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Note

Purification of long-chain, saturated, free fatty acids

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Saturated, radiolabeled, free fatty acids (FFAs) are often contaminated with fatty acids which are desaturated or of different chain length. To remove these materials, most purification schemes require conversion of the FFAs to the methyl ester, followed by various thin-layer chromatography (TLC) or column liquid chromatography (LC) separations[1]. We wished to avoid the esterification step since our intention was to synthesize the fatty acyl coenzyme A thioester from the free acid[2]. More recently, methods have been reported for the separation of FFAs by high-performance liquid chromatography (HPLC)[3,4]. However, purification of highly radiolabeled material by HPLC requires extensive clean-up of equipment normally used for analytical work. Also, critical pairs of FFAs may not be completely resolved[1].

The two-step procedure reported here offers a practical alternative to an HPLC purification of highly radiolabeled, saturated, FFAs. Unsaturated FFAs were easily eliminated by treatment with osmium tetroxide followed by silica gel column chromatography. Saturated FFAs of different chain lengths were then purified and recovered in high yield by preparative TLC using C_2 reversed-phase plates.

EXPERIMENTAL

Various lots of radiolabeled [1-^{14}C]palmitic acid and [1-^{14}C]stearic acid, 50–60 μCi/μmol, were obtained from ICN (Irvine, CA, U.S.A.), Amersham (Arlington Heights, IL, U.S.A.), and New England Nuclear (Boston, MA, U.S.A.). Stock solutions were prepared in acetone–1 *M* hydrochloric acid (100:1). (The inclusion of hydrochloric acid was essential for avoiding loss of material due to adherence of FFAs to glass tubes and pipets.) Unlabeled FFAs were obtained from Sigma (St. Louis, MO, U.S.A.). Osmium tetroxide was obtained from Ted Pella (Redding, CA, U.S.A.). All solvents were glass-distilled from Baxter, Burdick & Jackson (Irvine, CA, U.S.A.). Silica gel 60 (250 μm thick), RP-2 (250 μm thick), and RP-18 (200 μm thick) TLC plates were obtained from EM Science (Cherry Hill, NJ, U.S.A.). Silica gel for column chromatography (SilicAR CC-7) was obtained from Mallinckrodt (Paris, KY, U.S.A.). Silver nitrate TLC plates were prepared by briefly immersing silica gel 60 plates into 15% silver nitrate in acetonitrile[5]. All plates were activated for 1 h at 70°C prior to use.

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Osmium tetroxide treatment and column chromatography

In a typical preparation, 10 μCi (approximately 50 μg) of radiolabeled FFA was placed in a test tube, the solvents evaporated, and the sample dried 1 h over phosphorus pentoxide *in vacuo*. To the residue was added 200 μl of osmium tetroxide in carbon tetrachloride (50 mg/ml). After 1 h at room temperature, the solvent was evaporated, and 2 ml of 1 *M* hydrochloric acid were added. The FFAs were extracted three times into 2.0 ml of diethyl ether.

The ether was evaporated, the residue dissolved in hexane–diethyl ether (90:10, v/v), and loaded onto a column of 5 g of activated silica gel. The column was flushed with 25 ml hexane–diethyl ether (90:10), and the product eluted with 50 ml hexane–diethyl ether (75:25), followed by 25 ml hexane–diethyl ether (50:50). The products were collected into 10-ml fractions. The solvents were evaporated, the residues redissolved in acetone–1 *M* hydrochloric acid (100:1), and the radioactivity determined. The two or three fractions containing the largest quantities of radioactivity were combined.

Reversed-phase TLC

RP-2 reversed-phase TLC plates were pre-run in dioxane–0.001 *M* hydrochloric acid (7:3). FFAs were streaked onto the plates using acetone–1 *M* hydrochloric acid (100:1). Plates were run in dioxane–0.001 *M* hydrochloric acid (7:3). Radiolabeled fatty acids were visualized by autoradiography (Kodak XAR-5 film). After scraping the gel from the plate, the product was extracted three times into 3 ml acetone–1 *M* hydrochloric acid (100:1).

Analytical methods

Radioactivity was determined by liquid scintillation counting in Triton–toluene scintillant[6]. One-dimensional TLC analysis of FFAs was carried out on silica gel 60 TLC plates with a solvent system of hexane–diethyl ether–acetic acid (10:10:0.5). Fatty acid methyl esters were prepared using boron trifluoride, 12% in methanol[7], and extracted into hexane. Fatty acid methyl esters were analyzed on silver nitrate TLC plates by running plates twice in toluene at −20°C[5]. Fatty acid methyl esters were also analyzed by reversed-phase TLC on RP-18 plates by running twice in acetonitrile at room temperature[8]. Radiolabeled products were visualized by autoradiography. NMR analysis was carried out on a Bruker MSL-300 spectrometer ([^{2}H]chloroform solvent). Gas–liquid chromatography (GLC) was carried out on a Varian Model 3700 chromatograph with a 6 ft. × $\frac{1}{8}$ in. I.D. stainless-steel column of 10% SP-2330 (Supelco, Bellefonte, PA, U.S.A.) operating at 185°C.

RESULTS AND DISCUSSION

Removal of unsaturated fatty acids and other contaminants

The thin-layer chromatogram in Fig. 1 illustrates some of the contamination problems that were encountered with commercially-available, radiolabeled, palmitic and stearic acids (lane 1). To remove the unwanted materials, FFAs were first treated with osmium tetroxide, which reacted with unsaturated sites to form osmate esters. Osmium treatment of unsaturated fatty acid reduced its mobility on TLC, as seen most clearly with the stearic acid sample (lane 2). Subsequent silica gel column chro-

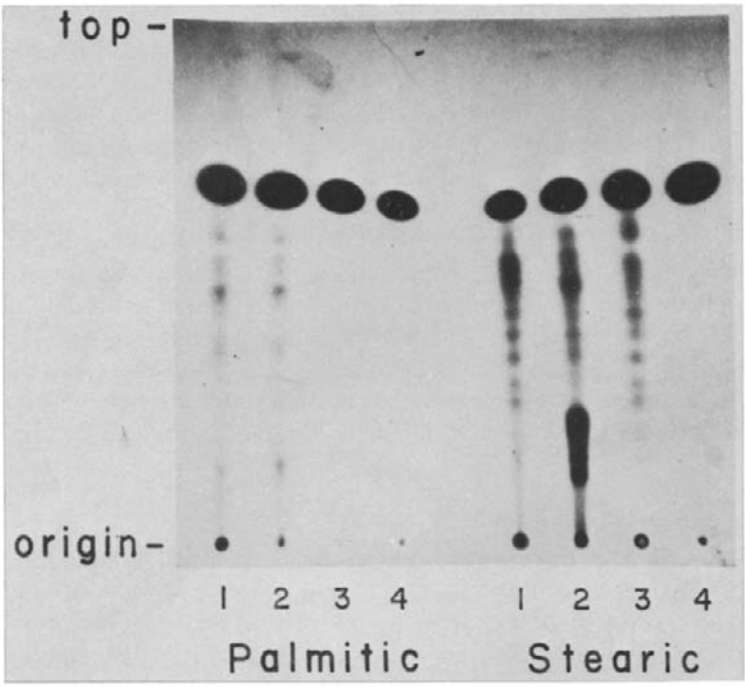

Fig. 1. TLC of radiolabeled palmitic and stearic acids on silica gel 60 TLC plates (hexane–diethyl ether–acetic acid, 10:10:0.5). Approximately 100 000 cpm ^{14}C per sample. (1) Untreated FFAs. (2) FFAs following treatment with osmium tetroxide. (3) FFAs after column chromatography only. (4) FFAs following treatment with osmium tetroxide and column chromatography.

matography successfully removed all contaminating material (lane 4). Recoveries of saturated FFAs following osmium treatment and column chromatography were on the order of 75–90% (Table I). Most of the loss occurred during column chromatography, due to the small quantities of fatty acid (< 50 μg) used for these experiments.

To confirm that unsaturated contaminants were eliminated, the FFAs were converted to methyl esters and analyzed by silver nitrate TLC. As seen in Fig. 2, the osmium tetroxide and column chromatography treatments efficiently removed unsaturated fatty acids.

TABLE I
RECOVERIES OF FREE FATTY ACIDS AFTER VARIOUS PURIFICATION STEPS

Treatment	*Recovery (%)*	
	[^{14}C]Palmitic acid	*[^{14}C]Stearic acid*
Osmium tetroxide only	97	97
Column chromatography only	86	79
Osmium tetroxide plus column chromatography	87	74
Preparative reversed-phase TLC (RP-2 plate)	93	94

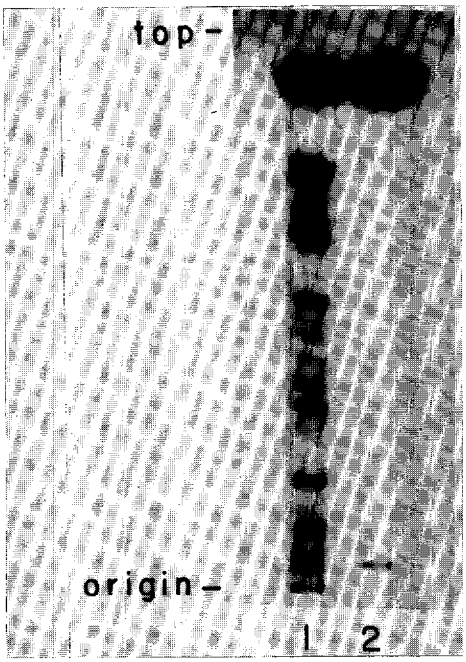

Fig. 2. Silver nitrate chromatography of purified, [^{14}C]stearic acid methyl ester. Stearic acids were converted to methyl esters, and approximately 100 000 cpm of material applied to a silica gel 60 plate impregnated with silver nitrate. Lane 1: untreated stearic acid. Lane 2: stearic acid following treatment with osmium tetroxide and column chromatography. (Material near the origin in lane 2 is a small quantity of free acid.)

Separation of saturated FFAs by chain length

Saturated FFAs were separated by preparative TLC on C_2 reversed-phase plates (silanized with dichlorodimethylsilane). Fig. 3 illustrates the separation of palmitic and stearic acids (after removal of unsaturated contaminants). Preparative TLC was successful when carried out with a solvent system of dioxane–dilute hydrochloric acid. Use of dioxane–water–formic acid mixtures, as reported elsewhere[9], were unsuccessful due to delamination of the reversed-phase material from the glass plate.

Recoveries of palmitic and stearic acids from the reversed-phase plates were excellent using a solvent system of acetone–1 *M* hydrochloric acid (100:1) (Table I). NMR analysis of the fatty acid revealed no detectable contamination from the silanized material.

To confirm that saturated free acids were successfully resolved by preparative TLC, the radiolabeled palmitic and stearic acids were converted to methyl esters and analyzed by reversed-phase TLC using RP-18 plates (data not shown). Neither the stearic nor palmitic acid samples contained saturated fatty acids of different chain length.

The capacity of the RP-2 plates (250 μm thickness) was determined for both palmitic and stearic acids by adding increasing amounts of unlabeled FFA to radiolabeled samples (data not shown). The maximum amounts of palmitic and stearic acids that could be streaked onto the plate without overloading were 200 μg/cm of

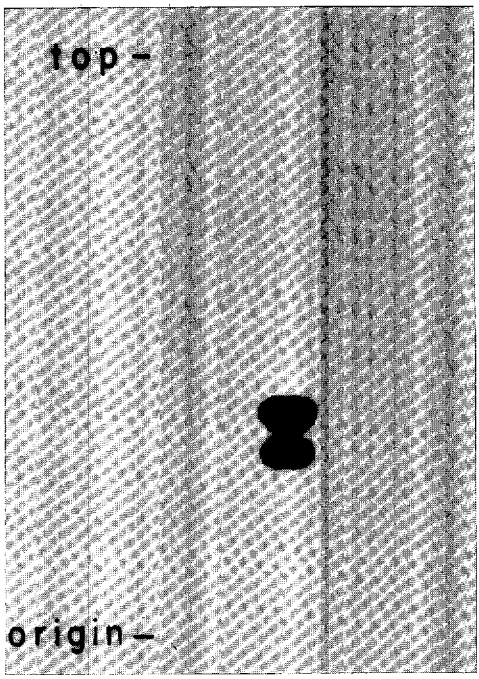

Fig. 3. Reversed-phase TLC of radiolabeled palmitic and stearic acids (following purification with osmium tetroxide and column chromatography). Approximately 100 000 cpm ^{14}C were applied to an RP-2 plate and run in dioxane–0.001 *M* hydrochloric acid (7:3). Upper band: palmitic acid. Lower band: stearic acid.

palmitic acid and 50 μg/cm of stearic acid. When larger quantities of material were applied, excessive spreading of the sample occurred during chromatography.

To confirm the advantages of these methods, fatty acid purity was also assessed on the basis of mass by GLC analysis with flame ionization detection. For these experiments, 200 μg of a mixture of palmitic, myristic, stearic, palmitoleic, and oleic acids (5:1:1:1:1) were prepared and the palmitic acid isolated using the purification procedures described above. (0.05 μCi of [1-^{14}C]palmitic acid was included to locate product.) Fig. 4A shows the GLC of the untreated mixture. Fig. 4B shows the saturated fatty acid fraction obtained after osmium tetroxide treatment and column chromatography. Following the osmium and column chromatography treatments, the palmitic acid was separated from the other saturated free acids by preparative reversed-phase TLC. As seen in Fig. 4C, contaminating fatty acids were below the level of detection.

Advantages of the method

Osmium tetroxide treatment followed by rapid column chromatography of FFAs required only a few hours to complete. The method was clearly faster than the time required to perform a more common approach of esterification, preparative silver nitrate TLC, and hydrolysis back to the free acid.

In our experience, saturated fatty acid contaminants in commercially-available samples differ by multiples of two carbon atoms. These free acids of undesired chain

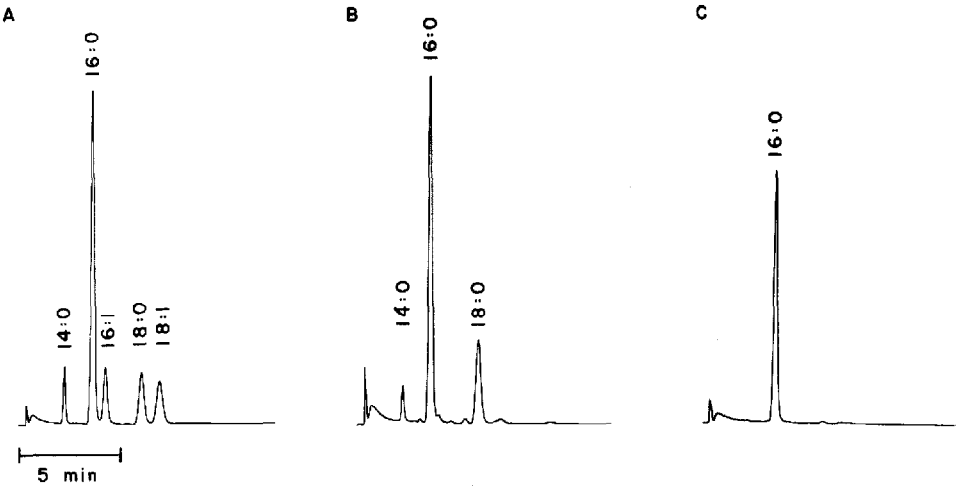

Fig. 4. GLC of a mixture of saturated and unsaturated fatty acids before and after purification steps to obtain palmitic acid. Palmitic, myristic, stearic, palmitoleic, and oleic acids were prepared in a ratio of 5:1:1:1:1. Approximately 200 μg of the mixture were treated with osmium tetroxide and the saturated fatty acids isolated by column chromatography. The palmitic acid fraction was then isolated by reversed-phase TLC. FFAs were converted to methyl esters prior to GLC analysis. (A) Fatty acid mixture before treatment. (B) Saturated fatty acids recovered after treatment with osmium tetroxide and isolation by column chromatography. (C) Palmitic acid fraction recovered after purification by preparative TLC.

length were easily separated by reversed-phase TLC. While a higher resolution can probably be achieved using preparative HPLC, radiochemical decontamination of HPLC equipment can be tedious, particularly when such equipment is usually used for analytical work. TLC offers the more convenient approach to purifying radiolabeled material on a preparative scale.

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