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A GAS–LIQUID CHROMATOGRAPHIC ASSAY FOR PLASMA FREE FATTY ACIDS

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SUMMARY

An operationally simple extraction–gas–liquid chromatographic assay has been developed for determining the quantity and composition of individual free fatty acids (FFAs, $C_{14:0}$–$C_{18:2}$) in plasma. FFAs in a dried extract of plasma are reacted with N,N'-carbonyldiimidazole and methanol to form methyl esters. The methyl esters are washed with base, analyzed by gas–liquid chromatography (GLC), and plasma concentrations of individual and total FFAs calculated. Values for total FFAs obtained by the thin-layer chromatographic (TLC) method did not differ from those where the samples were initially purified by TLC and then measured by the GLC method. Extraction–titration assays for FFAs in plasma were 17–30% higher than the GLC values; but after TLC, the average titratable acidity from eluted FFA zones was not significantly different from total FFA determined by the TLC method. The data support the hypothesis that the extraction procedures used extract significant quantities of titratable acids (or bases) from plasma which are not the "normal" fatty acids ($C_{14:0}$–$C_{20:4}$).

INTRODUCTION

Plasma levels of total free fatty acids (FFAs) have been measured as indicators of fat mobilization or of physiological normalcy of the animal. Extraction–titration methods[1–5] of measuring fatty acids in body fluids or tissue are subject to the interference of other titratable substances which are also extracted from the sample. Thus, the physiological significance of the quantity of the acids titrated is more or less uncertain. It was desirable, therefore, to develop a method which not only would be specific for fatty acids of interest but also simple and rapid enough to be used for routine analyses. Gas chromatography has been used for some time to determine the FFA composition of tissues and sera[6,7]. A specific extraction–gas–liquid chromatographic (EGLC) method has been developed to determine individual and total FFAs.

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MATERIALS

N,N'-Carbonyldiimidazole (CDI). CDI was prepared daily as a 0.325 *M* solution in freshly distilled chloroform (ethanol-stabilized chloroform extracted with water) or in hydrocarbon-stabilized chloroform.

Extraction mixture. 600 ml of heptane mixed with 1400 ml of isopropyl alcohol.

Triethylamine. Triethylamine (TEA) 10% in absolute methanol.

Sodium hydroxide wash. 1.0 *N* aqueous sodium hydroxide for washing fatty acid methyl esters was saturated with carbon tetrachloride.

Silicic acid. Silicic acid (sieved to contain 100–150 mesh), obtained from J. T. Baker, Phillipsburg, N.J., U.S.A., was washed with heptane and activated at 110° overnight.

Standard compounds. Fatty acids and methyl esters used as standards in the study were obtained from Applied Science Lab., State College, Pa., U.S.A. with a purity of at least 99% as measured by gas–liquid (GLC) and thin-layer chromatography (TLC). Mixtures of FFAs simulating the composition of human plasma were prepared in extraction mixture.

The same concentration of internal standard, pentadecanoic acid, was used in the extraction mixture for samples of plasma, standards, and blanks.

METHODS

Special cleaning procedures

All glassware used in the experiments were washed sequentially with a low foaming alkaline detergent (TERJ*: 4 oz. per gallon), acid (TART*: 25% phosphoric acid, final concentration) and rinsed successively with hot tap water, hot deionized water, and finally with chloroform.

Interfering substances eluted by chloroform from unwashed polyethylene caps of 1-dram vials were detected by GLC. To remove these interfering substances, the polyethylene caps for the 1-dram vials were rinsed with several washes of chloroform and then stored in chloroform until just before use (no evidence of deterioration of the caps could be detected during storage).

EGLC assay for FFAs

Fresh plasma or serum (0.1–1.0 ml) is added to 4 ml of extraction mixture containing the internal standard, pentadecanoic acid ($C_{15:0}$), in a 15-ml glass centrifuge tube (glass-stoppered). For standardization, the extraction mixture also contains fatty acids in amounts simulating the composition of human plasma. 0.033 *N* H_2SO_4 is added to make the total plasma plus acid volume 3 ml (sufficient H_2SO_4 has been included to permit efficient extraction of FFAs from 1.0 ml or less of plasma). The phases in the tube are mixed vigorously for at least 10 sec with a Vortex mixer and then allowed to separate on standing. Approximately 1 ml of the heptane phase (upper) is pipetted into a 1-dram vial containing 20–50 mg of silicic acid. The vial is rotated and vibrated for about 10 min. Most of the heptane phase (upper) is then decanted into a

* Further technical information on these two products may be obtained from the manufacturers, DuBois Chemical Company, Seventh and Broadway, Cincinnati, Ohio, U.S.A.

clean vial and dried 20 min with a nitrogen flow-rate of 2 ft.^{3}/ h per sample vial. 0.1 ml of CDI solution is added to the dried sample and the vial rolled on its side so that all of the dried extract is dissolved. After about 1 min, 0.1 ml of TEA is added and mixed in the same manner. 3 ml of sodium hydroxide wash are added 60 sec (or more) after adding TEA. The vials are immediately capped and shaken for 2 min on a mechanical shaker. The caps are punctured with a needle to relieve the pressure before removal from the mechanical shaker. The vials are centrifuged a few seconds to form a drop of chloroform on the bottom. 1–5 μl of the chloroform phase is removed by a 10-μl syringe (Precision Series C with plunger guide or Hamilton No. 701) and injected into the gas chromatograph.

TLC system for studying interferences in the EGLC assay (TLC–EGLC)

Total and individual FFAs of human plasma were determined by the EGLC assay after TLC. TLC was introduced in the following way. Extraction of plasma (with internal standard added in the extraction mixture), adsorption of phospholipids, and drying with nitrogen were carried out as in the EGLC procedure. The residue was dissolved in 30-μl aliquots of diethyl ether and spotted on a thin-layer plate (silica gel GF). Known fatty acids were spotted in another portion of the plate to serve as a marker. The thin-layer plate was developed with hexane–diethyl ether–acetic acid (82:18:1). The silica gel zone corresponding to fatty acids was vacuumed from the plate into separate centrifuge tubes containing chloroform and centrifuged, the supernatant decanted, and the chloroform evaporated by nitrogen gassing. The subsequent imidazolide formation and esterification were carried out as in the EGLC procedure.

Titration assays for FFAs

Method I. Add 0.5 ml of plasma to 4 ml of extraction mixture (isopropyl alcohol–heptane (7:3)) and 2.5 ml of 0.033 *N* H_2SO_4. Mix for 10 sec on a Vortex mixer. After the phases separate, 1.0 ml of the upper phase is titrated with 0.01 *N* tetrabutylammonium hydroxide with thymolphthalein (0.01 % in spectroscopic grade acetone) as indicator. During titration, the solutions were mixed by nitrogen gassing[5].

Method II. Conditions described by Dole and Meinertz[1] were employed for extraction; 3.0 ml of the heptane phase were titrated with 0.01 *N* tetrabutylammonium hydroxide, with thymolphthalein as indicator. During titration, the solutions were mixed by nitrogen gassing.

Isolation of FFAs by TLC and their titration (TLC–titration)

Plasma was extracted on a large scale analogous to the EGLC procedure. 1-ml aliquots of the heptane phase were titrated and an aliquot of heptane phase (31 ml) was dried (Buchi Rotavapor) and reconstituted with 5.5 ml of cold methanol–chloroform (1:2). 1-ml aliquots of the reconstituted residue were dried with nitrogen and the residue was streaked on a thin-layer plate (silica gel GF) with three 0.05-ml washes of methanol–chloroform (1:1). One blank, one standard, and one extracted plasma sample were run on a given thin-layer plate. In this manner, extracts of plasma from three species, human, rabbit, and dog, were applied to thin-layer plates. The plates were developed by the TLC solvent system of Habermann *et al.*[8] (chloroform–methanol–water (65:25:4)). After development of the plates the extracts were resolved

into four major zones corresponding to: I, lecithins; II, cephalins; III, FFAs; IV, cholesteryl esters, cholesterol, triglycerides, and diglycerides. The zones corresponding to the fatty acids, fatty acid blanks, and fatty acid standards were scraped from the plate.

The fatty acids were eluted with a mixture of 4 ml of extraction mixture and 3 ml of 0.033 *N* H_2SO_4; 1.0 ml of the heptane phase was titrated with 0.01 *N* tetrabutylammonium hydroxide with 0.1 ml of thymolphthalein as indicator (0.01% in acetone which had been purified by activated alumina chromatography). Nitrogen gassing mixed the solution and removed CO_2 (*cf.* ref. 5).

Conditions for GLC

All samples were run on an F & M Model 402 gas chromatograph with a flame ionization detector. Although columns, helium flow-rates, and temperatures were adjusted slightly in some experiments, the following set of conditions are representative: column temperature, 185°; injection port temperature, *ca.* 210°; detector temperature, *ca.* 200°; carrier gas, helium with a flow-rate of *ca.* 150 ml/min; detector gases, hydrogen with a flow-rate of *ca.* 38 ml/min and oxygen with a flow-rate of *ca.* 500 ml/min (ref. 9); column packing, 6% LAC-728 (diethylene glycol succinate) on Diatoport S, 80–100 mesh in a 6-ft. silanized glass U-tube (3 mm I.D.).

Treatment of data

To speed up the gathering and treatment of data, peak heights instead of peak areas were measured and computations calculated by digital computer. The peak heights read on the chromatograms are entered sequentially onto IBM cards in addition to other pertinent data (*e.g.*, amount of fatty acid standards). The digital printout from the computer gave concentrations (mg/ml and μmoles/ml) and percent composition of each component fatty acid and the total concentration of all FFA components (mg/ml and μmoles/ml).

RESULTS AND DISCUSSION

The EGLC assay for individual and total FFAs in plasma was validated for the conditions of (a) extraction, (b) esterification of fatty acids (formation of imidazolides of the fatty acids and alcoholysis of the imidazolides), and (c) reduction of interfering substances. The specificity of the assay was examined by using TLC in conjunction with EGLC or extraction–titration. These studies are described in the following sections.

Extraction of plasma

Fatty acids are extracted from plasma by a modified Dole extraction method similar to that previously described[5]. Recovery of normal FFAs are shown in Table I. About 10% of the phospholipids are extracted and 90% of these are removed by adsorption onto activated silicic acid.

Esterification of FFAs

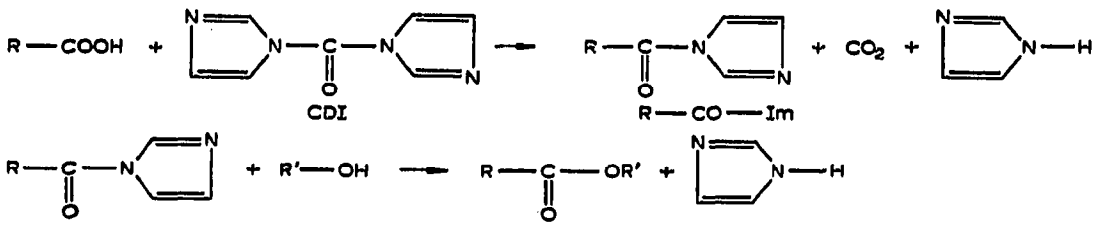
The reactions for esterifying the fatty acids were chosen particularly for their mildness and speed. The reactions go to completion within a few minutes at room

TABLE I

RECOVERY OF FREE FATTY ACIDS BY THE EGLC EXTRACTION SYSTEM

Fatty acid	*Percent recovery* ± *S.E. (N = 8)*
$C_{14:0}$	82.1 ± 1.7
$C_{15:0}$	86.1 ± 1.8
$C_{16:0}$	91.3 ± 2.0
$C_{16:1}$	84.2 ± 1.5
$C_{18:0}$	94.4 ± 2.9
$C_{18:1}$	90.7 ± 2.4
$C_{18:2}$	87.5 ± 1.7

temperature but are sufficiently gentle that triglycerides and cholesteryl esters are not transesterified and phospholipids are less than 20% transesterified. The general reaction scheme[10] is as follows:



Forming the imidazolide (R–CO–Im) of fatty acids with CDI allows one to make many esters of differing chemical and physico-chemical properties. Alcohols may thus be chosen so that both the alcohols and the esters synthesized will have desired properties (solubility and retention time). In the development of the assay endpoint, the properties of various alcohols and their corresponding linolenate esters were studied (Table II). Methanol was found to be optimal with respect to the following set of properties: (a) methanol is sufficiently polar so that most of it can be removed by an aqueous extraction and (b) the retention times of the fatty acid methyl esters are relatively short.

TABLE II

PROPERTIES OF DIFFERENT ALCOHOLS USED IN ESTERIFICATION OF FREE FATTY ACIDS

Column temperature, 180°; helium flow-rate, *ca.* 60 ml/min. The other conditions were the same as in Methods.

Alcohol used for esterification	*Retention time for linoleate ester (min)*	*Solubility of alcohol in 1.0 N NaOH*
Methanol	7.0	Soluble
Ethanol	7.4	Soluble
Trifluoroethanol	4.0	Soluble
Pentafluoropropanol	2.6	Insoluble
Heptafluorobutanol	2.2	Insoluble

Of the remaining alcohols shown in Table II, trifluoroethanol is also desirable in that fatty acid trifluoroethyl esters have shorter retention times than the corresponding methyl esters under the same GLC conditions. If the ester of the acid is thermally unstable, the short retention times of trifluoroethyl esters offer the advantage of GLC resolution at a lower temperature. Whether trifluoroethanol remaining in the chloroform phase after a single aqueous wash can appreciably transesterify triglycerides on column should be determined before this alcohol is employed for FFA analyses. The esters of other alcohols either had longer retention times than the corresponding methyl esters or could not be efficiently extracted from chloroform by water. For quantitation of FFAs, methanol and methyl esters had satisfactory properties and were therefore employed.

Since we employ a two-step process in esterifying fatty acids by the above reaction, the optimal conditions for each step of the process, imidazolide formation and methanolysis, were determined.

Imidazolide formation

As seen in Fig. 1, about 0.325 *M* N,N'-carbonyldiimidazole (CDI) is sufficient to obtain maximal esterification; up to 1 *M* CDI may also be used but this results in a higher background in the gas chromatogram. In studying the kinetics of the imida-

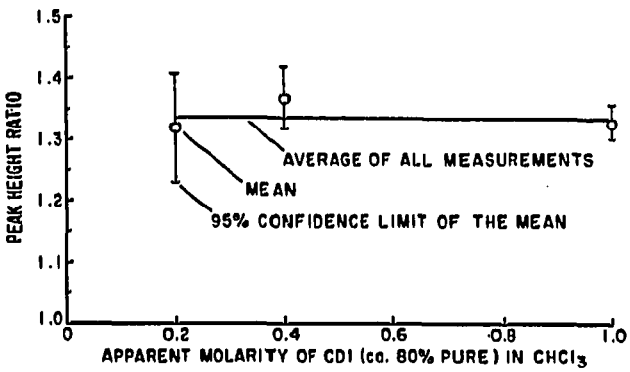


Fig. 1. Palmitic acid esterification as a function of N,N'-carbonyldiimidazole (CDI) concentration.

zolide formation using 0.325 *M* CDI, we found that a maximum had been attained at 30 sec and that at 60 sec the maximum was unaltered (Fig. 2). After 5 h the amount of ester observed was still about 95% of the maximal value. Thus, the imidazolide is sufficiently stable that methanolysis can be initiated in the following few minutes (but at least one) after adding CDI.

Methanolysis

As seen in Fig. 3, the presence of small amounts of triethylamine (TEA) increased the amount of ester formed, but an excess of TEA resulted in reduced ester formation. With the use of 10% TEA in methanol (after reacting the fatty acid for 5 h with 0.325 *M* CDI) esterification was essentially complete by 60 sec (Fig. 2) and the same maximum was achieved as that when 60 sec were employed for imidazolide

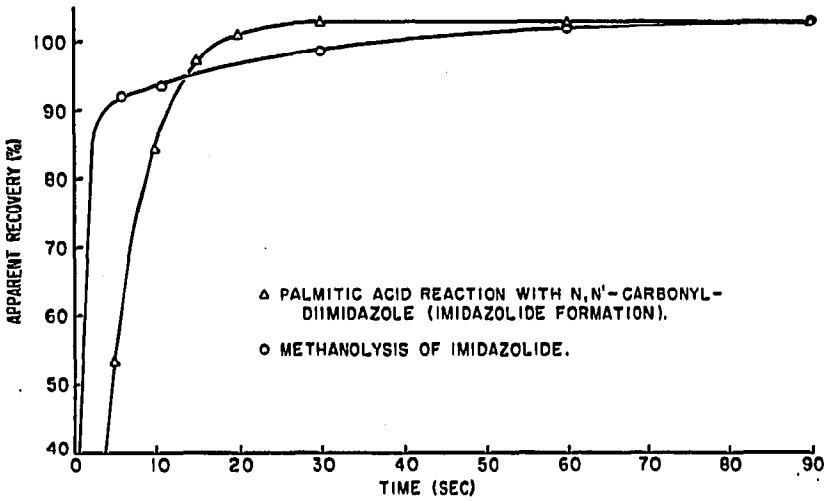


Fig. 2. Kinetics of esterification of palmitic acid with optimum concentrations of N,N'-carbonyldiimidazole (0.325 *M*) and triethylamine (10%) in methanol. Reaction kinetics with N,N'-carbonyldiimidazole (CDI): 0.325 *M* CDI reacted with palmitic acid for various times, then 10% triethylamine (TEA) in methanol reacted with the imidazolide for 30 sec before 1.0 *N* NaOH was added. Kinetics of methanolysis: 0.325 *M* CDI reacted with palmitic acid for 5 h; 10% TEA in methanol reacted with the imidazolide for the times shown before 1.0 *N* NaOH was added.

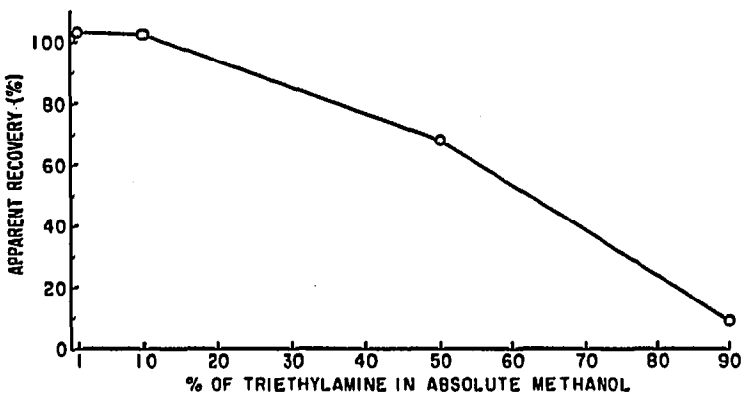


Fig. 3. The effect of triethylamine (TEA) in methanol on the esterification of palmitic acid. Condition for esterification (a) 20- min reaction with 0.325 *M* CDI, (b) 1-min methanolysis with TEA in methanol.

formation and 30 sec for methanolysis. Reaction times of about 60 sec for imidazolide formation and for methanolysis were therefore adopted in the assay.

Minimization of interference from other reaction products

In applying the foregoing reactions in an assay for FFAs and triglycerides, substantial interferences were observed in the region of $C_{14:0}$ methyl ester and a broad diffuse peak emerged in the $C_{18:0}$ region. Much of these interferences could be removed by a 1.0 *N* NaOH wash of the reaction mixture prior to injection into the gas chroma-

tograph. The interferences could also be reduced by lowering the amount of CDI used in the reaction (from 1 *M* to 0.325 *M*). The 1.0 *N* NaOH backwash also removes methanol which transesterifies triglycerides (from extracts of previous runs) on the gas chromatographic column (the methanol can also be removed by drying the sample, but in this procedure some ester is lost and longer processing times are required). To minimize the standard deviation (*cf.* Fig. 1) of the amount of ester formed and to reduce the interference resulting from high levels of CDI, 0.325 *M* CDI was chosen. The interference was reduced to about 1% or less.

Internal standard

An internal standard was used with all samples. By adding the internal standard at the extraction step, changes in volume transferred and, to some extent, sublimation and side reaction losses are compensated for throughout the remainder of the procedure. The concentration of the internal standard (pentadecanoic acid) is kept constant in the extraction mixture (heptane–isopropanol) used both for the plasma samples and for the standard samples of FFAs. The peak height ratio (with respect to the internal standard) of the methyl ester derived from any given fatty acid can thus be directly related to the peak height ratio of the same methyl ester derived from the corresponding standard fatty acid.

Quantitation of FFAs

Standard mixtures of FFAs were added to the heptane–isopropanol extraction mixture and their analyses were carried out by the procedure described in Methods. Standard curves constructed (Fig. 4) from these data compensate for losses such as those from sublimation, oxidation, or column adsorption. Linear least square fits of data to different levels of the fatty acids were calculated. After blanks were subtracted, the intercepts of the linear least square lines were not significantly different from zero ($p>0.05$). If blanks were not subtracted, $C_{14:0}$ and $C_{16:0}$ fatty acids had intercepts

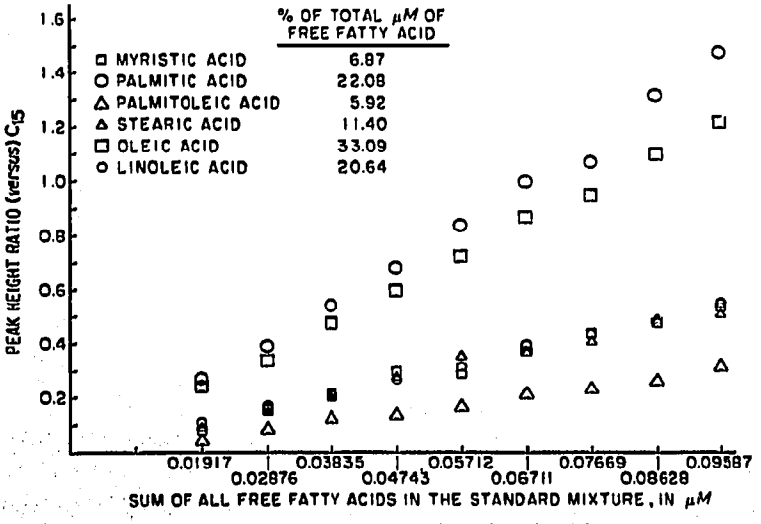


Fig. 4. Standard curves for FFAs at low levels (blanks subtracted).

significantly different from zero ($p<0.05$): the blanks correspond to about 4.6% and 3.0%, respectively, of the acids normally found in 1 ml of human plasma.

The EGLC method for determining FFAs was applied to plasma of three species: human, dog, and rabbit. Determinations of 0.207 (rat) and 0.031 (rabbit) μmoles of total free fatty acids in 0.5 ml of plasma had relative standard deviations of 2.5 and 7.6%, respectively.

The total amount of FFAs determined by the EGLC procedure is much lower (Table III) than that obtained by an extraction–titration procedure previously reported[5], Method I. The discrepancy between the two methods may be due to the possibility that the extraction–titration procedure is subject to large interferences from acids (or bases) in plasma which do not constitute normal fatty acids such as $C_{12:0}$–$C_{20:4}$.

TABLE III

QUANTITATION OF SERUM FFAs BY TITRATION AND BY THE EGLC METHOD

Species	*μmoles FFA per liter of serum*			
	EGLC	*S.E. (N = 4)*	*Titration***	*S.E. (N = 4)*
Human	376.1*	8.9	585.4	6.4
Rabbit	62.9*	2.4	210.1	2.3
Dog	84.9*	3.3	184.8	3.1

* $p < 0.05$ for the difference between the means obtained by the EGLC method and by titration.
** Method I.

To check the extent the EGLC assay is subject to interference, the fatty acids of human serum were isolated by TLC (TLC–EGLC). The TLC system resolved fatty acids, triglycerides, cholesteryl esters and phospholipids as pure lipid classes. Comparisons using the EGLC procedure were thus made in the presence or absence of other lipid classes. As seen in Table IV, no significant difference ($p>0.05$) was observed between the values obtained for the FFAs with or without TLC. These results indicated that the EGLC assay for FFAs was essentially free of interferences from other classes of lipids.

TABLE IV

EGLC DETERMINATION OF TOTAL FFAs BEFORE AND AFTER TLC OF A HUMAN SERUM EXTRACT

TLC conditions: silica gel F plates developed by hexane–diethyl ether–acetic acid (82:18:1).

Procedure	*FFAs (μequiv./ml) ± S.E. (N = 3)*
No TLC	0.2907 ± 0.0021
TLC	0.2844 ± 0.0058

To determine whether the extraction–titration assay for FFAs also measures additional interfering acids and hence results in higher values than those found by the EGLC method, TLC[8] was also used to separate plasma lipids into classes. In this TLC system, sufficiently low titration blanks were obtained and the fatty acids eluted from the plate could be titrated (TLC–titration). The fatty acids from identical aliquots

of plasma were also measured without TLC by the EGLC procedure and by two extraction–titration methods (I and II). No significant difference ($p > 0.05$, Table V) was observed between the two methods, EGLC *vs.* TLC–titration, of measuring total FFAs in the plasma of three species (human, dog, and rabbit). Titratable material of unknown structure was also present in each of the other TLC zones, but since standards were not available, the extent of their recovery (or stability) by elution from the thin-layer plate was uncertain. The extraction–titration methods (I and II) again gave significantly higher values ($p < 0.05$) as compared with those from EGLC or TLC–titration (Table V).

TABLE V

THE MEASUREMENT OF EXTRACTED FATTY ACIDS BY TITRATION BEFORE AND AFTER TLC AND BY GLC

Species	*μmoles fatty acid per liter ± S.E. (N = 4)*			
	Titration		*TLC*–Titration*	*EGLC*
	Method I	*Method II*		
Human	426 ± 1.9	520.7 ± 4.7	259 ± 1.6	251 ± 7.2
Rabbit	204 ± 1.9	—	80.2 ± 1.4	80.6 ± 2.0
Dog	340 ± 3.2	—	300 ± 14	290 ± 7

* The zone corresponding to fatty acids was eluted and titrated (see text).

Interferences have previously been observed in extraction–titration assays. To reduce interferences from lactic acid and phospholipids, plasma extracts were washed with aqueous acid[2,3] or the lower aqueous phase[1] and then titrated. The titration method developed by Trout *et al.*[3] gave plasma FFA values similar to those by the Gordon method[2]. To eliminate factors that interfere with titrimetric and colorimetric methods, FFAs were isolated by TLC, eluted and determined as a copper complex[11]. After separating lipids of plasma by TLC, Schlierf and Wood[4] quantitated the FFAs by measuring zone diameters. The TLC zone diameter method tended to give lower results than the titration procedure of Dole and Meinertz[1], particularly at low FFA levels. Good agreement with the zone diameter method was obtained when the FFAs of plasma were isolated by TLC and then titrated. Thus, all the results support the hypothesis that the heptane–isopropanol–water extraction systems used[1,5] also extract titratable acids or base(s) from plasma that are not the long-chain fatty acids ($C_{12:0}$, $C_{14:0}$, $C_{20:4}$) observed in the EGLC assay. The nature of these acid(s) or base(s) remains to be determined.

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