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Note

Direct quantitation of free cholesterol in total serum lipid extracts by computer-assisted gas—liquid chromatography

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The estimation of total cholesterol in serum can generally be made in two different ways: (a) by direct analysis [1—3], with certain drawbacks when hemolytic sera are used [1, 2], and (b) by using serum extract, notably the Abell—Kendall extract [4], which can be analyzed by a number of different, mostly chromatographic, techniques [5—7]. Amongst these techniques the gas chromatographic procedure is still one of the simplest, most sensitive and fastest methods by which quantitations are generally made by the internal standard procedure with and without silylation of the compound [8]. Some of the chromatographic methods also have the advantage of allowing the determination of both free and esterified cholesterol [7]. In this study it was found that free cholesterol can be quantified directly from a Folch extract of serum or any other material, e.g. of ascites tumor cells and their subcellular fractions, without interference of the other lipids. By using different extraction solvents including saponification of the material [4] we were able to determine the amount of cholesterol originally present in both its free and esterified form. These data are compared with the data obtained by two other procedures [1—3].

MATERIAL AND METHODS

Reagent grade cholesterol and 3,5-cholestadiene were purchased from Serva, Heidelberg, G.F.R., and used as standard solution for the gas—liquid chromatographic (GLC) analyses in comparison to the other procedures. In general, standard solutions of cholesterol between 1 and 4 mg/ml dissolved in chloroform or isopropanol were used. The enzymatic determinations were performed with a commercial kit (Boehringer, Mannheim, G.F.R.) according

to the description of Röschlau et al. [3] with isopropanol as solvent; the colorimetric measurements were made by the Liebermann—Burchard reaction [1, 2].

Samples

Serum was either prepared fresh from horse blood obtained from a slaughter house in Speyer, G.F.R., or was bought in a frozen state from Boehringer, Mannheim, G.F.R. The sera were kept frozen until used for the lipid extraction experiments. Lipid extracts of ascites tumor cell material were kept at 35°C under nitrogen.

Sample preparation

For free cholesterol determinations by our GLC method, serum lipid extraction was performed according to the Folch procedure [9] and by using butanol—diisopropyl ether [10] or isopropanol. Aliquots of 0.5—1 μl of the extract with a concentration of not less than 0.001% were injected into the gas chromatograph with the aid of a 1-μl Terumo micro syringe without any previous treatment. For analyses of total cholesterol the serum samples were saponified and extracted according to the Abell—Kendall method [4].

Gas chromatography

The analyses were performed on a Becker gas chromatograph, Model 419 (Packard Instruments), to which a CDS-111 data system (Varian, Darmstadt, G.F.R.) was connected. A 1.80 m × 2 mm stainless-steel column was packed with 3% OV-17 on Chromosorb Q (100—120 mesh), nitrogen was used as carrier gas. The gas flow-rate was about 55—58 ml/min and the column temperature 255°C. The temperature of the injector was 300°C and of the detector 280°C. The sensitivity of the electrometer ranged between 0.1 and $4 \cdot 10^{-10}$ A. Before each series of analyses the instrumental conditions were checked by using the standard cholesterol solution. The determinations of a particular sample were made at different sensitivities of the detector at least in duplicate or until reproducibility of the results was attained. Completion of a single analysis was usually reached after 10 min.

Calculation

The calculation factor (CF) is defined according to the formula

$$CF = \frac{\text{amount } (\mu g) \times \text{amount sample } (\mu l)}{\text{area}} \times 10{,}000$$

Having determined the calculation factor the amount of a sample can be obtained by rearranging the above equation

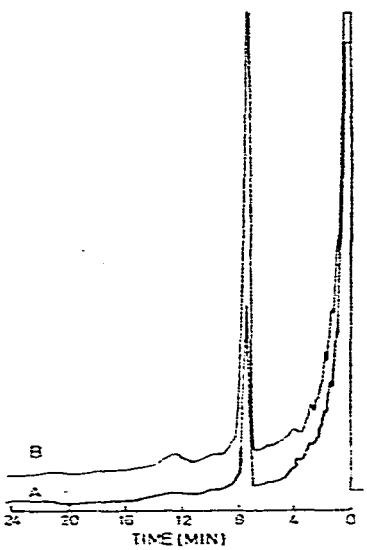
$$\text{Amount } (\mu g) = \frac{\text{area} \times CF}{\text{amount sample } (\mu l)} \times \frac{\text{scalar}}{10{,}000}$$

Since the expression scalar/10,000 is for instrumentation purposes, the equation can be simplified to amount (μg) = area × CF, if the amount of sample (μl) is equal to 1.

If the sample volume is different from 1 this has to be considered in the calculation. Since in most cases the calculated values are a fraction of 1 we have set the calculator print-out to four decimal points for reasons of greater accuracy. The print-out is given in g per 100 ml. Further details of the calculation procedure are written in the instructions for the instument.

RESULTS AND DISCUSSION

The advantage of our procedure is based on the observation that cholesterol can be determined gas chromatographically from Folch lipid extracts directly without any interference from other components. Therefore, there is no need to separate cholesterol from other lipids by, for example, thin-layer chromatography which has been done in the past prior to its quantitation. Fig. 1 shows a typical gas chromatogram of a serum lipid extract. Several peaks arising shortly after the solvent peak and which were of no interest in this context have not been identified. In a few samples we observed a peak with the retention time of 3,5-cholestadiene. We have reason to believe that this compound must be inherent within the particular sample and cannot be a degradation product of cholesterol during GLC analysis since we never observed such a peak when we analyzed the cholesterol standard. From these chromatograms cholesterol was quantified by the external standard procedure using the Chromatography Data System (CDS) 111. To perform the calcula-



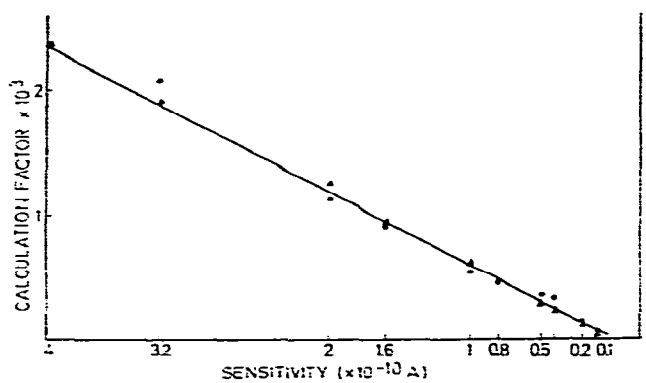


Fig. 1. Gas chromatograms of a total lipid extract of horse serum obtained by the Folch procedure. The samples were injected into the column (A, 0.5 μl; B, 1 μl) at a sensitivity of $0.2 \cdot 10^{-10}$ A, and analyzed according to the conditions described under Material and Methods.

Fig. 2. Dependence of the calculation factor upon the sensitivity of the electrometer. A range between 0.1 and $4 \cdot 10^{-10}$ A was tested using a cholesterol standard solution of 4 μg/μl. (●), Values for the 1-μl injections; (▲), data for the 0.5-μl injections. $y = 5.97 \cdot 10^{-13} x + 1.11 \cdot 10^{-5}$.

tions we determined the calculation factors which at constant amount and volume of the sample become dependent only upon the sensitivity of the electrometer since the area recorded is a function of the sensitivity.

In Fig. 2 the relationship between these two parameters is shown for 1- and 0.5-μl injections, giving an inverse correlation with a high degree of accuracy (r = 1.00). By using this standard curve cholesterol determinations can be made from any sample with a cholesterol concentration as low as 10 ng/μl by computerizing the appropriate calculation factor relative to the sensitivity applied. During the numerous injections made on one GLC column we have never observed an increase in the background signal. Occasionally, a few peaks with long retention times appeared which, however, did not impair the subsequent analyses.

In order to assess the accuracy and reproducibility of our procedure we have analyzed a cholesterol standard and horse serum extracted by different solvents and compared our data with the results obtained by two other procedures (Table I). Free serum cholesterol can be determined gas chromatographically both from Folch and isopropanol extracts. Total cholesterol analyses can be made from Abell—Kendall extracts by all three procedures. The

TABLE I

CHOLESTEROL QUANTITATION OF A STANDARD SOLUTION AND VARIOUS HORSE SERUM EXTRACTS BY GLC COMPARED TO TWO OTHER METHODS

Sample	Gas chromatographic analysis			Enzymatic procedure			Liebermann—Burchard reaction		
Cholesterol standard (yield, %)	95.9	± 7.0	(14)*	93.30	± 8.6	(17)	95.1	± 11.0	(18)
Serum extracts (mg/ml)									
Folch	0.13	± 0.017**	(9)	0.94	± 0.05	(4)	0.80	± 0.13	(6)
Abell—Kendall	1.22	± 0.23	(2)	1.12	± 0.23	(4)	1.15	± 0.18	(4)
Isopropanol	0.136	± 0.033	(3)	0.149	± 0.014	(3)	0.139	± 0.010	(3)

*Duplicate analyses of the number of samples given in parentheses.
**Free cholesterol.

TABLE II

COMPARISON BETWEEN THE GLC AND ENZYMATIC CHOLESTEROL QUANTITATION OF VARIOUS FOLCH LIPID EXTRACTS OF ASCITES TUMOR CELLS

The data are expressed as a percentage of the enzymatic analyses (= 100%).

Sample	Yield (%)
Ascites cells	
Homogenate	99.27 ± 9.15 (7)*
Microsomal fraction	99.38 ± 11.66 (4)
Culture cells	99.68 ± 6.43 (6)

*Number of experiments analyzed in duplicate.

data show a good correlation between the different methods. In Table II results are presented on the analysis of various Folch lipid extracts of ascites tumor cells by our procedure in comparison with the enzymatic method. Again, the values show a high degree of correlation, making our procedure an uncomplicated valuable contribution to the determination of cholesterol from any extract of biological material.

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