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QUANTITATIVE DETERMINATION OF CHOLESTERYL 14-METHYL-HEXADECANOATE BY RADIO-GAS CHROMATOGRAPHY

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SUMMARY

A method was developed for the quantitative determination of radioactive cholesteryl 14-methylhexadecanoate at picomole levels. Intact cholesteryl esters are separated by gas–liquid chromatography on OV-1 silicone, one portion of the effluent being led to the flame ionization detector and the other portion combusted to $^{14}CO_2$. For qualitative analyses the radioactivity is assayed by continuous gas flow counting. For quantitative purposes the radioactive gas is trapped in ethanolamine, fractions are collected according to the mass record and the radioactivity is determined by liquid scintillation counting. The latter technique is able to detect 1–2 pmoles of radioactive cholesteryl esters with a reproducibility within ± 10% and is suitable for the quantitative determination of these compounds enzymatically synthesized *in vitro*.

INTRODUCTION

Several papers from this laboratory demonstrated the fundamental role of cholesteryl 14-methylhexadecanoate in gene expression (see Hradec[1] for a recent review). Further studies on the biosynthesis of this compound required the development of a quantitative method for the determination of this ester at pmole levels. Radio-gas chromatography seemed to be the method of choice.

The method for quantitative determination of cholesteryl 14-methylhexadecanoate in biological materials developed earlier[2] was apparently not suitable for these purposes, as severe losses of radioactivity may occur during the rather complicated procedure. Thus the development of a method requiring no preliminary hydrolysis of cholesteryl esters seemed to have several advantages. Kuksis[3] was the first who described the gas–liquid chromatography (GLC) of intact cholesteryl esters. The separation was based primarily on the carbon number or molecular weight of the material and the procedure did not require any preliminary treatment of the sample. A similar technique was described by Swell[4] for the separation of radioactive cholesteryl esters prepared enzymatically. Kuksis *et al.*[5] later reported on an improvement of their original procedure permitting a direct examination of total lipid extracts and quantitative estimations of individual steryl esters. Ikekawa *et al.*[6] described a simultaneous

determination of cholesterol and cholesteryl esters by GLC. They found this rapid, sensitive and simple method useful for routine clinical analysis.

In this paper we describe a method for the quantitative determination of cholesteryl 14-methylhexadecanoate at pmole levels in the presence of other cholesteryl esters. The method is relatively simple, has good reproducibility, and is suitable for the quantitative determination of this cholesteryl ester produced by enzymatic reactions *in vitro*.

MATERIALS AND METHODS

Chemicals and radiochemicals

Non-radioactive cholesteryl esters were prepared from commercial fatty acids (Koch-Light, Colnbrook, Bucks., Great Britain) and cholesterol (purified as the dibromide) as described by Hradec and Sommerau[7]. All solvents were redistilled before use. The OV-1 silicone and Chromosorb W-HP were purchased from Carlo Erba (Milan, Italy). Dimethylchlorosilane was from Calbiochem (Los Angeles, Calif., U.S.A.). [4-^{14}C]Cholesterol (59.2 mCi/nmole) and [^{14}C]hexadecane (1.06 μCi/g) were obtained from Radiochemical Centre (Amersham, Great Britain).

Preparation of radioactive cholesteryl esters

Rat liver homogenate or cytosol was incubated with labelled cholesterol and total lipids were extracted with chloroform–methanol (2:1) mixture. This extract was supplemented with 10 μl of a standard solution of cholesteryl esters (containing 7.5 μg of cholesteryl laurate, 13.2 μg of myristate, 18.2 μg of palmitate, 21.4 μg of margarate, and 23.4 μg of stearate) and esters of cholesterol with saturated fatty acids were separated by TLC on silica gel G impregnated with $AgNO_3$ (ref. 8). Spots of esters detected in UV light[2] were eluted with ether and the eluate was condensed under a stream of nitrogen to a final volume of about 20 μl.

Apparatus

A Chrom III chromatograph (Laboratorní Přístroje, Prague, Czechoslovakia) was modified for the present purposes. The instrument was equipped with a dual glass column system, permitting two analyses to be run simultaneously or as a single temperature-programmed run. At the inlet side the column begins immediately beneath the septum, thus permitting on-column injections. At the outlet side each column is coupled to a flow splitter assembly with a nominal split of 1:8–1:10. The minor effluents are led to a modified flame ionization detector and the major effluents are led into a stainless-steel combustion tube (10 × 140 mm) containing cupric oxide wire and heated to 750–780° by a variable transformer. The radioactive gas is cooled by passage through a 10-cm long stainless-steel tube of 2-mm I.D. This is connected to a glass tube (6 × 100 mm) filled with magnesium perchlorate. Through a silicone connecting tube the gas is fed to a proportional gas flow counter equipped with facilities for continuous recording. The connection between the splitter assembly and the combustion tube is made from one piece of stainless-steel tubing (2 mm I.D.) with silanized inner walls. Alternatively, the radioactive gas after having been dried is trapped in monoethanolamine solution using a collection assembly attached to a conventional scintillation vial (Fig. 1). A schematic diagram of the whole radio-gas chromatographic assembly is given in Fig. 2.

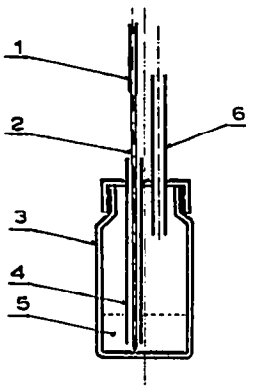


Fig. 1. The vial used for trapping $^{14}CO_2$ in ethanolamine for liquid scintillation counting. 1 = Silicone rubber connecting tube for the gas chromatograph outlet, 2 = stainless-steel capillary, 3 = conventional scintillation vial, 4 = inlet glass pipe, 5 = trapping solution, and 6 = outlet glass tube.

Procedure

The solution of cholesteryl esters is injected into the gas chromatograph and separated on columns (3 × 2100 mm) filled with Chromosorb W-HP, 100–120 mesh, coated with 3% of OV-1 silicone. If gas flow counting is required, 20% CO_2 in purified argon is used as the carrier gas, pure nitrogen being used if the radioactivity is assayed by liquid scintillation. The carrier gas flow-rate is 35 ml/min with an inlet pressure of 3.2 atm. The mass detector is fed with hydrogen (20–25 ml/min) and air

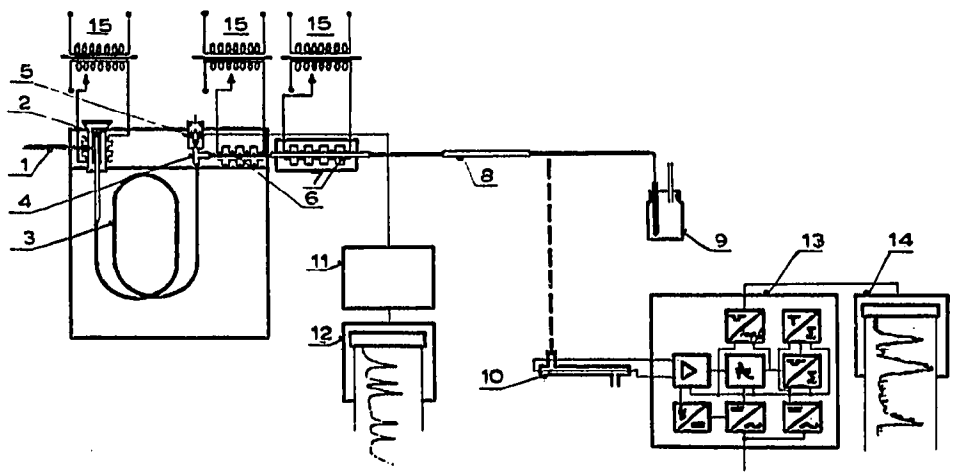


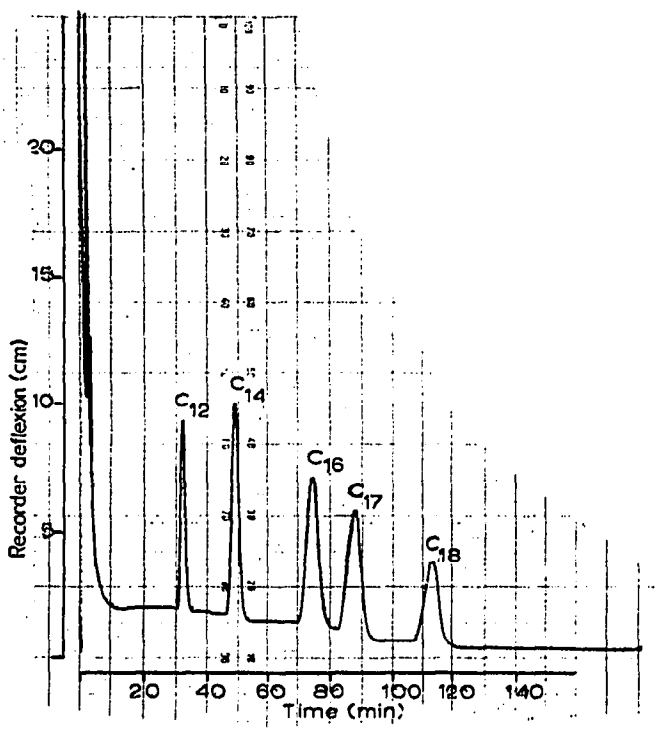
Fig. 2. Schematic diagram of the assembly for radio-gas chromatography. 1 = Carrier gas inlet, 2 = septum, 3 = glass column, 4 = splitter assembly, 5 = flame ionization detector, 6 = outlet pipeline, 7 = combustion tube, 8 = drying tube with magnesium perchlorate, 9 = vial for $^{14}CO_2$ trapping, 10 = proportional gas flow counter, 11 = electronics for the flame ionization detector, 12 = mass recorder, 13 = electronic equipment for the gas flow counter, including amplifiers and integrator, 14 = radioactivity recorder, and 15 = variable transformers for inlet and outlet heating and for the combustion furnace.

(about 250 ml/min). The inlet temperature is 305° while the columns are kept isothermal at 295° and pipelines between the splitter assembly and the combustion furnace are heated to 320–340°. If gas flow is used for the assay of the radioactivity, the counter tube has 2120 V and the rate meter is attenuated to give 180–600 dpm for total deflection of the recorder. If the radioactivity is counted by liquid scintillation, the vials contain 3 ml of a solution of monoethanolamine in methyl cellosolve (1:2). The time required for the collection of individual fractions is determined from the appearance of corresponding peaks resulting from the mass detector response (the delay of the radioactivity *versus* the mass elution is 20 sec). After trapping, each vial is supplemented with 15 ml of a scintillation mixture (5.5 g of 2,5-diphenyloxazole in 1000 ml of a toluene–methyl cellosolve (2:1) mixture)[9], the contents are thoroughly mixed and the radioactivity is counted in a Nuclear-Chicago Mark II liquid scintillation spectrometer with an efficiency of about 81% (Nuclear-Chicago, Des Plaines, Ill., U.S.A.). The counting efficiency was determined by the channel ratio method.

RESULTS

Separation of cholesteryl esters

The GLC procedure described above gives an excellent separation of cholesterol esters with C_{17} acids from cholesteryl palmitate and stearate (Fig. 3). However, no



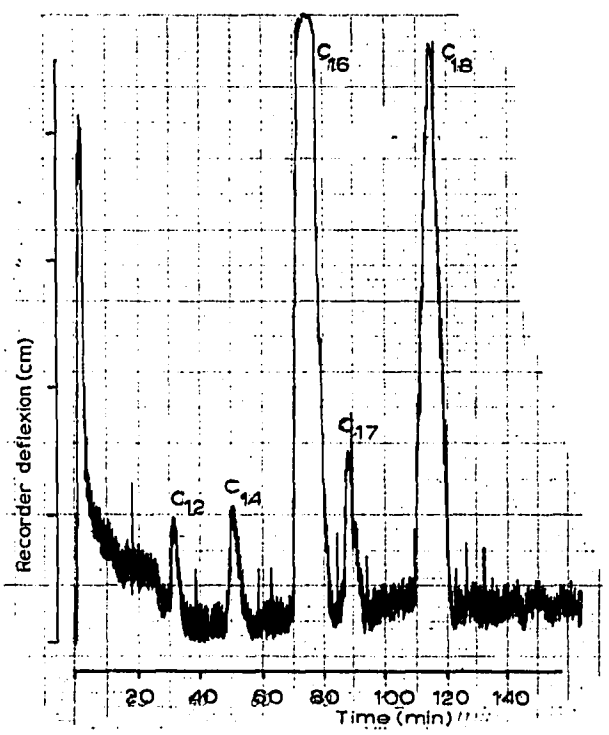


Fig. 3. Gas chromatogram of cholesteryl esters synthesized by rat liver cytosol.

Fig. 4. Radio-gas chromatogram of the same cholesteryl ester mixture as given in Fig. 3.

TABLE I

COMPOSITION OF CHOLESTERYL ESTERS SYNTHESIZED *IN VITRO* BY RAT LIVER CYTOSOL

Results were obtained with five different incubations of the same subcellular preparation.

Fatty acid component of the ester	*Elution time* (min)*	*Cpm** (mean ± S.D.)*	*Quantity detected (pmole)*	*% of total cholesteryl esters****
C_{12}	5	275 ± 43.0	2.11	1.89
C_{14}	10	356 ± 27.4	2.73	2.45
C_{16}	15	6164 ± 174.8	47.32	42.47
C_{17}	15	684 ± 52.3	5.25	4.71
C_{18}	20	5534 ± 253.3	42.49	39.26

* Time required for elution of total fraction.
** Values corrected for efficiency, as described in Results.
*** Lower esters up to and including decanate are not included.

separation of homologues with the same carbon number can be achieved. Thus cholesteryl palmitate is eluted in the same volume as cholesteryl palmitoleate and cholesteryl stearate has the same retention volume as cholesteryl oleate. The quantitative composition of cholesteryl esters in rat liver analysed by the technique described, together with the elution times of individual fractions, is given in Table I. It is apparent that one analysis of the total cholesteryl ester pattern may be completed within 200 min. The radioactivity pattern obtained by using the gas flow counter is presented in Fig. 4.

Efficiency of the system

As determined by repeated injections of [^{14}C]hexadecane, 52.2–62.9% of the total injected radioactivity is recovered by liquid scintillation. The actual efficiency for each run can be precisely determined as follows:

$$\text{Efficiency } (\%) = \frac{\Sigma F_{(n)} - (BKG \times t)}{S} \times 100$$

where $\Sigma F_{(n)}$ is the sum of radioactivities (dpm) collected in individual fractions, *BKG* is the background radioactivity (dpm/min of elution) and *S* is the total radioactivity (dpm) injected into the gas chromatograph. The collecting of fractions is started at the moment of sample injection and lower esters (up to and including cholesteryl decanate) are usually collected together in a single vial. After finishing the analysis, the effluent is trapped in ethanolamine for another 60 min. The contents of this vial are then counted and the background radioactivity is thus determined. For the determination of total radioactivity injected into the instrument, the radioactivity of an aliquot of the sample to be injected is counted.

The trapping of radioactive CO_2 in monoethanolamine is quantitative, as shown by experiments in which additional vials containing the trapping mixture were connected to the first vial.

Reproducibility

When the same mixture of radioactive cholesteryl esters was subjected to repeated analyses, good reproducibility of the procedure used was demonstrated. The reproducibility is different for different cholesteryl esters but lies within ± 10% for most of them. These data are given in Table II.

TABLE II

REPRODUCIBILITY DETERMINED FROM FIVE REPLICATE ANALYSES OF THE SAME SAMPLE

Values of individual experiments are net total cpm present in a particular fraction.

Fatty acid component of the ester	*Experiment No.*					*Relative error (%)*
	1	*2*	*3*	*4*	*5*	
C_{12}	206	261	293	308	307	15.6
C_{14}	325	348	386	337	382	7.6
C_{16}	6208	6304	6164	5869	6278	2.8
C_{17}	675	668	737	610	732	7.6
C_{18}	5240	5309	5568	5755	5798	4.6

Sensitivity

When determining the minimum radioactivity detectable by the procedure described, the background radioactivity of the system must be taken into account. This value is relatively constant (4.5 ± 2.5 cpm/min of elution). The minimum detectable radioactivity for the system using the gas flow counter is about 60 dpm/min of elution. Assuming the average elution volume of a given component and the overall efficiency of the system, the minimum detectable radioactivity for this procedure seems to be about 300 pCi.

Far more favourable values are obtained if liquid scintillation is used for the assay of radioactivity. Owing to shorter elution volumes of cholesteryl esters with lower fatty acids these compounds may be determined with far better sensitivities than esters with fatty acids containing more carbon atoms. Nevertheless, at least one third of the minimum quantity of cholesteryl 14-methylhexadecanoate detectable by the gas flow counting may be determined using liquid scintillation. These results are shown in Table III.

TABLE III

MINIMUM QUANTITIES OF INDIVIDUAL CHOLESTERYL ESTERS DETECTABLE BY THE LIQUID SCINTILLATION TECHNIQUE

Fatty acid component of the ester	*Minimum detectable quantity*	
	pCi	*pmole**
C_{12}	36	0.61
C_{14}	72	1.21
C_{16}	108	1.82
C_{17}	108	1.82
C_{18}	144	2.42

* Based on the specific radioactivity of the [^{14}C]cholesterol used.

DISCUSSION

Cholesteryl esters having high boiling points may condense in the outlet tubings if these are not sufficiently heated. This difficulty was overcome in our system by keeping the whole outlet at 320–340°. It is advisable to make the connection between the splitter assembly and the combustion furnace from one piece of stainless-steel tubing. The seal between the glass columns and the splitter assembly is another part of the system which is difficult to make. Several materials have been tested for this seal, including asbestos impregnated with the liquid phase[10]. Conical seals made from PTFE appeared to be the only solution of this problem. These seals may be easily tightened by screw-nuts to yield close-fitting connections. Because the long columns used for the separation are relatively heavy it is essential to support them by a steel frame.

It is necessary to inject the relatively small samples of cholesteryl esters obtained by biosynthesis in a minimum volume of solvent. To eliminate losses occurring during the injection of liquid samples the method of solid injection was tested. However, cholesteryl esters were decomposed by the contact with hot metal surfaces and thus an on-column injection of the sample in a minimum volume of ether seemed to be the only solution of this difficulty.

Our technique gives separation of individual cholesteryl esters superior to that described in earlier papers[3–6]. This is probably due partly to the longer columns used in our procedure and partly to the use of the new silicone phases of the OV series, which yield far better separations than the silicones used previously. It was unfortunately not possible to further increase the efficiency of the separation by decreasing the column diameter because relatively large masses of cholesteryl esters are to be injected into the gas chromatograph. It is necessary to add these large masses of non-radioactive esters to the extract to facilitate the detection of spots on thin layers. However, even our efficient GLC system is not capable of separating esters of cholesterol with straight-chain fatty acids from those with branched-chain or unsaturated fatty acids of the same carbon number. This disadvantage is not critical if cholesteryl 14-methylhexadecanoate is to be determined. Unsaturated C_{17} fatty acids are extremely rare in biological systems and the same is true for margaric acid[2]. Thus, in most cases the ester of cholesterol with a C_{17} fatty acid is cholesteryl 14-methylhexadecanoate. It is advisable, however, to check the fatty acid composition of the cholesteryl esters if an unknown material is to be analysed.

Several techniques have been used during past years for the radioactivity assay of GLC effluents (see Karmen[11] for a review). These include ionization chambers[12], scintillation counting[13] and gas flow counting[14]. Improvements to these techniques were recently suggested, including the hydrocracking of radioactive compounds for continuous radio-gas chromatography[15] and collection of effluents on a moving strip of paper[16]. Also a method has been described for continuous mixing of the effluent from the gas chromatograph with the scintillator solution[17]. However, it seems that no satisfactory and universal method has been developed so far for these purposes. For qualitative analyses we prefer continuous gas flow counting. The use of CO_2 as the quenching gas, as suggested by Strong *et al.*[18], seems to be safer than the use of inflammable quenching gases such as methane[19]. Gas flow counting, however, does not seem very suitable for quantitative analyses. For the detection of low radioactivities,

as are usual for products of enzymatic reactions, it is essential to use highly sensitive counting equipment, which involves a considerable noise on the baseline. For quantitative purposes we therefore prefer the trapping of radioactive CO_2 in monoethanolamine[9]. This technique is very sensitive, as larger fractions are collected and it also has good reproducibility. It is, however, suitable only for $^{14}CO_2$ and cannot be used for tritiated compounds.

The actual efficiency of the system as revealed by our experiments is somewhat lower than expected for the splitting ratio used. This is probably due to several factors, such as losses of radioactivity during the injection, radiochemical impurities present in the sample and decomposition of radioactive compounds during chromatography. Radioactive materials may also be continuously adsorbed on the tubing[18]. However, no increase of the background value, indicating a slow desorption of these materials, was found when working with the present system for several months. Nevertheless, it seems advisable to flush the pipelines with an appropriate solvent and to change the columns after four to five weeks of uninterrupted work.

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