QUANTITATIVE DETERMINATION OF CHOLESTEROL IN AUTO-OXIDATION MIXTURES BY THIN-LAYER CHROMATOGRAPHY

CSABA HORVATH*
Physics Research Laboratory, Massachusetts General Hospital, Boston, Mass. (U.S.A.)
(Received September 13th, 1965)

An analytical method was needed to follow the degradation of crystalline cholesterol in a study of its thermal and radiation induced decomposition, the results of which will be reported at a later date.

Cholesterol readily undergoes auto-oxidation and decomposition by heat or by irradation[1]. Some of the products have been identified as 7-ketocholesterol, the α- and β-7-hydroxycholesterols, cholestan-3β,5α,6β-triol, $\Delta^{3,5}$-cholestadien-7-one and 25-hydroxycholesterol.

As the thin layer chromatogram of our samples demonstrates (Fig. 1) a great number of products arose from the decomposition of crystalline cholesterol. Presumably, most of the products are closely related to cholesterol. No simple and reliable method has been reported for the quantitative determination of cholesterol in such mixtures.

Although NORCIA[2] has suggested the SCHOENHEIMER-SPERRY method, modified by SPERRY AND WEBB[3] for quantitative determination of cholesterol in auto-oxidation mixtures the specificity of this technique was doubtful in the present case. Values of cholesterol were expected to be higher than the actual ones, since large amounts of the epimeric 7-hydroxycholesterols can interfere[4,5] and some of the unknown degradation products could also precipitate with digitonin and react with the Liebermann-Burchard reagent. Although gas chromatography is being successfully used for the determination of cholesterol, this method might result in unreliable low cholesterol values for our application[6]. Some of the degradation products, particularly peroxides and hydroperoxides may react with cholesterol at high temperatures in the gas chromatograph decreasing the cholesterol values.

As a conclusion, it was necessary to develop a specific analytical method in order to obtain reliable values for cholesterol in mixtures which contain both its oxidation and degradation products. Thin-layer chromatography (TLC) has been found to be an expeditious and simple method for separation of cholesterol from these products. The color reaction with the perchloric acid–phosphoric acid–ferric chloride (PPF) reagent introduced by MOMOSE *et al.*[7] is relatively insensitive to the impurities of the reagents and gives a simple method for the quantitative determination of the separated cholesterol.

The method described here utilizes a combination of this color reaction with TLC.

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* Present address: Yale University School of Medicine, Department of Internal Medicine, 333 Cedar Street, New Haven, Conn.

EXPERIMENTAL

Materials

Cholesterol, reagent grade, was recrystallized from glacial acetic acid and then from ethyl acetate. Silica Gel H and aluminium oxide H were supplied by E. Merck AG (Germany). Reagent grade solvents and $SbCl_3$ were used throughout the study.

Preparation of the samples

Five different samples prepared under the conditions given in Table I were analyzed. Crystalline cholesterol was placed in glass vessels of 20 mm inner diameter and air, saturated with water at 20°, was passed through the vessel at a slow flow rate.

TABLE I

DECOMPOSITION OF CHOLESTEROL

Sample	*Treatment*	*Temperature (°C)*	*Approx. dose (mrads)*	*Duration of treatment (h)*	*Remaining cholesterol[a] (%)*
A	Thermal	105	—	171.5	52.0
B	Thermal	98	—	143.0	45.7
C	γ-irradiation	50	51	89.4	15.0
D	γ-irradiation	50	79	142.3[b]	15.4
E	γ-irradiation	50	24.2	43.0[c]	74.2

[a] Determined by the present method.
[b] In air saturated with water and hydrogen peroxide.
[c] In dry air.

Each batch containing 3 g cholesterol was either heated in an oil bath or irradiated in a ^{60}Co-irradiator[8]. The samples were then dried *in vacuo* and powdered. Solutions were made up in chloroform which contained about 15 mg of the solid material per ml.

Separation of the samples

In order to obtain information about their composition, the samples were separated by the thin-layer chromatography (TLC) technique successfully used by VAN DAM *et al.*[9] to separate some oxidation products of cholesterol. For our purpose, a mixture of chloroform and acetone, 5:2 by volume, was found to be a suitable solvent system for plates coated with silica gel H. Fig. 1 shows the separation of the five different samples A–E.

The plate was divided in 20 mm strips and for optimal separation of the products the strips were narrowed to a width of 5 mm below the starting line by removing excess silica gel.

The plate was activated at 120° for 2 h and allowed to cool in air. The separation was obtained at approximately 27° and 40 % relative humidity. 25 μl samples of the chloroform solutions containing 360–380 μg of auto-oxidized cholesterol were applied 30 mm from the bottom edge. The plate was irrigated with the above mentioned solvent system in a lined chamber filled to a height of 1 cm. The solvent was allowed to migrate 13 cm from the starting line (55 min) and the plate was dried at 100° for 3 min. The irrigation and drying were repeated in order to obtain a better separation.

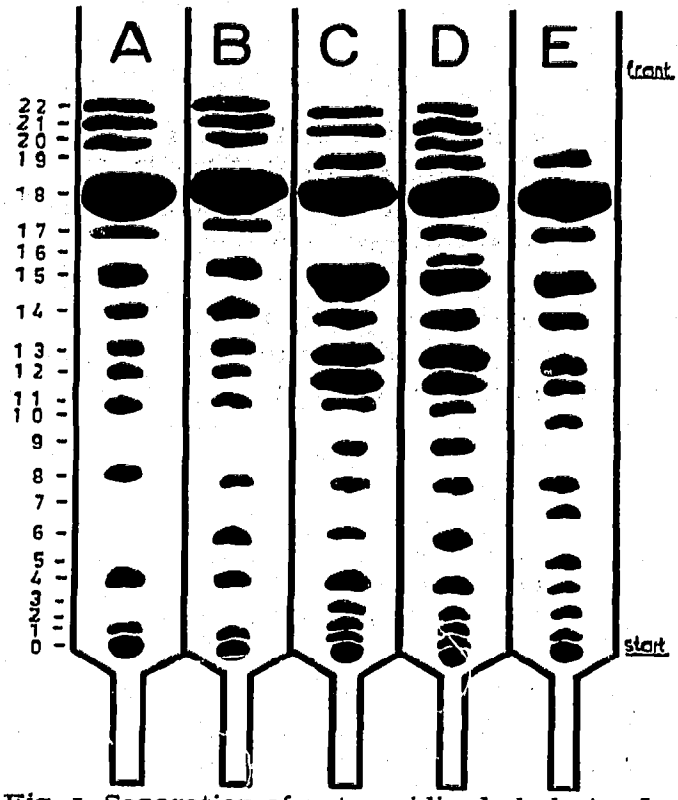

Fig. 1. Separation of auto-oxidized cholesterol samples A–E.

The components were detected by spraying with a saturated solution of $SbCl_3$ in chloroform, and after recording the color the plate was heated at 100° for 3 min. A 300 μg sample of the original cholesterol proved to be pure by this procedure.

The R_{Chol}-values, *i.e.* the R_F-values related to cholesterol, and the colors of the spots are given in Table II. Although the goal of the present work was not the identification of the products, some information has been obtained by using reference substances and detection methods for peroxides. Spot No. 18 was identified as cholesterol and spot No. 4 as cholestane-3β,5α,6β,-triol. Most probably, spots No. 12–13 represent the epimeric 7-hydroxy-cholesterols and No. 15 the cholesterol-5-hydroperoxide. The last one might arise from rearrangement of cholesterol-5-hydroperoxide in chloroform[10]. 7-Ketocholesterol and $\Delta^{3,5}$-cholestadien-7-one have been found in samples A and B using H_2SO_4 charring for detection. The position of these compounds corresponds to that of the spots No. 21–22 in Fig. 1. The presence of peroxidic compounds was confirmed by spraying with potassium iodide and starch solutions[11] or with a mixture of ferro-ammonium-sulfate and ammonium-thiocyanate-solutions[12].

PROCEDURE FOR QUANTITATIVE ANALYSIS

Reagents and solutions

Mixture of chloroform and acetone, 4:1 by volume. Glacial acetic acid containing 0.1% $FeCl_3 \cdot 6H_2O$. Color reagent: 4 g of $FeCl_3 \cdot 6H_2O$ dissolved in a mixture of 100 ml of 70% perchloric acid and 300 ml of 85% phosphoric acid. Standard cholesterol stock solution prepared by dissolving 500 mg of cholesterol in 50 ml chloroform.

TABLE II

R_{Chol}-VALUES AND COLORS OF THE SPOTS IN FIG. 1

Colors were observed immediately after spraying with $SbCl_3$ solution and after heating the plate at 100° for 3 min. The results of the second observation are given in brackets. The peroxide reactions were obtained on other plates. The U.V. fluorescence was observed in long-wave ultraviolet light.

No.	R_{Chol}[a]	*Colors of the spots from samples*		
		A and B	*C and D*	*E*
0	0.00	blue (gray)[c]	orange (brown)[b, c]	pink (violet)[b, c]
1	0.04	— —	pink (brown)[b]	pink (violet)
2	0.07	— —	pink (brown)[b]	— —
3	0.09	— —	pink (brown)[b]	pink (yellow)
4	0.15	blue (yellow)[d]	blue (yellow)[d]	blue (yellow)[d]
5	0.20	— —	— —	— —
6	0.24	— —	pink (violet)	— —
7	0.31	— —	— —	— (brown)
8	0.37	blue (brown)	blue (brown)	— (brown)
9	0.46	— —	blue (brown)	— —
10	0.52	— —	— —	— (brown)
11	0.55	blue (brown)	— (violet)	— —
12	0.60	blue (blue)	blue (blue)	blue (blue)
13	0.65	blue (blue)	blue (blue)	blue (blue)
14	0.75	pink (brown)	pink (violet)	— (violet)
15	0.82	blue (blue)[b, c]	blue (blue)[b, c]	violet (violet)[b, c]
16	0.86	— —	— (brown)	— —
17	0.93	— (violet)	blue (brown)[c]	pink (violet)
18	1.00	orange (maroon)	orange (maroon)	orange (maroon)
19	1.08	— —	[d]	— (crimson)
20	1.12	blue (blue)	blue (blue)[d]	— —
21	1.16	violet (maroon)	— (maroon)[d]	— —
22	1.19	blue (violet)	violet (violet)[d]	— —

[a] R_F value related to cholesterol.
[b] Peroxidic reaction with KI–starch[11].
[c] Peroxidic reaction with Fe^{2+}–SCN^-.[12]
[d] Fluorescence in U.V.

Thin-layer chromatography

Glass plates 200 × 200 mm were coated uniformly with silica gel H to a thickness of 0.4 mm according to the procedure suggested by the supplier. After coating, the plates were dried in the atmosphere. Beginning at 10 mm from one edge, 1 mm channels spaced 20 mm apart were engraved with a jeweler's screwdriver. Nine strips of 20 mm were obtained, allowing nine separate samples to be run simultaneously. The plates were washed overnight in the usual manner in a chamber with ethanol and activated at 100° for 2 h. Immediately after cooling, the samples containing 20–200 μg cholesterol in a solution of chloroform were applied at a point in the center of each strip, 30 mm from the lower edge. For sampling, 50 μl Hamilton syringes with Chaney-adaptors were used and heat was applied to the bottom of the plates for samples larger than 20 μl in order to facilitate the evaporation of the solvent.

The plates were placed in a chromatography jar lined on three sides by filter paper and immersed to a depth of 10 mm from the lower edge in a mixture of chloroform–acetone, 4:1 by volume. The solvent was allowed to rise to a height of 160 mm above the starting points. This development required about 35 min. The plates were

removed and dried at 100° for 5 min and upon cooling were sprayed with distilled water. The spraying was started at a safe distance in order to avoid the peeling of dry silica gel by the air stream. Upon spraying, the cholesterol spots ($R_F \sim 0.6$) became visible by viewing the plate on black paper. From the center of the spots, distances of 8 mm in the starting direction and 12 mm in the flow direction were marked off on the strips. The marked parts of the wet silica gel strips were then transferred into 15 ml centrifuge tubes by using a micro spatula which had been ground flat at the end and bent 5 mm from this edge. The silica gel remaining on the spatula was removed with a stirring rod which was put in each centrifuge tube. Using two spatulas, one bent and one straight, the operation could be carried out more quickly. The centrifuge tubes were dried for 10 min at 100° and the silica gel scraped from the walls to the bottom by the stirring rods. To avoid the oxidation of the finely dispersed cholesterol, the samples must be worked up immediately.

Color development

2 ml of glacial acetic acid containing 0.1 % ferric chloride were added to each tube and the mixture was stirred vigorously with the stirring rod. The tubes were then heated in a water bath at 80° for 6 min.

After cooling, 1 ml color reagent was added to each tube and the contents were mixed thoroughly. The tubes were heated in an oil bath at 100° for 20 min while the liquid level was kept above the surface of the contents of the tubes. The tubes were allowed to cool in cold water and after removing the glass rods they were centrifuged at 3000 r.p.m. for 15 min. The supernatant solution was poured carefully into glass cuvettes of 10 mm path length and the absorbance was read using a Beckmann DK-2 spectrophotometer against a reagent blank at 450 mμ. The absorbance remained constant for about 2 h. The reagent blank was made from 2 ml of glacial acetic acid containing 0.1 % ferric chloride and 1 ml color reagent in the previously described manner omitting the centrifugation. From each batch of thin-layer plates, blanks were made with silica gel, which was scraped from a 4 cm^2 area at the edge of the plate. The absorbance of these blanks was found to be equal to that of the reagent blanks at 450 mμ.

Calibration curve and calculation

Solutions were prepared by diluting 2,4,6 and 8 ml of standard cholesterol stock solution to 10 ml with chloroform, and 20 μl samples containing 20, 40, 80, 120, 160 and 200 μg cholesterol were applied. The absorbances were measured after the described procedure above. The calibration curve obtained by plotting absorbance *versus* micrograms of cholesterol is a straight line passing through the origin. The cholesterol values of the samples were calculated from the calibration curve or conveniently from the ratio of the absorbance of the unknown sample to that of a standard cholesterol sample.

RESULTS AND DISCUSSION

Fig. 1 shows the pattern of the products in samples oxidized under different conditions such as elevated temperature or gamma irradiation. It is seen that the number of the degradation products is much greater than has been assumed on the

basis of previously reported results. However, there may be additional products which have not been separated or detected with the present technique. Although the qualitative and quantitative make-up of the samples varies with the conditions, a large number of identical products are present in each sample. This indicates that the present method also could be used for cholesterol determination in samples arising from other autoxidation processes. The separation of the samples shown in Fig. 1 has been achieved on specially prepared plates and with double irrigation in a tedious procedure. The quantitative determination of cholesterol does not require the separation of the products from each other but that of cholesterol from the products. The procedure used for this purpose is simple and gives reliable results as shown by the following tests.

Marking the spot area

For an equal amount of cholesterol the visible spot area is smaller after spraying with water than with $SbCl_3$-solution. Spots representing 80–300 μg of cholesterol were examined in order to mark that part of the silica gel strips which had to be transferred. After spraying with water, the spot lengths in the flow direction varied from 6 to 11 mm when the plates were examined on underlying black paper.

Spot lengths of 15–20 mm were measured after drying and spraying with $SbCl_3$-solution in transparent light on the same plate. The centers of the colored spots were generally 2 mm closer to the front of the spots. In agreement with this observation up to 250 μg of cholesterol can be transferred quantitatively after spraying with water if 20 mm of the strip beginning 8 mm below the center of the spots are scraped off. This order of marking has been verified by the following tests. 360–380 μg of samples A–E were separated. Silica gel areas containing cholesterol from triplicate samples were combined and extracted with 1.5 ml of chloroform. The solutions were concentrated to about 60 μl in a water bath and 25 μl samples were applied on two TLC-plates. One of them was coated with silica gel H, the other with aluminium oxide H. The plates were prepared and irrigated as described above. After treatment with $SbCl_3$, no spots were found close to that of cholesterol. However, spots were found with R_{Chol}-values of 0.63, 0.82 and 1.17 which correspond to those of spots No. 12–13, No. 15 and No. 21 in Fig. 1. These compounds should have been separated from cholesterol in the normal procedure in consideration of their R_{Chol}-values. The areas of these spots were significantly larger when the silica gel containing cholesterol was stored in air at room temperature for several days. Compounds showing the same spots arose from pure cholesterol which was adsorbed on silica gel, exposed to air at room temperature for a week and chromatographed as above. All these indicate that they are the primary autoxidation products of cholesterol. Because of the rapid autoxidation of cholesterol adsorbed on silica gel, the separated cholesterol must be processed immediately.

The existence of cholesterol outside of the marked area has been investigated. The contiguous 10 mm parts of the silica gel strips on both sides of the removed cholesterol spot areas were scraped off, extracted and chromatographed as above. No cholesterol was detected on spraying with $SbCl_3$ solution. Absorption spectra of the developed color between 300–1000 mμ were checked in the quantitative analysis of the five samples. In all cases, the shape of the spectrum was the same as that of the standard cholesterol samples. As expected, absorption spectra of the five samples

obtained by the color reaction without TLC separation were significantly different from that of cholesterol.

Effect of the sample volume

Chloroform solutions of sample A (see Table I) containing 0.8 μg, 3.2 μg and 40.0 μg solid material per μl were prepared. Sample volumes of 3000 μl, 75 μl and 6 μl, respectively were applied on TLC plates and analyzed in the described manner. The starting spot areas were less than 7 mm in diameter. Each sample contained 240 μg of sample A, *i.e.* 124.8 μg of cholesterol. The relative standard deviation of the measurement was 1.9% from 10 determinations.

Reproducibiltiy of the measurement

Chloroform solutions of samples B and C (see Table I) containing 231 μg and 91.8 μg cholesterol in 30 μl were analyzed. The fractional standard deviations of each cholesterol value at 7 and 6 degrees of freedom were 1.81 and 1.75 %.

However, this loss and the decrease of color by silica gel are proportional to the amount of cholesterol in the range of 20–250 μg as indicated by good linearity *i.e.*, 0.34 % relative standard deviation of the calibration curve.

Comparison of different methods

The five samples examined have been analysed for cholesterol by three other methods which are:

(a) The SCHOENHEIMER-SPERRY method modified by SPERRY AND WEBB[3] which involves a digitonin separation.

(b) The BARR method[13] which is essentially the Liebermann-Buchard color reaction.

(c) The PPF-color reaction[7].

Microliter volumes of the chloroform solutions of samples A–E and of the standard cholesterol solutions used in the foregoing study were evaporated. The residues were dissolved in the particular solvent of each method and processed according to the descriptions.

Table III shows the results of these analyses compared with those obtained by the method described in this paper. As expected, the color reaction of cholesterol

TABLE III

CHOLESTEROL VALUES DETERMINED BY DIFFERENT METHODS

The figures in brackets represent the obtained values normalized to those measured by the present method.

Method	*Cholesterol (wt. %)*				
	A	*B*	*C*	*D*	*E*
SCHOENHEIMER-SPERRY method modified by SPERRY AND WEBB	57.6 (1.11)	53.3 (1.16)	17.7 (1.18)	21.4 (1.39)	82.6 (1.11)
BARR method	76.2 (1.46)	67.2 (1.26)	39.8 (2.65)	34.1 (2.21)	91.2 (1.22)
Direct color reaction with the PPF-reagent	63.6 (1.20)	61.9 (1.35)	34.4 (2.29)	19.4 (1.26)	80.6 (1.09)
Present method	52.0	45.7	15.0	15.4	74.2

separated with thin-layer chromatography results in lower values than the other methods where cholesterol was not or only partly separated. The extent of the differences depends on the qualitative and quantitative composition of the samples and varies with the methods. Generally, the variation is smaller in case of samples of high cholesterol content as the relative amount of interfering products is smaller. The best agreement with the results of the present method has been obtained by the SCHOENHEIMER-SPERRY method probably due to the separation by the digitonin precipitation. However, none of the compared previously known methods can be considered as reliable for analyzing such mixtures.

ACKNOWLEDGEMENTS

The author gratefully acknowledges and appreciates the assistance of G. L. BROWNELL under whose direction this work was performed.

This work was supported by the American Cancer Society, Grant No. T-82F and the U.S. Public Health Service, Grant No. 5T1 GM889-08.

SUMMARY

A method is presented for the quantitative determination of cholesterol in the presence of its oxidation and degradation products arising from thermal and radiation induced decomposition. The samples are chromatographed on silica gel coated thin layer plates with a chloroform–acetone mixture. Cholesterol becomes visible upon spraying with water. The spot is scraped off and transferred with the wet silica gel into a centrifuge tube. After drying, glacial acetic acid containing ferric chloride is added, and color is developed with perchloric acid–phosphoric acid–ferric chloride reagent in the slurry. After centrifugation the absorption of the liquid is measured at 450 mμ. The method can be used for the determination of 20–200 μg cholesterol with less than 2 % error.

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