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CRYSTALLINE STEROLS OBTAINED BY GAS CHROMATOGRAPHY

JOHAN E. VAN LIER AND LELAND L. SMITH

Department of Biochemistry, University of Texas Medical Branch, Galveston, Texas 77550 (U.S.A.)

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SUMMARY

A gas chromatographic method utilizing capillary tube collection of eluted sterols has been examined for preparative use. A variety of sterols may be collected in a state of high purity after gas chromatography, their high purity being demonstrated by melting point and thin-layer and other gas chromatographic evidences.

Use of gas chromatographic methods for analysis has far outpaced use for preparative purposes. Although preparative gas chromatographic methods have been utilized for some time with simple molecules and special preparative instruments are offered commercially, only relatively recently has there developed a serious interest in the application of gas chromatographic techniques for the isolation of small quantities of highly pure steroids and related natural products.

The methods already described for collection of components resolved by preparative gas chromatography include: simple cooled traps[1–5]; capillary tubes[3,6–10]; electrostatic precipitators[11–14]; centrifugal coolers[15]; traps with specific thermal gradients[7,8,16,17]; traps with solvent[18,19], glass or cotton wool[20,21], potassium bromide[22–24], solid or liquid scintillator[25–28], or treated diatomaceous earth[29]; thermoelectric cooled devices[30]; Millipore filters[31]; and cellulose acetate cigarette filters[32]. The many recent reports wherein gas chromatographic effluents are analyzed by mass spectrometry, by infrared absorption spectrophotometry, by thin-layer chromatography, or by radioactivity assay are not regarded here as preparative methods.

Our own experiences[33] together with those of others[4,19,34–38] demonstrate the potential of preparative gas chromatography of steroids. We have made additional relevant observations on the glass capillary collection technique which we describe herein.

EXPERIMENTAL

Melting points of chromatographed sterols were determined on a Kofler block under microscopic magnification. Infrared absorption spectra were obtained on samples incorporated into 0.5 or 1.5 mm potassium bromide disks (*ca.* 5 μg of sample with 0.5 mg of spectral grade potassium bromide for a 0.5 mm disk, 20–30 μg of sample with 2 mg of potassium bromide for a 1.5 mm disk). The gas chromatographed sample was

dissolved in chloroform and applied to the potassium bromide in a 1 cm diameter boron carbide morter, the mixture dried under an infrared lamp for five minutes, ground, and pressed. Spectra (4000–400 cm^{-1}) were obtained on a Perkin-Elmer Model 337 infrared spectrophotometer equipped with a beam condenser. Ultraviolet absorption spectra of gas chromatographed samples were recorded on 10–20 μg/ml solutions in 95% ethanol using a Cary Model 14 spectrophotometer. Thin-layer chromatographic examination of gas chromatographed sterols was conducted on 250 μm. Silica Gel HF_{254} chromatoplates as previously described[39]. All gas chromatographed sterol samples were routinely checked for their identity and homogeneity by both thin-layer and additional analytical gas chromatography.

Analytical gas chromatography was accomplished on 3% SE-30 and on 3% QF-1 columns in the manner previously described[33,40]. Preparative gas chromatography was conducted on an F and M Model 400 gas chromatograph as previously described[33] and on an F and M Model 402 dual column gas chromatograph (Hewlett-Packard Corp., Avondale, Pa.) equipped with a stream splitter. Mass detection was by means of a hydrogen flame detector standard with this instrument. Preparative columns 1.8 m long of 6 mm I.D. silanized glass were packed with 3% QF-1 on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories, State College, Pa.) with a funnel through which a micro-pipette was inserted. Gentle squeezing of air in and out of the column, with gentle tapping of the column a few times on a wooden block, gave a uniform and satisfactory filling. The packed column was conditioned at 260° with nitrogen carrier gas flowing for 12 h before use. In addition to our earlier preparative work[33] where simultaneous detection and collection was not done, we also used a stream splitter attachment in the present studies with a stream split ratio variable from 1:2 to 1:20. Nitrogen was used as carrier throughout at a flow rate of 30 ml/min (cylinder pressure of 40 lbs./sq.in.). The oven temperature was 230°, the flash heater was 250°, and the detector was 240° for preparative runs.

A sample of sterol to be chromatographed preparatively, 1–2 μg dissolved in 1–2 μl of chloroform–methanol (9:1), was first analyzed qualitatively on either a 3% QF-1 or 3% SE-30 column in the same oven as the preparative column (carrier gas at 20 ml/min). For preparative chromatography 0.1–1 mg of the sterol preparation was injected on column in as small a volume (5–20 μl) of chloroform–methanol (9:1) as possible. When a sought component began to elute from the column, as witnessed by an appropriate recorder pen excursion at the previously determined retention time, a 2 × 150 mm or 2 × 300 mm melting point capillary tube was inserted in a Teflon sleeve placed on the effluent end of the stream splitter. Capillary tubes were not inserted until emergence of the desired component in order to minimize contamination of the collected sterol by column bleeding or trace impurities in the injected sample. Condensation of eluted sterol usually begins as a streak of amorphous material on the capillary wall within 1 cm of the end of the Teflon sleeve, at about 3 cm from the inserted end of the capillary. Massive crystallization occurs rapidly at the cooler upper end of the initial streak, with crystals grown to the center of the lumen of the capillary (Fig. 1). The crystalline mass develops just before the recorded maximum of the peak on the elution curve. As the desired component being eluted begins to diminish in amount but before the recorder tracing has returned to base line the initial capillary may be removed and a second capillary inserted to condense the tailing portion of the eluted component. Maximum purity obtains in this fashion.

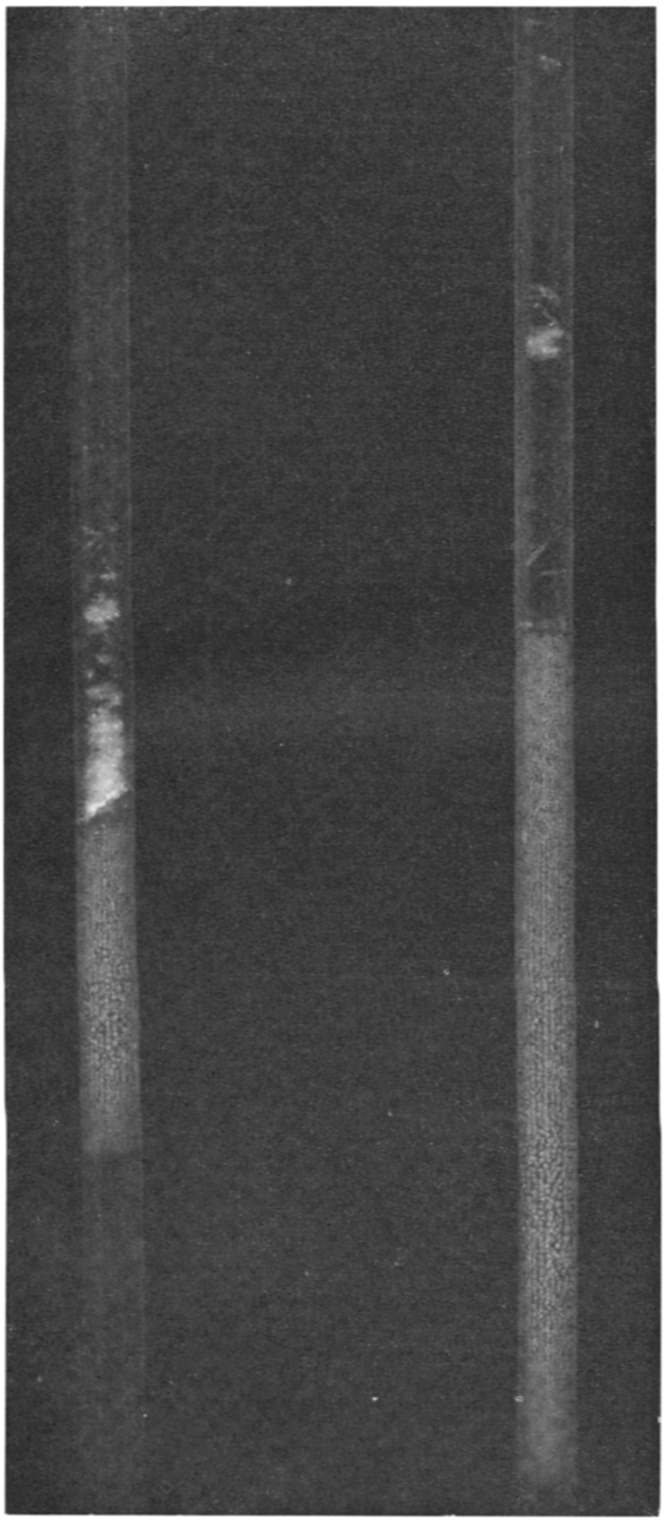

Fig. 1. Capillary collection of cholesterol without applied temperature gradient (left) and with applied temperature gradient (right).

The time involved between initial visible crystallization in the capillary and optimal recovery of pure material is relatively short (about 30 sec for 500 μg of cho-

lesterol with retention time of 7.4 min on 3% QF-1 columns), and the amount of impurities condensed on the crystals is minimal. Collection of different portions of the eluted component in separate capillary tubes in order to obtain a fraction of higher purity is also possible. Removal of one capillary and insertion of a second can be done within one second with little apparent loss of eluted component.

In certain cases where the amount of sample is large or the carrier gas flow too great, some of the crystals from the condensed crystalline cluster may be blown from the capillary and lost. Thus attention at the time of crystallization is of importance, not only to insert and remove capillary tubes at the appropriate times but also to view the actual crystallization process.

Recovery of the condensed sterols may be made in one of two ways. For total recovery of the sterol for repetitive preparative gas chromatography or for other analytical purposes all of the condensed sterol is removed from the capillary by use of a solvent, preferably chloroform–methanol (9:1).

However, for retention of the high purity obtained by crystallization in the capillary removal of the crystalline mass separately from the amorphous material is

TABLE I

MELTING POINT BEHAVIOR OF STEROLS PURIFIED BY GAS CHROMATOGRAPHY

Sterol	*Melting point*		
	Before gas-chromatography	*Purified by gas chromatography*	*Literature*[a]
Category A (high melting point, no decomposition)			
1. 5α-Cholest-7-en-3β-ol	95–100°	120–122°	118–127°
2. Cholesta-5,24-dien-3β-ol	115–120°	116–120°	117–147°
3. Cholest-5-ene-3β,25-diol	176–180°	188–189°	172–183°
4. (25*R*)-Cholest-5-ene-3β,26-diol	172.5–174.0°	174.5–176.5°	177–178°
5. 5α-Cholestane-3β,5,6β-triol	201–209°	239–240°	236–241°
6. 3β-Hydroxypregn-5-en-20-one	181–194°	195–197°	188–194°
Category B (high melting point, some decomposition)			
7. Cholest-5-en-3β-ol			
(a) Commercial sample	145–149°	149–152°	145–151°
(b) Aged sample	105–142°	142–144°[b]	
(c) Very pure sample	152.0–153.5°	149–152°	
8. 3β-Hydroxycholest-5-en-7-one	175–176°	173–175°	165–173°
Category C (low melting point with decomposition)			
9. 5α-Cholestane	79–82°	81–82°	76–82°
10. 5β-Cholestan-3α-ol	115–117°	94–96°	107–118°
11. Cholesta-3,5-dien-7-one	112–114°	81–96°	111–115°
Recovery by rinse and crystallization			
12. Cholest-5-en-3β-ol acetate	110–112°	109–111°	111–116°
13. 3β-Acetoxycholest-5-en-7-one	—[c]	157–159°	155–163°
14. 3β-Acetoxycholest-5-en-25-ol	—[c]	138.5–139.5°	138–142°
15. (25*R*)-Cholest-5-ene-3β,26-diol 3β,26-diacetate	—[c]	127–128.5°	128–129°

[a] All m.p. taken from JACQUES *et al.*[42], are composite ranges taken from the literature.
[b] Rinse of the capillary walls gave sterol, m.p. 139–142°.
[c] Non-crystalline material was gas chromatographed. No m.p. recorded.

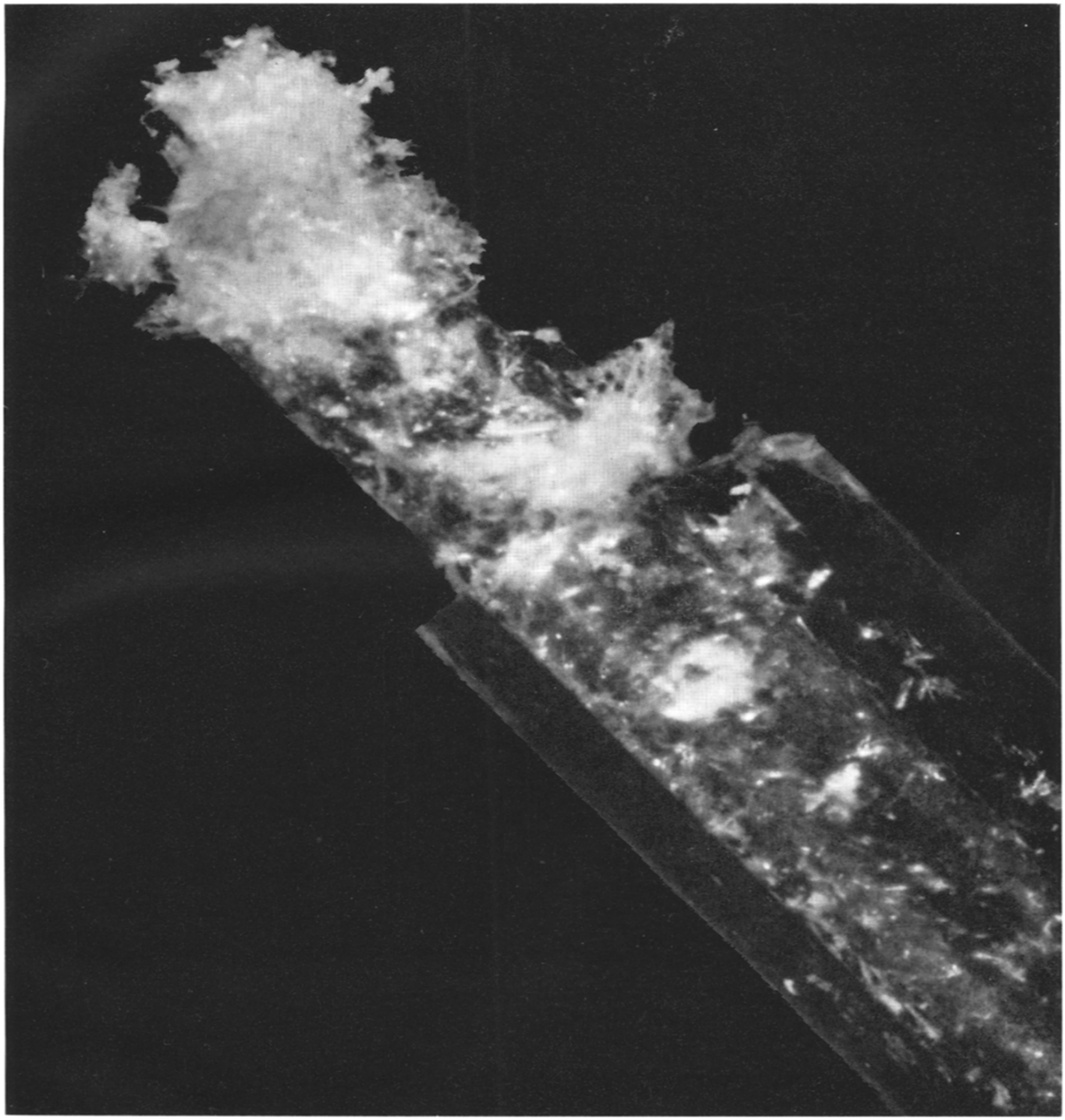

Fig. 2. Crystalline cholesterol removed by micro probe from capillary collection after gas chromatography on 3% QF-1.

necessary. The capillary is broken near the crystalline cluster and a fine metal or glass probe is carefully inserted into the capillary to extrude the crystalline mass without disturbing the non-crystalline material adhering to the capillary walls (Fig. 2). The resultant purification is supported by the improved melting point (No. 7b of Table I).

Where total recovery of eluted and condensed sterol component is of importance

collection using the recommended metal sleeve for generating a thermal gradient is possible. We have used a copper tube 7 mm in diameter and 8 cm long for the imposition of a suitable thermal gradient on the glass capillary collection tube.

In some cases our earlier technique in which the hydrogen flame was not used and the burner tip replaced by a sleeve and the capillary inserted directly on top of the column (without a stream splitter) gave better results. We consider that these cases were the result of an immediate condensation and crystallization of the eluted sterol in the capillary with less chance of earlier condensation as a fog during passage of the stream splitting unit.

The components of a sterol mixture may be collected in the same manner as for a homogeneous sample, by collection of each eluted component in its separate capillary tube. By laying out the desired number of capillaries before operation of the preparative column the resolved components may be collected in their order of elution without difficulty. Under column loadings suitable for preparative gas chromatography the elution curve shape is not ideal, and component peak skewing is regularly observed[4,41]. This skewing of the elution curve becomes critical for mixtures of sterols with similar retention times.

A 1 mg sample of a 1:9 mixture of 5β-cholestan-3β-ol and cholesterol dissolved

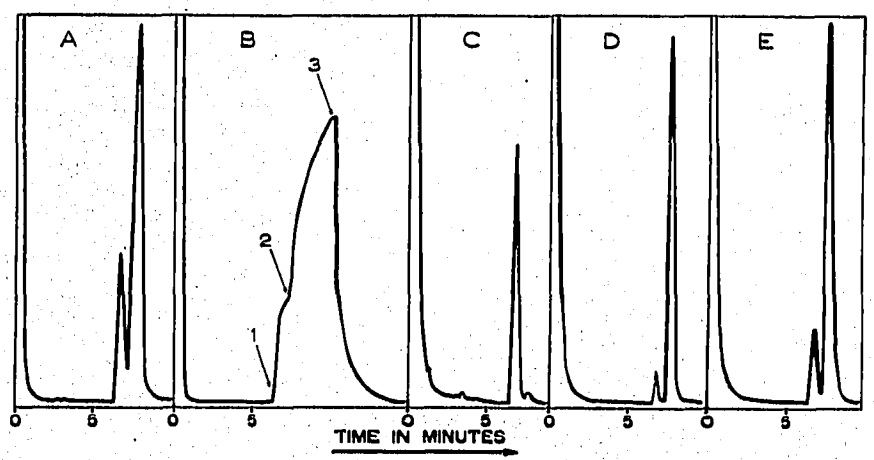

Fig. 3. Gas chromatographic separation of cholesterol and 5β-cholestan-3β-ol on preparative 3%; QF-1 columns. (A) original mixture (4 μg) on analytical QF-1 column; (B) preparative gas chromatographic elution curve of 700 μg sample (first capillary inserted at 1 and removed at 2, second capillary inserted at 2 and removed at 3); (C) analysis of the 5β-cholestan-3β-ol fraction; (D) analysis of the cholesterol crystalline cluster removed from capillary by probe; (E) analysis of chloroform rinse of capillary after removal of crystalline cluster of cholesterol.

in 10 μl of chloroform–methanol (9:1) was chromatographed on a 3% QF-1 6 mm preparative column. On analytical 3% QF-1 columns these sterols are easily resolved in our hands[40]. Using our standard procedure the crystalline product was collected, removed with a fine probe from the broken capillary tube, and analyzed by gas chromatography. In addition the condensate on the capillary walls was dissolved in solvent and analyzed. The crystalline product analyzed 6% 5β-cholestan-3β-ol, the amorphous wall condensate 11%. Elution curves for this preparative fractionation are presented in Fig. 3.

A 1:1 mixture of an authentic reference sterol and a suspect sterol whose identity

is to be established by mixture melting point is gas chromatographed preparatively in the usual manner and the suitably collected sterol sample used for melting point determination. Gas chromatography under these circumstances not only assures identical chromatographic properties of the two sterols but also the intimate mixing fundamentally necessary for proper mixture melting point determinations. Examples of our successes in use of this procedure for mixture melting point determinations include cholesterol, cholest-5-ene-3β,25 diol, (25R)-cholest-5-ene-3β,26-diol, 3β-hydroxycholest-5-en-7-one, androst-5-ene-3β,17β-diol, and 3β-hydroxypregn-5-en-20-one. For this latter example we obtained m.p. 191.0–192.0° for the authentic sample, 190.5–192.0° for the suspected sample, and 191.0–192.0° for the mixture melting point.

RESULTS

We have applied these preparative methods to a variety of sterols and related steroids with different degrees of success. In all cases the purified steroid was satisfactorily collected in the capillary tube without resort to cooling, use of thermal gradient, or other treatment. However, not all gas chromatographed sterols could be collected as crystalline products even though high purity sterol samples were used in the attempt. A set of examples is collected in Table I.

In all instances the collected sterol was homogeneous by thin-layer chromatography and by analytical gas chromatography, except as noted. However, neither of these chromatographic techniques actually measures high purity of the crystalline sterol as sensitively as does the common melting point, and we have applied this simple criteria as a reliable measure of purity of the gas chromatographed sterols.

Three categories of melting point behavior were obtained on free sterols prepared by gas chromatography. Category A includes sterols of relatively high melting point, above about 120°, with no significant decomposition by melting point or analytical gas chromatographic criteria. These sterols rapidly formed crystalline clusters in the capillary tubes and were repeatedly readily collected in this manner.

An excellent example of Category A behavior is found in our experience with cholest-5-ene-3β,26-diol[33]. This sterol diol is well separated on preparative columns from the other polar sterols present in mixed aortal polar trace sterol preparations (cholest-5-ene-3β,25-diol, 3β-hydroxycholest-5-en-7-one, cholesta-3,5-dien-7-one), and very good results have been obtained in tissue extracts which have been partially purified to remove the massive amounts of cholesterol commonly present.

Catogory B contains those sterols of relatively high melting point as in Category A, except that some decomposition was evinced by the melting point behavior. Such evidence usually involved a lowering melting point by a degree or so after gas chromatographic preparation. The diminished melting point can be attributed to trace impurities formed on gas chromatography not separated in the capillary collecting tube. For these compounds it is important to remove the capillary tube directly upon crystallization in order to minimize the effects of these impurities. Cholesterol is classified in this group (Table I) because of the relatively minor lowering of the melting point of a very high purity sample of m.p. 152.0–153.5° as a consequence of gas chromatography. In fact the melting point of the collected sample, m.p. 149–152°, is as high as most literature values but suffers from a decrease of 1.5° from the melting point of the pure sample prior to gas chromatography. Less pure samples of cholesterol

are generally purified by preparative gas chromatography, so that an elevation of melting point is observed.

Our experience with 3β-hydroxycholest-5-en-7-one has been discussed previously[33]. We consider that minor dehydration regularly accompanies any heating of this sterol, whether it be during the warming period following spraying with 50% sulfuric acid for standard visualization of the sterols (3β-hydroxycholest-5-en-7-one does not give a beige coloration itself but its dehydration product cholesta-3,5-dien-7-one does), or during gas chromatography. We estimate the degradation rate of the 7-ketone to be 0.25% per min on analytical gas chromatography on 3% QF-1 columns. However, because of the effects of selective crystallization of the sterol in the capillary collecting tube the melting point of the 7-ketone is not greatly depressed.

Category C includes those sterols of relatively low melting point, less than about 120°. These sterols will not crystallize but condense as droplets on the capillary wall. In this case the non-crystalline sterol is better recovered with a solvent and recrystallized by appropriate micro methods since high purity as evinced by sharp, high melting point will not be obtained by physical removal of the condensed sterol.

Interestingly, steryl acetates, even those of relatively high melting point, are not readily crystallized in capillary following gas chromatography. Recovery of steryl acetates by means of solvent rinsing and subsequent crystallization affords satisfactory, highly pure crystalline steryl acetate preparations for melting point and other identifications. The enhanced thermal stability of steryl acetates in general has greatly facilitated recovery of high purity samples despite the inherent loss of the advantage of selective crystallization from the gas phase obtained with the sterols themselves.

Trimethylsilyl ethers of sterols can also be utilized for preparative purposes, except that the ether is not particularly useful as a derivative for characterization of unidentified sterols because of their relative instability and sensitivity to moisture. Thus, the steryl trimethylsilyl ethers recovered from preparative gas chromatography may be hydrolyzed by a simple exposure of the ether to dilute methanolic alkali. The hydrolyzed sterol of high purity is obtained and can be crystallized or recrystallized directly.

Thus the steryl acetate ester and trimethylsilyl ether derivatives may be classified as Category C, mainly because of a lack of massive crystallization in the collecting capillary. The acetates are readily recovered from a chloroform rinse of the capillary by evaporation of the solvent, with recovery of high purity crystalline derivative. In this respect the acetates are also differentiated from the free sterols, which may not be so readily recovered as high purity crystalline products from the solvent rinse by mere evaporation.

DISCUSSION

As already noted, the preparative gas chromatography of steroids has now become a recognized and useful method for work with limited amounts of sample. However, the purity of the steroids recovered from preparative gas chromatograms has not been adequately studied. Our own interest in recovery of certain known sterols from human tissue[33] for rigorous identification purposes required that we

examine the purity of the sterols so recovered. No other workers have as yet addressed themselves to this problem.

The relatively insensitive spectral methods mentioned elsewhere in connection with preparative gas chromatography of steroids[43] do not afford a suitable measure of high purity nor is there usually enough sample for adequate or extensive spectral or optical purity determinations on high concentration solutions. Necessarily thin-layer and gas chromatographic analysis of the gas chromatographed steroids are appropriate techniques and are indeed required for the minimum assessment of purity. Mass spectral analysis is of obvious relevance and effectiveness in such matters, but the capital expense of the instrument and its operational expenses limit its availability. Reliance on other less expensive but equally sensitive methods must ordinarily be had.

In casting about for such means we found that traditional melting point behavior is a quite acceptable means of establishing purities, the notorious difficulties encountered in melting point behavior of sterols notwithstanding. Noteably, we are unaware of any prior publication which examines this obvious approach. Since purity of recovered sample is of critical importance to the matter of rigorous identification, this approach should be well received and easily accommodated in most laboratories undertaking such problems.

In fact the application of preparative gas chromatography to the free sterols affords not only a pure sample for identification purposes, but the very process of crystallization in the collecting capillary affords the final purification by differential condensation of very high purity sterol from trace impurities and amorphous portions of the sterol preparation. Maximum advantage of the differential condensation of impurities on the walls and of crystallization of the major sterol component within the lumen of the capillary tube is obtained by abrupt air cooling of the capillary without using the often recommended thermal gradient generating metal sleeves. In distinction to the differential crystallization obtained without an imposed thermal gradient, application of a thermal gradient causes condensation to occur over a longer region of the capillary, with attendant increased contamination of crystalline material by amorphous material. The photographic evidence previously published[8] upholding the merit of use of applied thermal gradients for recovery of greater amounts of collected product, together with our own present photographs of Fig. 1, also support our present contention that the added advantage of differential crystallization in capillary is readily achieved in the absence of an applied thermal gradient.

However, the use of a thermal gradient for increased recovery of eluted sterol is of importance where quantitative recovery rather than high purity is wanted. The melting points of sterols recovered with chloroform from glass capillaries using the metal sleeve thermal gradient technique can be depressed as much as ten degrees over the melting point of the same sterol sample collected as crystalline material without use of the thermal gradient. In Fig. 4 infrared absorption spectra of high purity cholesterol are compared with spectra of the crystalline cluster obtained with pure cholesterol and with spectra of that portion of the gas chromatographed cholesterol which condensed on the capillary wall. It may be seen that no major differences are obtained by use of either technique.

An additional advantageous application of these preparative gas chromatographic methods involves the determination of the melting point of deliberately mixed

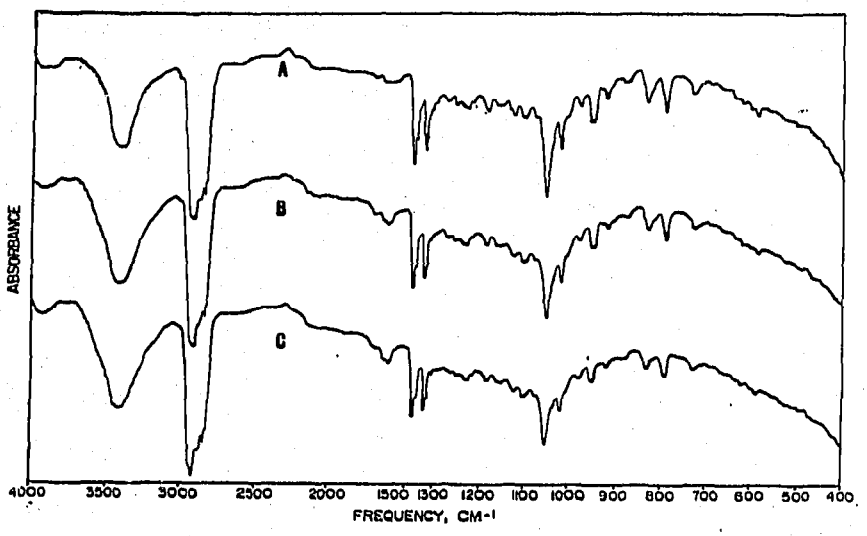

Fig. 4. Infrared absorption spectra of: (A) reference high purity cholesterol, m.p. 152.0–153.5°; (B) crystalline cholesterol from collection in capillary, recovered by differential removal of the crystalline cluster from the capillary, m.p. 149.0–152.0°; (C) cholesterol condensed on the capillary wall, recovered by chloroform rinse after initial removal of the crystalline cluster as in (B) m.p. 147.0–149.0°.

reference and test sterol. The traditional mixture melting point procedure as applied to sterols is fraught with pitfalls. However, where proper characterization and analysis has been accorded the sterol sample, it is reasonable to rule out likely contributions of sterol epimers or other closely related steroid contaminations where classically the difficulties of solid solution are encountered and where use of the mixture melting point for identification purposes may fail.

Correct experimental procedure for determination of mixture melting points requires admixture of equal amounts of reference and test sterol, fusion and resolidification or recrystallization, and determination of melting point. Practically the fusion of the two samples is rarely done, and reliance is made on a mere physical mixture of the reference and test sample. Accordingly, mixture melting point data in the literature are not fully trustworthy, witness the reluctance of authors to quote precise values for mixture melting points determined in such matters.

Whereas reliance on physical admixture of the reference and test sample may be justified with sensitive sterols which decompose on fusion in the usual treatment, conclusions from such data are clearly compromised and require great care to avoid error. This mixing problem is solved by co-gas chromatography of the two samples, with recovery of the crystalline sterol product mixture and melting point determination in the usual fashion. In this manner we have made reliable identifications of test sterol samples with reference sterols.

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