The quantitative determination of cholesterol in brain lipid extracts using gas–liquid chromatography

The large number of methods for the quantitative assay of cholesterol may be divided into three main groups. In the least sensitive procedures, cholesterol is measured gravimetrically after it has been separated from other lipids in an extract. The sterol may be isolated as a digitonide or by using column chromatography[1,2]. More sensitive assays depend upon the property of cholesterol to react with a variety of strong acids to give coloured products[3–6]. The best known of these is the LIEBERMAN-BURCHARD reaction[3]. Among the infinite number of modifications of this procedure, probably the most sensitive is the fluorometric adaption of ALBERS AND LOWRY[7]. The fluorometric peak has been shown to remain much more stable than the colorimetric one and the assay can measure samples down to 0.1 μg of cholesterol. Fluorimetry is not without problems, however, and furthermore in most assays, the cholesterol must be separated from the other lipids (as the digitonide; *e.g.*, see ref. 5).

Recently cholesterol has been assayed using gas–liquid chromatography (GLC). The first separation of sterols by GLC was described by VANDENHEUVEL *et al.*[8] and these workers have since demonstrated excellent separation of several sterols using similar techniques[9]. FUMAGALLI *et al.*[10] used this method to determine the ratio of cholesterol to desmosterol in developing brain as well as in certain pathological conditions. In their procedure, sterols are chromatographed on 6 ft. glass columns and thus have a relatively long retention time. Furthermore, the peaks are not well defined unless the sterols are converted to the trimethylsilyl derivatives[11]. Using a high gas flow with a very short glass column and adding cholestane as an internal standard, SCHMIDT AND MATHER[12] assayed serum cholesterol quantitatively by gas chromatography. They used cholestane as an internal standard and compared the ratio of the peak heights of cholestane and cholesterol in their unknown samples to ratios obtained with known standard mixtures. WILLIAMS *et al.*[13] have subsequently examined this method and found a much better reproducibility when they compared ratios of peak areas using a disk integrator. Employing a shorter column and a very high carrier gas flow, the method has now been adapted for the rapid and simple determination of cholesterol in lipid extracts of cerebral tissue.

Brain tissue was extracted by a modified FOLCH procedure[14,15] with chloroform–methanol (2:1, v/v) and the volume of the washed lower phase carefully measured. Suitable aliquots of the lower phase, containing 100–200 μg of cholesterol, were dried in a stream of nitrogen at 60° in a small vial. To each dried aliquot, 200 μl of a 0.100 % solution of cholestane was added. Samples of 1–3 μl of this solution were injected into the gas chromatograph. Chromatography was performed with an F & M Model 400 Gas Chromatograph using a 9 in. U-shaped glass column, of 0.3 mm I.D. The column was packed in the middle 4.5 in. with 3.8 % SE-30 on Diatoport S (80–100 mesh). The temperatures utilized were: column 235°; injection port 250°; detector 250°. When the carrier gas flow was 100 ml/min (40 p.s.i.g.), cholestane and cholesterol appeared as two distinct, sharp peaks within 45 sec of sample injection (see Fig. 1).

Cholesterol content was determined by comparing the cholestane–cholesterol peak height ratio to the ratio of peak heights from known standard mixtures. These were prepared by carefully weighing cholestane and cholesterol (Applied Science Labs.) in a series of different proportions (*e.g.*, 1:1, 2:1, etc.). The samples are made

up in chloroform to a final concentration of about 1 mg/ml. The cholestane internal standard was made by dissolving 10 mg in 10 ml of chloroform. It was necessary to keep this solution in a tightly stoppered flask and to prepare a fresh solution frequently in order to avoid errors due to evaporation.

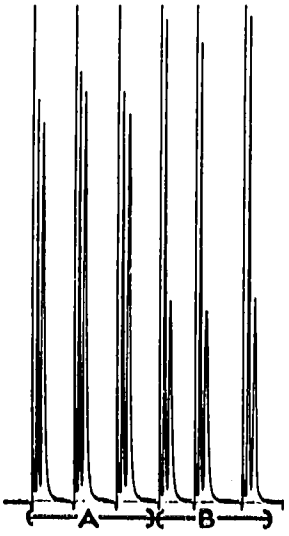

Fig. 1. Gas–liquid chromatography of cholesterol in lipid extracts. All samples chromatographed on an F & M Model 400 gas chromatograph 9 in. U-shaped glass column, 0.3 mm I.D., packed with 3.8 % SE-30 on Diatoport S (80–100 mesh). Column temperature 230°. Injection port 250°. Detector 250°. Hydrogen flame ionization detector. Carrier gas, helium at 100 ml/min. (A) Standard mixture cholestane–cholesterol, weight ratio = 0.478. (B) Standard mixture cholestane–cholesterol, weight ratio = 1.042.

Chromatography of the total mixture of brain lipids in the lower phase of FOLCH-type extracts resulted in considerable tailing of the cholesterol peak, due to the presence of other non-volatile lipids. Furthermore, after 50–75 such samples had been chromatographed, the column performance was usually so affected that the peak height ratios of the standard mixtures began to rise and the results were no longer reproducible. In order to avoid the rapid deterioration of the column, dried aliquots of the lower phase were dissolved in chloroform and passed through a small (0.5 g) silicic acid column which was then eluted with about 10 ml of chloroform–methanol (50:1, v/v). The entire eluate (chloroform and chloroform–methanol) was dried and the cholestane in chloroform added as the internal standard. Recovery of standard cholesterol was 97 % from these columns (see Table I). Samples which had been previously chromatographed on silicic acid showed little or no tailing on GLC and allowed the operator to inject a new sample into the column every 90 sec (see Fig. 1). The lifespan of columns used only for the more purified samples was several hundreds of injections.

The sensitivity of the present method compares favourably with that of the fluorometric procedure of ALBERS AND LOWRY[7] in that a 1 μl sample of the standard mixture contains about 1 μg of cholesterol and gives a good detector response. The method has been scaled down by a factor of 10 by simply diluting the standards and decreasing the attenuation (from 10^2 to 10 on the F & M Model 400). In this manner

TABLE I

QUANTITATIVE DETERMINATION OF CHOLESTEROL BY GAS–LIQUID CHROMATOGRAPHY

Sample[a]	*μg cholesterol*[b] *assayed*	*Recovery* (%)
Cholesterol, 230 μg	231 ± 3	100
Cholesterol after silicic acid column chromatography	223 ± 7	97
Lipid extract (ox brain)	60.5 ± 3	
Lipid extract + 95 μg cholesterol	154 ± 7	98

[a] Sample dissolved in 200 μl chloroform containing 200 μg cholestane as the internal standard. Three aliquots of 1–3 μl from each sample were chromatographed.

[b] Values represent the mean (± S.D.) for six separate samples.

we have been able to assay the small quantities of cholesterol in 1 ml samples of CSF (0.1–0.7 μg, depending on age, etc.).

WILLIAMS *et al.*[13] present evidence that cholesterol–cholestane ratios in their system may be determined more accurately using a disk integrator to measure peak areas instead of peak heights. Lacking this instrument, we have measured peak heights by reading units measured directly from the chart paper. The procedure is much faster than calculating peak areas without instrumentation. Table II indicates that the method is also considerably more reproducible than WILLIAMS *et al.*[13] suggest. The high carrier gas flow and very short retention time may account for part of this difference.

TABLE II

REPRODUCIBILITY OF PEAK HEIGHT RATIOS FOR WEIGHED STANDARD MIXTURES OF CHOLESTANE AND CHOLESTEROL

Cholestane–cholesterol	*Standard mixtures*						
	1	*2*	*3*	*4*	*5*	*6*	*7*
Weight ratio	3.290	1.643	1.095	0.822	0.548	0.411	0.274
Peak height ratio[a]	6.65 ± 0.13	3.40 ± 0.02	2.23 ± 0.03	1.650 ± 0.020	1.084 ± 0.008	0.821 ± 0.006	0.550 ± 0.011
Range of values for peak height ratio	4.2%	1.7%	3.8%	2.4%	1.6%	0.9%	2.5%

[a] Values represent mean ratios of 5 consecutive chromatograms (± S.D.).

Table II compares the peak height ratios with weight ratios for standard mixtures of cholestane and cholesterol over a wide range of values. The range, calculated from five consecutive injections varies more when there is a large difference in the peak heights. When cholestane and cholesterol are present in nearly equivalent proportions, however (*e.g.*, 2:1 to 1:2) the range of values for peak height ratios is small enough to be insignificant in most cholesterol determinations.

Brain tissue from adult animals contains only minute amounts of esterified cholesterol. Total cholesterol determinations can be made, however, if the samples

are hydrolysed in 2 *N* KOH in 50 % ethanol and subsequently extracted into light petroleum (boiling range 60–80°) before the internal standard is added.

The cholesterol content of lipid extracts may thus be measured rapidly, simply and reproducibly using gas–liquid chromatography.

The author is indebted to Miss M. A. LaRAMÉE for technical assistance. This work was supported in part by Operating Grant MA 1602 from the Medical Research Council of Canada. The author is a fellow of The Helen Hay Whitney Foundation.

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Received August 29th, 1966