# Purity of cholesterol to be used as a primary standard

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SUMMARY Cholesterol preparations from 14 commercial sources were purified by the dibromide method. The cholesterol contents of the original and purified preparations were determined by gas–liquid chromatography. Calculation of the results of the gas chromatographic analyses from the areas of the peaks rather than from their heights gave more precise results. Purified samples had higher contents of cholesterol, higher melting points, and narrower melting point ranges, and exhibited fewer contaminants on thin-layer chromatoplates than did the original preparations.

KEY WORDS cholesterol · primary standard · purification · cholesterol dibromide · quantitative gas–liquid chromatography · thin-layer chromatography

DIFFERENCES IN THE PURITY of cholesterol used by various laboratories as a primary standard may contribute to the differences in levels of serum total cholesterol reported by such laboratories. The dibromide method (1) is widely used for the purification of this steroid. It would be of interest to determine the purity of the various commercial preparations of cholesterol which might be used as primary standards for colorimetric procedures. In this paper are reported an evaluation, using gas–liquid and thin-layer chromatography, as well as melting point determinations, of the purity of 14 commercial preparations of cholesterol and the efficacy of the dibromide procedure in their purification.

## MATERIALS AND METHODS

Cholestane was obtained from Applied Science Laboratories, State College, Pa.[1]

[1] The use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health, Education, and Welfare.

### *Commercial Sources of Cholesterol*

*1*, General Biochemicals, U.S.P., Lot 51615. *2*, Sigma Chemical Co., U.S.P., Lot C92B-244. *3*, Pfanstiehl Laboratories, Inc, C.P., ash free, 6259. *4*, Pfanstiehl Laboratories, Inc., C.P., 6513. *5*, California Corp. for Biochemical Research, U.S.P., C. grade, Lot 34875. *6*, Chemed, Inc., purified. *7*, Fisher Scientific Co., Lot 720287. *8*, Nutritional Biochemicals Corp., U.S.P., Control No. 6349. *9*, Mann Research Laboratories, Inc., U.S.P., C.P., 1374, E 1908. *10*, K and K Laboratories, Inc., Lot 44480. *11*, Eastman Organic Co., 909. *12*, Armour Pharmaceutical Co., U.S.P., Lot Y 32812. *13*, Matheson Coleman and Bell, CX 1530, 5177. *14*, L. Light and Co., Ltd., England.

### *Purification of Cholesterol*

The method of purification of cholesterol by preparation of the dibromide as the acetic acid complex and subsequent debromination with zinc dust was adapted from that of Fieser (1). No attempt was made to collect a second crop of cholesterol from the residual mother liquors. The final product was collected in a fritted glass filter, washed with cold methanol, air-dried overnight, dried in vacuo at 45° for 4 hr and stored under nitrogen over phosphorus pentoxide at −20°.

Original samples listed in the above section are indicated in the text by their numbers, and the corresponding purified products by the letter "a." Other letters indicate deviation from the standard procedure of purification: 5b, the ethereal solution of the preparation was not washed with water; 12d and 14d, the ethereal solutions were dried over sodium sulfate after washing with water.

### *Thin-Layer Chromatography*

The plates were spotted with 1% sample solutions in redistilled chloroform and then developed either in

petroleum ether–2-butanone–glacial acetic acid 85:15:1 or in hexane–diethyl ether–acetic acid 70:30:1. The plates were sprayed with 50% sulfuric acid and placed in an oven at 180° for 10 min or until spots were visible. The developed plates were photographed with a Polaroid Land camera.

### *Melting Point*

The Fisher-Johns melting point apparatus was used for the determination of melting points. After the melting point block was heated to 130°, the sample was placed on the block between 2 microcover glasses. Then the temperature was increased slowly, and for the last 5° before the expected melting point the rate of temperature increase was kept at about 1°/min. The thermometer was calibrated against the melting point of primary standard grade benzoic acid.

### *Gas–Liquid Chromatography (GLC)*

The instrument was equipped with a hydrogen flame ionization detector and a recorder with a disk integrator. The reproducibility of the integrator throughout the range, except at zero, was within ±0.01% of full scale. The integrator showed high sensitivity and there was virtually no lag between the movement of the recorder pen and the corresponding motion of the integrator except at zero, where there was a lag of 0.2%. A glass column 1.5 ft × 1/4 inch o.d. was packed with 3.8% silicone gum rubber SE-30 on 80–100 mesh Diatoport S. The column was conditioned at 275° for 64 hr. The temperatures utilized were: column 220°, injection port 270°, and the detector 240°. Carrier gas helium flow was at 60 ml/min. There were no adsorption losses of cholesterol noted in preliminary runs. Cholestane used as an internal standard showed no contaminants on the gas–liquid chromatograms. Equal volumes of a 2% solution of each sample or standard in chloroform and a 1% solution of the internal standard cholestane were combined and 0.6–0.8 μl of each combined solution was injected. The standard mixture was repeatedly chromatographed in order to have at least one standard run for four adjacent samples, two on each side before and after the standard run. The cholesterol content of the samples was calculated from the ratio of the integrated peak areas of cholestane to those of cholesterol in the sample as compared to the ratio of areas of both components in the adjacent standard run.

## RESULTS AND DISCUSSION

### *Evaluation of Gas–Liquid Chromatographic Method*

The gas–liquid chromatographic method used in this study was adapted from that developed by the biomedical research group of F and M Scientific Corporation (2). In this procedure cholestane is used as an internal standard, and the level of cholesterol is calculated from the ratio of the peak heights of the two compounds. However, in this laboratory, as is shown in Table 1, much more precise results were obtained if the cholesterol contents were calculated from the ratio of the integrated areas of the peaks rather than from the ratios of the peak heights, although the ratios of peak heights of cholestane to cholesterol were well below the upper limit of 2.75 suggested by the F and M group (2). For seven runs of a cholesterol standard, assumed to be 100% pure (see next section), the range was 7.7% and the coefficient of variation ±2.3%, when the results were calculated from the ratios of the peak heights. On the other hand, the range was 0.8% and the coefficient of variation ±0.3% when the results of this same experiment were calculated from the ratios of the peak areas, a tenfold increase in precision. Therefore, in the present study all results were calculated on the basis of ratios of peak areas.

TABLE 1 COMPARISON OF CHOLESTEROL DETERMINATIONS CALCULATED FROM PEAK HEIGHTS WITH THOSE CALCULATED FROM PEAK AREAS*

| Run Number | Ratio Cholestane/Cholesterol | | % Cholesterol | |
|---|---|---|---|---|
| | Based on Peak Heights | Based on Peak Areas | Based on Peak Heights | Based on Peak Areas |
| 1 ("Standard") | 2.545 | 0.552 | (100.0) | (100.0) |
| 2 | 2.556 | 0.552 | 99.5 | 100.0 |
| 3 | 2.438 | 0.552 | 104.4 | 100.0 |
| 4 | 2.609 | 0.554 | 96.7 | 99.6 |
| 5 | 2.597 | 0.550 | 98.0 | 100.4 |
| 6 | 2.512 | 0.554 | 101.3 | 99.6 |
| 7 | 2.579 | 0.550 | 98.8 | 100.4 |

* The concentration of cholestane was one-half that of cholesterol. Hence the values of the peak heights of cholestane were multiplied by 2 in order to obtain ratios which are comparable to those given by the F and M biomedical group (2).

### *Results of Purification of Cholesterol*

The cholesterol content determined by GLC and the melting points of the original samples and of the preparations which had been subjected to the dibromide procedure are shown in Table 2. Preliminary experiments on thin-layer plates with three of the commercially available preparations which were claimed to be of high purity showed more impurities than other cholesterol samples which had been subjected to the dibromide procedure. Therefore, preparation 12d was arbitrarily selected as the reference standard, and calculations were made on the assumption that this preparation contained 100% cholesterol. All results of the gas chromatographic method were calculated relative to the cholesterol content of this sample including as a control, sample 12d.

TABLE 2 CHOLESTEROL CONTENT AND MELTING POINT OF COMMERCIAL PREPARATIONS OF CHOLESTEROL BEFORE AND AFTER PURIFICATION BY DIBROMIDE PROCEDURE

| Sample Number | % Cholesterol | % Difference (Purified − Original) | Corrected Melting Point | Range ° | ° Difference (Purified − Original) |
|---|---|---|---|---|---|
| 1 | 98.7 | | 147.8–149.0 | 1.2 | |
| 1a* | 98.8 | 0.1 | 149.4–149.9 | 0.5 | 1.3 |
| 2 | 99 7 | | 148.2–149.6 | 1.4 | |
| 2a | 101.3 | 1.6 | 148.9–149.8 | 0.9 | 0.5 |
| 3 | 98.0 | | 148.7–149.4 | 0.7 | |
| 3a | 100.4 | 2.4 | 149.3–150.2 | 0.9 | 0.7 |
| 4 | 97.4 | | 147.0–148.4 | 1.4 | |
| 4a | 98.2 | 0.8 | 149.6–150.4 | 0.8 | 2.3 |
| 5 | 96.7 | | 147.6–148.9 | 1.3 | |
| 5a | 100.9 | 4.2 | 149.6–150.1 | 0.5 | 1.6 |
| 5b | 100.5 | 3.8 | 149.2–150.1 | 0.9 | 1.4 |
| 6 | 99.0 | | 147.9–149.1 | 1.2 | |
| 6a | 101.4 | 2.4 | 149.1–150.0 | 0.9 | 1.1 |
| 7 | 99.7 | | 147.3–148.9 | 1.6 | |
| 7a | 101.4 | 1.7 | 149.2–150.1 | 0.9 | 1.6 |
| 8 | 99.1 | | 149.0–149.7 | 0.7 | |
| 8a | 102.1 | 3.0 | 150.0–150.6 | 0.6 | 1.0 |
| 9 | 95.8 | | 147.4–148.7 | 1.3 | |
| 9a | 98.5 | 2.7 | 149.1–150.4 | 1.3 | 1.7 |
| 10 | 100.3 | | 146.7–148.4 | 1.7 | |
| 10a | 103.5 | 3.2 | sample lost | | |
| 11 | 99.0 | | 147.9–149.2 | 1.3 | |
| 11a | 100.4 | 1.4 | 149.6–150.2 | 0.6 | 1.4 |
| 12 | 96.8 | | 148.1–149.6 | 1.5 | |
| 12a | 99.0 | 2.2 | 149.1–150.0 | 0.9 | 0.7 |
| 12d | 100.1 | 3.3 | 149.3–150.3 | 1.0 | 1.0 |
| 13 | 97.3 | | 148.2–149.1 | 0.9 | |
| 13a | 101.8 | 4.5 | 149.1–149.8 | 0.7 | 0.8 |
| 14 | 94.5 | | 146.8–149.4 | 2.6 | |
| 14a | 99.9 | 5.4 | 149.7–150.3 | 0.6 | 1.9 |
| 14d | 103.5 | 9.0 | 148.9–149.8 | 0.9 | 1.3 |

* Preparation numbers followed by letters are samples subjected to the dibromide treatment as described in the section *Purification of Cholesterol*. Sources of the samples are given under *Commercial Sources of Cholesterol*. Sample 12d was arbitrarily selected as reference.

The melting points of all preparations of cholesterol were increased in amounts ranging from 0.5° (No. 2) to 2.3° (No. 4) by the dibromide procedure. The melting point ranges of the samples which had been subjected to the dibromide procedure were in general narrower than those of the original commercial preparations. The range of melting points of all samples treated by the dibromide method was 148.9–150.6° with an average melting point of 149.8°, which compares very well with the melting point range of 149.5–150.0° for cholesterol purified by recrystallization from acetic acid as listed by Bladon (3) or for the identical range found by Fieser (1) for cholesterol purified by the dibromide method.

The cholesterol content as measured by GLC of 12 of the 14 samples was increased in amounts ranging from 1.4 (No. 11) to 9.0% (No. 14) by the dibromide procedure. In the two other samples (No. 1 and No. 4), the increase in cholesterol content was within the range of variation found in GLC.

The results of thin-layer chromatography, illustrated in Fig. 1, indicate that some impurities present in the original preparations were removed by the dibromide procedure. In general, there was a fair correlation between the increase in cholesterol content after treatment with bromine, as determined by GLC, and the amount of impurities in the original preparation as revealed by thin-layer chromatography. This point is illustrated nicely with preparation No. 14 or preparation No. 5. It appears that chromatography in the thin-layer system described, together with melting point determinations, might provide a useful guide as to whether dibromide purification should be carried out on a given preparation.

The increase in purity of cholesterol and the accompanying rise in melting point of commercial sources of cholesterol after treatment by the dibromide method found in the present study are in agreement with the previous studies of Radin and Gramza (4), in which molar absorptivities of the Liebermann-Burchard or ferric chloride reactions were used as criteria of purification. The same conclusion can be drawn from their data as from the results of the present study, namely,

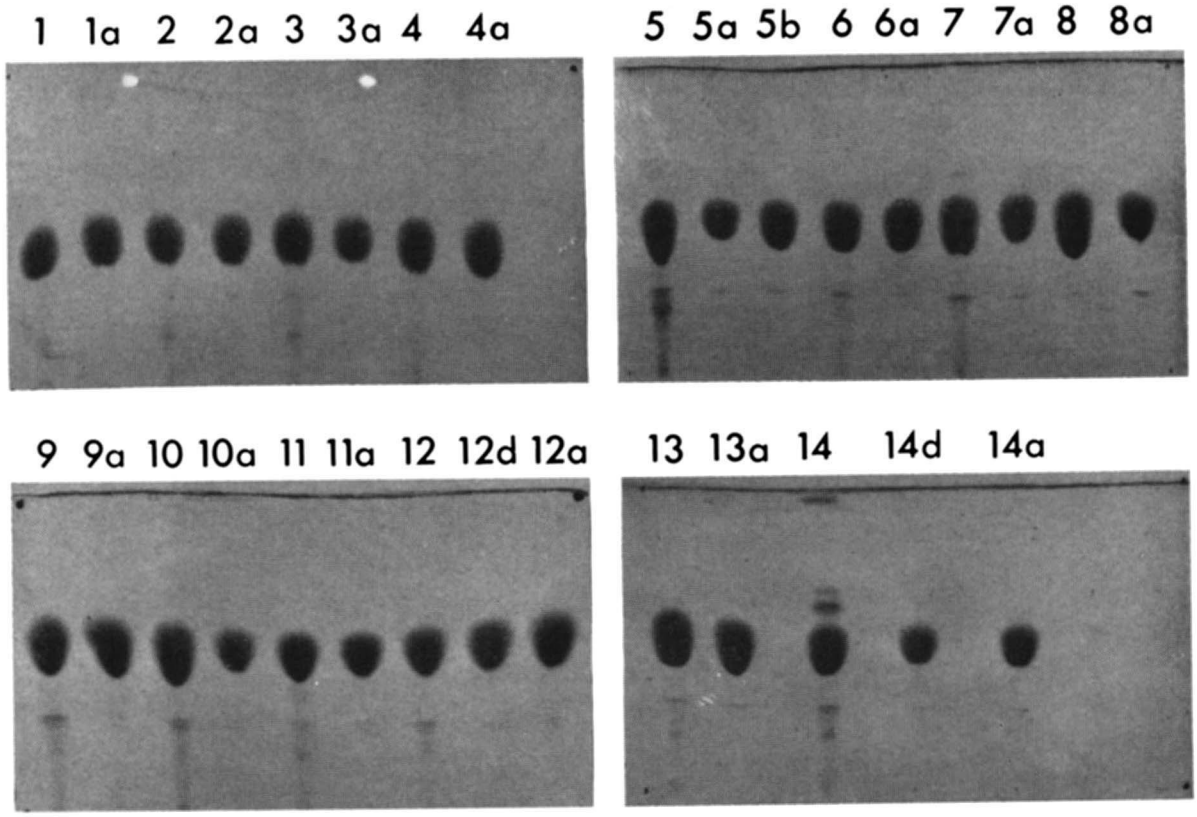


FIG. 1. Thin-layer chromatography of cholesterol preparations. Solvent: petroleum ether–2-butanone–acetic acid 85:15:1 (v/v/v). Developed by charring with 50% sulfuric acid spray. The identification of sample numbers and letters is given under *Commercial Sources of Cholesterol* and *Purification of Cholesterol*.

that the dibromide procedure may not remove all impurities from commercial samples of cholesterol.

Information about the nature of some of the impurities in the commercial preparations can be ascertained from the results of thin-layer or gas–liquid chromatography. The impurities with an $R_F$ of 0.35 or less, which were found in all commercial preparations except No. 8, are believed to be oxidation products. When solutions of cholesterol were allowed to stand for 8 weeks on the shelf in the laboratory, constituents were formed with mobilities in the before-mentioned range, and a known oxidation product of cholesterol, 7-dehydrocholesterol, moved with an $R_F$ equal to that of the slowest moving of these impurities. The faint spot just above cholesterol in preparation No. 5 had an $R_F$ value identical with that of fatty acids; the faint spot below the cholesterol spot in preparation No. 7 exhibited the $R_F$ value of diglyceride, and the moderately heavy spot near the front in preparation No. 14 ran to the $R_F$ range of cholesterol esters.

Cholestanol, which has been found in some cholesterol preparations (5, 6), has the same retention time ($t_R$) on SE-30 columns at 220° (7) and exhibits the same $R_F$ values as cholesterol in the solvents used. Lathosterol, also reported to be a contaminant of such preparations (5, 6), differs in $t_R$ value from cholesterol by only 10% and its $R_F$ value is just slightly lower than that of cholesterol in the gas–liquid or thin-layer chromatographic systems employed. It is doubtful whether lathosterol in amounts of less than 5% could have been detected in the gas chromatographic system.

However, the dibromide procedure is known to remove both cholestanol and lathosterol (5, 6). Hence, it is probable that these two sterols, if present in the commercial preparations, would have been removed by the dibromide procedure. The increase in melting point of all preparations after purification is in agreement with this idea.

The results obtained in the present study indicate that the degree of purity of the commercial preparations of cholesterol used as a primary standard could be an important factor leading to differences in results among laboratories or variation in results within the same laboratory. A difference of 4% or more in degree of purity, as found with three preparations (Nos. 5, 13, and 14), is within the limits of detection by most colorimetric methods, particularly if large numbers of determinations are run, as would be the case with a primary standard.

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