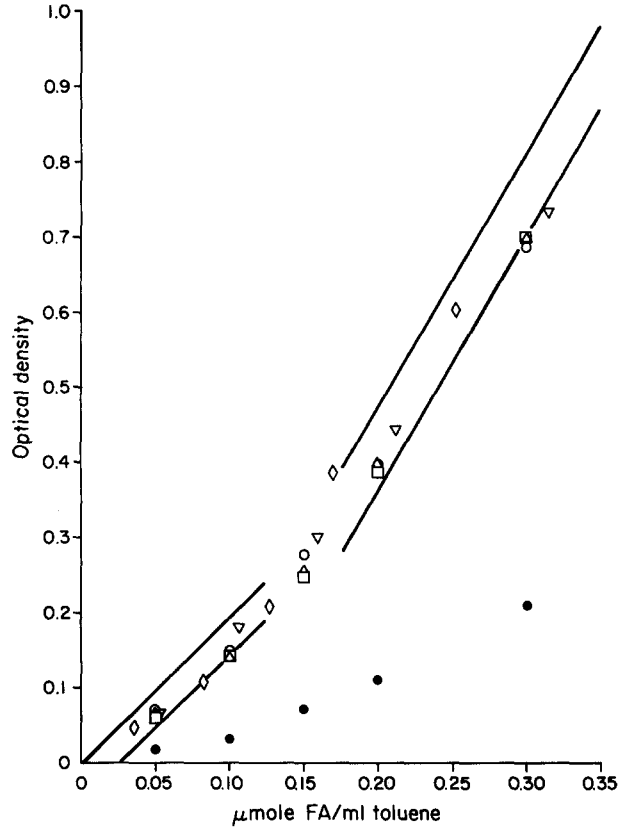

FIG. 7. Optical density vs. concentration for various fatty acids (FA). 95% confidence limits (lines) for palmitic acid are taken from Fig. 3. ○, Palmitic; □, stearic; △, myristic; ◇, oleic; ▽, linolenic; and ●, caproic.

remove color-producing detergents, etc., from glassware.

Mineral, acetic, and lactic acids produce no color in the toluene phase. They do not interfere in the analysis of fatty acids unless very large relative quantities are present. Up to 2 μmoles of acetic acid per ml of toluene produced no change in color yield of palmitic acid, but above 2 μmoles/ml there was a slow loss in color yield up to 8 μmoles/ml, and then an increased loss above 8 μmoles/ml. The mineral acids hydrochloric, nitric, and sulfuric acid did not change color yield significantly up to 1 μmole/ml, an amount unlikely to be extracted with the fatty acids. These acids will form salts with Rhodamine B that are soluble in water, but not in the aromatic hydrocarbon.

Purified soya bean lecithin produced a colored complex soluble in toluene. The spectrophotometer gives identical absorption spectra for lecithin and fatty acid complexes. However, fatty acid complexes have an orange fluorescence (activation wave length 350 mμ, fluorescent wave length 575 mμ) which lecithin complexes do not have. Thus visual appearances of the two types of complex are different. Concentrations as low as 0.01 μmole of lecithin per ml of toluene give a significant color yield. Therefore, phospholipids will interfere with the determination of fatty acids both by absorption and fluorescent methods (because of quenching) and must be removed before analysis.

Phospholipid Removal

Phospholipids are partially extracted from plasma by the procedure described, but could be eliminated by treatment of the plasma with zeolite (11, 12). The ability of zeolite to remove phospholipid, but not fatty acid, was tested. Palmitic acid and (or) lecithin, dissolved in petroleum ether, was placed in an extraction tube and

the solvent was evaporated. 1 g of zeolite was added followed by 1 ml of water, with mixing. The methylal–methanol–petroleum ether extraction procedure was then applied, and color developed on the extracts. Table 1 shows that this procedure removed the lecithin without removing palmitic acid.

The effect of zeolite treatment was determined for plasma of dog, rat, and man. 1 ml of plasma was added to 1 g of zeolite, and extraction and color development carried out as before. In addition, the same samples were analyzed without zeolite treatment, and extracts of treated and untreated samples were titrated according to Dole (1). Results are given in Table 2. Zeolite pretreatment resulted in decreased values by colorimetry and titration, which indicated that interfering materials, presumably phospholipids, were removed. When this was done, essential agreement between the two methods of measurement was obtained. These values also agree with expected values for the species and conditions used. On the basis of the change due to zeolite treatment, the amount of phospholipid removed was calculated (as lecithin), using a standard curve for lecithin in the colorimetric procedure. Table 2 shows that only a small fraction of the phospholipid available in the various plasmas carries over into the extract, but that it is sufficient to produce significant interference in dog and human plasma, but relatively little interference in rat plasma. It should be noted that phospholipid interference is greater in the colorimetric procedure than in the titration method, because of the fact that phospholipids (at least lecithin) produce a greater color yield than fatty acids.

TABLE 1 REMOVAL OF PHOSPHOLIPID FROM FREE FATTY ACIDS BY ZEOLITE

Amount Added		Recovered*	
FFA	Phospholipid	Untreated	Zeolite-Treated
μmoles		*μmoles FFA*	
1.0	0	1.04	1.04
		1.01	1.03
0	2.0	0.86	0.01
		0.85	0.02
1.0	2.0	1.8	1.05
		1.9	1.06

* Recoveries in the extract were calculated in terms of FFA from the FFA calibration curve. The phospholipid used was purified soybean lecithin.

Comparison With Titration

Various amounts of palmitic acid in petroleum ether were added to a series of tubes, in quadruplicate. The solvent

TABLE 2 PHOSPHOLIPID INTERFERENCE IN FFA ANALYSIS FOR PLASMA OF DIFFERENT SPECIES. COMPARISON OF COLORIMETRIC AND TITRIMETRIC PROCEDURE

Species	FFA Estimated in Untreated Plasma (Avg. of Duplicates)		FFA Estimated in Zeolite-Treated Plasma (Avg. of Duplicates)		Phospholipid Extracted (Calculated)*	Total Phospholipid in Plasma†
	Colorimetric	Titration	Colorimetric	Titration		
			μmoles/ml plasma			
Dog (fed)	1.29	0.40	0.30	0.28		
	1.64	0.52	0.28	0.26		
	2.00	1.15	0.39	0.39		
	2.00	1.19	0.70	0.52		
	1.75	0.46	0.34	0.28		
	1.14	0.62	0.44	0.40		
	1.63	0.72	0.41	0.36	0.51	3.38 ± 0.20
Rat (fed)	0.74	0.76	0.64	0.64		
	0.54	0.54	0.43	0.44		
	0.73	0.72	0.66	0.70		
	0.44	0.38	0.30	0.26		
	0.56	0.49	0.46	0.43		
	1.27	1.27	1.20	1.16		
	0.71	0.69	0.62	0.61	0.07	1.64
Human (fasted)	0.75	0.46	0.39	0.38		
	0.78	0.54	0.44	0.38		
	1.02	0.98	0.54	0.45		
	0.98	0.78	0.72	0.64		
	0.94	0.76	0.70	0.64		
	0.89	0.70	0.56	0.50	0.16	2.49 ± 0.16

Mean values ± SEM.

* Determined by calculating the difference in optical density between treated and untreated plasma and assuming that the difference was due to phospholipid removed by zeolite.

† Method of Zilversmit and Davis (15). Average values for species determined in this laboratory.

was evaporated to dryness in a water bath at 60°C, rat plasma was added, and the contents were extracted by the method of Mendelsohn (7). Each extract was divided into two equal parts; one part was assayed by the colorimetric method, and the other was dissolved in heptane and titrated according to Dole (1). The same procedure was repeated by using the heptane–isopropyl alcohol–sulfuric acid extraction system with an acid wash as described by Trout et al. (6). The quantities of fatty acid were known: 1.0, 2.0, 4.0, 6.0, and 8.0 μmoles palmitic acid per tube. Data are reported in Table 3. Recovery with the colorimetric procedure was better following the petroleum ether–methylal–methanol–phsophoric acid extraction system (Mendelsohn) than after the heptane–isopropyl alcohol–sulfuric acid extraction system (Dole). A comparable difference was not seen with the titration assay. The combination of colorimetry with the petroleum ether–methylal–methanol–phosphoric acid extraction system produced the same recovery as titration.

A comparison of the colorimetric and titration assay procedures with rat, dog, and human plasma, with and without zeolite treatment as discussed above, are shown in Table 2. Further assays of plasma from these three species with zeolite treatment are reported in Table 4. The first sample of each species was assayed 10 times, for determination of the precision of both methods. Rats were either fed ad libitum (Purina Lab Chow) or fasted by removing food at 4:30 p.m. on the day before the blood was taken. Dogs were fasted in the same manner or fed (Wayne dog food) within 4 hr of taking blood. Human blood samples were drawn under various conditions: "fasting" refers to a 16 hr fast, and "fed" refers to blood sampling 1–2 hr after a meal. A few of the human samples were from patients with abnomal lipid patterns. These are indicated in the table. These patients were fed a low fat meal and blood was taken 5 hr later.

Though rat and dog samples showed excellent agreement between colorimetric and titrimetric procedures, this was not so with all human plasmas. In general, the colorimetric method gave slightly higher values with human plasma. Since there was no pattern in terms of fatty acid level and disagreement between methods, no reason for these discrepancies can be given.

DISCUSSION

Rhodamine B forms colored complexes with many acidic materials, especially when uranyl ion is present. According to Feigl (10), uranyl ion forms a complex with fatty acids in which the fatty acid is completely ionized. The proton released from the fatty acid changes Rhodamine B to the cationic form, which complexes with the uranyl–fatty acid ion. Ramette and Sandell (13) discuss the chemistry of Rhodamine B. We have found that when the fatty acid has a chain length of four carbons or more, the complex has some solubility in benzene and toluene; if the chain is longer than C_{10}, the complex has no detectable solubility in water and is completely extracted into the aromatic hydrocarbon phase. Duncombe (4) has reported similar solubility relationships for the copper soaps of fatty acids used in his analytical method. If the acid is mineral, acetic, lactic, etc., the Rhodamine B complexes will remain in the aqueous phase, no color being produced in the aromatic phase. Phospholipids produce colored complexes that are soluble in the aromatic hydrocarbon, and therefore they must be removed before fatty acid analysis. If color fades, it may be regenerated to its original intensity if the solution is shaken again with fresh uranyl acetate solution.

The calibration curve for Rhodamine B–uranyl ion–fatty acid complex in toluene does not obey Beer's law over the full range, but is made up of two straight-line portions; the slope of the upper portion is twice that of the lower. This must reflect a change in complex composition. Such a phenomenon is mentioned by Hiskey (14). The workable range of fatty acid concentrations in the method

TABLE 3 Recovery of Added Palmitic Acid from a Plasma System by the Dole and Mendelsohn Extraction Procedures Followed by Colorimetric or Titrimetric Method

FA Added	Mendelsohn Extraction			Dole Extraction		
	Avg. Recovery ± SEM*		Ratio Colorimetric: Titration	Avg. Recovery ± SEM*		Ratio Colorimetric: Titration
	Colorimetric	Titration		Colorimetric	Titration	
μmoles	%			%		
1.0	79.8 ± 2.4	79.7 ± 3.8	1.00	90.0 ± 5.5	97.0 ± 4.6	0.927
2.0	89.8 ± 4.6	96.2 ± 1.3	0.933	82.8 ± 5.5	83.2 ± 4.6	0.995
4.0	98.4 ± 3.4	91.3 ± 3.3	1.08	78.0 ± 1.5	87.7 ± 0.7	0.889
6.0	95.7 ± 0.6	91.2 ± 1.1	1.05	81.7 ± 1.2	89.1 ± 1.1	0.917
8.0	94.6 ± 2.0	93.4 ± 0.5	1.01	85.8 ± 1.6	91.1 ± 0.9	0.941
Total average	91.6 ± 1.9	90.9 ± 1.5	1.01	83.7 ± 1.7	89.7 ± 1.6	0.933

* n = 4 for individual average values.

TABLE 4 COMPARISON OF COLORIMETRIC AND TITRIMETRIC ASSAYS OF FREE FATTY ACIDS FROM PLASMA OF DIFFERENT SPECIES. EXTRACTED WITH PETROLEUM ETHER–METHYLAL–METHANOL–PHOSPHORIC ACID SYSTEM; PHOSPHOLIPID REMOVED WITH ZEOLITE

Species	Condition	Sample No.	Free Fatty Acid		
			Colorimetric	Titrimetric	Difference (C – T)
			μmole/ml plasma		
Dog (mongrel)	fasted	1 (10)*	0.72 ± 0.01	0.74 ± 0.02	−0.016 ± 0.03
		1† (3)	0.70	0.73	
Dog, beagle	fasted	2 (3)	1.35	1.32	
	fasted (8)	4–11 (2)	1.22 ± 0.13	1.14 ± 0.14	0.08 ± 0.07
	fed (8)	12–19 (2)	0.44 ± 0.02	0.50 ± 0.03	−0.05 ± 0.03
Rat	fasted (15 pooled plasmas)	1 (10)	0.72 ± 0.01	0.75 ± 0.04	−0.24 ± 0.10
	fed	2 (3)	0.18	0.17	
	fed	3 (3)	0.32	0.26	
	fasted (8)	4–11 (2)	0.67 ± 0.04	0.66 ± 0.04	0.012 ± 0.05
Human normal	fasted	1 (10)	0.81 ± 0.01	0.79 ± 0.02	0.017 ± 0.039
normal	fed	2 (1)	0.39	0.36	
		2† (1)	0.36	0.40	
normal	fed	3 (3)	0.44	0.22	
endogenous hyperlipoproteinemia (alcoholic)		4 (3)	1.01	1.01	
normal	fasted	5 (3)	0.33	0.29	
normal	fed	6 (2)	0.66	0.25	
endogenous hyperlipoproteinemia (diabetic)		7 (3)	0.69	0.57	
familial hypercholesterolemia		8 (3)	1.07	0.84	
normal	fasted	9 (3)	0.71	0.57	
endogenous hyperlipoproteinemia (diabetic)		10 (2)	0.68	0.50	
familial hypercholesterolemia		11 (2)	0.84	0.74	
endogenous hyperlipoproteinemia		12 (2)	0.74	0.68	

Mean values ± SEM.
* Number of determinations in parentheses.
† Redetermined from same plasma about 10 days later.

described is 0.05–0.4 μmole/ml. Since there are two different straight-line portions (the slope changing between 0.15 and 0.2 μmole/ml), we suggest that concentrations below 0.15 and above 0.2 be used for determination of unknown samples.

The recommended procedure works well with a plasma–fat emulsion system for lipase assay. However, the assay procedure is adaptable to other systems with different sensitivity requirements, if the dependence of color intensity on Rhodamine B concentration (Fig. 6) is utilized. An example is the use of a Rhodamine B concentration of 3 mg/ml to assay plasma, which allows greater sensitivity at low concentrations of fatty acid.

The comparison of the colorimetric procedure with titration gave good agreement with rat and dog plasmas. Although the majority of human plasmas agreed quite well, a few did not. Further work will be required to determine the cause of difference in these samples.

The use of Rhodamine B in this two-phase system has several advantages over the titration and *p*-rosaniline (7) procedures. It is more specific for acid complexes that are soluble in the aromatic solvent; *p*-rosaniline and titration methods detect any acid present in the extract. The sensitivity of the method is equal or superior to those reported previously (1, 4, 5, 7). The procedure is rapid and the steps are simple; a large number of samples can therefore be assayed in a short time. Interference from phospholipid is greater in this procedure than in the titration procedure, but this source of error can be removed.

By comparison with the copper and cobalt soap methods (4, 5), the present procedure requires one less step; there is no danger of chromogen contamination from the aqueous phase (16), and the method is therefore less subject to mechanical error.

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