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SEPARATION OF [^{3}H]CHOLESTERYL ESTERS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the determination of [^{3}H]cholesteryl esters separated by reversed-phase high-performance liquid chromatography on C_{18}-bonded silica gel. The procedure readily separates esters of saturated fatty acids from those of acids with various degrees of unsaturation. Saturated homologues of straight-chain fatty acids are completely separated from esters of branched-chain higher fatty acids. Picomole amounts of tritiated cholesteryl esters can be detected with a high precision, and the method is suitable for the determination of such compounds synthesized enzymatically *in vitro*.

INTRODUCTION

Studies on the biogenesis and mode of action of cholesteryl 14-methylhexadecanoate, an apparently important compound in mammalian protein synthesis (see Hradec[1,2] for reviews), require methods for the determination of picomole amounts of this ester. Such methods should also be suitable for the detection of radioactive cholesteryl esters.

Radio-gas chromatography of cholesteryl esters developed in this laboratory[3] permitted the determination of picomole quantities of [^{14}C]cholesteryl 14-methylhexadecanoate. However, it was not possible to separate homologous cholesteryl esters of saturated fatty acids from those of unsaturated or branched-chain fatty acids. Moreover, the determination of [^{3}H]cholesteryl esters by this technique was very difficult because of problems involved in the assay of ^{3}H in chromatographic effluents.

Recent developments in high-performance liquid chromatography (HPLC) of lipids suggested that this method may be suitable for the separation of tritiated

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cholesteryl esters. For example, by this means it is possible to separate in a single run different lipid classes and even cholesteryl esters of fatty acids with various degrees of unsaturation[4]. However, no method has as yet been reported for the separation of individual cholesteryl esters within a homologous series.

In this paper an HPLC procedure is described for the separation of cholesteryl esters. Its efficiency is significantly better than that of gas–liquid chromatography (GLC), it has high sensitivity and good reproducibility. Determination of [^{3}H]cholesteryl esters by this technique is accurate and relatively simple.

MATERIALS AND METHODS

Chemicals and radiochemicals

Synthetic [^{3}H]cholesteryl esters were prepared as described by Helmich and Hradec[5]. 14-Methylhexadecanoic acid was purified from wool fat[6]. The other fatty acids were purchased from Serva (Heidelberg, G.F.R.). Natural mixtures of [^{3}H]cholesteryl esters were kindly prepared by Dr. J. Kvíčala of this laboratory by incubation of generally labelled [^{3}H]cholesterol (3.4 Ci/mmol; Institute for Nuclear Research, German Academy of Sciences, Dresden, G.D.R.) with rat liver homogenates[7].

Apparatus

The HPLC apparatus consisted of a VLD 30 high-pressure pump (Workshops of the Czechoslovak Academy of Sciences, Prague, Czechoslovakia), glass columns (300 × 3.8 mm)[8] filled with C_{18}-bonded spherical silica gel[9] and a RIDK 101 differential refractometer with a TZ 4221 line recorder (both supplied by Laboratory Instrument Works, Prague, Czechoslovakia). Methanol–acetone (85:15) was used for the elution. The flow-rate was 0.85 ml/min and the column temperature was maintained at 40°C by a constant temperature water-bath.

Assay of radioactivity

Fractions collected by a fraction collector in scintillation vials were supplemented with 6 ml of a toluene-based scintillation mixture and radioactivity was counted in a Nuclear Chicago Mark II liquid scintillation spectrometer, with an efficiency of 40–42% for ^{3}H as determined by the channel-ratio technique.

RESULTS

Separation of different groups of cholesteryl esters

HPLC analysis of model mixtures containing non-labelled cholesteryl laurate, myristate, palmitate, 14-methylhexadecanoate and stearate together with [^{3}H]- and [^{14}C]cholesteryl 14-methylhexadecanoate revealed not only an excellent separation of esters differring by two carbon atoms in their molecules but even a good separation of homologues containing straight carbon chains (palmitate) from those with a branched chain (14-methylhexadecanoate). The elution pattern was very sharp and no substantial tailing of radioactivity occurred (Fig. 1).

Essentially the same results were obtained with natural mixtures of cholesteryl esters of saturated fatty acids from incubates of rat liver after preliminary separation from esters of unsaturated fatty acids by thin-layer chromatography (TLC) on silica gel G impregnated with $AgNO_3$[10] (Fig. 2).

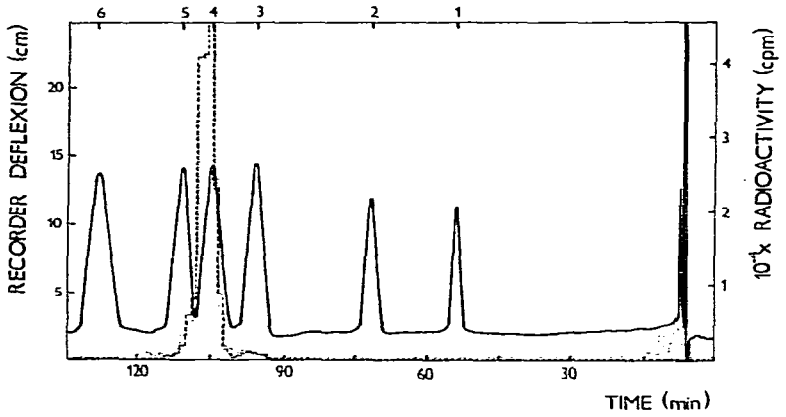

Fig. 1. Separation of a model mixture of cholesteryl laurate (1), myristate (2), palmitate (3), 14-methylhexadecanoate (4), margarate (5) and stearate (6). The mixture comprised 15–30 μg of each non-labelled cholesteryl ester and contained, in addition, [^{14}C]cholesteryl 14-methylhexadecanoate (– – –) and [^{3}H]cholesteryl 14-methylhexadecanoate (· · ·).

If natural cholesteryl esters with monounsaturated fatty acids (again obtained from incubates of rat liver with [^{3}H]cholesterol by separation on $AgNO_3$-impregnated silica gel) were analysed by HPLC, the retention volumes of cholesteryl oleate and palmitoleate were significantly smaller than those of their saturated homologues (stearate and palmitate). They were eluted even significantly earlier than saturated homologues with chains two carbon atoms shorter (palmitate and myristate) (Fig. 3).

Further evidence that the degree of unsaturation in the fatty acid moiety determines the retention volume of the cholesteryl ester was provided by separations of natural mixtures of esters with double-unsaturated fatty acids. Cholesteryl linolenate was eluted earlier than oleate and preceded the elution of cholesteryl myristate. It would thus seem that each double bond present in the fatty acid results in the elution

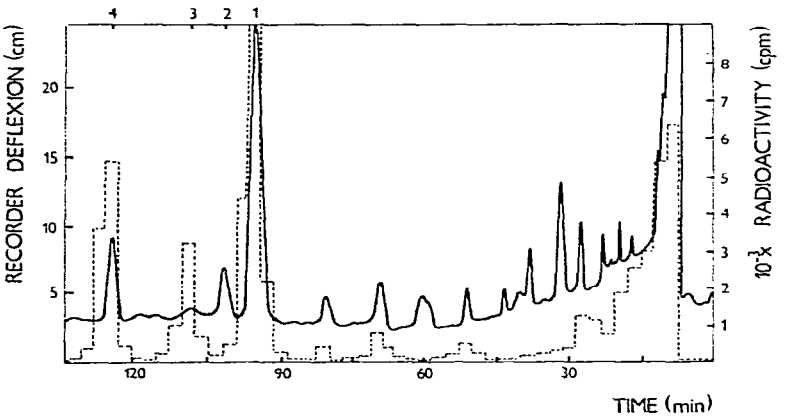

Fig. 2. Separation of a natural mixture of [^{3}H]cholesteryl esters obtained from rat liver homogenate after its incubation with [^{3}H]cholesterol[7]. Peaks: 1 = cholesteryl palmitate; 2 = 14-methylhexadecanoate; 3 = margarate; 4 = stearate.

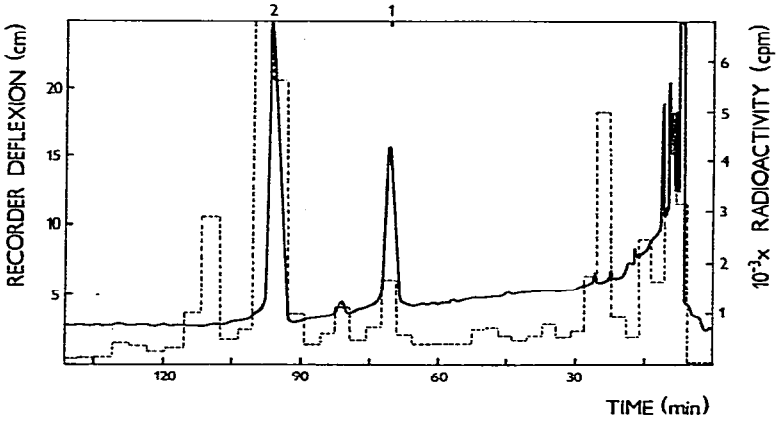

Fig. 3. Separation of natural cholesteryl esters with monounsaturated fatty acids. Peaks: 1 = cholesteryl palmitoleate; 2 = oleate.

of its cholesteryl ester ahead of the saturated homologue two carbon atoms shorter (Fig. 4).

Effect of column temperature

As expected, an increase in column temperature resulted in a more rapid elution of individual cholesteryl esters and the time required for the analysis of a model mixture at 40.4°C was less than one half of that at 25.6°C. Even more important, increased temperature significantly improved the separation of the "critical pair" cholesteryl palmitate and 14-methylhexadecanoate. Relative to palmitate, the retention time of 14-methylhexadecanoate was significantly increased at higher temperatures, whereas the retention volumes of cholesteryl esters of straight-chain saturated fatty acids remained essentially unaltered (Table I).

Further increases in column temperature were found impractical for our purposes since the peak of cholesteryl 14-methylhexadecanoate began to merge with that

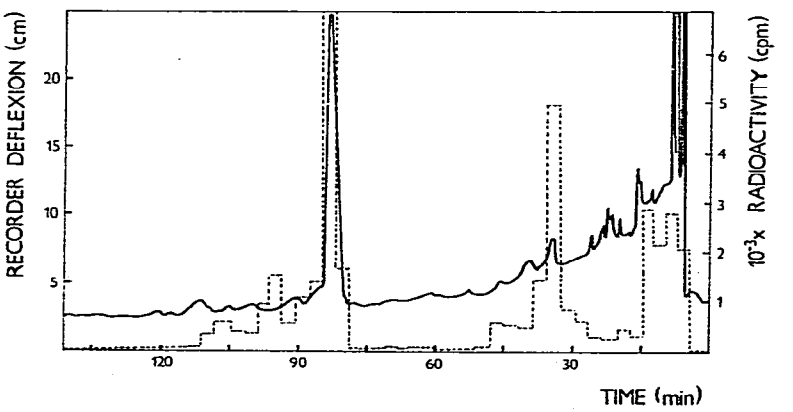

Fig. 4. Separation of a natural mixture of [^{3}H]cholesteryl esters with polyunsaturated fatty acids. Cholesteryl esters were obtained from rat liver homogenate incubated with [^{3}H]cholesterol and were subjected to a preliminary separation on thin layers of silica gel impregnated with $AgNO_3$[10].

TABLE I

EFFECT OF COLUMN TEMPERATURE ON THE RETENTION OF INDIVIDUAL CHOLESTERYL ESTERS PRESENT IN A MODEL MIXTURE

All values are relative to the retention volume of cholesteryl palmitate.

Temperature (°C)	*Retention volume of cholesteryl*					
	Laurate	*Myristate*	*Palmitate*	*14-Methyl-hexadecanoate*	*Margarate*	*Stearate*
25.6	0.605	0.745	1	1.048	1.138	1.290
35.2	0.608	0.755	1	1.070	1.140	1.315
40.4	0.615	0.763	1	1.085	1.145	1.305

of margarate. However, for other analyses, the separations could be significantly affected and probably also improved at even higher column temperatures.

Sensitivity and reproducibility

The minimum amounts of individual cholesteryl esters which could be detected by the differential refractometer were *ca.* 10 μg. Samples were usually dissolved in benzene and 2–4 μl of this solution were injected. However, the sample volume could easily be increased to 30 μl (corresponding to 200 μg of individual esters in the mixture) without any harmful effects on the separation. This indicates that the method could be adapted for micropreparation.

If, however, the determination of cholesteryl esters is based on their radioactivity, more favourable results are obtained and quantities corresponding to *ca.* 0.5 pmole may be accurately estimated. In this case, addition of suitable amounts of non-labelled cholesteryl esters is advisable because of the low sensitivity of refractometer detectors.

The reproducibility of the retention volumes of individual cholesteryl esters (in repeated analyses of the same model mixture) was very good and differences did not

TABLE II

REPRODUCIBILITY OF RETENTION VOLUMES AND RADIOACTIVITY ASSAYS IN ANALYSES OF MODEL MIXTURES OF CHOLESTERYL ESTERS

Seven separate analyses were made in each case.

Cholesteryl ester	*Retention volume (ml)*		*Total radioactivity (cpm)*	
	Mean	*S.D.*	*Mean*	*S.D.*
Laurate	47.6	0.3	–	–
Myristate	60.6	0.3	–	–
Palmitate	78.8	0.5	6070	511
14-Methylhexadecanoate	84.8	0.5	61,028	2181
Margarate	89.8	0.5	6829	476
Stearate	102.6	0.4	–	–

exceed 0.6% (Table II). Repeated determinations of [^{3}H]cholesteryl esters showed a low standard error even when relatively low radioactivities were used (Table II), indicating tnat no tailing of the radioactivity takes place. Almost quantitative recoveries of the radioactivity were found in the column eluate, indicating that no substantial amounts of radioactive materials become irreversibly retained on the column during the separation.

DISCUSSION

The efficiency of the separation of cholesteryl esters by HPLC significantly exceeds that obtained by GLC. Whereas only separations of groups of esters having the same carbon number are possible with the latter technique[3,11] and homologues of unsturated fatty acids cannot be distinguished from those of saturated ones, the present procedure enables not only the separation of homologues according to the degree of unsaturation but also of homologues differing in the branching of the fatty acid chain. Comparable efficiences can be obtained by GLC only for methyl esters of fatty acids[12], but this method fails if higher-molecular-weight esters, such as cholesteryl esters, are analyzed.

The results of the present study indicate that simple mixtures of cholesterol esters of natural fatty acids differing in their extents of saturation could probably be separated in a single run. However, if more complex mixtures and those of an unknown composition are analyzed, a preliminary separation of cholesteryl esters on thin layers impregnated with $AgNO_3$ seems advisable. Moreover, the results reported allow one to predict the retention volumes of esters of fatty acids with varying degrees of unsaturation and thus a tentative identification of unknown components.

When compared with GLC, the sensitivity of detection of cholesteryl esters by the present HPLC procedure is significantly lower. This is due to the fact that the sensitivity of the refractometer is much lower than that of flame ionization detectors used in GLC. This disadvantage may probably be overcome by using a variable wavelength detector[4]. However, if assay of radioactivity is used for the detection, considerably lower quantities of cholesteryl esters can be precisely determined than by our previous GLC procedure[3] because tritiatec esters have much higher specific radioactivities than those labelled with ^{14}C.

Although several techniques have been used for the detection of ^{3}H in effluents from gas chromatographs, including improved hydrocracking[13] or a continuous mixing of effluents with the scintillator solution[14,15], none of these methods seems to be complete satisfactory and, in particular, these procedures are not compatible with the most sensitive and simple assay of radioactivity by liquid scintillation counting. These difficulties are overcome by HPLC where the counting of ^{3}H radioactivity in collected fractions is very simple and efficient. HPLC is thus the method of choice for the separastion and precise determination of tritiated lipids of biological interest.

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