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Quantitative isolation of sterols*

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SUMMARY

Procedures are described for the quantitative gravimetric isolation of cholesterol and cholestan-3β-ol as the digitonides and for the quantitative recovery of these sterols from their digitonides in the range of 0.5–4.0 mg.

In 1909, Windaus published the important discovery that cholesterol, sitosterol, and β-cholestanol form sparingly soluble addition compounds with digitonin, whereas α-cholestanol and cholesterol esters do not (1). Ever since, this reaction has been a major tool of steroid chemists; it has been used in many methods for cholesterol determination and in many separations of steroids with the beta configuration at carbon 3 from those with the alpha configuration. In 1937, I reported experiments in which this reaction had been employed in various ways in a study of the cholesterol of the blood serum (2). One of these (Procedure F_1) was the method used by the late Rudolph Schoenheimer and which I applied under his guidance. It had been developed from the experience of Schoenheimer and Dam (3), and it involved precipitation in 80% ethanol by digitonin in at least 50% excess, filtration, washing with 80% ethanol and ether, drying, and weighing. In preparing to apply this method to the unsaponifiable lipids of brain, I tested it on pure cholesterol with only two changes: (*a*) the amount per sample was decreased from an average of 13 mg to about 2 mg, and (*b*) centrifugation instead of filtration was used in the isolation and washing of the precipitate. Unexpectedly, recoveries were not quantitative; the digitonide weights accounted for only about 90–95% of the cholesterol taken for analysis. A series of experiments, in which several variations of the procedure were tried, led to the finding of conditions under which satisfactory results were consistently obtained. Good recoveries of sterols from the digitonides were also obtained with a modification of the pyridine-ether method of Schoenheimer and Dam (3). These procedures are described in this communication.

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PROCEDURE

Special Apparatus.

Test tubes, 13 x 100 mm, selected for maximal internal diameter, cut to a length of about 7.6 cm, fire-polished, and numbered.

Stirring Rods. Sheet aluminum, 0.3 mm thick, is cut into strips 0.4–0.5 cm wide and 7.2 cm long. The edges are smoothed, and the ends are rounded with a file. The rods are numbered to correspond to the tubes. In the procedure, the rods are always handled with a hemostat clamped on the upper end. To remove a rod from a tube, one jaw of the hemostat is pressed against the rod with the other jaw outside the tube, and the rod is raised until the hemostat can be clamped on it.

Apparatus C, Fig. 1, modified by drawing Tube S, made of 3-mm tubing, to a tip about 1 mm o.d. The overall length of the side arm is about 12 cm. Corks, instead of rubber stoppers as previously described (4), are used in making apparatus C.

Semimicro Balance. Since sterol digitonides are hygroscopic, rapid weighing is essential. Sartorius Selecta balances were used in this work.

Dri-Jar Desiccators (Bethlehem Apparatus Co., Hellertown, Pa.). Drierite is used as the desiccant.

Reagents.

Acetone–Absolute Ethanol 1:1 (*v/v*). The acetone used in this work was distilled through an Oldershaw 40-plate column, according to the general practice of

purifying solvents in this laboratory, and the ethanol used in part of the work was distilled in the same way. Such high purity is probably not necessary for use in this method.

Ethanol, 80%. Absolute ethanol–water 4:1 (v/v).

Ethyl Ether. Baker's reagent grade anhydrous ether was distilled at a slow rate through a long column to remove a trace of residue in this product. It was stored in a brown bottle at 4° and tested occasionally for peroxides (5). None was found even after storage for several months.

Digitonin Solution, 2% in 80% ethanol. The Hoffmann-La Roche product, used in this work, dissolves readily with gentle heating on the steam bath. The warm solution is filtered through Whatman No. 1 filter paper to remove a small amount of insoluble material. The filtrate is collected and stored in a glass-stoppered Erlenmeyer flask. Some of the digitonin precipitates on standing but it dissolves quickly to a clear solution on the steam bath and remains clear for several hours at room temperature.

Pyridine, distilled from and stored over BaO.

Quantitative Isolation of Digitonides.

Preparation and Weighing of Tubes. The tubes are rinsed successively with acetone–ethanol, 80% ethanol, and ether and allowed to dry. The outside walls are rinsed with distilled water and wiped dry with a clean towel. The rods are treated in the same way and each is placed in the tube with the corresponding number. The tubes are kept overnight *in vacuo* over Drierite, transferred to Dri-Jar desiccators, one on its side in each pan, and left at least 30 min before weighing. One of the four tubes in each desiccator is weighed as a control and returned to the desiccator.

Precipitation. The tubes are placed in a rack, and the rods are removed and stored in a covered beaker. Into each tube is pipetted 4 ml of an acetone–absolute ethanol 1:1 solution containing 0.5–4.0 mg of precipitable sterols; 1 ml of the digitonin solution is added, followed at once by 1 ml water. Neither heating nor stirring is necessary. The tubes are placed in a small beaker in a pint fruit jar containing some of the solvent mixture (acetone–ethanol–water 2.0:2.8:1.2), and the cover is screwed tightly on.

Centrifugation and Removal of Supernatant Solution. Next day, the tubes are transferred to a rack. The walls of the tubes just above the surface of the liquid are scrubbed with the corresponding rods to wet and loosen any precipitate that might have dried there. The rods are placed on a wire rack in positions corresponding in order to those of the tubes. The tubes are centrifuged 15 min at about 2,300 rpm. A tube is clamped to the upright rod of a Lab-jack, and the supernatant solution is removed by modified apparatus C with suction just sufficient to fill tube S and start siphoning. The tube should be well illuminated and watched closely, preferably with a magnifier. The precipitate usually has an irregular surface and frequently some is lodged in clumps against the wall

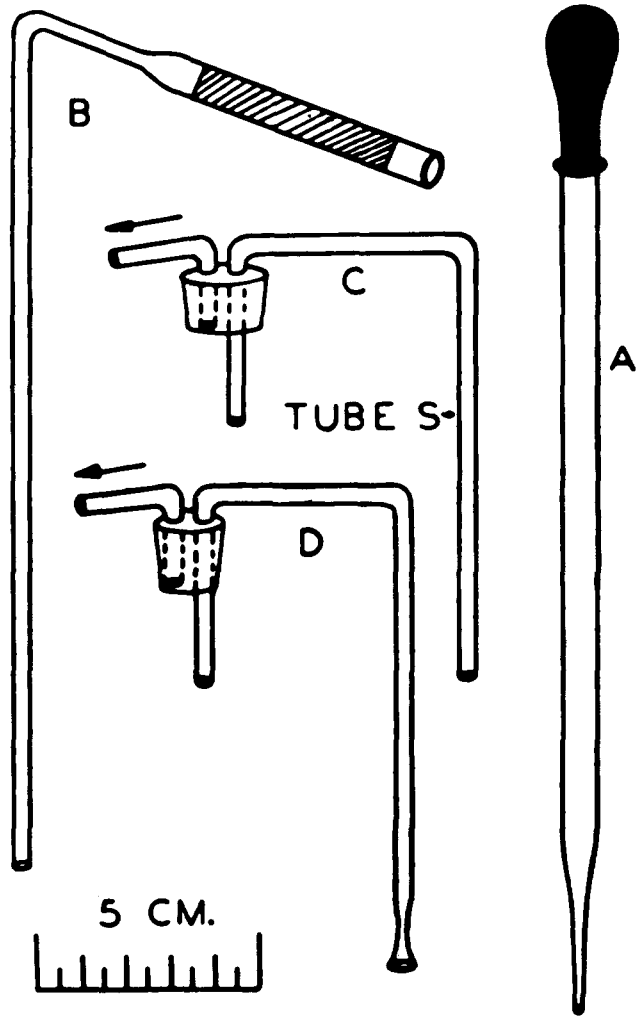

FIG. 1. A, the solvent pipette. The tube should be at least 15 cm long to avoid contact of the solvent with the rubber bulb. B, the tube for admitting inert gas; glass wool is placed in the inlet arm. C, apparatus for transfer of lipid solutions. Gentle suction, controlled by a screw clamp, is applied as indicated by the arrow. The inlet arm of the tube S should extend at least 2 cm below the cork to avoid any contact between the latter and the solution. D, filter stick. A small wedge-shaped piece of filter paper is twisted into the constriction and crumpled into the small cup formed by flaring the end. Enough of a suspension of shredded asbestos in water is sucked onto the paper to make the filter sufficiently retentive, but not so much as to make the filtration unnecessarily slow. The proper amount is soon learned by practice. A filter, once prepared, may be used for many filtrations. The apparatus is attached to a 5-ml volumetric flask by means of a short piece of rubber tubing, which fits snugly over the neck of the flask and the cork. (Reprinted from *J. Biol. Chem.* **209**: 377, 1954 [4].)

of the tube. The danger of loosening and sucking out such precipitate can be avoided by turning the tube and moving the Lab-jack so the tip of tube S is directed into the deepest pocket in the surface of the precipitate. The tip must be kept in the solution until the transfer is completed. This is particularly important as the surface of the liquid approaches the surface of the precipitate, since the agitation caused by emergence of the tip may stir some of the precipitate into suspension. It is best to raise the tube at the start to the final position of the tip, near the surface of the precipitate, and to leave it there until the transfer is completed. The tube is lowered quickly just as, or slightly before, the surface of the solution reaches the tip. A few very small particles adhering to the upper wall of the tube are sometimes sucked out; such losses do not appear to have a significant effect on the result.

If the supernatant solutions and washes are to be collected quantitatively and separately from each tube for further study, it is desirable to equip each of the requisite number of 50-ml Erlenmeyer flasks with a modified apparatus C and to clamp them in order around a ring stand. The suction line is transferred from one to the next, the stand is turned, and the Lab-jack is raised or lowered as necessary. Before the suction line is removed, a small amount of solvent is sucked over from a 1-ml beaker to avoid loss of the solution which "refluxes" in tube S.

Washing. About 1.5 ml of 80% ethanol is added to each tube with a transfer pipette (Fig. 1). The tip of the pipette is held against the wall just below the top and the tube is turned as the solvent is expelled so that the entire wall is washed. The precipitate is stirred into suspension with the appropriate rod, which is rinsed with a few drops of solvent as it is withdrawn and returned to the wire rack. The tubes are centrifuged 5 min, and the supernatant solutions are removed as before. The precipitate packs better than at the first centrifuging, and there is little danger of loss at this stage. The precipitate is washed twice more with 80% ethanol and three times with ether in the same way except that the suction must be decreased. The precipitate is easily resuspended in ether, and particular care must be taken to avoid loss. The suction is adjusted so that it is just sufficient to start siphoning of ether before it is applied to the sample.

Drying and Weighing. The rods are placed in the tubes, and the residual ether is removed by a gentle stream of nitrogen. The outside walls are cleaned as before the tare weighings, and the tubes are placed in an oven at 110° for 1 hr, and *in vacuo* over Drierite overnight. The same timing and sequence are used in tare and gross weighings. The weights are corrected for any change in weight of the control tube. This is usually small, but it may be significant.

Isolation of Sterols from Digitonides.

The tubes are heated 1 hr at 110° and cooled in a desiccator over Drierite. To each tube, 0.3 ml of pyridine is added. After the bulk of the digitonide at the bottom of the tube has dissolved, the rod is clamped in an hemostat, and a drop of the solution is drawn up the wall almost to the top and allowed to run back. This is repeated until the entire wall has been washed and the digitonide has all dissolved. Three milliliters of ether are added and the suspension of released digitonin is thoroughly stirred. The rod is removed, rinsed with a few drops of ether, and placed on a wire rack. The tube is centrifuged 5 min at about 2,300 rpm. The supernatant solution is transferred to a 50-ml Erlenmeyer flask by the procedure and with the precautions described above for the transfer of ether solutions. The precipitate of digitonin is washed three times with ether with the procedure used in washing digitonides. The inner arm of tube S is rinsed with a few drops of ether, and a little ether is sucked through.

The ether is removed at about 30° with a stream of nitrogen (4), and the pyridine is removed by leaving the flasks over H_2SO_4 *in vacuo* overnight. The sterols are dissolved in a little ether added in a continuous film down the wall of the flask. A small amount of material, presumably digitonin, usually remains undissolved. The suspension is filtered with five washes into a tared 5-ml volumetric flask with apparatus D (Fig. 1). The ether is removed by a stream of nitrogen, and the flask is cleaned, dried, and weighed.

EXPERIMENTAL METHOD

The method was tested with pure cholesterol and cholestan-3β-ol in several series of experiments carried out at widely separated times with different preparations of reagents. It was also applied to duplicate analyses of 18 unsaponifiable fractions obtained from brain lipids with an unpublished procedure.

RESULTS

The method was originally devised for application to samples expected to contain about 2 mg of precipitable sterols, and it was tested most extensively at this level (Table 1). The recovery as digitonide was satisfactory at all levels tried except for values that were a little low at the 4-mg level for both sterols. The differences between the 4-mg and 2-mg average percentage recoveries, although small, are significant ($p < 0.01$) and probably due to the use of too small an excess of digi-

TABLE 1. RECOVERY OF STEROLS AS DIGITONIDES AND AS STEROLS FROM DIGITONIDES

Sterol	Amount in Sample*	Sterols Recovered as Digitonides				Sterols Recovered from Digitonides			
		N†	Amount	Sterol Equivalent§	Recovery	N†	Amount	Recovery from Sample	Recovery from Digitonide Equivalent
	mg		*mg*	*mg*	*%*		*mg*	*%*	*%*
Cholesterol	0.5	11	2.09 ± 0.07‡	0.50 ± 0.02	100.9 ± 3.2				
"	1.0	14	4.14 ± 0.06	1.00 ± 0.02	100.1 ± 1.6				
"	2.0	31	8.26 ± 0.08	2.00 ± 0.02	99.8 ± 0.9	23	2.02 ± 0.05	101.0 ± 2.5	101.3 ± 2.4
"	4.0	15	16.24 ± 0.15	3.92 ± 0.04	98.1 ± 1.0	8	3.88 ± 0.05	97.1 ± 1.2	99.1 ± 1.3
Cholestanol	0.5	5	2.10 ± 0.05	0.51 ± 0.01	101.9 ± 1.1				
"	1.0	5	4.15 ± 0.04	1.01 ± 0.01	100.5 ± 1.1				
"	2.0	7	8.27 ± 0.08	2.00 ± 0.02	100.1 ± 1.1	5	2.10 ± 0.10	105.1 ± 4.7	105.5 ± 4.6
"	4.0	5	16.16 ± 0.08	3.91 ± 0.02	97.9 ± 0.9	5	4.07 ± 0.16	101.8 ± 4.0	104.0 ± 4.0

* The actual amounts per sample varied within the following ranges in the order of entry: 0.480–0.505, 0.96–1.01, 1.92–2.36, 3.93–4.21, 0.516–0.518, 1.030–1.035, 1.88–2.07, 4.13–4.14. To facilitate comparison of the data, the weights of digitonide and of sterol recovered from digitonide were calculated to the even amounts shown.

† Numbers of determinations.

‡ Standard deviations.

§ To obtain the data shown in this column, the digitonide weights were divided by 4.14 for cholesterol and 4.13 for cholestanol.

tonin in the analyses at the 4-mg level. In two experiments, the recoveries of 0.245 and 0.240 mg of cholesterol as digitonides were 95.6% and 96.8%, respectively.

The recoveries of cholesterol from the digitonide were also satisfactory although the data were more variable, as would be expected since the amounts weighed were small. In a series of three experiments, the recoveries of cholestanol from the digitonide were all high; whereas in two experiments, carried out at a later time, the recoveries averaged 100.6 ± 0.8%. Some recoveries from digitonides at levels of 0.5 and 1.0 mg were attempted. The results averaged 98.7% for both sterols but were quite variable.

The duplicate determinations of digitonin-precipitable sterols in 18 unsaponifiable fractions from brain lipids provide a basis for estimating the precision of the method as applied in practice. The standard deviation of an analysis[1] was 0.104 mg of digitonides, which was 0.73% of the average digitonide weight. The maximal difference between duplicate weights was 2.0% of their average. In these experiments, the average recovery of sterol (calculated as cholesterol) from digitonides by the pyridine-ether technique was 98.4 ± 3.6%.

DISCUSSION

For the quantitative, gravimetric isolation of sterols as digitonides, the excess digitonin must be removed without loss of digitonide dissolved in the solvents used for washing. There is danger that retention of digitonin may approximately balance loss of digitonide with misleading results. For this reason, the procedure should be tested either by recovery of sterols from the digitonides, as was done in this work by means of the pyridine-ether method, or, where applicable, by a colorimetric method. If the Liebermann-Burchard reaction is used, the latter test must be applied with caution when more than one sterol is present since unsaturated sterols vary widely in their responses to the Liebermann-Burchard reagent and saturated sterols do not react.

The Schoenheimer-Sperry (7) method for cholesterol was not designed for the quantitative isolation of sterol digitonides free from digitonin, and I doubt very much that it, or the Sperry-Webb (5) modification of it, can be used for that purpose. On the contrary, a major feature of the method, as it evolved originally in the fertile mind of Rudolph Schoenheimer, was the absence of any need to wash out excess digitonin, which gives no significant color with the Liebermann-Burchard reagent in the portion of the spectrum where readings are made. No attempt to wash out all unreacted digitonin is made in the method, and it is doubtful that all is removed.

Since the procedure is empirical and was evolved by trial and error, it should be tested on pure sterols by any investigator who intends to use it. Such tests are certainly essential before the method is applied to sterols other than the two, cholesterol and cholestanol, that were used in developing the procedure.

[1] Calculated from the equation: S.D. = $\sqrt{\Sigma d^2/2n}$, in which d is the difference between duplicate weights of digitonide and n is the number of samples (6).

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