(22) J. O. Hoppe, and S. Archer, *Am. J. Roentgenol.*, **69**, 630 (1953).
(23) C. Hansch, A. R. Steward, S. M. Anderson, and D. L. Bently, *J. Med. Chem.*, 11, 1 (1968).
(24) J. O. Hoppe, in "Medicinal Chemistry," vol. 6, E. E. Campaigne and W. H. Harting, Eds., Wiley, New York, N.Y., 1963, p. 343.

ACKNOWLEDGMENTS

The author thanks Dr. W. S. Bucke and Dr. A. L. Kunz for valuable discussion and support, Barbara Behrmann and Leonard Klein for technical assistance, and Wilma Lotts for help in manuscript preparation.

Quantitative GLC Analysis of Sterols in Biological Samples

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Received May 23, 1977, from the *College of Pharmacy, Faculty of Health Professions, Dalhousie University, Halifax, Nova Scotia, Canada B3H 3J5.* Accepted for publication December 6, 1977.

Abstract □ A GLC method for the quantitative analysis of cholesterol, β-sitosterol, stigmasterol, campesterol, 7-dehydrocholesterol, and dihydrocholesterol in biological samples was developed to screen serum and lipid extracts of heart and liver tissue for these sterols precisely. The addition of the internal standard, cholestane, at the beginning of the procedure led to a reduction in the required sample size and the elimination of several steps. The only critical measurements are those of the biological samples and internal standard.

Keyphrases □ Sterols, various—GLC analyses in biological fluids and extracts □ GLC—analyses. various sterols in biological fluids and extracts

A study involving the feeding of cholesterol and other sterols to large numbers of chicks required a simple, precise method for the qualitative and quantitative analysis of the sterol content of serum, heart, and liver samples. The commonly employed colorimetric methods are subject to several limitations, not least of which is the lack of discrimination among two or more sterols in a single sample (1).

Several GLC methods were reported for the determination of serum cholesterol (2–8); however, a procedure was required to determine quantitatively cholesterol and other sterols in a single sample. Additionally, a small sample size and the least number of manipulations consistent with precise and accurate analysis were desirable objectives.

EXPERIMENTAL

Materials and Chemicals—All solvents were reagent grade. Cholestane[1] and the sterols were dissolved in chloroform, and their purity was checked by GLC. Accuracy was monitored by the daily use of commercial controls[2].

Apparatus—Chromatographs A[3] and B[4], equipped with dual hydrogen flame-ionization detectors and glass columns (3 mm × 2 m), were connected to a recorder fitted with an integrator[5] or an automatic programmable integrator[6] Nitrogen was the carrier gas. All gas flows were adjusted according to recommendations of the manufacturer.

The injection port and detector were set at 300°. Columns were packed with 3% OV-17 on 100–120-mesh Gas Chrom Q[7], 3% SE-30 on 100–120-mesh Gas Chrom Q[8], or 3% SP-2401 on 100–120-mesh Supelcoport[9]. Column temperatures (250–280, 280–300, or 240–250°, respectively) were adjusted to give retention times of less than 10 min for cholesterol. All analyses were isothermal, and the attenuation was adjusted to give near full-scale deflection for the internal standard.

Serum Analysis—Fresh serum (0.1 ml), cholestane standard (200 mg/100 ml, 0.1 ml), 33% KOH aqueous solution (0.2 ml), and ethanol (2.0 ml) were measured into a screw-capped test tube (13 × 100 mm). After mixing[10], the tubes were placed in a shaking water bath[11] at 60° for 2 hr. Following hydrolysis, water (0.5 ml) was added and the cholesterol and cholestane were extracted with hexane (2.0 ml). The hexane fraction was subjected directly to GLC analysis.

Tissue Extracts—Total lipid was determined quantitatively by extracting the weighed hearts and livers according to the method of Bligh and Dyer (9). The lipid subsequently was dissolved in chloroform (5 ml), 0.5 ml was immediately transferred to a screw-capped test tube, and the chloroform was evaporated in a stream of dry nitrogen. The analysis was continued as for serum, care being taken that the amount of lipid was small enough (less than 200 mg) to be saponified by the amount of potassium hydroxide being used. Values were then related to the weight of the tissue lipid.

GLC Analysis—GLC standards containing known amounts of cholestane, cholesterol, and/or β-sitosterol, campesterol, stigmasterol, dihydrocholesterol (cholestanol), and 7-dehydrocholesterol were prepared and analyzed[4,5] at the beginning and end of each day. A correction factor was determined by dividing the sterol concentration found by GLC with the known concentration. Sterol concentration of the serum and tissue samples was determined as follows:

Table I—Analysis of Control Serums

Control Serum	Number of Analyses	Mean ± *SE*
1[a]	41	174.7 ± 2.0
2[b]	37	135.8 ± 3.0

[a] Choles-Trol; reported mean value was 196/100 ml when assayed by the Anderson and Keys modification of the Abell method. [b] Moni-Trol I; reported mean value was 151 mg/100 ml when assayed by the Abell method.

[1] Aldrich Chemical Co., Milwaukee, WI 58233.
[2] Choles-Trol and Moni-Trol I, Dade Division, American Hospital Supply Corp., Miami, FL 33152.
[3] Hewlett-Packard model 5710A.
[4] F&M Scientific 700 laboratory chromatograph.
[5] Fisher Recordall series 100 fitted with disk chart integrator.
[6] Hewlett-Packard model 3380A.
[7] Chromatographic Specialties, Brockville, Ontario, Canada.
[8] Applied Science Laboratories, State College, PA 16801.
[9] Supelco, Inc., Supelco Park, Bellefonte, PA 16823.
[10] Vortex-Geni, Fisher Scientific Co.
[11] Magni Whirl model MSB-1122AA-1.

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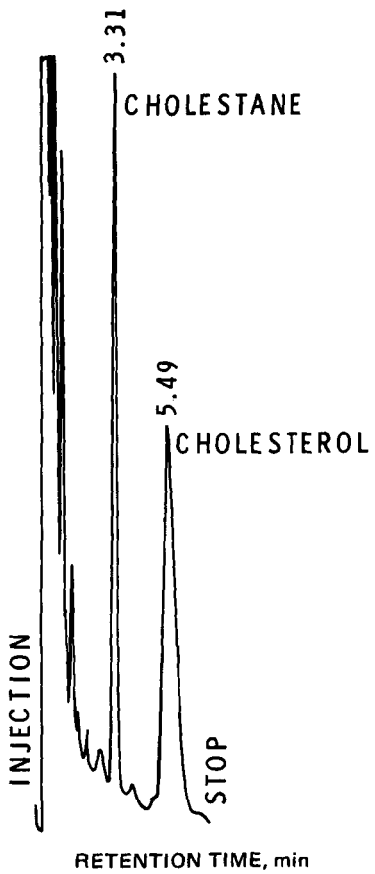

Figure 1—*GLC analysis of heart cholesterol using 3% SE-30 and Chromatograph A.*

$$\text{sterol (mg/100 ml)} = \frac{\text{cholestane concentration (ml/100 ml)}}{\text{counts of GLC peak (cholestane)}} \times \frac{\text{counts of GLC peak (sterol)}}{\text{correction factor}} \quad \text{(Eq. 1)}$$

GLC standards also were injected[3] to program the integrator[6] and then to check the accuracy of the equipment. The integrator subsequently printed the sterol concentrations, in terms of milligrams per 100 ml in the original sample, for all subsequent injections.

RESULTS AND DISCUSSION

Commercially available cholesterol control serums were analyzed with each batch of samples. Values obtained over 15 months with two gas chromatographs, three column phases, and 10 columns are summarized in Table I and testify to the reproducibility of this procedure.

Columns packed with SE-30 (Fig. 1) or OV-17 (Fig. 2) were used interchangeably. While relative retention times differed, there was no evident interference from other components (Fig. 3), except during experiments involving 7-dehydrocholesterol and dihydrocholesterol. Both of these sterols had retention times nearly identical to cholesterol under the experimental conditions. For these sterols, columns packed with SP-2401 were employed (Figs. 4 and 5).

Since a rapid, precise method was needed to conduct numerous, routine determinations of total cholesterol and/or other sterol levels, a programmable automatic integrator[6] was employed. With this apparatus, repeated injections of the same sample varied by less than 1%. The earlier use of a disk-type integrator[5] required more instrument tending and value judgment by the operator; consequently, variations of 5% were observed between repeated injections.

Cholesterol levels as determined by GLC are usually lower than those determined by the standard colorimetric methods, partly because of the greater specificity of the GLC technique (10, 11). The column absorption phenomenon noted previously (2, 8, 11) was observed in this study, even after conditioning and many injections of cholesterol. Consequently, a correction factor, based on the analyses of standards, was determined

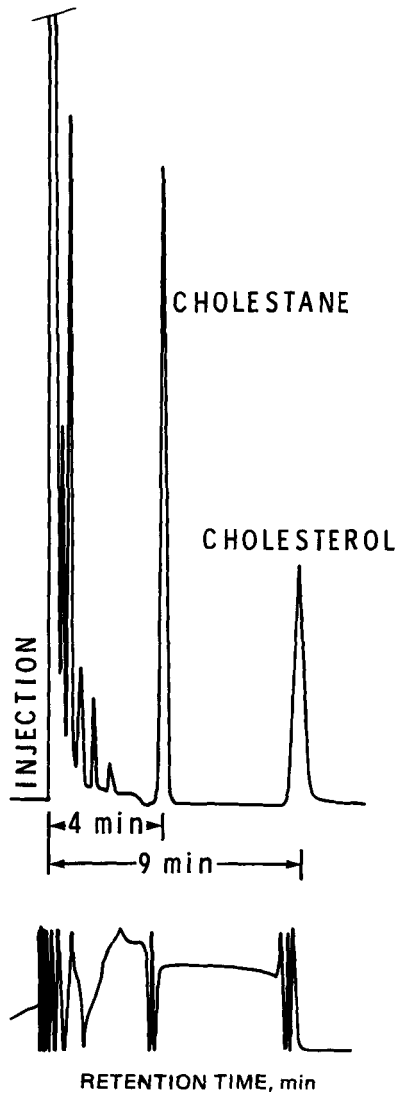

Figure 2—*GLC analysis of chick serum cholesterol using 3% OV-17 and Chromatograph B.*

for each day's set of samples and was further checked during and after each series of analyses. This factor ranged between 0.75 and 0.90 for the disk-type integrator[5]. The calibration of the programmable integrator[6] adjusted the correction factor automatically. When it was determined separately, it was constant at 0.85. Correction factors were similar for the other sterols.

Both samples and standards were kept tightly covered and refrigerated when not in use. Under these conditions, both samples and standards were stable for several weeks, in agreement with those values stated by Tonks (1).

While the use of cholestane as an internal standard was reported previously for cholesterol in serum, it was used only in the final step, necessitating careful measurement of the extraction solvent and extreme care in ensuring complete extraction of cholesterol. In Blomhoff's method (2), the extraction solvent was measured, transferred, and reduced to dryness under nitrogen; the internal standard then was added in chloroform to redissolve the cholesterol. It was found that by adding the cholestane to the accurately measured sample of serum or lipid extract (in the cases of heart and liver tissues), these steps could be eliminated. The complete analysis involved only one test tube and no reduction of solvent. Injections from hexane rather than chloroform also resulted in a cleaner detector and longer column life.

In normal circumstances the hydrolysis time could be greatly decreased (11); however, this project was dealing with artificially high cholesterol levels, and the prolonged hydrolysis period was required. Free sterol concentrations, if desired, could be determined by GLC analysis of the samples prior to hydrolysis.

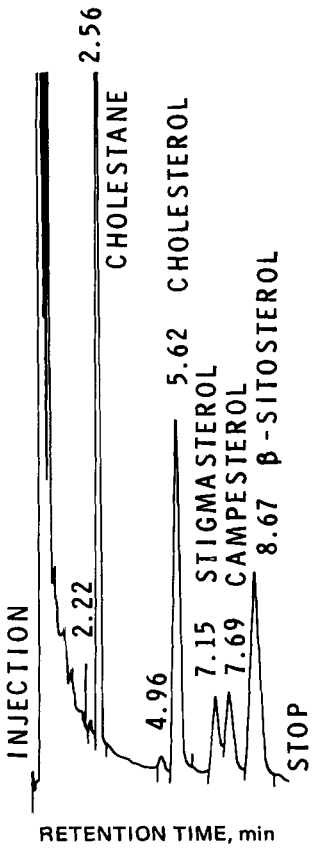

Figure 3—*GLC analysis of a standard solution of some sterols using 3% SP-2401 and Chromatograph A.*

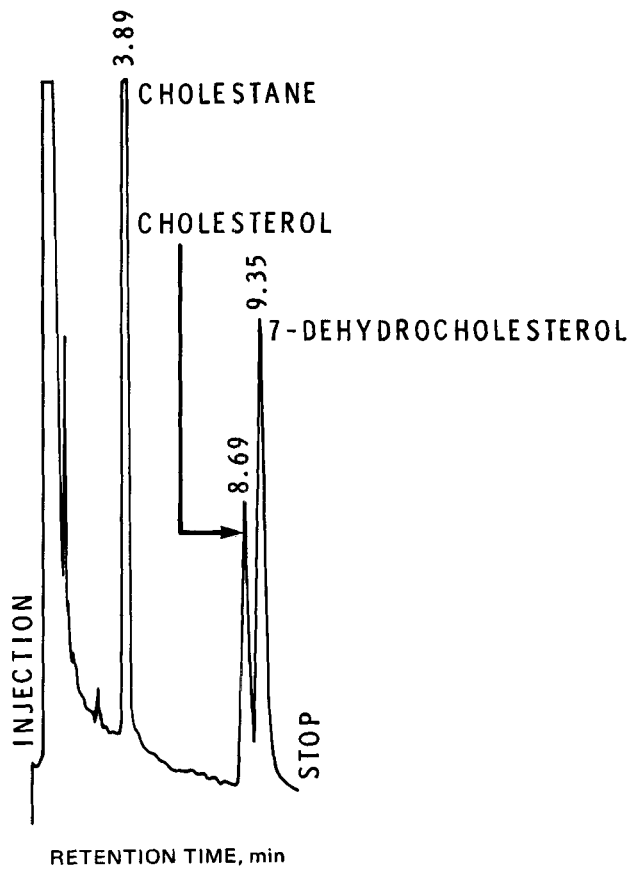

Figure 4—*GLC analysis of 7-dehydrocholesterol and cholesterol in chick serum using 3% SP-2401 and Chromatograph A.*

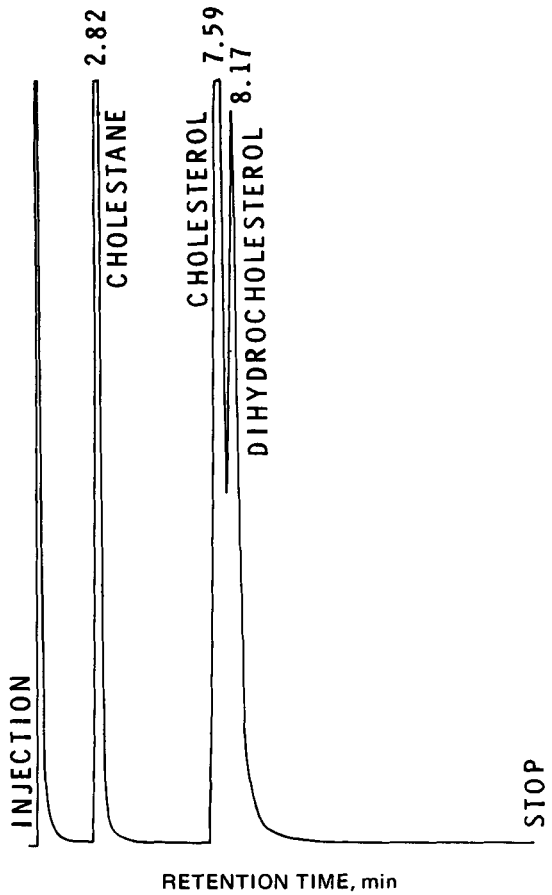

Figure 5—*GLC analysis of dihydrocholesterol and cholesterol in chick serum using 3% SP-2401 and Chromatograph A.*

This method is rapid, precise, and flexible for the qualitative and quantitative analysis of cholesterol and other sterols in biological materials. Except for the amounts of biological sample and internal standard, no other measurements are critical.

REFERENCES

(1) D. B. Tonks, *Clin. Biochem.*, **1**, 12 (1967).

(2) J. P. Blomhoff, *Clin. Chim. Acta*, **43**, 257 (1973).

(3) L. P. Crawley, B. O. Musser, S. Campbell, and W. Faucette, *Am. J. Clin. Pathol.*, **39**, 450 (1963).

(4) H. C. Von Curtius and W. Burgi, *Z. Klin. Chem.*, **4**, 38 (1966).

(5) J. L. Driscoll, D. Aubuchon, M. Descoteaux, and H. F. Martin, *Anal. Chem.*, **43**, 1196 (1971).

(6) J. MacGee, T. Ishikawa, W. Miller, G. Evans, P. Steiner, and C. J. Glueck, *J. Lab. Clin. Med.*, **82**, 657 (1973).

(7) T. T. Ishikawa, J. MacGee, J. A. Morrison, and C. J. Glueck, *J. Lipid Res.*, **15**, 286 (1974).

(8) P. A. Harris and K. L. Harris, *J. Pharm. Sci.*, **63**, 781 (1974).

(9) E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.*, **37**, 911 (1959).

(10) D. J. Munster, M. Lever, and R. W. Carrell, *Clin. Chim. Acta*, **68**, 167 (1976).

(11) L. Lillienberg and A. Svanborg, *ibid.*, **68**, 223 (1976).

ACKNOWLEDGMENTS

Presented in part at the 23rd Canadian Conference on Pharmaceutical Research, Saskatoon, Canada, 1976, and at the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Orlando meeting, 1976.

Supported by the Medical Research Council of Canada, Grant MA-3539.

The authors thank Eli Lilly and Co. Ltd. for the generous supply of cholesterol and β-sitosterol NF.