# A CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE DETERMINATION OF CHOLESTEROL 14-METHYLHEXADECANOATE (CARCINOLIPIN) IN BIOLOGICAL MATERIALS

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## INTRODUCTION

Carcinolipin (cholesterol (+)-14-methylhexadecanoate[1]) has been found to be present in several biological materials[2]. This substance is carcinogenic[3] and stimulates protein synthesis *in vitro*[4]. Because of this rather unique biological activity a more thorough investigation into its presence and quantitative distribution in various animal tissues seemed to be of interest. Development of a procedure for the quantitative assay of this substance in biological material was desirable for these purposes.

A preliminary group separation of cholesterol esters from other lipids present in tissue extracts is necessary if individual esters are to be determined. This may easily be achieved by adsorption chromatography[5]. Separation of the single fatty acid esters with cholesterol represents a far more difficult problem. Liquid–solid chromatography was found suitable only for the separation of cholesterol esters with saturated higher fatty acids from those with unsaturated acids; this occurred with both thin layer[6] and paper[7] techniques. For the partition of esters with individual saturated fatty acids liquid–liquid chromatography with reversed phases is usually used. MICHALEC *et al.* reports satisfactory results obtained by such a technique on both paper[8] and thin layers[9]. KAUFMANN *et al.*[10] describe a good separation of single cholesterol esters by two-dimensional TLC*. Separation of cholesterol methylhexadecanoate from palmitate and stearate may be also achieved by our method using gradient-elution partition chromatography with reversed phases[7].

However, the complete separation of single cholesterol esters as required for quantitative purposes is not obtained by either of these techniques. The quantity of sample applied, which seems to be of a critical importance at least when using some of these methods[7,10], is a further severe disadvantage if only traces of the substance to be determined are present. Moreover, additional difficulties arise from the quantitative evaluation of individual spots in paper chromatography or TLC.

GLC of cholesterol esters does not give satisfactory separations of esters with saturated and unsaturated homologous fatty acids[11] and could therefore scarcely be expected to separate a branched-chain fatty acid ester from those with a straight chain.

Hydrolysis of cholesterol esters and separation of 14-methylhexadecanoic

* Abbreviations: GLC = Gas–liquid chromatography; TLC = thin-layer chromatography; ai-$C_{17}$ = anteiso margaric (14-methylhexadecanoic) acid; *n*-$C_{17}$ = margaric acid.

acid seemed to be the method of choice although it renders the procedure more time consuming. Furthermore, the quantitative determination of fatty acid methyl esters by GLC presents no serious problems today and has become a routine procedure in many laboratories. A method for the quantitative determination of Carcinolipin based on these principles is described in this paper.

## MATERIALS

Silicic acid for chromatography (Silikagel CH, 150/300 mesh) was a product of Spolana, Velvary, Czechoslovakia. Before use for TLC it was activated in a drying cabinet at 120° for at least 48 h. The petroleum ether used was the fraction boiling between 35 and 50°. All solvents were redistilled before use. Margaric acid was obtained from Koch-Light Laboratories, Colnbrook, England and purified by preparative GLC. (+)-14-Methylhexadecanoic acid was isolated from beef liver cholesterol esters and purified as described earlier[12]. Methyl esters of both these fatty acids were prepared by esterification with diazomethane. Cholesterol was a commercial preparation purified as the dibromide[12]. Cholesterol margarate and 14-methylhexadecanoate were synthesized from the corresponding acyl chlorides as described by KRITCHEVSKY AND ANDERSON[13]. The standard solution of cholesterol esters was prepared by dissolving 5 mg of cholesterol margarate and 2.5 mg of cholesterol 14-methylhexadecanoate in chloroform–methanol 2:1 and diluting with the same solvent mixture up to 1 l.

## PROCEDURE

### *Extraction of biological materials*

0.5 ml of human blood serum (or 1 ml of rat serum) is added dropwise with constant mixing to a mixture of 6 ml of chloroform–methanol 2:1 and 4 ml of the standard solution of cholesterol esters in a 100 ml flask. This suspension is gently and mechanically shaken for 1 h[14]. After this, 10 ml of water are added and contents of the flask are carefully mixed without excessive shaking to avoid the formation of an emulsion. The mixture is then centrifuged at 4,000 r.p.m. until a clear separation of both phases is obtained (usually 5–10 min). The lower phase is carefully aspirated through a long needle into a 20 ml syringe, transferred into a conical test tube and evaporated to dryness under a stream of nitrogen. Tissue homogenates are processed in the same way as blood serums.

When whole tissue is used for the determination, it is weighed, homogenized in a 20-fold volume of chloroform–methanol 2:1 (containing 4 ml of the standard solution of cholesterol esters) in a blendor and the resulting suspension filtered. 20 ml of water are then added to the filtrate and samples processed in the same way as blood serum.

### *Separation of cholesterol esters*

Unbound layers of silicic acid are prepared by spreading this material on glass plates 12 cm wide and 20 cm long with a glass rod as described by MOTTIER AND POTTERAT[15]. The residues of the lipid extracts in the conical test tubes are dissolved in 0.3–0.5 ml of petroleum ether–ether 1:1 and applied on the thin layers by means of

a micropipette. Four samples are usually run on one plate. Layers are dried at 120° in a drying cabinet for 10 min and then developed with petroleum ether–ether 95:5. Layers are then dried at room temperature for 1–2 h and cholesterol esters detected by their bluish fluorescence in ultraviolet light from a Philips Philora HPW 125 mercury lamp. Suitable standards of cholesterol esters are run on the same plate as the unknown samples to simplify the detection. The portions of the thin layers containing cholesterol esters are marked, the silicic acid is sucked off and eluted with 5 ml of ether using the elution device shown in Fig. 1. Samples are evaporated to dryness under a stream of nitrogen in the same tubes as were used for the elution.

*Hydrolysis of cholesterol esters*

4 ml of 96 % ethanol followed by 1 ml of 33 % potassium hydroxide in water (freshly prepared) are added to each test tube and this mixture heated at 55° in a constant-temperature bath for 20 min[16]. After cooling 2 ml of petroleum ether are added, the test tubes are stoppered and their contents thoroughly shaken The upper phase is sucked off with a syringe, 2 ml of petroleum ether are added to the ower phase and the extraction repeated twice more in the same way. The petroleum

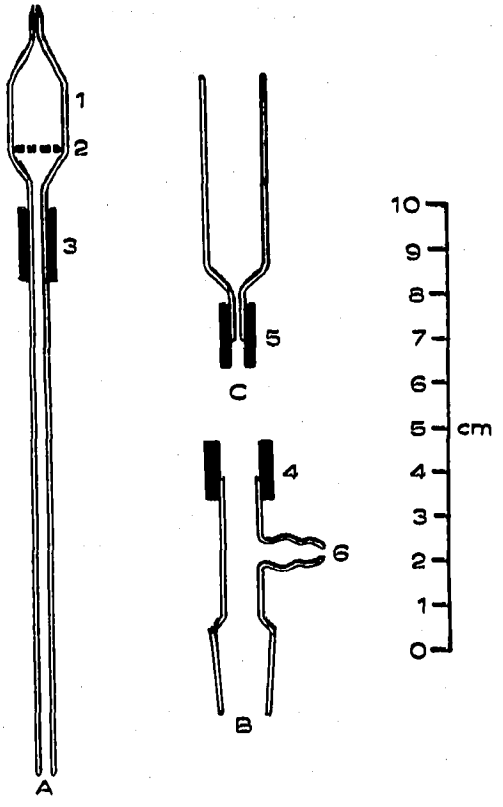


Fig. 1. Device for sucking-off and elution of silicic acid from thin layers. Lower end of the sucking pipette (A) is attached to a vacuum pump and silicic acid sucked into the bulb (1) equipped with a porous glass disc (2). The pipette is turned over and inserted by its polyethylene ring (3) to fit closely into the enlarged polyethylene part (4) on the top of the glass connecting piece (B). The whole device is then put into a test tube equipped with a conical joint (12/21). The funnel (C) is attached by means of a polyethylene tube (5) to the top of the sucking pipette (A) and vacuum pump connected to the side tube (6) of the connecting piece (B). Solvent is then poured into the funnel (C) and sucked-off by vacuum through the layer of silicic acid in the bulb (1) of the sucking pipette (A).

ether extracts are pooled, when necessary, evaporated to dryness under a stream of nitrogen and used for the determination of cholesterol if desired. The aqueous phase is diluted with 3 ml of water and acidified with 5 *N* hydrochloric acid to acid reaction (indicator paper). It is then extracted by three successive portions (2 ml each) of petroleum ether in the same way as above. Extracts are pooled and an ethereal solution of diazomethane added. After 10 min the volume of the samples is reduced to 0.5–1 ml under a stream of nitrogen and the samples are transferred by a syringe equipped with a long needle from normal test tubes to small conical tubes (diameter 5 mm, length 20–30 mm). Solvent residues are evaporated to dryness under a stream of nitrogen.

*GLC of fatty acid methyl esters*

The residues in the conical tubes are quantitatively dissolved in 5–10 $\mu$l of ether and the whole sample injected into the gas chromatograph. The chromatogram is run until methyl arachidonate has emerged from the column. For the present experiments a Chrom II Gas Chromatograph (Laboratorní přístroje, Prague) was used with columns 2 mm in diameter and 800 mm long filled with Chromosorb W, 80/100 mesh, coated with 15 % Apiezon L grease. The column temperature was 198°, carrier gas nitrogen, inlet pressure 0.55 atm., hydrogen 40 ml/min, air 600 ml/min, and sensitivity 1:50. One analysis was completed in about 70 min (see Fig. 2).

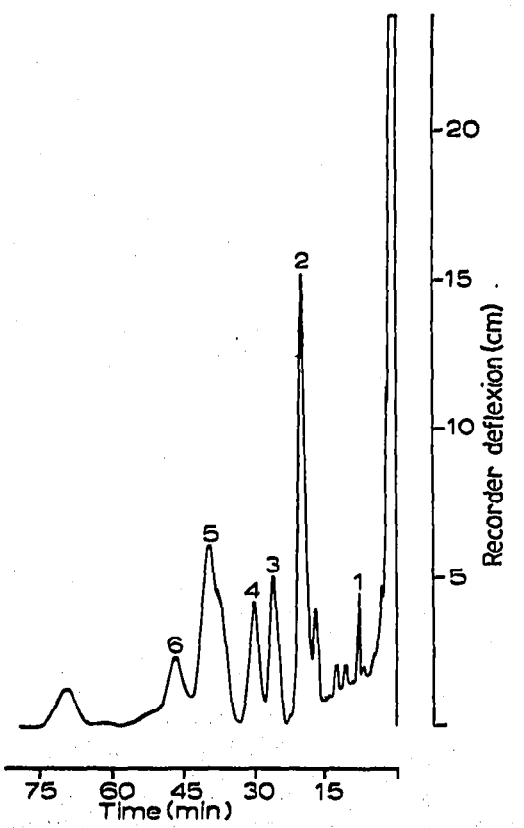


Fig. 2. Gas chromatogram of fatty acid methyl esters present in cholesterol esters from rat liver tissue (1 g). The composition of individual peaks is as follows: 1 = myristate; 2 = palmitate; 3 = 14-methylhexadecanoate; 4 = margarate; 5 = oleate; 6 = stearate.

*Calculation*

The base line is constructed by leading a tangent from the lowest point on the curve between methyl palmitate and 14-methylhexadecanoate, and methyl margarate and oleate. Peak height in millimetres of methyl margarate and methyl 14-methylhexadecanoate is then measured from this base line and the peak height of methylhexadecanoate divided by that of margarate[16]. This ratio is multiplied by 20 ($\mu$g of cholesterol margarate in the volume of standard solution added). The quantity of cholesterol-14-methylhexadecanoate added with the standard solution (10 $\mu$g) is subtracted from this result. This final value is the quantity ($\mu$g) of cholesterol 14-methylhexadecanoate present in the sample analyzed.

## RESULTS

*Linearity of the detector response*

Known amounts of a solution of cholesterol 14-methylhexadecanoate were mixed with the solvent mixture used for the extraction and 0.5 ml of the same human blood serum added. Samples were processed by the method described and after GLC the ratio ai-$C_{17}$/$n$-$C_{17}$ was determined. There was a linear response up to at least 100 $\mu$g of cholesterol 14-methylhexadecanoate added, as seen in Fig. 3.

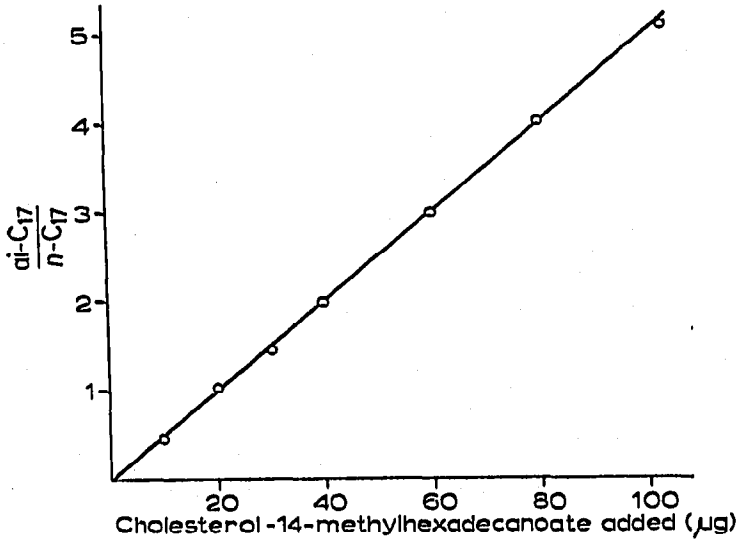


Fig. 3. Relation between the quantity of Carcinolipin added and the ai-$C_{17}$/$n$-$C_{17}$ ratio.

Methyl esters of both margaric and 14-methylhexadecanoic acid gave the same response to the flame ionisation detector used. This was checked by a mixture of known composition of both these esters. This check was repeated every time after conditions of analysis had been changed (exchange of columns).

*Reproducibility of results*

Samples (0.5 ml) of the same human blood serum were analyzed by two technicians and results obtained are given in Table I.

TABLE I

REPRODUCIBILITY OF THE CHOLESTEROL 14-METHYLHEXADECANOATE DETERMINATION IN SAMPLES OF THE SAME HUMAN BLOOD SERUM

| *Technician* | *Number of determinations* | *Mean (μg)* | *μg found in the range* | *Probable error* |
|---|---|---|---|---|
| A | 5 | 19.9 | 18.9–20.9 | ±0.65 |
| B | 6 | 20.1 | 19.2–20.8 | ±0.62 |

*Recovery*

Known quantities of cholesterol 14-methylhexadecanoate standard solution were mixed with the solvent mixture used for extraction and 0.5 ml of the same human blood serum added. Results of these experiments are given in Table II.

TABLE II

RECOVERY OF CHOLESTEROL 14-METHYLHEXADECANOATE ADDED TO HUMAN BLOOD SERUM SAMPLES

| *Experiment No.* | *μg of cholesterol methylhexadecanoate* | | *% Recovery* |
|---|---|---|---|
| | *Added* | *Found* | |
| 1 | 20.0 | 19.7 | 98.5 |
| 2 | 40.0 | 41.4 | 103.4 |
| 3 | 60.0 | 58.3 | 97.2 |
| 4 | 80.0 | 78.8 | 98.4 |
| 5 | 100.0 | 103.3 | 103.3 |

## DISCUSSION

Losses of material analyzed seem to be inevitable during the rather complicated procedure. It was felt desirable, therefore, to add internal standards as early as possible. Addition of the standard solution at the first stage of the method avoids errors due to losses during all subsequent steps, in particular during TLC where a complete recovery could hardly be expected. Extraction of lipids with chloroform–methanol mixtures is frequently used and seems to yield quantitative recoveries[17]. Our results support this opinion.

Cholesterol margarate seems to be very suitable as an internal standard since we have found no detectable quantities of this ester in biological materials studied so far (human blood serum, rat serum and tissues, subcellular fractions of rat liver cells.) However, this point should be checked before an unknown material is analyzed. Addition of known quantities of cholesterol 14-methylhexadecanoate into internal standards was found advantageous since small amounts of this substance are usually

present in most biological materials which would yield only low peaks in GLC records. Considerable errors may arise in the quantitative evaluation of such peaks.

For rapid routine separations of fatty acid methyl esters the use of polar phases is usually recommended[18]. However, methyl palmitoleate which is present in relatively high quantities in most biological materials has a very similar retention volume on polyester columns to methyl 14-methylhexadecanoate. Non-polar phases must therefore be used to obtain satisfactory separations. However, the use of short and narrow columns gives relatively short elution times.

Several methods are used for the quantitative evaluation of GLC records. When complicated mixtures are analyzed the determination of the peak area either by manual techniques or by electronic integration seems to be the most precise procedure[19]. The peak height is dependent not only on the mass of the substance present, but also on its elution volume[20]. However, if peaks of closely similar retention times are to be evaluated this disadvantage has to be avoided practically. However, if relatively non-polar material such as fatty acid methyl esters are chromatographed symmetrical peaks are obtained[20] and the measurement of the peak height then gives satisfactory results. It is also the simplest procedure of obtaining quantitative results.

The procedure described here may be used for the determination of Carcinolipin in any biological material if at least 2.5–3 $\mu$g of this substance is present in the sample analyzed. The upper limit rather depends upon the quantity of cholesterol palmitate contained in the material. If high amounts of this ester are present a steep slope of the base line in the GLC record results and relatively high errors are then involved in the determination of peak heights. However, base line slopes up to about 30° were found to have no significant effect on the accuracy of results.

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SUMMARY

A procedure is described for the quantitative determination of Carcinolipin (cholesterol 14-methylhexadecanoate) in 0.5–1 g samples of biological materials (blood serum and tissues). The material is extracted with chloroform–methanol 2:1, internal standards of cholesterol margarate and 14-methylhexadecanoate are added and cholesterol esters separated by thin layer chromatography on silicic acid using petroleum ether–ether 95:5 for the development. Cholesterol esters are hydrolyzed; the free fatty acids extracted, esterified with diazomethane and separated by gas–liquid chromatography. The quantity of Carcinolipin present is calculated on the basis of peak heights of methyl margarate and 14-methylhexadecanoate. The method described shows a good reproducibility and may be used for the determination of 2.5–100 $\mu$g of Carcinolipin in the sample.

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