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Note

Isolation of 14-methylhexadecanoic acid from wool fat by preparative gas–liquid chromatography

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Several papers from this laboratory have reported that cholesteryl 14-methylhexadecanoate is present as an essential component in some enzymes and factors required for protein synthesis, and that this compound appears to be closely involved with malignant growth (see refs. 1 and 2 for reviews). The original method for the preparation of this ester involved separation of the 14-methylhexadecanoic acid from ox liver[3], its purification[4] and chemical synthesis of the cholesteryl ester from this purified acid and cholesterol[5]. This technique was time-consuming with low yields of the final preparation because of the very low contents of 14-methylhexadecanoic acid in the liver tissue.

Branched-chain saturated higher fatty acids were identified in several naturally occurring lipids[6]. Lipids extracted from *Listeria monocytogenes* contain up to 40% of 14-methylhexadecanoic acid[7]. Also liver oil from various fishes is relatively rich in branched-chain fatty acids[8]. Wool fat contains almost exclusively this type of fatty acid, including 14-methylhexadecanoic acid[9]. Because it is readily obtainable and relatively inexpensive, wool fat seemed to be the most suitable starting material for the isolation of this acid.

The method for the isolation of 14-methylhexadecanoic acid described here was developed for the purification of larger amounts of this acid which are not readily available commercially. With minor modifications, this technique may be generally useful for the isolation of branched-chain fatty acids.

MATERIALS AND METHODS

Wool fat was a commercial product of pharmaceutical grade. All solvents were redistilled before use.

For the preparative gas–liquid chromatography (GLC), the LPCH 1 automatic gas chromatograph (Workshops of the Czechoslovak Academy of Sciences) was used. Methyl esters of fatty acids were separated on columns (2.5 m × 6 mm I.D.) of silanized Chromosorb W (60–80 mesh) coated with 25% Apiezon L. The column temperature was 215°, and the inlet temperature 280°; the thermal conductivity detector was maintained at 250° and outlet tubing at 175°. No condensation occurred in the outlet tubing at this temperature. Higher temperatures of the outlet lead to aerosol formation and losses of the separated material. The carrier gas was helium at *ca.* 2 atm inlet pressure yielding a flow-rate of 50 ml/min.

For analytical GLC, the Chrom 41 gas chromatograph (Laboratory Instruments, Prague, Czechoslovakia) was used. For the separation of fatty acid methyl esters, glass columns (1.2 m × 4 mm I.D.) of Chromosorb W AW (80–100 mesh) coated with 15% Apiezon L were used. The column temperature was 195° and the inlet temperature 270°; the carrier gas was nitrogen at a flow-rate of 30 ml/min, and flame ionization detection was used.

RESULTS AND DISCUSSION

Wool fat was hydrolysed by refluxing it with an equal volume of 20% KOH in ethanol for 2 h. After partial cooling, two volumes of 60% ethanol were added and the mixture was left to cool at room temperature. The insoluble portion was removed by low-speed centrifugation and the precipitate was washed twice with 60% ethanol. Pooled supernatants were acidified with dilute HCl to pH 3. Fatty acids, appearing as a dark surface layer, were separated in a separatory funnel and washed twice with water. Thereafter they were dissolved in light petroleum, and water was separated in a separatory funnel. Light petroleum was removed using a rotary vacuum evaporator, and fatty acids were converted into methyl esters by refluxing in a 20-fold molar excess of absolute methanol containing 1 ml of H_2SO_4 per litre in a dry atmosphere. After 5 h of refluxing, the mixture was diluted 1:1 with water and extracted with three successive portions of light petroleum (b.p. 35–50°). The light petroleum phases were pooled, washed with water and dried ovenight over $CaCl_2$. The solvent was removed in a rotary evaporator. Thie yield in this step was 120–150 g of crude methyl esters of fatty acids per kilogram of wool fat.

Fractional distillation was used earlier for the purification of branched-chain fatty acids[6], but resulted inevitably in significant losses of the material particularly if good purification was required. Therefore preparative GLC seemed to be the best method for the purification of 14-methylhexadecanoic acid from wool fat hydrolysates. However, this technique is of limited capacity, and relatively low yields of the methyl ester of this fatty acid were obtained if the crude material (Fig. 1a) was directly separated by GLC because of the low content of 14-methylhexadecanoic acid in this material.

It was therefore necessary to enrich the starting material in 14-methylhexadecanoic acid methyl ester by two preliminary distillations. In the first, fatty acid methyl esters from the hydrolysate were distilled in the temperature range 65–275° *in vacuo* (6 mmHg). The distillation-rate was maintained to give a flow-rate of 0.5–3 ml/min. Methyl esters with boiling temperatures up to 160° were discarded, and the distillate passing between 175 and 220° was collected in small fractions (4–6 ml) and analysed by GLC. Maximum 14-methylhexadecanoic acid methyl ester contents in these fractions were 28.5%. Fractions containing more than 3% of this ester were pooled and in this way *ca.* 36 g of fatty acid methyl esters per kilogram of wool fat, containing on average 9.5% of the methyl ester of 14-methylhexadecanoic acid, were obtained.

For further enrichment, this material was distilled through a 1-m long fractionation column with automatic reflux ratio control. The distillate in the range 185–195°/6 mmHg was collected in 4–6-ml fractions and these were again analysed by GLC. Fractions containing at least 70% of 14-methylhexadecanoic acid methyl ester

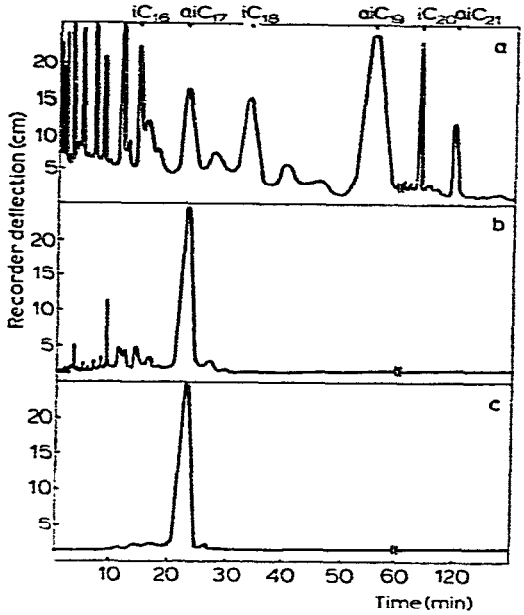

Fig. 1. (a) Fatty acid composition by GLC of wool fat hydrolysate; (b) The material after two fractional distillations and (c) after preparative GLC. The recorder speed was reduced to 1:10 of the original speed after 60 min. The position of the methyl ester of 14-methylhexadecanoic acid is designated as "aiC_{17}" (*anteiso*-margaric acid), "iC_{16}, iC_{18} and iC_{20}" stands for *iso*-acids (14-methylpentadecanoic, 16-methylheptadecanoic and 18-methylnonadecanoic acids) and "aiC_{19} and aiC_{21}" for *anteiso*-acids (16-methyl-octadecanoic and 18-methyleicosanoic acid) methyl esters. The following total amounts of fatty acid methyl esters were injected: (a) *ca.* 4 mg; (b) 0.8 mg; (c) 0.5 mg.

(maximum 91.5%) were pooled and an average yield of 1.8 g per kilogram of wool fat was obtained. Because of its high content of the 14-methylhexadecanoic acid methyl ester (Fig. 1b) this material was suitable for the final purification by GLC.

Using preparative GLC, the methyl ester of 14-methylhexadecanoic acid may be readily purified to at least 97%. During this step, methyl esters of fatty acids with 15, 16 and 17 carbon atoms representing the principal impurities (Fig. 1c) were removed. Under the conditions used, up to 250 μl of the crude methyl ester of 14-methylhexadecanoic acid obtained by the distillation were injected and the separation from impurities was completed in *ca.* 140 min, after which the next portion of the material to be separated was injected automatically.

Fractions from GLC containg the pure methyl ester were refluxed with a five-fold molar excess of 10% KOH in ethanol in a nitrogen atmosphere. After cooling, the reaction mixture was diluted with five volumes of water and acidified with dilute HCl to pH 3. The liberated fatty acid was extracted with three successive portions of diethyl ether. After drying overnight over $CaCl_2$, the solvent was removed in a rotary vacuum evaporator and 14-methylhexadecanoic acid was crystallized from the residue as described earlier[4]. After two recrystallisations the melting point was constant (37.2–37.9°) and the purity was better than 98% as checked by GLC. The overall yield was 0.85 g of 14-methylhexadecanoic acid per kilogram of wool fat.

More than 100 g of pure 14-methylhexadecanoic acid were prepared by using

the procedure described here. Because preparative GLC is involved, the isolation is rather time-consuming, in particular with large amounts. However, the automatic instrument may be run uninterrupted for several weeks with little attention. Moreover, several pure branched-chain fatty acids may be obtained as by-products. This procedure may be thus used as a general technique for the isolation of minor fatty acid constituents of animal fats.

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