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Note

Capillary gas chromatographic investigation of plasma lipid alcoholysis during alcohol extraction

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Alcoholysis of lipids has been known since 1846 when Rochleder [1] obtained fatty acid methyl esters using castor oil, methanol and dry hydrochloric acid. Other methods for alcoholysis have been described using hydrochloric acid methanol [2], hydrochloric acid and sulfuric acid [3], boron trifluoride methanol [4], potassium carbonate [5], sodium methoxide [6] and potassium hydroxide ethanol [7].

Alcohols are commonly used as solvents for extraction of plasma and tissue lipids. Chloroform—methanol extraction is most commonly used and is known as the method of Folch et al. [8]. Several authors have reported the formation of fatty acid alcohol esters as an artifact when using alcohols for extraction of lipids [9—14]. Using capillary gas chromatography (GC) we investigated the formation of fatty acid alcohol esters when treating plasma with alcohol. The high efficiency of the capillary columns enabled us to separate the various derivatives of fatty acids.

EXPERIMENTAL

Materials

Lauric acid (dodecanoic), myristic acid (tetradecanoic), palmitic acid (hexadecanoic) and stearic acid (octadecanoic) were purchased from Poly Science (Niles, IL, U.S.A.). Pentadecanoic acid, linoleic acid (9,12-octadecadienoic), oleic acid (*cis*-9-octadecanoic) and arachidonic acid (6,10,14,18-eicosatetraenoic) were obtained from Sigma (St. Louis, MO, U.S.A.). Chloroform, ethanol, ethyl acetate, methanol and propanol were obtained from Spectrum Chemical Manufacturing (Redondo Beach, CA, U.S.A.). N,O-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were from

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Pierce (Rockford, IL, U.S.A.). L-α-Lecithin (bovine liver) and L-α-dipentadecanoyl lecithin were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Tripentadecanoin and cholesteryl pentadecanoate were from Nu-Chek-Prep (Elysian, MN, U.S.A.).

Methods

Standard solutions of free fatty acids were prepared by dissolving 20 mg of free acid in 20 ml of ethyl acetate. Standard solutions of triglycerides, phospholipids, and cholesterol esters were prepared by dissolving 10 mg of the lipid in 1 ml of chloroform. All standard solutions were stored below 0°C.

Different amounts (10, 25 and 50 μg) of lauric (C_{12}), myristic (C_{14}), palmitic (C_{16}), linoleic ($C_{18:2}$), oleic ($C_{18:1}$), stearic (C_{18}), and arachidonic ($C_{20:4}$) acids were derivatized with 25 μl of BSTFA—TMCS (10:1). A 2-μl aliquot of the resulting solution was injected into the capillary column of the gas chromatograph and peaks were obtained for each acid as trimethylsilyl (TMS) derivatives.

We used a Model 5880A gas chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.) equipped with two split-mode capillary injection ports and two flame ionization detectors. The chromatograph was controlled by a Level 4 Hewlett-Packard computer terminal. We used a 25 m × 0.31 mm I.D. cross-linked fused-silica capillary column, with a 0.52-μm thick film, and a stationary phase of 5% methyl silicone (Ultra-1). The flow-rate (linear velocity) of the carrier gas helium was 25 cm/sec. The injection split ratio was 1:100, and the injection port and detector temperatures were 250°C and 300°C, respectively.

The initial programmed oven temperature was 150°C, increasing by 5°C/min to a final temperature of 275°C, after which the oven temperature was increased to 325°C and held for 15 min before cooling to 150°C.

Retention times and peak areas were determined by the computer. Methylene units were calculated with a program stored on tape.

Heparinized blood was obtained by venipuncture from laboratory personnel. The plasma was immediately separated and if not analyzed, stored at −70°C. A 1-ml sample of plasma was slowly mixed with 6 volumes of alcohol (methanol, ethanol, or propanol). After centrifugation, the alcoholic supernatant was dried under a stream of nitrogen at 50°C. To the dry residue, 1 ml of deionized water was added and the fatty acids were extracted with 3 ml of ethyl acetate. The ethyl acetate layer was separated by centrifugation and dried under nitrogen. The dry residue was derivatized with 25 μl of BSTFA—TMCS (10:1) at 80°C for 15 min. A 2-μl aliquot of the resulting solution was injected into the capillary column of the gas chromatograph.

Ethanol precipitation was used for the following investigations:

In order to check whether esterification of free fatty acids occurs, different amounts (10, 25 and 50 μg) of long-chain fatty acids were added to plasma which was treated as described above. The peak areas of the fatty acids TMS and ethyl ester derivatives were compared to those of the plasma with no fatty acids added to assess whether any of the added fatty acids was converted to ethyl ester. Recovery percentages of the free fatty acids added to plasma were calculated.

In order to find out which lipid class is affected by transesterification, we added to three 1-ml aliquots of the same plasma, 1 mg of tripentadecanoin,

1 mg of L-α-dipentadecanoyl lecithin and 1 mg of cholesteryl pentadecanoate. The samples were then treated as described above and we looked for TMS and ethyl ester derivatives peak areas of pentadecanoic acid on the chromatograms to assess the degree of alcoholysis (pentadecanoic acid, being an odd number carbon fatty acid, was not detected in normal plasma with our method).

The effect of room temperature on plasma lipid alcoholysis was determined by extracting two 1-ml aliquots of the same plasma. One sample was dried at room temperature while the other was dried at 50°C (drying time 2 h). We compared the peak areas of the TMS and ethyl ester derivatives of the fatty acids in both samples.

We prepared three 0.5-ml aqueous solutions to each of which we added 1 mg of L-α-lecithin (bovine liver); to two of these we added 2.5 mg of sodium carbonate and adjusted the pH of one to neutrality with 1 *M* hydrochloric acid solution. These samples were treated as plasma and were analyzed by capillary GC.

Finally, we analyzed two 1-ml aliquots of the same plasma. To one of the samples we added 0.05 mequiv. of hydrochloric acid to eliminate sodium bicarbonate; then we added to both samples 1 mg of L-α-dipentadecanoyl lecithin and treated them as described before. All identifications of the fatty acid derivatives were confirmed by mass spectrometry. All samples were tested in duplicate.

RESULTS

Fig. 1 shows the chromatogram obtained from free fatty acids standards as their TMS derivatives on the Ultra-1 column. Fig. 2 shows three chromatograms of plasma to which L-α-dipentadecanoyl lecithin was added and which was treated with methanol (a), ethanol (b), or propanol (c). Methyl, ethyl, and propyl esters were obtained for palmitic, linoleic, oleic, stearic acids (derived from plasma lipids) and for pentadecanoic acid (derived from L-α-dipentadecanoyl lecithin added). TMS derivatives of lauric, myristic, palmitic, linoleic, oleic, stearic, arachidonic and pentadecanoic acids were also obtained. No ethyl esters were obtained from free fatty acids added to plasma. These acids were recovered exclusively as TMS derivatives and the recovery percentage was close to 100%.

It appears that most of the fatty acid TMS and alcohol ester derivatives were obtained from phospholipids, as added L-α-dipentadecanoyl lecithin resulted in much larger peaks of pentadecanoic TMS and pentadecanoic ethyl ester than added tripentadecanoin, the ratio being 30:1. We obtained no pentadecanoic acid peaks from cholesteryl pentadecanoate.

Table I compares the peak areas obtained for the fatty acid derivatives when the extract was dried at room temperature and at 50°C. Avoiding the warming of the extract did not result in less alcoholysis.

In aqueous solutions, alcoholysis occurred only when we added sodium carbonate to the solution (pH 11). When we adjusted the pH to 7 with hydrochloric acid the reaction did not occur.

Adding 0.05 mequiv. of hydrochloric acid to plasma before alcohol extraction (pH 5.5) resulted in no ethyl esters formation and no hydrolysis of phos-

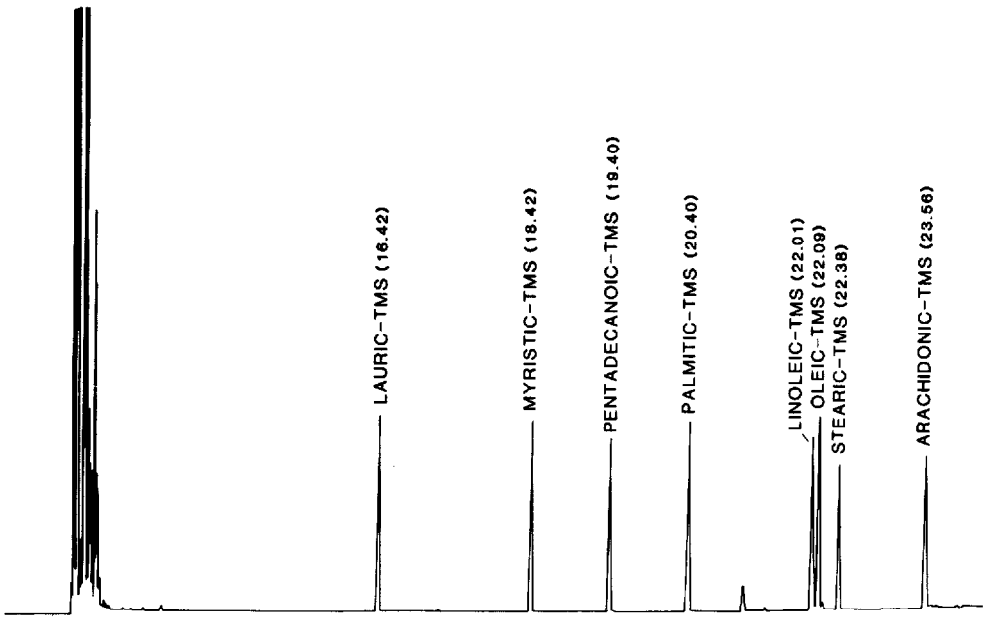

Fig. 1. Long-chain fatty acids as their TMS derivatives separated on a 5% methyl silicone (Ultra-1) capillary column temperature program 150°C to 275°C at 5°C/min. The number in brackets indicates the methylene units of the acid derivative.

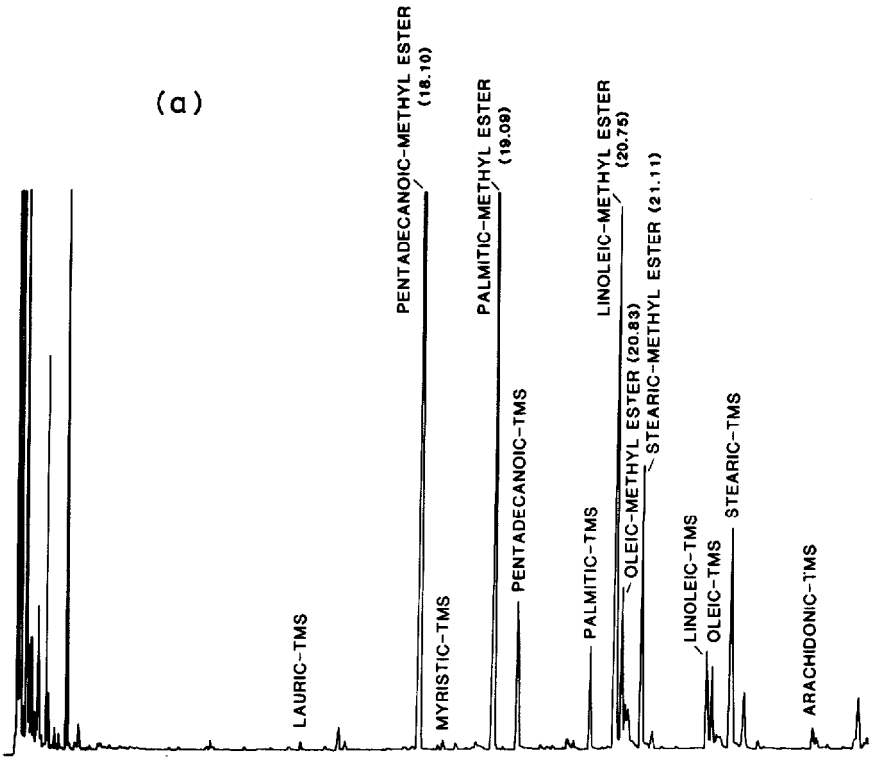

Fig. 2.

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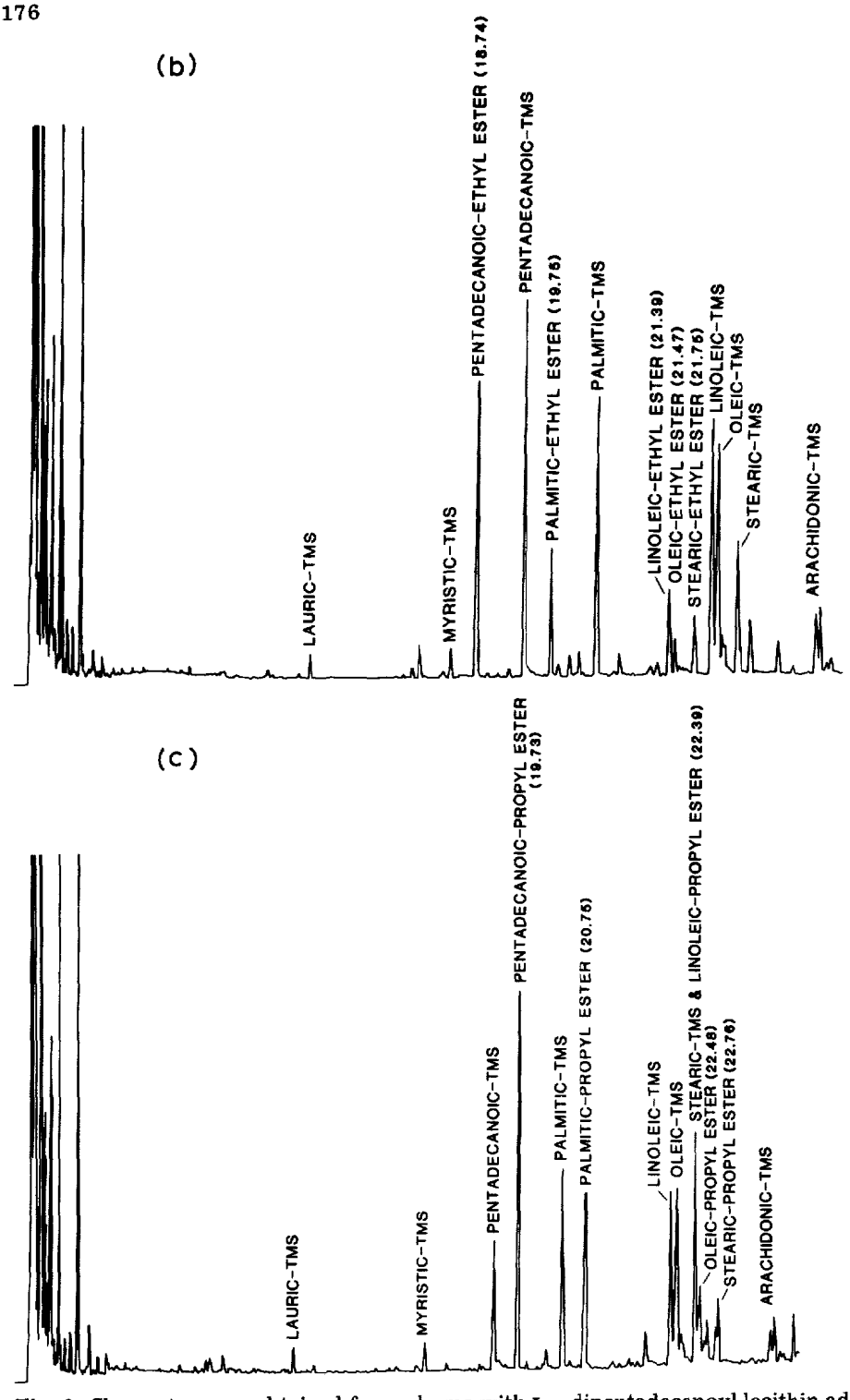

Fig. 2. Chromatograms obtained from plasma with L-α-dipentadecanoyl lecithin added, using methanol (a), ethanol (b), and propanol (c). The chromatograms were obtained on an Ultra-1 capillary column. The number in brackets indicates the methylene units of the acid derivative.

TABLE I

FATTY ACID ESTERS OBTAINED FROM THE SAME PLASMA EXTRACTED AT DIFFERENT TEMPERATURES

Fatty acid derivative	Peak areas	
	Room temperature	50°C
Palmitic ethyl ester	36.30	25.29
Palmitic TMS	185.32	170.29
Linoleic ethyl ester	29.01	25.12
Linoleic TMS	236.37	236.27
Oleic ethyl ester	8.30	5.54
Oleic TMS	237.68	218.48
Stearic ethyl ester	19.44	11.97
Stearic TMS	72.33	71.91

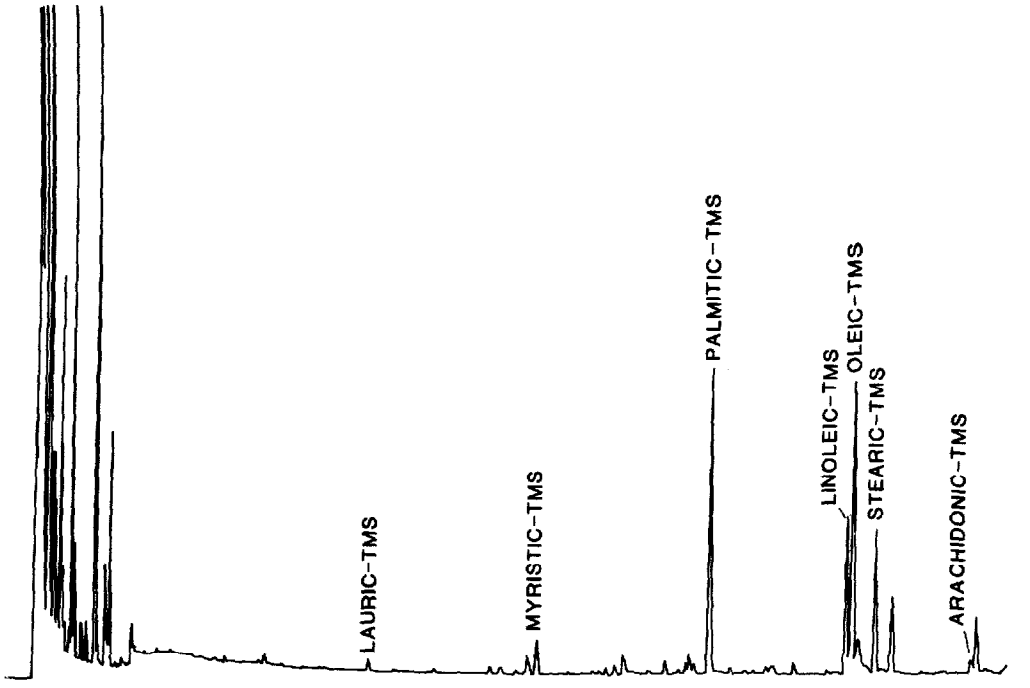

Fig. 3. A chromatogram obtained from plasma with L-α-dipentadecanoyl lecithin added, eliminating the plasma sodium bicarbonate prior to extraction.

pholipids and free fatty acid release as indicated by the lack of pentadecanoic acid peaks.

In this experiment we obtained peaks of free fatty acids as their TMS derivatives that were probably the original plasma free fatty acids. Fig. 3 displays the chromatogram obtained.

DISCUSSION

Several authors have noticed that during extraction of plasma or tissue lipids with alcohols, alcoholysis of these fats occurs. Newsome and Rattay [15] sug-

gested that free fatty acids may be esterified in the presence of alcohol by an enzymatic process. We did not obtain alcohol esters from free fatty acids added to plasma, thus precluding alcohol esterification of free fatty acids as a major source of this artifact.

Our experiments show that hydrolysis and transesterification of fatty acids from phospholipids, and to a much lesser extent, from triglycerides, are the explanation of this observation. No hydrolysis or transesterification from cholesterol esters occurred. These observations are contradictory to those of Lough et al. [10] who obtained most of the alcohol esters from triglycerides.

Analysis of our samples involved drying at 50°C, but when the extraction was performed at room temperature, there was no decrease in the extent of hydrolysis or transesterification of plasma fats.

Lough et al. [10] and Fukuda et al. [11] obtained transesterification of triglycerides by adding sodium carbonate. They postulated that heating the plasma or lymph lipid extracts produces sodium carbonate from sodium bicarbonate the former catalyzing the reaction.

We obtained significant hydrolysis and transesterification of phospholipids in plasma or in aqueous solutions with sodium carbonate added; this effect disappeared when hydrochloric acid was added to plasma or when the solution pH was neutralized with hydrochloric acid probably due to conversion of sodium carbonate to carbon dioxide. Gordon et al. [13], reported alcoholysis when storing lipids in acid alcohol solutions which precludes sodium carbonate as a catalyzing factor in that case.

It is possible that other yet unknown factors present in plasma or tissues are involved in alcoholysis of lipids. The fact that no alcoholysis of lipids was obtained after eliminating sodium bicarbonate with hydrochloric acid favors the findings of Lough et al. [10] and Fukuda et al. [11]. The present study indicates that it may be possible to determine plasma free fatty acids from alcoholic extracts using a direct method, providing the alcoholysis of plasma lipids can be avoided.

CONCLUSIONS

Artifact formation of free fatty acids and fatty acid alcohol esters occurs when plasma is treated with alcohols. This alcoholysis occurs mainly from phospholipids, and to a much lesser degree, from triglycerides. This can be avoided by pre-eliminating plasma sodium bicarbonate using hydrochloric acid.

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