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Letter to the Editor

Simple and rapid thin-layer chromatographic method for quantitative measurement of free cholesterol in serum

Sir,

The investigation of free cholesterol in serum is of clinical importance in the diagnosis of hepatic diseases and the study of clinical pathology [1,2].

Many methods for the determination of cholesterol in serum have been reported. Among these, the colorimetric method [3] is the most practical, but it suffers from interferences. The precipitation with reagents such as digitonine [4] or tomatine [5] is accurate, but the procedure is cumbersome, time-consuming and hardly meets the needs of simplicity and rapidity for clinical determination. Thus it often serves as a reference procedure for the more commonly used routine procedures.

A thin-layer chromatographic (TLC) method for separating cholesterol in serum with a silica gel CMC Na (sodium carboxymethylcellulose) plate is described in this paper. The separated spot is distinct and the R_F value is suitable. After it has been sprayed with a solution of sulphuric acid–vanillin, the cholesterol spot can be quantitatively detected with a CAMAG TLC scanner. This method is rapid and accurate, and the results of clinical applications are reported.

EXPERIMENTAL

Apparatus

TLC plates were scanned with a CAMAG TLC scanner II, which was controlled by an IBM-XT computer. Samples were applied to the plate with a CAMAG LINOMAT IV applicator. TLC plates were prepared with an automatic TLC plate coater.

Reagents

Cholesterol standard solution (1 mg/ml) was prepared by dissolving 100 mg of purified cholesterol (dried at 75°C for 2 h) in 100 ml of ethanol; it was stored in a refrigerator. The solvent system was petroleum ether–ethyl acetate–glacial acetic acid (80:20:1, v/v). The spraying reagent was prepared by dissolving 1 g of vanillin in 100 ml of sulphuric acid.

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All chemicals, except where otherwise indicated, were of analytical grade.

Procedure

To prepare the TLC plates, 20 g of silica gel (CP, Qing dao) were mixed with 60 ml of the solution of 0.5% CMC Na. The thick slurry was spread onto glass plates (20 cm × 20 cm, 0.4-mm layer) with an automatic TLC plate coater. After drying at room temperature, the plates were activated in an oven at 100°C for 60 min.

A 100-μl aliquot of serum was pipetted into a 5-ml test-tube, then 2.4 ml of ethanol were added to the tube to precipitate serum proteins. The tube was capped and vortex-mixed for 1 min. The tube was kept at room temperature for 10 min and vortex-mixed for 30 s again. Then the sample was centrifuged (3200 *g*, 5 min) and 15 μl of the supernatant were analysed.

Standard and sample solutions were applied with a CAMAG LINOMAT IV applicator connected to a nitrogen supply by spray-on technique at 2 cm from the edge of the plate. The band length was 5 mm and the space between two bands was 5 mm.

The plates were developed in a twin-trough chamber to a height of 8 cm above the origin (*ca.* 15 min) at room temperature, then allowed to dry in a ventilated cabinet at room temperature. Spots were visualized by spraying with a sprayer connected to nitrogen supply, then heating in an oven at 85°C for at least 6 min.

The stained plate was measured quantitatively by a CAMAG TLC scanner connected to an IBM-XT computer. The TLC conditions were as follows: detection wavelength, 525 mm; bandwidth, 10 nm; slit width, 0.3 mm; slit length, 4 mm; scanning speed, 6 mm/s. The data were collected and processed by the computer, and quantitative results were obtained by using external calibration.

RESULTS AND DISCUSSION

Selection of development solvent system

Under the same conditions, four solvent systems for separating cholesterol in

TABLE I

R_F VALUES OF CHOLESTEROL IN DIFFERENT SOLVENT SYSTEMS

A = *n*-hexane; B = diethyl ether; C = water; D = glacial acetic acid, E = methanol; F = ethyl acetate, G = chloroform, H = petroleum ether

Solvent system	R_F value
A–B–D (90:10·1)	0 14
H–F (8:2)	0.49
H–B–D (90 10.1)	0.08
G–E–C (65.25 4)	0.74

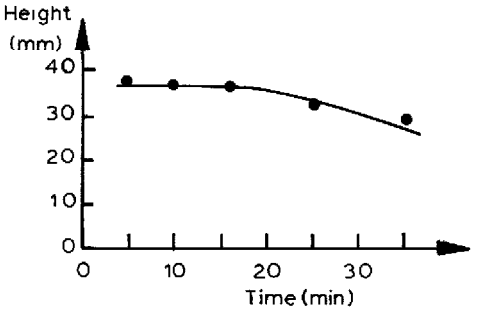

Fig. 1 Stability of staining of cholesterol

serum were tested (Table I). The solvent system chosen was petroleum ether–ethyl acetate. Adding a small amount of glacial acetic acid to the solvent system restrains the diffusion of the spot. Thus the development solvent system selected was petroleum ether–ethyl acetate–glacial acetic acid (80:20:1, v/v; R_F = 0.52).

Selection of spraying reagent

The mixtures phosphoric acid–sulphuric acid–ethanol, phosphomolybdic acid–ethanol–perchloric acid and sulphuric acid–vanillin were tested for staining of cholesterol. The results showed that the sulphuric acid–vanillin system was the most sensitive and the background was colourless.

Stability of colouration

Fig. 1 shows that the colour of cholesterol spot was stable for *ca.* 20 min.

Reproducibility

The reproducibility of the method was assessed from repeated analyses of the spots containing 0.5 μg of cholesterol per spot. Intra-plate reproducibility studies, evaluated from eight spots each of 0.5 μg of cholesterol per spot, gave a coefficient of variation of 2.4%. Inter-plate reproducibility, calculated by assaying the same amount and spots on six plates, was 7.4%

TABLE II

ANALYTICAL RECOVERY OF FREE CHOLESTEROL FROM SERUM

No	Amount present (mg/dl)	Amount added (mg/dl)	Amount measured (mg/dl)	Recovery (mean ± S.D., $n = 5$) (%)
1	36.9	50.0	86.7	99 7 ± 7 4
2	51 2	40.0	92.1	102 3 ± 4 9
3	48 2	60.0	109.9	102 8 ± 6 4

Relationship between peak area and concentration

Under selected conditions the relationship between the peak area and amounts of cholesterol was studied. The results showed that the area was linearly related to the cholesterol amounts in the range 80–700 ng per spot. The equation of the line was $y = 36.8 + 2.67x$, the coefficient of correlation was 0.998, and the detection limit was 40 ng per spot.

Recovery

Serum samples and the serum samples to which a known amount of cholesterol had been added were applied simultaneously to the plates to test the analytical recovery. The formula used was $(A - B)/S \times 100\%$, where A is the amount measured, B is the amount present and S is the amount added. Table II shows the recovery of free cholesterol from serum. The average recovery of free cholesterol from serum was 102%.

Determination of cholesterol in normal subjects

Blood samples from 26 healthy adult fasting subjects were collected to determine free cholesterol. The subjects were divided into three groups on the basis of the age: 19–34 years, 35–49 years and above 50 years. The analytical results were as follows: the first group ($n = 10$), mean ± S.D. = 40.6 ± 10.1 mg/dl; the second group ($n = 8$), mean ± S.D. = 45.2 ± 6.9 mg/dl; the third group ($n = 8$), mean ± S.D. = 53.8 ± 7.2 mg/dl. The average value was 46.1 ± 9.8 mg/dl. The results agree well with those reported by previous investigators [1,6–8].

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1 L. Chang, P Clifton, P Barter and M. Mackinnon, *Hepatology*, 6 (1986) 46.
2 D B Zilversmit, *Am J Cardiol*, 35 (1975) 559
3 G Vanzetti, *Clin Chim. Acta*, 10 (1964) 389
4 B. Sahagian and V E Levinet, *Clin Chem*, 10 (1964) 116
5 J J Kabara, T.J. McLaughlin and C. A. Riegel, *Anal. Chem.*, 33 (1961) 305.
6 W Richmond, *Clin. Chem.*, 22 (1976) 1579
7 S Samuels and C Fisher, *J Chromatogr*, 71 (1972) 297
8 T. Yao, M. Sato, Y. Kobayashi and T. Wasa, *Anal Biochem*, 149 (1985) 387

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