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Note

Potential application of thin-layer chromatography and thin-layer chromatography with flame ionization detection of cholestanol in the diagnosis of cerebrotendinous xanthomatosis

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Cerebrotendinous xanthomatosis (CTX) is a rare familial sterol storage disease which results from a block in bile acid synthesis and is characterized by the accumulation of cholestanol (5α-cholestan-3β-ol) in blood and tissues[1], particularly in the tendons and the nerve tissues. On the other hand, the concentration of cholesterol (5α-cholesten-3β-ol) in the serum of CTX patients is usually normal or even lower than that in healthy subjects. A definite diagnosis of this disease could be made after the determination of serum cholestanol concentration. However, the amount of cholestanol in serum is much lower than that of cholesterol and, accordingly, the determination of cholestanol is difficult owing to the similarity of the molecular structures of the two compounds.

Several gas–liquid chromatographic (GLC) methods for the determination of cholestanol in serum have been reported[2–6]. It seems that the GLC of cholestanol is not completely suitable for screening for CTX owing to an inability to analyse many samples in a limited time. Matsuoka *et al.*[7] reported the use of high-performance liquid chromatography (HPLC) with fluorimetric detection. Recently, Iwata *et al.*[8] described a highly sensitive HPLC method for the determination of cholesterol and cholestanol in human serum after conversion into fluorescent carbamic esters by treatment with 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl azide.

Thin-layer chromatography (TLC) has rarely been used; *e.g.*, Kasama and Seyama[9] developed a quantitative procedure for the determination of cholestanol in serum involving reversed-phase TLC after conversion of cholesterol to epoxides with 3-chloroperbenzoic acid. To the best of our knowledge, TLC with flame ionization detection (TLC–FID) has not previously been used.

This paper presents an improved TLC and HPTLC method and reports the use of the TLC–FID technique for the detection and determination of cholestanol in human serum.

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EXPERIMENTAL

Sample preparation

A 1-ml volume of human serum was saponified with 9 ml of 1 *M* potassium hydroxide solution in 90% methanol for 30 min at 100°C. After cooling, 5 ml of water and 20 ml of *n*-hexane were added and the mixture was agitated vigorously for 2 min in a separating funnel. The upper phase was transferred into a 50-ml vessel. The lower phase was extracted twice with 20 ml of *n*-hexane. The combined upper phases were evaporated to dryness under a stream of nitrogen in a glass test-tube. The residue was dissolved in 1 ml of chloroform.

Oxidation of cholesterol with peracetic acid

A 0.5-ml volume of the chloroform extract solution was evaporated to dryness under a stream of nitrogen in a glass test-tube. To the residue 2.5 ml of 3.5% peracetic acid in methanol were added and, after thorough agitation, the reaction mixture was left for 4 h at 37°C. Subsequently, 5 ml of 2% sodium hydrogensulphite solution and 0.25 ml of 10 *M* potassium hydroxide solution were added. The oxidized sample was extracted three times with 5 ml of *n*-hexane. The hexane phases were filtered and evaporated to dryness and the residue was dissolved in 0.5 ml of chloroform.

Thin-layer chromatography

Silufol sheets (10 × 10 cm) (Kavalier Glassworks, Votice, Czechoslovakia) or silica gel 60 HPTLC plates (10 × 10 cm) (Merck, Darmstadt, F.R.G.) were used. Chromatography was carried out in *n*-heptane–ethyl acetate (60:40). The spots were located by spraying the chromatogram with a 5% solution of phosphomolybdic acid in 2-propanol or 50% sulphuric acid.

The blue spots after the reaction with phosphomolybdic acid were measured by densitometry using an ERI 10 apparatus (Zeiss, Jena, G.D.R.).

Thin-layer chromatography with flame ionization detection

An Iatroscan TH 10 Analyser Mark IV connected with a Philips PV 4850 Video Chromatography Control Centre computing system was used. The samples (1 μl of a 2% solution in chloroform) were applied on Chromarods S III (Iatron Laboratories, Tokyo, Japan) and separated by two subsequent elutions [8 min with *n*-pentane ethyl acetate (50:50) and 30 min with *n*-pentane ethyl acetate (90:10)]. After drying at 60–65°C, the rods were scanned in the flame ionization detector.

RESULTS AND DISCUSSION

A typical scheme for the separation of cholestanol and the oxidation products of cholesterol is given in Fig. 1 and 2.

Under above-described reaction conditions, the oxidation of cholesterol with peracetic acid proceeded quantitatively and cholestanol was not affected. We compared the oxidation reactions with 3-chloroperbenzoic and peracetic acid and the results obtained were found to be nearly identical.

Kasama and Seyama[9] reported that on reversed-phase TLC the linearity of the coloration of cholestanol using phosphomolybdic acid was satisfactory in the

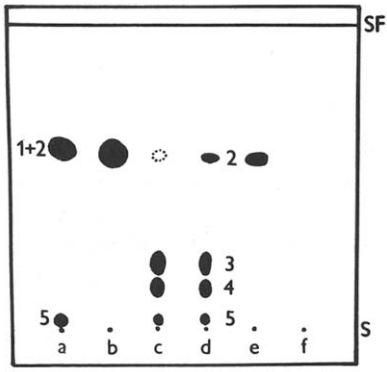

Fig. 1. TLC on Silufol sheets with *n*-hexane–ethyl acetate (60:40). (a) Standard mixture of cholesterol (1), cholestanol (2) and 3β,5α,6β-cholestantriol (5); (b) extract of hydrolysed human serum; (c) oxidized extract of hydrolysed human serum (normal); (d) oxidized extract of hydrolysed human serum (patient with CTX) showing cholesterol epoxides, (3) and (4); (e) cholestanol standard; (f) blank extract of the reaction mixture. Detection with phosphomolybdic acid.

range 100–1000 ng when measuring with a TLC scanning densitometer at a wavelength of 630 nm. Using the same experimental conditions, we could not obtain satisfactory results. Nevertheless, in accordance with Kasama and Seyama, we can

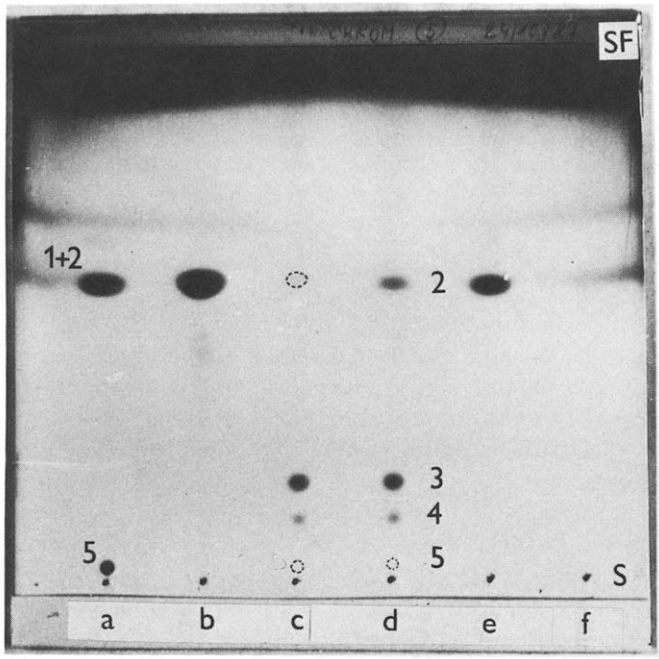

Fig. 2. HPTLC on silica gel 60 (Merck) plates with *n*-hexane–ethyl acetate (60:40). (a) Standard mixture of cholesterol (1), cholestanol (2) and 3β, 5α,6β-cholestantriol (5); (b) extract of hydrolysed human serum; (c) oxidized extract of hydrolysed human serum (normal); (d) oxidized extract of hydrolysed human serum (patient with CTX) showing cholesterol epoxides, (3) and (4); (e) cholestanol standard; (f) blank extract of the reaction mixture. Detection with 50% sulphuric acid.

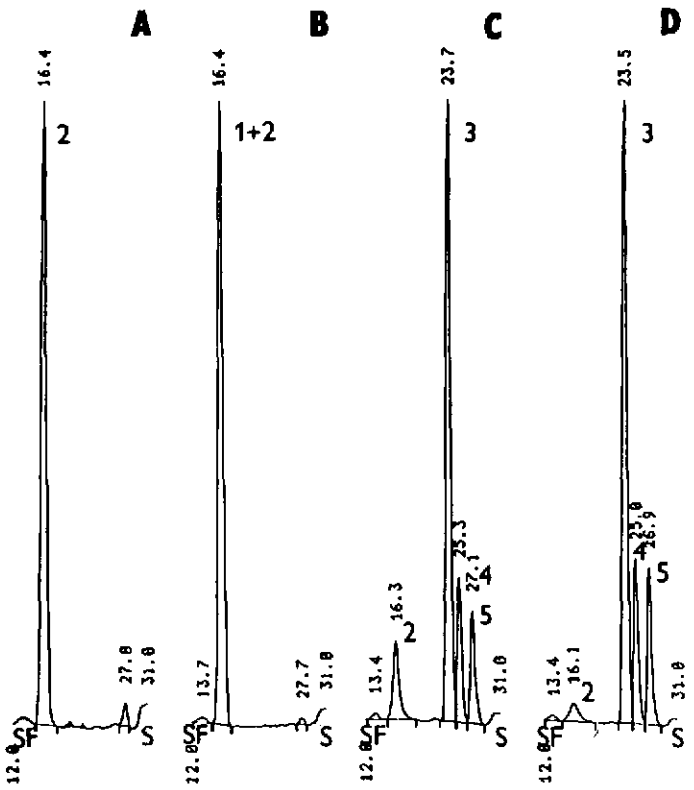

Fig. 3. TLC–FID on Chromarods S III. (A) Cholestanol standard; (B) extract of hydrolysed human serum; (C) oxidized extract of hydrolysed human serum (patient with CTX); (D) oxidized extract of hydrolysed human serum (normal). 1 = Cholesterol; 2 = cholestanol; 3 5 = products of oxidation of cholesterol.

confirm that the slope of the calibration graph varies with different plates and depends strongly on the colouring conditions.

Our results suggest that the densitometric determination of cholestanol in the TLC zone after reaction with phosphomolybdic acid can serve only as a semi-quantitative indicator of an increased concentration of cholestanol in serum.

We have found that TLC–FID is a time-saving and suitable quantitative screening method for serum cholestanol. The use of a new kind of thin layer (Chromarods S III) gives a better reproducibility than the older S II type. The separation of the cholesterol–cholestanol peak from the oxidized products is satisfactory (Fig. 3).

Further investigations on the application of these methods to biological samples are in progress and will be reported in the near future.

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