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Method for isolation of non-esterified fatty acids and several other classes of plasma lipids by column chromatography on silica gel

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ABSTRACT

A method is described for isolation from human plasma of non-esterified fatty acids, cholesteryl esters, triglycerides, cholesterol and diglycerides, monoglycerides, and some phospholipids by extraction and silica gel column chromatography. All of these lipid classes except diglycerides and cholesterol were separated cleanly in seven elution steps. Diglycerides and cholesterol were isolated together. Recovery of model compounds which represent the most significant classes of plasma lipids during the column chromatographic step was nearly complete. The overall recovery of added heptadecanoic acid from plasma specimens was 81% after both sample isolation steps. The overall recovery of added synthetic pentadecanoic acid and heptadecanoic acid ester lipid homologues from plasma was 80–91% after both sample preparation steps. About 6 h are required for extraction and isolation in duplicate of these lipid classes from twenty plasma specimens. Alternatively, non-esterified fatty acids can be isolated from twenty plasma specimens in duplicate within 4 h by a variation of the full procedure.

INTRODUCTION

Studies of fatty acid distribution among plasma and tissue lipid classes require specific analytical techniques. This requirement is most rigorous for accurate determination of non-esterified fatty acids (NEFAs). NEFAs are present in plasma and tissues at concentrations far lower than those of cholesteryl esters, triglycerides, and total phospholipids [1]. Hydrolysis of a small fraction of any major fatty acid ester lipid class during sample preparation results in substantial error in the determination of sample NEFAs. Workers interested in detailed study of lipid metabolism have sought to minimize this error either through experimental systems which exploit lipid class-specific chemical reagents or enzymes, or through chromatographic separation of lipid classes before determination of the constituent fatty acids. Total lipid extraction [2–5] and subsequent direct determination of NEFAs [6–11] are attractive insofar as they circumvent a time-consuming chromatographic sample preparation step. However, chromatographic isolation of sample lipid classes before study of their constituent fatty acids can eliminate any question concerning the actual sample lipid class source of these fatty acids.

Thin-layer chromatography (TLC) [12–15] liquid column chromatography [12], and high-performance liquid chromatography (HPLC)

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[16–20] are available for separation of lipid classes before detailed study. TLC involves difficulties inherent in mechanical and extractive recovery of separated lipid classes from chromatographic plates. Some workers have described losses of certain lipid classes during recovery of those lipids from TLC plates [21]. The time and equipment costs of HPLC limit its use in studies which involve many samples.

Liquid column chromatography is a convenient alternative to TLC for lipid class separation in that eluent fractions are collected quantitatively in tubes used for subsequent sample preparation steps. Carbon [22], anion-exchange cellulose [23,24], anion-exchange resin [25], octadecyl-bonded silica [26], and aminopropyl-bonded silica stationary phases [27–31] have been used for this purpose. Kaluzny *et al.* [27] described successive separation of cholesteryl esters, triglycerides, cholesterol and diglycerides, NEFAs, monoglycerides, and total phospholipids on three columns of aminopropyl-bonded silica. This technique since has been applied to analysis of plasma cholesteryl esters and triglyceride fatty acids [28] and determination of tissue NEFAs [29]. It has been extended to separation of some neutral lipids from acidic lipids, with subsequent intraclass separation of certain phospholipids [30,31]. Column chromatography on silica has been used to separate sample lipid classes [21,32–37].

We undertook the work described here in anticipation of need for specific isolation of NEFAs and some other plasma lipid classes from many specimens during fatty acid turnover studies. We employed a chromatographic sample preparation step to eliminate error in non-esterified fatty acid determination resulting from hydrolysis of fatty acid ester lipids during sample preparation.

EXPERIMENTAL

Equipment

Gas chromatography (GC) of cholesteryl esters, triglycerides, diacylglyceride acetates, and acylglyceride diacetates was accomplished with a Perkin-Elmer (Norwalk, CT, USA) Model 3920B gas chromatograph equipped with an on-column injection port and a flame ionization detector. GC of fatty acid methyl esters was accomplished with a Hewlett-Packard (Avondale, PA, USA) Model 5890 instrument equipped with a splitless capillary injection port, two flame ionization detectors, and a Model 7673 automatic liquid sampler. Hewlett-Packard Model 3354 and Model 3365 chromatographic data systems were used for collection and reduction of chromatographic data. A DuPont-Sorvall Model GLC-4 tabletop centrifuge (DuPont Laboratory Instruments, Newtown, CT, USA), a Big Vortex unit (Glas-Col Apparatus, Terre Haute, IN, USA), an Evapo-Rac sample evaporation unit (Cole-Parmer Instruments, Chicago, IL, USA), and a Multi-Blok heater (Labline Instruments, Melrose Park, IL, USA) were used for preparation of samples. All glassware was rinsed thoroughly with HPLC-grade methanol or absolute ethanol and dried before use.

Materials

E. Merck silica gel type 60 (230–400 mesh, EM Science catalog No. 9385-3) was obtained from Curtin-Matheson Scientific (Cleveland, OH, USA). Isooctane (HPLC grade), ethyl acetate, chloroform, methanol, and glacial acetic acid were obtained from Fisher Scientific (Cleveland, OH, USA). Ethanol was obtained from the hospital pharmacy. Pyridine, 2,2-dimethoxypropane, and acetic anhydride were obtained from Aldrich (Milwaukee, WI, USA), and they were distilled before use. Fatty acid standard mixtures were obtained from Nu-Check Prep (Elysian, MN, USA). All other synthetic and natural lipids used in this work were purchased from Sigma (St. Louis, MO, USA), and used without purification.

All solvent mixtures used in this work were prepared by additive combination of the named solvents in the stated proportions by volume (*e.g.* isooctane–ethyl acetate 20:1, v/v was prepared by combination of 20 volume units of isooctane with 1 volume unit of ethyl acetate).

Glyceryl diester acetate and glyceryl ester diacetate derivatives for use as external standards in diglyceride and monoglyceride lipid class isolation experiments were prepared from commercial

1,3-diglycerides and 1-monoglycerides by reaction with acetic anhydride in pyridine [38]. The reaction products were dissolved in 2 ml of isooctane–ethyl acetate (20:1, v/v), applied to a 15 cm × 0.8 cm I.D. column of silica gel, and eluted from this column with 25 ml of isooctane–ethyl acetate (20:1 v/v). The products were subjected to GC as described below. Each product was eluted from the GC column in a single peak which contained more than 99% of total determined area of peaks which eluted after the solvent front.

Water was purified by passage through a MilliQ apparatus (Millipore, Bedford, MA, USA). The 0.67 *M* phosphate buffer solution was prepared by combining 4.56 g of KH_2PO_4 and 5.58 g of K_2HPO_4 with purified water to give 100.0 ml of solution. The solution pH was adjusted to 6.2 with a small amount of dilute HCl if necessary. Chloroform–methanol solution (3:1, v/v) used for lipid extraction from plasma samples was prepared daily.

Preparation of silica gel

Silica gel was placed in a clean glass beaker, suspended in two volumes of methanol, and allowed to settle. The methanol was decanted, and this operation was repeated four times. Most residual methanol was evaporated under a gentle stream of clean, filtered compressed air. The beaker then was covered, and it was placed in a desiccator under vacuum until the silica behaved as a free-flowing powder.

Sample preparation columns

Chromatographic columns were made from 14.5 cm × 0.5 cm I.D. Pasteur pipettes by plugging their constricted ends with glass wool. Silica gel was suspended in isooctane, and it was transferred to the columns with a 10-ml glass pipette. Columns were packed to a bed height of 5 cm above the glass wool plug. They were rinsed twice with 1 ml of isooctane–ethyl acetate (80:1, v/v) prior to sample application.

Identification of eluates for lipid class isolation

Standard solution aliquots of 0.2 ml which contained 0.05–0.1 mg of model lipid compound were applied directly to the silica gel columns. The columns were eluted with 1.0-ml aliquots of selected eluent solutions, and fractions of 1 ml were collected in 75 mm × 12 mm glass test tubes. Each collected discrete milliliter was prepared for chromatographic analysis according to the procedures described below. Experiments were conducted in groups of six to ten columns for each model lipid compound studied.

Experimental external standard solutions were used for sample reconstitution and normalization of results in GC experiments. For experiments with cholesteryl esters, triglycerides, diglycerides, carboxylic acids, and monoglycerides, the GC flame ionization detector response was calibrated by replicate preparation as appropriate for the studied lipid class, reconstitution in external standard solution, and GC analysis of ten lipid standard solution aliquots of 0.2 ml. The experimental compound/external standard detector response ratios obtained were averaged to establish the response ratio expected for quantitative recovery. For experiments with natural phospholipids, the largest lipid-derived fatty acid methyl ester peak in the gas chromatogram obtained after saponification and derivatization of standard solution aliquots was selected as the basis for recovery estimation. Recovery of model lipid in each column fraction was determined by comparison of the model compound/external standard peak-height ratio calculated from each column fraction with the mean peak-height ratio calculated from the set of calibration standard injections. These fractional recoveries were summed to estimate total model compound recovery from each column in an experimental group.

Fractions collected during experiments with cholesteryl esters or triglycerides were evaporated to dryness under a stream of filtered compressed air. The residues obtained were reconstituted with 0.2 ml of ethyl acetate which contained about 0.2 mg/ml homologous cholesteryl carboxylate or glyceryl tricarboxylate external standard. Sample aliquots of 1 μl of these solutions were injected into the gas chromatograph.

Fractions collected during experiments with diglycerides, monoglycerides, or cholesterol were

evaporated to dryness. The residues obtained were dissolved in 0.5 ml of pyridine and treated with 0.5 ml of acetic anhydride at 40°C for 1 h [38]. The samples then were evaporated to dryness under compressed air, and the residues were dissolved in 0.2 ml of ethyl acetate which contained about 0.2 mg/ml cholesteryl butyrate, a homologous diglyceride acetate, or a monoglyceride diacetate external standard. Sample aliquots of 1 μl were injected into the gas chromatograph.

Fractions collected during experiments with carboxylic acids were evaporated to dryness and treated with 0.5 ml of methanol, 0.5 ml of 2,2-dimethoxypropane, 0.05 ml of water, and 0.02 ml of 12 *M* HCl. The tubes were covered with marbles and left to stand for 30 min at room temperature [6]. The samples were concentrated to about 0.1 ml total volume, diluted with 0.2 ml of water, and vortex-mixed with 0.1 ml of isooctane which contained about 0.2 mg/ml of an appropriate homologous carboxylate methyl ester external standard. The samples were centrifuged at 1200 *g* for 5 min to effect phase separation, and 0.05 ml of the organic phase was transferred to glass autosampler vial inserts. The vials were sealed with PTFE-lined rubber septa. Aliquots of 1 μl were analyzed.

Fractions collected during experiments with phospholipids were evaporated to dryness under compressed air and subsequently treated in closed tubes with 0.3 ml of 0.5 *M* KOH in 95% ethanol at 85°C for 30 min. Samples were vortex-mixed once after half of this reaction time had elapsed. The samples were cooled, acidified by addition of 0.22 ml of 0.12 *M* HCl, and extracted with 1.0 ml of isooctane. The organic phase was transferred to 100 mm × 13 mm test tubes and evaporated to dryness under a stream of compressed air. These residues were derivatized as described for carboxylic acids, and the last extraction of methyl esters from the derivatization reaction mixture was made with an isooctane solution of carboxylate methyl ester external standard. Aliquots of 1 μl were analyzed.

Gas chromatography

GC separations of cholesteryl esters, triglycerides, diacylglyceride acetates, and acylglyceride diacetates were accomplished on a 50 cm × 2 mm I.D. glass column packed with 1% Dexsil 300 carborane silicone on 100–120 mesh Supelcoport (Supelco, Bellefonte, PA, USA) [39]. Helium was used as the carrier gas at a flow-rate of 20 ml/min. Chromatography of the following model lipid compounds (and homologous experimental external standard compounds) was carried out at the indicated column oven, injection port, and detector interface zone temperatures: cholesteryl acetate (cholesteryl butyrate), 275, 300, 295°C; 1-monocapryloyl-*rac*-glycerol-2,3-diacetate (1-monodecanoyl-*rac*-glycerol-2,3-diacetate), 185, 200, 200°C; 1-monopalmitoyl-*rac*-glycerol-2,3-diacetate (1-monostearoyl-*rac*-glycerol-2,3-diacetate), 250, 280, 270°C; 1,3-dipalmitin-2-acetate (1,3-distearin-2-acetate), 320, 340, 340°C; cholesteryl palmitate (cholesteryl stearate), 350, 365, 370°C; tricaprylin (tricaproin), 280, 300, 300°C; tricaprylin (tricaprin), 280, 300, 300°C; and trimyristin (tripalmitin), 355, 360, 360°C.

Gas chromatography of carboxylate methyl esters was accomplished with the Hewlett-Packard instrument simultaneously on two 15-m fused-silica capillary columns. One of these contained SP-2330 cyanopropyl polysiloxane (0.25 mm I.D., 0.20 μm film thickness, Supelco). The second column contained HP-1 dimethyl polysiloxane (0.2 mm I.D., 0.33 μm film thickness, Hewlett-Packard, Kennett Square, PA, USA). Helium was employed as the carrier and flame ionization detector make-up gas at flow-rates of 1.5 and 36 ml/min, respectively. The injection port and detector temperatures were 230 and 250°C, respectively. The initial column oven temperature was 60°C. Sample injection started a programmed column oven temperature increase by 30°C/min to a final temperature of 240°C. The column oven was maintained at 240°C for 5 min to complete the separation of fatty acid methyl esters.

Procedure for extraction of plasma lipids

Plasma lipids were extracted according to a modification [40] of a frequently used procedure [2]. A 0.2-ml volume of plasma, 0.3 ml of 0.67 *M*

phosphate buffer solution, and 2.0 ml of chloroform–methanol (3:1, v/v) were combined in 100 mm × 13 mm glass test tubes. The tubes were vortex-mixed for 2 min, and then they were centrifuged at 1200 *g* for 5 min to effect phase separation. The aqueous layer was removed with a glass Pasteur pipette and discarded. The organic layer was transferred to a 100 mm × 13 mm glass test tube with a glass Pasteur pipette and evaporated to dryness under a stream of compressed air.

Procedure for isolation of plasma lipid classes

Sample residues obtained after plasma lipid extraction were extracted twice in succession with 0.5 ml of isooctane–ethyl acetate (80:1, v/v). These extracts were applied to prepared silica gel columns. Cholesteryl esters were eluted from the columns by 4.5 ml of isooctane–ethyl acetate (80:1, v/v) (fraction 1). Triglycerides then were eluted by 5 ml of isooctane–ethyl acetate (20:1, v/v) (fraction 2). The solid residues remaining in the sample tubes then were extracted twice in succession with 0.5 ml of isooctane–ethyl acetate (75:25, v/v), and these extracts were applied to the silica gel columns. Cholesterol and diglycerides were eluted with 4.5 ml of isooctane–ethyl acetate (75:25, v/v) (fraction 3). Fatty acids were eluted next with 4 ml of isooctane–ethyl acetate–acetic acid (75:25:2, v/v/v) (fraction 4). Monoglycerides were eluted by another 8 ml of isooctane–ethyl acetate–acetic acid (75:25:2, v/v/v) (fraction 5). The solid residue remaining in the original sample tube was extracted twice with 0.5 ml of methanol, and these extracts were applied to the silica gel columns. Some phospholipids including ethanolamine phospholipids were eluted from the columns by 4 ml of methanol (fraction 6). Some other phospholipids including choline phospholipids were eluted by an additional 4 ml of methanol (fraction 7).

Alternate procedure for rapid isolation of plasma long chain length NEFAs

The dry residue obtained from the plasma lipid extraction was extracted twice successively with 0.5 ml of isooctane–ethyl acetate (75:25, v/v) by vigorous vortex-mixing for 2 min. Both extracts were transferred by glass pipette to the silica gel columns. Apolar sample constituents were eluted from the columns by 4.5 ml of isooctane–ethyl acetate (75:25, v/v). Long-chain NEFAs then were eluted from the column by 4 ml of isooctane–ethyl acetate–acetic acid (75:25:2, v/v/v). This fraction was dried under compressed air, derivatized, and the methyl esters were chromatographed as described above.

Recovery of model lipid compounds from plasma

Synthetic pentadecanoic acid or heptadecanoic acid ester lipids were used for estimation of recovery of lipid classes except monoglycerides and some minor phospholipid classes from plasma specimens. A single model lipid compound was added to plasma specimens. Total lipid extracts from those plasma specimens were separated on the silica gel columns. Fractions of 1 ml were collected, treated with a standard solution of heptadecanoic acid or pentadecanoic acid, saponified, and derivatized as described above. Standard curves of chromatographic peak-height ratio *versus* concentration were established for both pentadecanoic acid and heptadecanoic acid in experiments in which one compound was used as internal standard for determination of the other. The determined quantity of pentadecanoic acid or heptadecanoic acid in each fraction was compared with the quantity of that compound added as a model lipid constituent to the plasma specimens. These fractional recoveries were summed to calculate the total recovery of lipid from each column.

Monoglyceride recovery from plasma was examined by standard addition of 1-monopalmitoyl-*rac*-glycerol to plasma and subsequent determination of monoglyceride-derived palmitic acid in the isolated monoglyceride fraction. The determined quantity of palmitic acid in fraction 5 was corrected for blank plasma monoglyceride palmitic acid contribution. This difference was compared with the quantity of 1-monopalmitoyl-*rac*-glycerol added initially to the experimental plasma specimens.

TABLE I

RECOVERY OF MODEL LIPID COMPOUNDS FROM SILICA COLUMNS

Standard lipid solution aliquots were applied to columns, eluted, and collected fractions were analyzed as described in the Experimental section of this report. These results are expressed as mean percent recovery and relative standard deviations (R.S.D.) for groups of six to ten replicates.

Compound	Fraction	Recovery (%)	R.S.D. (%)	*n*
Cholesteryl acetate	1	92	3	10
Cholesteryl *n*-octanoate	1	98	1	10
Cholesteryl *n*-hexadecanoate	1	95	6	10
Tricaprylin	2	101	3	6
Trimyristin	2	94	4	10
Cholesterol	3	99	3	10
1,3-Dipalmitin	3	113	4	10
Octanoic acid	4	86	16	10
Heptadecanoic acid	4	101	4	10
1-Monopalmitoyl-*rac*-glycerol	5	90	6	10
Diheptadecanoyl-L-α-phosphatidylethanolamine	6	96	23	10
Dipalmitoyl-L-α-phosphatidyl-N-monomethylethanolamine	6	72	5	10
Dipalmitoyl-L-α-phosphatidyl-*n*,*n*-dimethylethanolamine	6	88	4	10
Dimyristoyl-L-α-phosphatidyl-D,L-glycerol	6	59	7	8
Cardiolipin (bovine heart)	6	76	11	10
L-α-Phosphatidylinositol	6	97	20	10
Sphingomyelin (bovine brain)	7	62	18	10
Dipentadecanoyl-L-α-phosphatidylcholine	7	95	12	10
Diheptadecanoyl-L-α-phosphatidylcholine	7	102	14	10

RESULTS AND DISCUSSION

Some time ago we planned a series of human subject fatty acid turnover studies. The study design required accurate and precise determination of stable isotope dilution in plasma NEFAs and certain plasma fatty acid ester lipids after infusion of stable isotope-labeled palmitic acid. We needed to distinguish the actual lipid class origin of determined fatty acid methyl esters. We rejected published methods which relied on TLC for lipid separation owing to practical difficulties involved in quantitative recovery of separated lipids and selective loss of polyunsaturated fatty acids or polyunsaturated fatty acid-rich lipids during lipid class isolation by TLC [21,41]. We considered some recently reported methods for plasma lipid class separation on commercially prepared columns containing reversed- or bonded polar-phase separation media [27–31]. None of these was fully satisfactory for this work owing to multiple column sequences required for complete lipid class separation [27], incomplete separation of certain lipid classes [28–31], large procedural blank values for the NEFA fraction when isolated with plastic sample preparation cartridges [27–30,36], and the expense of commercial sample preparation columns.

We devised a plasma lipid isolation scheme based on earlier work [34,35] which employed glass chromatographic columns and silica gel sorbent. We found that a Merck 230–400 mesh silica preparation offered useful mechanical properties. This material was transferred easily to chromatographic columns as a slurry in hydrocarbon solvent. It settled immediately. Eluent applied to columns of this sorbent drained to the top of the chromatographic medium and no farther. This was convenient during the handling of large numbers of samples. We used mixtures of isooctane and ethyl acetate for separations of lipids less polar than phospholipids. These mixtures

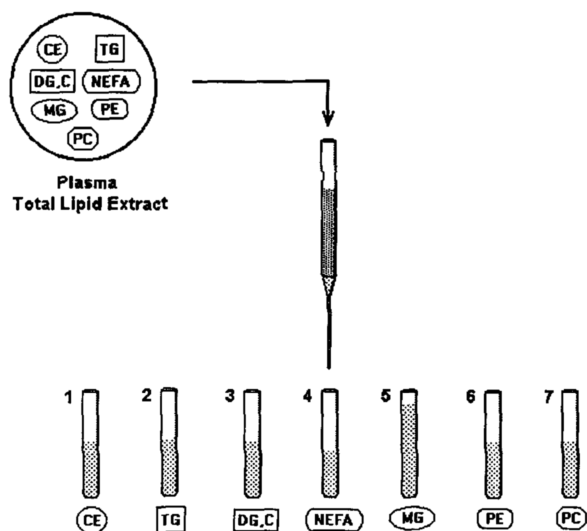

Fig. 1. Schematic representation of lipid separation procedure. Abbreviations used: CE, cholesteryl esters; TG, triglycerides; C, cholesterol; DG, diglycerides; NEFA, non-esterified fatty acids; MG, monoglycerides; PE, ethanolamine phospholipids; PC, choline phospholipids. Numeric fraction identifiers correspond with those used in text.

evaporate readily during subsequent concentration steps.

We found a series of five eluents for separation of seven major plasma lipid classes in seven elution steps (Fig. 1, Table I). Cholesteryl esters were eluted sharply by 5 ml of isooctane–ethyl acetate (80:1, v/v) (fraction 1). We did not attempt to characterize the elution of plasma lipid components less polar than cholesteryl esters. Triglycerides were eluted by 5 ml of isooctane–ethyl acetate (20:1, v/v) (fraction 2). Cholesterol and the 1,3-diglycerides which we studied were eluted together by 5 ml of isooctane–ethyl acetate (75:25, v/v) (fraction 3). We did not study the retention behavior of 1,2-diglycerides. Cholesterol and diglycerides can be determined in subsequent analytical steps.

NEFAs were isolated cleanly after elution by 4 ml of isooctane–ethyl acetate–acetic acid (75:25:2, v/v/v) (fraction 4). In experiments with fatty acid ester lipid model compounds we subjected fraction 4 to a saponification step before preparation of methyl ester derivatives. No methyl ester was detected in this fraction. This suggests that the defined NEFA fraction does not contain other fatty acid ester lipids at concentrations detectable by our experimental methods. Also, we detected no cholesterol in this fraction.

Model 1-monoglycerides were eluted after nonesterified fatty acids by 8 ml of isooctane–ethyl acetate–acetic acid (75:25:2, v/v/v) (fraction 5). Retention of 1-monoglycerides by this sorbent depended on the eluent concentration of ethyl acetate in isooctane. Retention of long chain-fatty acids depended on the eluent concentration of acetic acid. Long-chain *n*-carboxylates which we studied were not eluted at detectable concentrations by isooctane–ethyl acetate (75:25, v/v). Long-chain *n*-carboxylates were eluted sharply by addition of two volume units of acetic acid to the eluent mixture. Retention of 1-monoglycerides by this silica was not affected by this addition of acetic acid to isooctane–ethyl acetate (75:25, v/v). We did not study the relative retention behavior of 1- and 2-monoglycerides.

Most of the phospholipid model compounds with which we experimented were eluted from the silica columns by 4 ml of methanol (fraction 6) after application to the column in two aliquots of 0.5 ml of methanol. Choline phospholipid model compounds were retained more strongly by the silica medium than were the ethanolamine phospholipids. Choline phospholipids and a natural sphingomyelin preparation were eluted by a second 4-ml portion of methanol (fraction 7). Samples prepared from the fourth, fifth, and sixth discrete milliliters of methanol collected during this elution step were found to contain collectively about 8% of total heptadecanoic acid methyl ester derived from diheptadecanoyl-L-α-phosphatidylethanolamine in experiments with that compound. The sample prepared from the fourth 1-ml fraction of methanol collected during this elution step contained about 4% of total heptadecanoic acid methyl ester derived from diheptadecanoyl-L-α-phosphatidylcholine. Most of the choline phospholipid model compounds were recovered in the fifth and sixth 1-ml fractions of methanol. We made no further attempt to define selective elution conditions for those compounds.

We considered the possibility of elution behavior changes for these lipid compound classes

when they were isolated from plasma extracts with comparatively large quantities of total sample lipids. In these experiments one pentadecanoic or heptadecanoic acid ester lipid homologue was added to 200 μl of plasma before total lipid extraction and lipid class fractionation. We collected and analyzed discrete 1-ml fractions of each eluent. We noticed no change in the chromatographic behavior of any of the apolar lipids or NEFAs with which we experimented in relation to their behavior when applied to separation columns as comparatively dilute standard solutions. We subjected each milliliter of eluent collected in fraction 4 to a saponification step before derivatization. This step was meant to disclose the presence of coeluted apolar lipid or phospholipid in the NEFA fraction. We detected no fatty acid methyl ester derived from the apolar lipid or phospholipid model compounds with which we experimented in fraction 4.

We modified the procedure to provide a quick method for selective isolation of NEFAs. Cholesteryl esters, triglycerides, diglycerides, and cholesterol were eluted together from the sample isolation columns by 4.5 ml of isooctane–ethyl acetate (75:25, v/v). NEFAs were eluted by 4 ml of isooctane–ethyl acetate–acetic acid (75:25:2, v/v/v) (Fig. 2). In experiments with standard solutions of apolar lipids, monoglycerides, and phospholipids, no fatty acid methyl ester was detected in the defined NEFA fraction after saponification and methyl ester preparation. We carried out similar experiments in which synthetic pentadecanoic acid or heptadecanoic acid ester lipid homologues were added to 200 μl of human plasma. The isolated NEFA fraction was saponified prior to derivatization to disclose the presence of coeluted fatty acid ester lipid. Pentadecanoic and heptadecanoic acid methyl esters were not detected in the NEFA fraction during these experiments. These results suggest that detectable quantities of fatty acid ester lipids do not coelute with the defined NEFA fraction during the two step elution procedure. The elution order and selectivity observed in these experiments conformed generally to those described earlier [21,32–37] (Fig. 3).

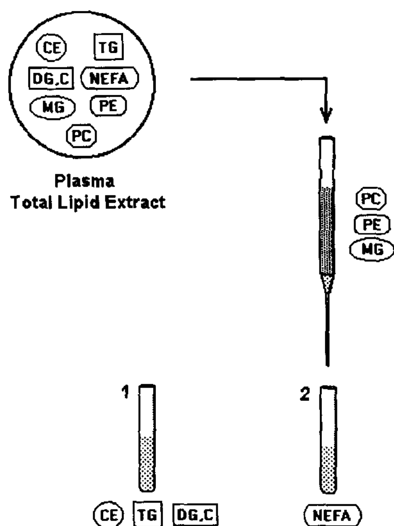

Fig. 2. Schematic representation of alternate lipid separation procedure for isolation of non-esterified fatty acids. Abbreviations used are as described for Fig. 1. Elution conditions were: (1) 4.5 ml of isooctane–ethyl acetate (75:25, v/v); (2) 4 ml of isooctane–ethyl acetate–acetic acid (75:25:2, v/v/v).

The overall recovery of added model lipid compounds from plasma is shown in Table II. During the plasma lipid extraction operation we were able to transfer about 90% of the organic phase to the next sample preparation step. Recovery of heptadecanoic acid and glyceryl-1-hexadecanoate from plasma was not improved by a second extraction. We did not attempt to find better extraction conditions for NEFAs and 1-monoglycerides. We experimented with nitrogen for evaporation of solvents from isolated fractions. Nitrogen did not improve recovery of the lipids with which we experimented. In our applied work with this procedure we add synthetic pentadecanoic acid or heptadecanoic acid ester lipid recovery standards to plasma samples.

We encountered the same difficulties with procedural blanks in NEFA determination noted by others [21,27,29] whose sample preparation conditions actually would reveal this problem. Fig. 4A represents the chromatogram obtained after chloroform–methanol extraction of standard heptadecanoic acid from a clean test tube, isolation of the NEFA fraction according to the two-elution step procedure, derivatization, and GC of

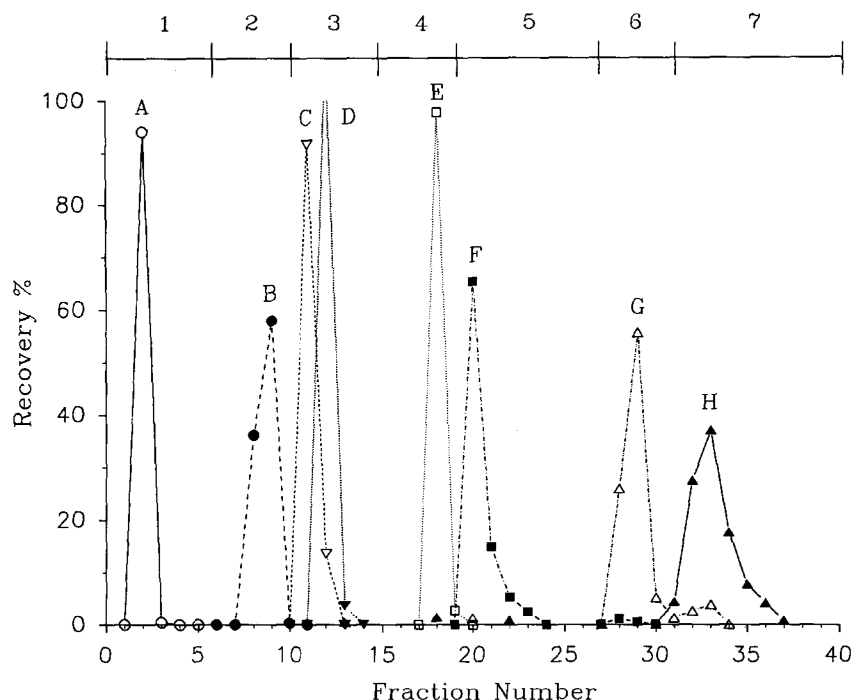

Fig. 3. Separation of several model lipid compounds on silica gel as described in text. Elution conditions were: (1) 4.5 ml of isooctane–ethyl acetate (80:1, v/v); (2) 5 ml of isooctane–ethyl acetate (20:1, v/v); (3) 4.5 ml of isooctane–ethyl acetate (75:25, v/v); (4) 4 ml of isooctane–ethyl acetate–acetic acid (75:25:2, v/v/v); (5) 8 ml of isooctane–ethyl acetate–acetic acid (75:25:2, v/v/v); (6) 4 ml of methanol; (7) 6 ml of methanol. Peaks: A = cholesteryl hexadecanoate; B = trimyristin; C = cholesterol; D = 1,3-dipalmitin; E = heptadecanoic acid; F = 1-monopalmitin; G = diheptadecanoylphosphatidylethanolamine; H = diheptadecanoylphosphatidylcholine. This figure was constructed from results obtained in experiments with one model compound and determined in a replicate group of six to ten separation columns.

TABLE II

OVERALL RECOVERY OF ADDED LIPID MODEL COMPOUNDS FROM HUMAN PLASMA AFTER ONE OR TWO EXTRACTION STEPS AND COLUMN CHROMATOGRAPHY ON SILICA GEL

Lipid model compounds were added to plasma, extracted, and isolated by silica gel chromatography as described in the text. Results are expressed as averages and relative standard deviations (R.S.D.) for six replicate experiments with each compound.

Compound	Recovery (%)			
	One extraction step		Two extraction steps	
	Mean	R.S.D.	Mean	R.S.D.
Cholesteryl-*n*-heptadecanoate	86	6	91	3
Glyceryl tripentadecanoate	88	5	97	3
Glyceryl-1,3-dipentadecanoate	80	2	96	1
Glyceryl-1-hexadecanoate	80	5	81	4
Heptadecanoic acid	81	9	79	8
Diheptadecanoylphosphatidylethanolamine	91	2	98	7
Diheptadecanoylphosphatidylcholine	88	6	90	5

the fatty acid methyl esters. The integrated signals for tetradecanoic, hexadecanoic, and octadecanoic acid methyl esters in this chromatogram are about 200, 30, and 100%, respectively, of typical values obtained after isolation, derivatization, and GC of the NEFAs in 200 μl of normal human plasma. We did not observe these peaks after concentration, reconstitution, and GC of collected fractions without a derivatization step. This result and capillary GC retention data relative to identified standards on two stationary phases suggest that these interfering compounds are long-chain *n*-carboxylates. In sectioning experiments intended to identify the relevant sources of these high procedural blank values, we found that about 80% of this procedural blank was contributed by plastic components of the solvent dispensing apparatus which we used early in this work. About 80% of the remaining blank contribution was introduced by the silica gel sorbent. Some of the remainder was contributed by every other item of material and equipment used for sample preparation. Long-chain *n*-carboxylates are constituents of surfactants and lubricants [42]. Use of glass containers and dispensing apparatus throughout the procedure, careful cleaning of all glassware with methanol, and preparation of the chromatographic silica by batch extraction with methanol achieved a reduction of the procedural NEFA blank to the tolerable level shown in Fig. 4B.

We considered that methanol treatment of the chromatographic silica gel might alter its selectivity for these lipid classes. We repeated the standard solution and plasma extract lipid separation experiments described above with methanol washed silica gel. We noticed no change in the elution behavior of these model lipid compounds in relation to that observed with unwashed silica gel during those experiments.

These plasma lipid isolation procedures succeed when they are carried out *exactly* as described with the sorbents, solvents, and glass apparatus identified in the Experimental section of

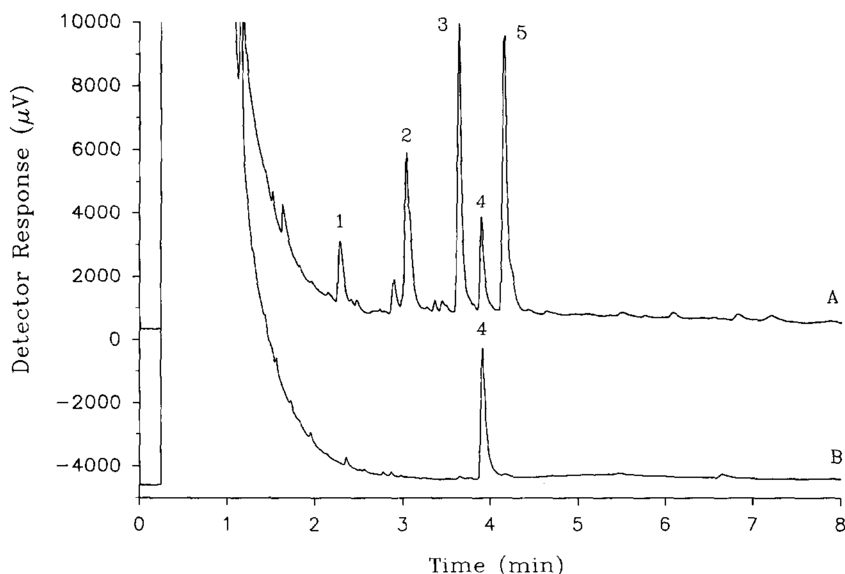

Fig. 4. (A) Chromatogram obtained after chloroform–methanol extraction of standard heptadecanoic acid from a clean test tube, isolation of the NEFA fraction from that extract according to the two elution step procedure, derivatization, and GC of resultant fatty acid methyl esters on HP-1 dimethylpolysiloxane. GC conditions were as described in the Experimental section of this report. Peaks were identified by retention time relative to the internal standard and coincident retention with authentic fatty acid methyl esters. Peaks: 1 = dodecanoic acid methyl ester; 2 = tetradecanoic acid methyl ester; 3 = hexadecanoic acid methyl ester; 4 = heptadecanoic acid methyl ester (internal standard); 5 = octadecanoic acid methyl ester. (B) Chromatogram obtained as described for (A) after procedural modification. Chromatographic conditions were as described for (A). The detector signal was offset arithmetically by $-4 \cdot 10^{-3}$ V for construction of this figure.

this report. Other solvents and other silica sorbent preparations show substantially different selectivity for the lipids with which we experimented. We prepare eluent mixtures accurately, and we carefully control the sorbent bed height within the columns. The solvents which we use in eluent mixtures are those which contributed least to the NEFA procedural blank among the brands and grades of solvents with which we experimented. We use glass apparatus throughout this procedure owing to discernible procedural fatty acid blank contribution by every piece of plastic apparatus which we examined. Sequential reconstitution and transfer of plasma lipid extracts to the separation columns must be carried out as described for efficient overall recovery of those lipids. The volumes of eluents used for those transfers affect the elution profile, and these volumes should not be altered without confirmation of the relevant portions of the lipid elution pattern. We did not experiment with plasma sample quantities larger than 200 μl. We do not reuse the sample preparation columns.

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