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QUANTITATIVE ANALYSIS OF HUMAN SERUM CHOLESTEROL BY THIN-LAYER CHROMATOGRAPHIC SPOT TEST

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SUMMARY

A fast, inexpensive, simple method is described for the determination of the total cholesterol concentration in human serum. The cholesterols (free and esterified) are extracted from serum by a double extraction procedure, using isopropanol—water—10 *N* sodium hydroxide (250:125:10) and then *n*-octane. An aliquot of the octane extract is spotted on a thin-layer plate and the cholesterol in the spot is rendered visible using an aqueous phosphomolybdic acid staining solution and a heat treatment for the color development. The colored spot is scanned with a densitometer.

INTRODUCTION

The significance of the cholesterol level in serum, due to its presumed relationship to atherosclerosis and other cardiovascular diseases, lead to the development of numerous laboratory tests for its quantitative determination. Among other methods, thin-layer chromatography (TLC) has also been applied for the analysis. In the direct method [1—7], cholesterol and its esters are separated from the other serum lipids on a silica gel coated thin-layer plate, the chromatogram is stained and the bands are scanned. In the indirect method [8—10], the separated cholesterol fractions are eluted from the silica gel and are quantitated by some conventional method. Both methods have their particular drawbacks: the direct method lacks the necessary precision and the indirect method is rather tedious. Both methods have an additional common drawback: the free and the esterified cholesterol fractions separate into two distinct bands and therefore both bands should be quantitated if the total cholesterol is to be determined.

The method described below permits the separation of the cholesterol and its esters from the other lipids by a double extraction, using a combination of alkaline isopropanol and *n*-octane. The octane extract is then applied to a thin-layer plate for the quantitative measurement of the total cholesterol.

EXPERIMENTAL

Procedure

Five hundred μl of the isopropanol—water—10 *N* sodium hydroxide mixture (250:125:10) are pipetted into a 12 × 75 mm glass tube. Twenty-five μl of serum are added to the tube and the contents are mixed on a Vortex-type mixer for 15 sec. After 2 min, 200 μl of *n*-octane are added and the contents are mixed again on a Vortex mixer for 30 sec. Octane separates from the lower isopropanol—water—sodium hydroxide mixture in 1 min. Ten μl of the upper octane phase are dispensed onto a 5 × 10 cm silica gel 60 coated glass plate with a 10-μl Eppendorf pipet. The octane forms a round spot on the plate with a diameter of about 10 mm. The spot dries in air in about 1 min, after which the plate is immersed in a staining solution. The staining solution is composed of 15 g of phosphomolybdic acid and 10 ml of perchloric acid dissolved in a mixture of 90 ml of water and 100 ml of ethanol. After the air bubbles have escaped (10 sec), the plate is taken out of the solution and blotted with a paper towel. The dried plate is placed in front of a forced air heater for 5 min at a distance at which the temperature does not exceed 50°. During the heat treatment the spot turns blue while the rest of the plate shows a pale yellow background. The color intensity of the spot is determined with a single-beam photodensitometer (Quick Scan; Helena Labs., Beaumont, Texas, U.S.A.). The spots are scanned and the readings are evaluated from a calibration curve. For the preparation of the calibration curve, solutions of 1, 2, 3, and 4 mg/ml cholesterol in isopropanol are prepared. The color of the spot and its intensity are stable for at least 1 h. To increase the sensitivity a 645-μm filter is used for the scanning.

RESULTS

The standard curve prepared from the spots of 1—4 mg/ml cholesterol solutions is shown in Fig. 1. The scanned values are the mean values of ten replicate measurements. The relative standard deviation (RSD) values are at 1 mg/ml: 8.7%, at 2 mg/ml: 7.9%, at 3 mg/ml: 8.6%, and at 4 mg/ml: 9.6%. The mean RSD is 8.7%.

Twenty serum specimens were analyzed by the conventional Liebermann—Burchard colorimetric method and by the micro-spot method, respectively. The scatter diagram is shown in Fig. 2.

It shows how well the scanning values compares with the Liebermann—Burchard test.

DISCUSSION

The color-producing reaction is not specific for cholesterol. Since the other lipid components of the serum (triglycerides, free fatty acids, phospholipids) also produce the same color, cholesterol must first be separated from these

components. The separation is carried out with a combination of an alkaline hydrolysis and an extraction with octane. When the serum is mixed with the alkaline isopropanol, the lipids are separated from their carrier proteins and simultaneously react with the alkali. Triglycerides and phospholipids are hydrolyzed and the unesterified and liberated fatty acids are neutralized. Accordingly, the alkaline isopropanolic extract contains sodium salts of the fatty acids, sodium phosphate, free glycerol and the free and esterified cholesterol

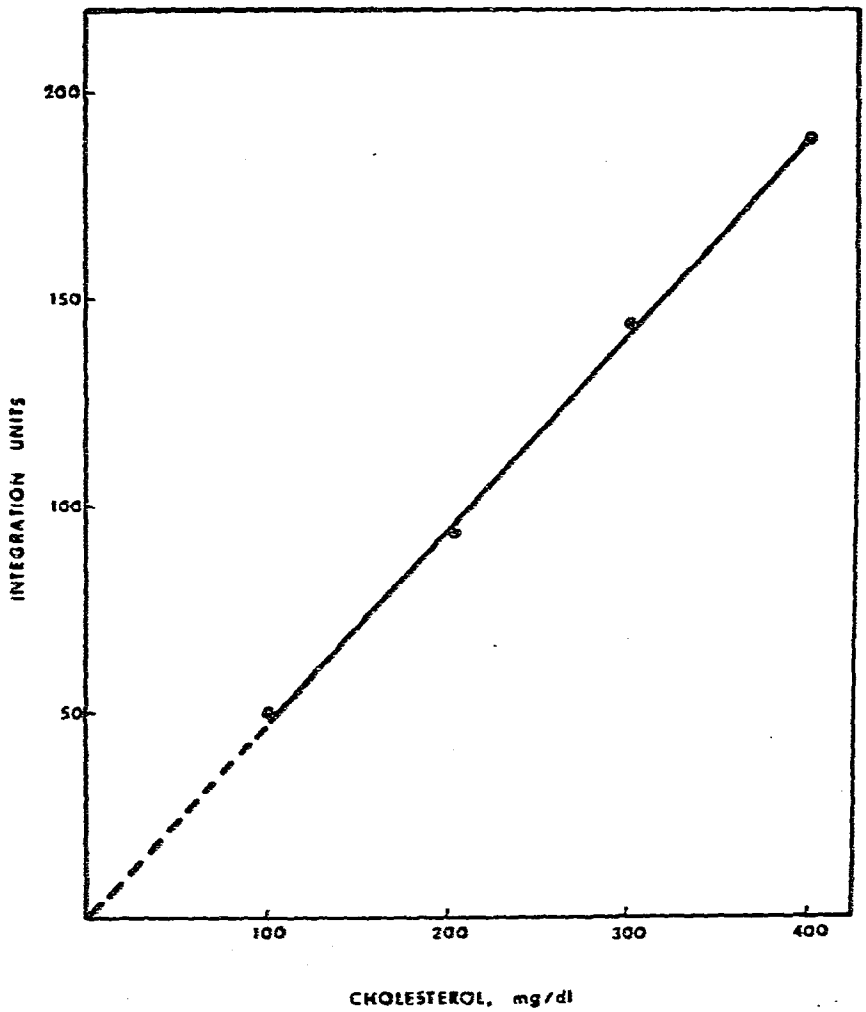

Fig. 1. Calibration curve constructed from mean values of 10 replicate densitometric measurements in reference solutions with 1—4 mg/ml cholesterol concentration. Scanner, Quick Scan; slit width, 5 mm.

Octane extraction of this mixture achieves the separation of free and esterified cholesterol from the other components, since only the cholesterol compounds are soluble in the apolar solvent. Octane separates rapidly from the aqueous isopropanolic phase, which contains all the other compounds present after the hydrolysis.

The effect of the alkaline treatment and of the octane extraction can be

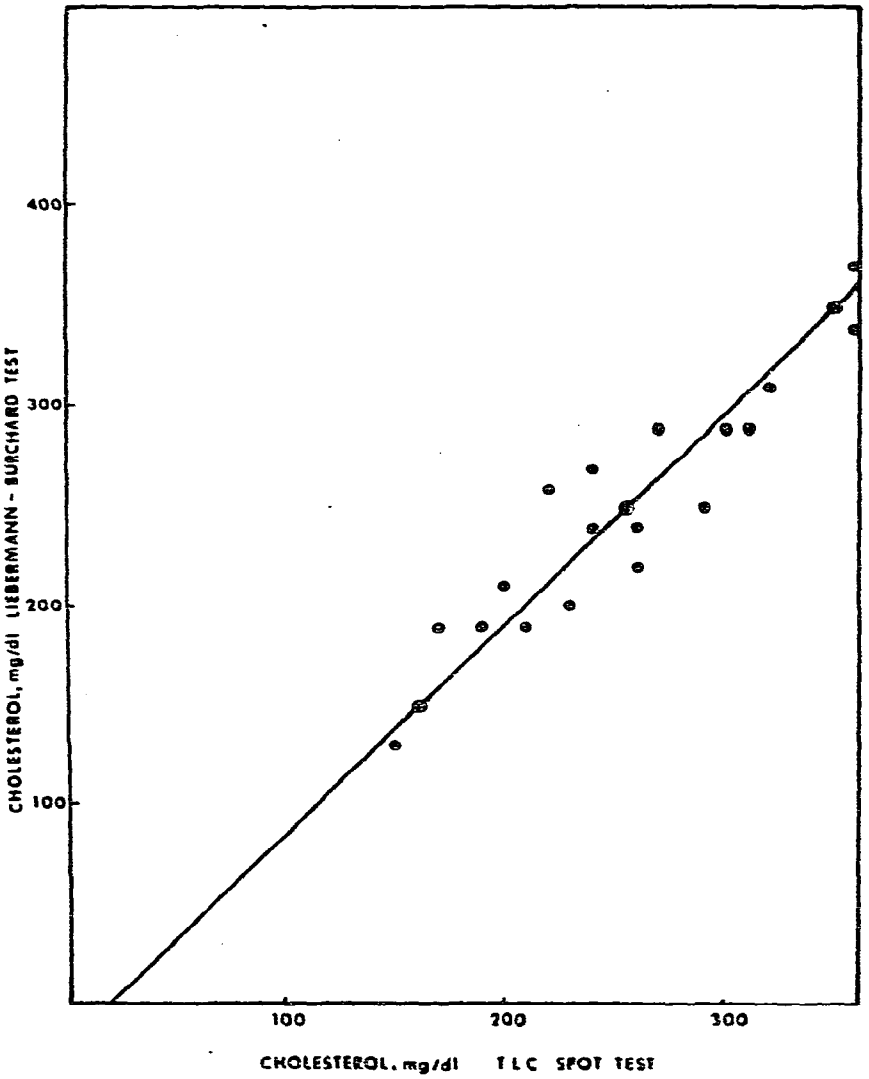

Fig. 2. Comparison of total serum cholesterol values obtained by the TLC-spot method and the Liebermann—Burchard test.

followed by TLC. Fig. 3 shows the composition of a conventional isopropanolic extract. Starting from the round shaped spot reading upwards, the chromatogram shows the five main lipid classes of serum; phospholipids, free cholesterol, free fatty acids, triglycerides, and cholesterol esters.

This chromatogram has been prepared from the extract of a pooled serum. Five hundred μl of isopropanol and 25 μl of serum were mixed on a Vortex mixer for 5 sec and the precipitated protein was separated by centrifugation. A 10-μl aliquot of the supernatant was applied to the TLC plate.

Two solvent mixtures were used for the separation. The development in the first mixture moved all the lipids except the phospholipids to the solvent front, concentrating them into a thin line 1 cm above the starting point. This

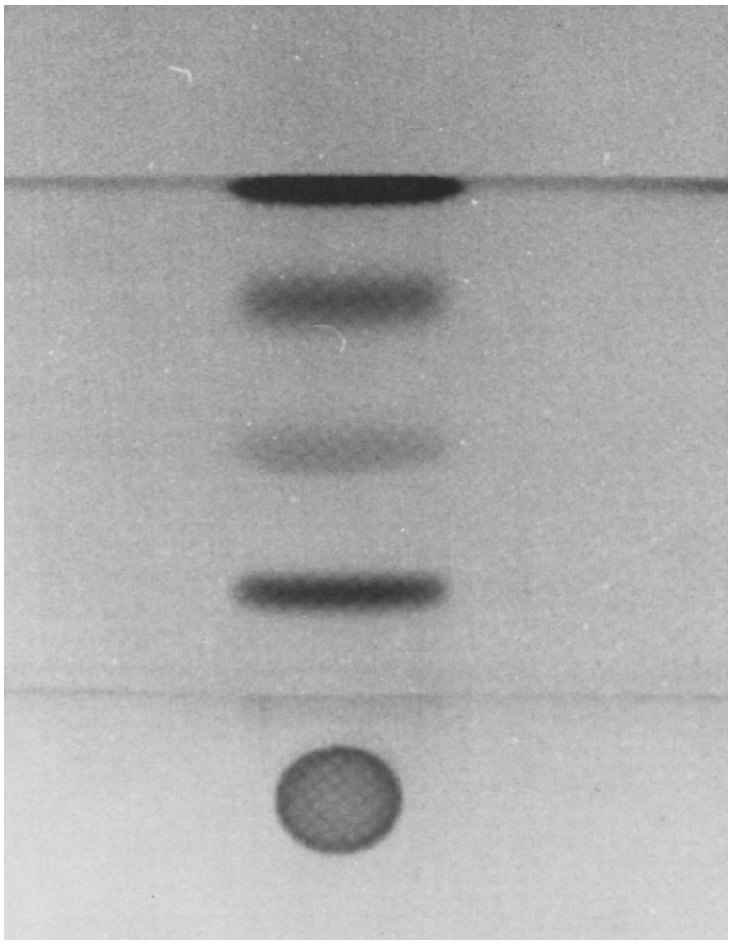

Fig. 3. Chromatogram of the isopropanolic extract of a pooled serum.

separation took 1 min. This made it possible to perform a fast and easy separation with the second solvent, for a 2-cm distance in 4 min. After it was removed from the first solvent, the plate was air dried and returned to the second solvent. This separated the four other lipids into distinct bands. The first solvent system contained methanol—chloroform—glacial acetic acid—water (50:50:5:5). The second solvent system consisted of light petroleum (b.p. 20—40°)—diethyl ether—glacial acetic acid (80:20:1).

Fig. 4 shows the effect of the octane extraction when there is no alkali present in the isopropanol. Twenty-five μl of the serum were added to 500 μl of isopropanol and the mixture was agitated for 15 sec. Two hundred μl octane were added and the contents were mixed on a Vortex again for 30 sec.

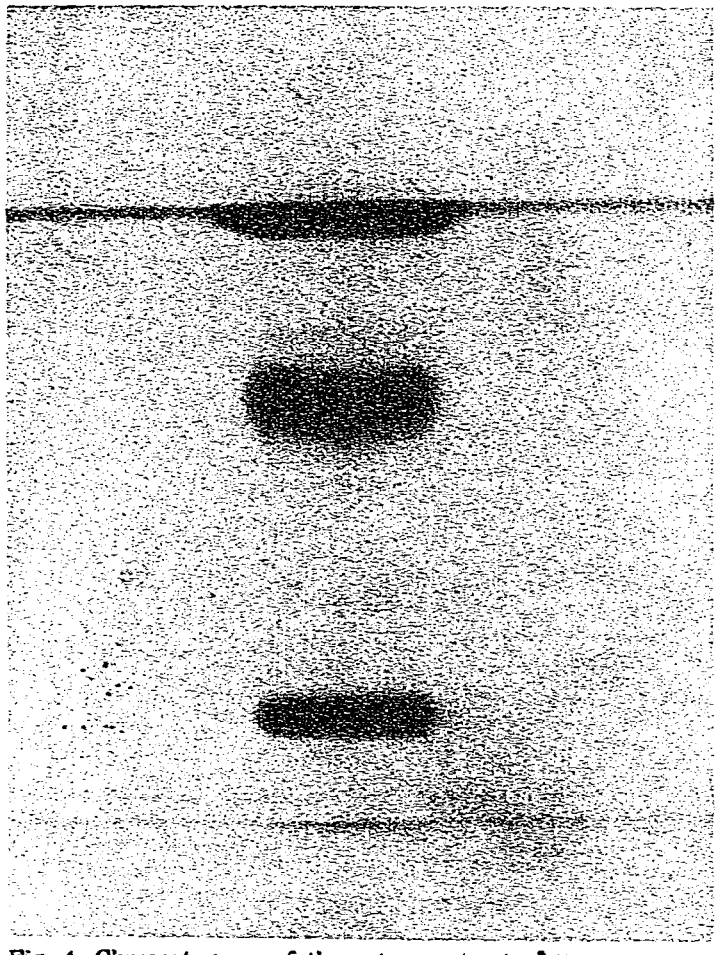

Fig. 4. Chromatogram of the octane extract of the isopropanolic solution of serum lipids.

After the two phases separated (2 min), 10 μl of the upper octane phase were applied to a TLC plate. The same two-step separation was carried out on the plate with the first and then with the second solvent system as before. The chromatogram shows that, although the octane extraction eliminates both the phospholipids and the free fatty acids (the two polar lipid species) in the absence of alkali, triglycerides are still present in the octane in addition to the cholesterols.

Fig. 5 shows the effect of alkali in the elimination of triglycerides. The alkaline isopropanolic extracting solvent hydrolyzed the triglycerides in 2 min and split them into free fatty acids and glycerol. Both the neutralized fatty

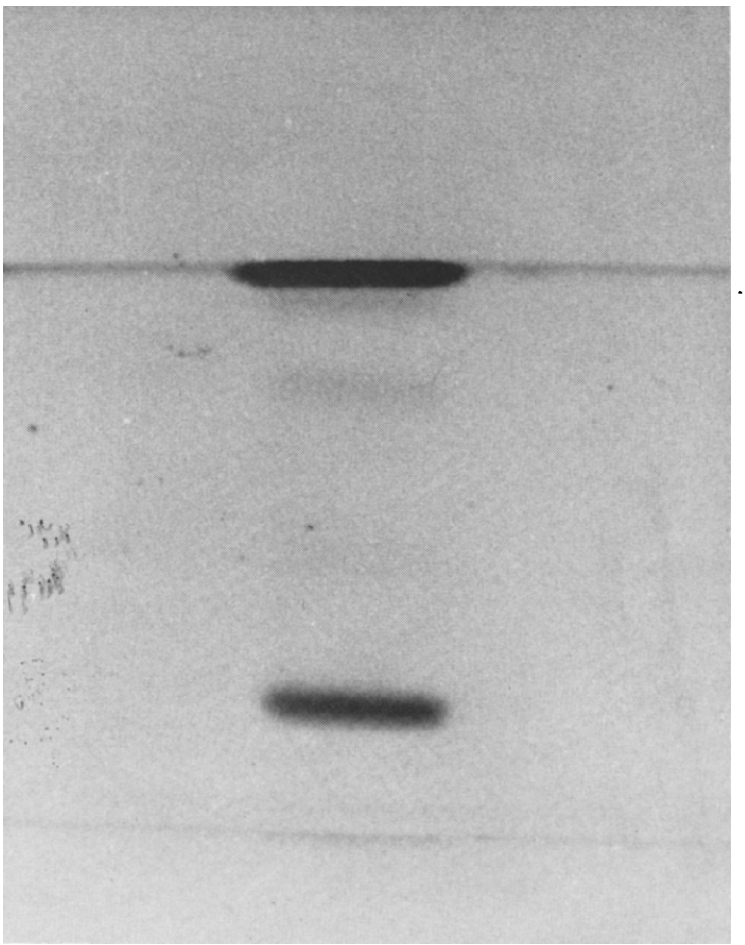

Fig. 5. Chromatogram of the octane extract of the alkaline isopropanolic solution of serum lipids.

acids and the glycerol remained in the isopropanolic phase after the octane extraction, as indicated in the chromatogram, which now shows the bands of free and esterified cholesterol and a very faint band representing an insignificant amount of unhydrolyzed triglyceride.

The advantage of the method is that it eliminates the need for separation of the lipids on the plate and the spots are much more uniformly stained. Therefore, the densitometric readings are much more reproducible and the results more reliable. The determination is rapid. It takes less than 10 min, including the evaluation and can be carried out with common laboratory equipment. It is relatively inexpensive and suitable for handling a large number of specimens, and thus can be used for screening purposes. If a semiquantitative evaluation is sufficient, the intensity of the blue color can be compared with the color intensity of a reference spot (e.g. an extract of a 2.5 mg/ml cholesterol solution) to determine whether the unknown spot has a higher or lower concentration than the reference spot.

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