A SIMPLE AND EFFICIENT SYNTHESIS OF [^{3}H]CHOLESTERYL 14-METHYL-HEXADECANOATE

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SUMMARY

The reaction of labelled cholesterol with a large excess of freshly prepared 14-methylhexadecanoyl chloride in a simple glass apparatus was used for the synthesis of [^{3}H]cholesteryl 14-methylhexadecanoate in microgram amounts. After purification of the crude product by thin-layer chromatography, the purity of the final preparation was better than 98% as checked by gas-liquid and high-pressure liquid chromatography. On average 90% of the input radioactivity was present in the ester. The same technique was also used for the preparation of [^{14}C]cholesteryl 14-methylhexadecanoate and [^{3}H]or [^{14}C]cholesteryl laurate, myristate, palmitate, margarate and stearate with similar yields. All these preparations were found suitable for biochemical studies.

Key words: Cholesteryl 14-methylhexadecanoate, Cholesteryl esters, Labelled cholesterol, Fatty acids

INTRODUCTION

The importance of cholesteryl 14-methylhexadecanoate for reactions involved in protein synthesis and its role in malignant growth have been demonstrated by several papers from this laboratory [see (1,2) for reviews]. For further studies on the mode of action of this compound its availability in a labelled form became essential.

Methods for the synthesis of milligram amounts of labelled cholesteryl esters were described using acyl chlorides of the

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fatty acids and cholesterol as starting materials (3-5). For the preparation of esters labelled in their fatty acid moiety, acylimidazole derivatives or fatty acids activated with dicyclohexylcarbodiimide were also used (6). Although some of these methods gave relatively good yields, no procedures suitable for the synthesis of cholesteryl esters on a microgram scale have been described which are required for the preparation of tritiated esters of a high specific radioactivity essential for some metabolic studies.

The rapid and simple procedure described in this paper may be useful for the preparation of labelled cholesteryl estersin general in microgram amounts with high yields and high specific radioactivities required for biochemical studies.

MATERIALS AND METHODS

14-Methylhexadecanoic acid was isolated from wool fat and purified by preparative gas-liquid chromatography as described elsewhere (7). Lauric, myristic, palmitic, margaric and stearic acids were products of SERVA, Heidelberg, West Germany. [G-^{3}H]Cholesterol (3.4 Ci/mmol) was obtained from the Institute for Nuclear Research, German Academy of Sciences, Dresden, GDR., and [4-^{14}C]cholesterol (59 mCi/mmol) was purchased from the Institute for Research, Production and Uses of Radioisotopes, Prague, Czechoslovakia.

Thin-layer chromatography of cholesteryl esters was performed using layers of Silicagel G (Merck, Darmstadt, West Germany) developed with petroleum ether (b.p.45°C) - ethylether mixtures (97.5 : 2.5, v/v). A Chrom 41 gas chromatograph (Laboratory Instruments Work, Prague, Czechoslovakia) was used for GLC analyses. Cholesteryl esters were separated on glass columns (inner diameter 4 mm, length 1600 mm) filled with Chromosorb W-HP (100/120 mesh) coated with 3% OV 101 silicone using a linear tem-

perature programme (1°C/min) in the range of 235-295°C and FID for the detection. Separations by HPLC were performed on glass columns (inner diameter 4 mm, length 300 mm) of a spherical silica octadecyl-bonded phase (average particle size 10 μm) (8,9) (marketed under the trade name Separon Si C18 by Laboratory Instruments Work, Prague, Czechoslovakia). Methanol- acetone mixtures (85:15) were used at 40.5°C for the elution with a flow-rate of 0.5 ml/min maintained by a VLD 30 high-pressure pump (Workshops of the Czechoslovak Acad.Sci., Prague, Czechoslovakia). A RIDK 101 differential refractometer (Laboratory Instruments Work, Prague) was used for the detection. Fractions (up to the elution of cholesteryl palmitate 7.5 ml, after that 0.75 ml each) were collected, the effluent was evaporated to dryness, residues were dissolved in a toluene-based scintillation mixture and the radioactivity was counted in a Nuclear Chicago Mark II System. The separation of cholesteryl esters by HPLC will be described in detail elsewhere (O.Helmich, in preparation).

RESULTS AND DISCUSSION

For the preparation of acyl chloride, dry 14-methylhexadecanoic acid was refluxed at approximately 85°C for 60 min with a 5-7-fold molar excess of freshly redistilled thionyl chloride. Excess of thionyl chloride was then removed in a stream of dry nitrogen, the complete removal of thionyl chloride being controlled by the indicator paper placed at the outlet tube of the reaction vessel.

For the synthesis of [^3H]cholesteryl 14-methylhexadecanoate a simple glass apparatus was used (Fig.1). Approximately 10 μl (33 μmol) of the freshly prepared acyl chloride were put into the siliconized micro test tube, the apparatus was closed and left at room temperature for approximately 30 min to obtain a

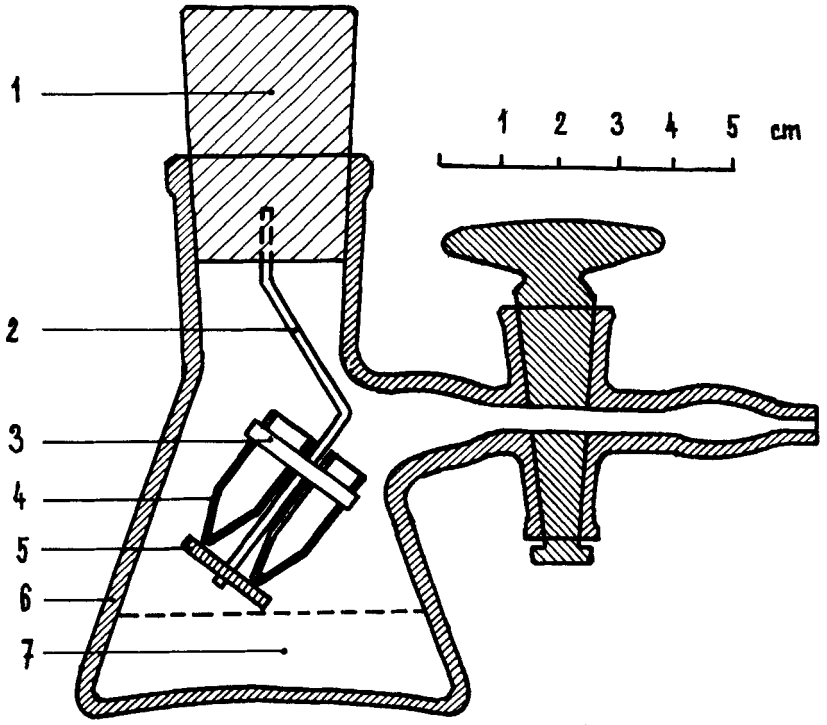

Fig.1. Apparatus used for the synthesis of [^{3}H]cholesteryl 14-methylhexadecanoate. 1, Rubber stopper, 2, stainless steel wire, 3, rubber ring, 4, silanized glass tube, 5, rubber disc, 6, Buechner flask, 7, solid NaOH

dry atmosphere within it. Thereafter 10 µl (7 nmol) of [^{3}H]cholesterol in benzene were added and the apparatus was evacuated to approximately 400 mm Hg at room temperature. After the evaporation of benzene, the synthesis was completed by heating the whole apparatus to 80°C for 2 h. After cooling, the product was dissolved in ethylether and applied on to thin layers of silicagel. A standard of cholesteryl stearate was run on the same layer. The portion of the layer containing the cholesteryl ester (as checked by the position of the standard ester after its detection by phosphomolybdic acid) was scrapped-off and eluted with ethylether.

The final product was completely free of [^{3}H]cholesterol which remained at the starting line during thin-layer chromatography and of the unreacted free fatty acid having an R_F= 0.2-0.4 in

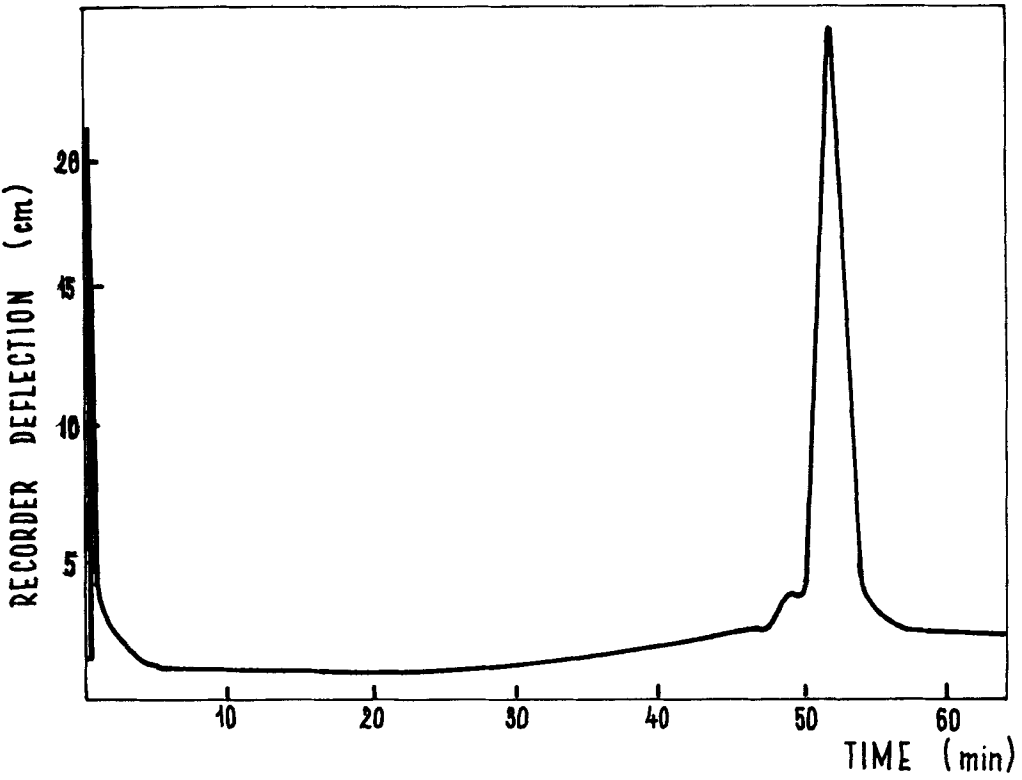

Fig.2. GLC analysis of a [^{14}C]cholesteryl 14-methylhexadecanoate preparation. 5 µg of the ester dissolved in ethylether were injected and analysis was performed as described in the Materials and Methods section

the solvent mixture used whereas the cholesteryl ester had an R_F= 0.7. GLC may be used to detect impurities in the final preparations of [^{14}C]cholesteryl esters only. Because of the high specific radioactivity of tritiated compounds the amount of radioactivity to be injected would be extremely high and severe losses would result. However, HPLC may be used to check the radiochemical purity of such preparations. Analysis of cholesteryl esters synthesized from a less purified fatty acid (approx. 95%) is presented in Figs. 2 and 3 to show that small quantities of impurities present in the final preparation are easily detected by both these techniques. It seems that the purity of the final

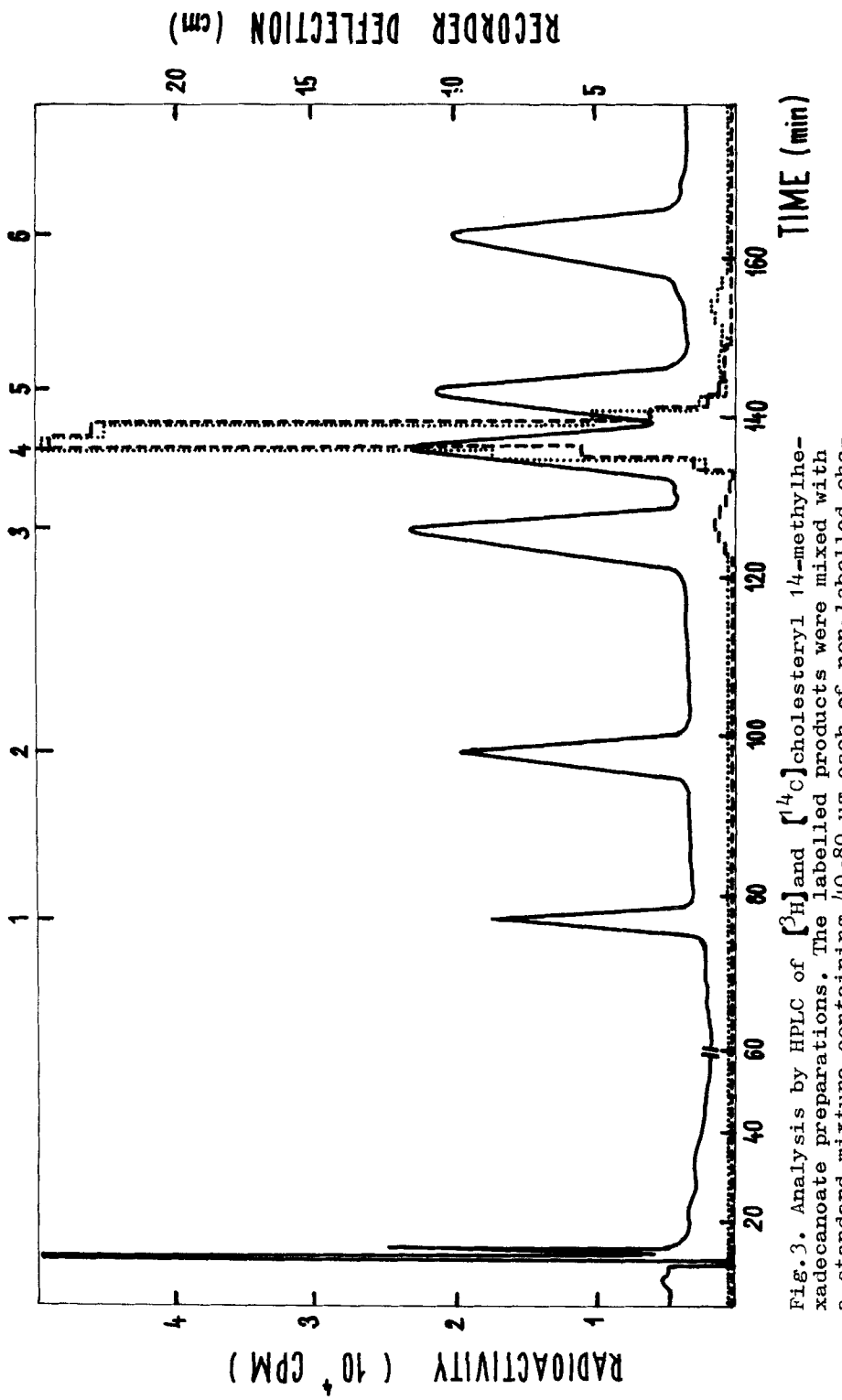

Fig.3. Analysis by HPLC of [^{3}H]and [^{14}C]cholesteryl 14-methylhexadecanoate preparations. The labelled products were mixed with a standard mixture containing 40-80 µg each of non-labelled cholesteryl laurate (1), myristate (2), palmitate (3), 14-methylhexadecanoate (4), margarate (5) and stearate (6). The analysis was performed as described in the Materials and Methods section. ——— , mass record, – – – – , ^{14}C, ·····, ^{3}H

preparation is determined mainly by the quality of the fatty acid used for the synthesis although the presence of minute impurities from labelled cholesterol cannot be excluded. When using fatty acids purified by repeated preparative GLC (7) , the purity of synthesized cholesteryl esters always exceeded 98%. If even higher purities are required, this may be easily achieved by HPLC.

As calculated from the radioactivity data, the yield of [^{3}H] cholesteryl 14-methylhexadecanoate represented on average 90% of the quantity of labelled cholesterol used for the synthesis.

The method described here may be used as a general procedure for the synthesis of labelled cholesteryl esters also on a milligram scale and ^{14}C- labelled esters of cholesterol with lauric, myristic, palmitic, margaric and stearic acid have been prepared in yields similar to those described for cholesteryl 14-methylhexadecanoate. Our technique seems to be less suitable for the synthesis of cholesteryl esters labelled in the fatty acid moiety since a very large excess of fatty acyl chloride is to be used to obtain satisfactory yields of the ester. This fact presents a relative disadvantage of this procedure. However, the unreacted fatty acid may be almost completely recovered from thin layers by elution with ethylether and may be readily used for the synthesis of further batches of labelled or non-labelled cholesteryl esters.

The method described here gives yields exceeding those reported for the other syntheses of labelled cholesteryl esters (3-6) and because of its simplicity the preparation may be rapidly performed in most laboratories. Furthermore, only microgram quantities of the ester required for immediate use may be easily prepared excluding thus the possibility of a partial decomposition of these compounds during a prolonged storage.

Labelled cholesteryl 14-methylhexadecanoate as well as other esters prepared by this method were found suitable for several biochemical studies. They are satisfactory substrates for cholesterol esterase (10) and were also used for the experiments on the binding of cholesteryl 14-methylhexadecanoate by some protein synthesis factors (Z.Tuháčková and J.Hradec, unpublished).

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