SEPARATION OF CHOLESTEROL ESTERS. A COMPARISON BETWEEN PAPER AND THIN LAYER CHROMATOGRAPHY

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SUMMARY

A method is presented for direct chromatography of serum on paper, treated with silicic acid, for the separation of cholesterol esters. No previous lipid extraction is necessary. A new solvent system for the thin-layer chromatographic separation of natural cholesterol esters is also discussed. The results obtained by paper chromatography and thin-layer chromatography are compared. Quantitative results obtained by thin-layer chromatography (heating with ammonium sulphate) show lower scatter, yet paper chromatography (staining with Rhodamine B) gives satisfactory results and both methods can be used for semiquantitative estimations. R_F values of both methods and their variations for the separate results are shown.

During the last few years a surge of interest in investigations of cholesterol ester (CH-esters) has become evident. A current extensive survey was presented by GOODMAN[4] in 1965, who, in his paper, collected most of the GLC data on the fatty acid composition of CH-esters. Comparatively simple methods have been developed for separation of natural cholesterol esters. Several authors have used paper chromatography (PC), usually treated with silicic acid[3,5,6,9–12,14]. Thin layer chromatography for the separation of cholesterol ester mixtures has been described by WEICKER[25] and developed by ZÖLLNER *et al.*[27–32] and others[2,8,13,15–17]. The cholesterol esters were separated by PC as well as TLC according to the degree of unsaturation of their fatty acids (FA). The chain length of the FA has been considered less important than the degree of unsaturation. In previous studies[14,22,23] use was made of MICHALEC's technique of CH-ester separation on paper impregnated with silica gel for lipid extract of biological fluids and tissue. Instead of using lipid extracts, we have now modified MICHALEC's technique and used total serum, spotting the sample directly on to the paper and deproteinizing at the origin. The TLC technique has been tested for a group of various solvents[24], resulting in the suggestion that a mixture of *n*-heptane–toluene (4:1 up to 6:2.5, v/v) is the most suitable.

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METHODS

Paper chromatography

Ten μl of serum is spotted directly by means of a 5 μl micropipette as a 2 cm long band on a 22.5 × 19 cm Whatman No. 3 paper, impregnated with sodium silicate. 20 μl of isopropanol–chloroform (1:1, v/v) are applied on the line of application for the purpose of breaking the lipoprotein linkage[19,20] and releasing the lipid components. Prior to ascending chromatography in 6 × 23 × 20 cm jars, with petrol ether (b.p. 80–110°) as a solvent, the paper is activated at 115–120° for a period of 2 min, the running time is about 33 min, and the distance run 18 cm (about 16 cm from the sample to the solvent front) at room temperature. Subsequent to evaporation of the solvent (which can be accelerated with a slight flow of warm air), the paper is rechromatographed in the same solvent. Staining is performed at room temperature on the dried chromatograms by dipping in a 1 mg% solution of Rhodamine B in 0.25 *M* K_2HPO_4 [18] prepared a day ahead. The excess of the dye is washed out in running water for exactly 15 min. The chromatogram is scanned on a Vitatron automatic densitometer (Cd–Hg lamp, filter 545, slit 2 × 6, recorder speed 8). The distribution of five visible fractions in percentage of the total amount is computed. The R_F values from 30 different chromatograms were collected.

Thin layer chromatography

A chloroform–methanol extract was prepared according to SVENNERHOLM[21] within a few hours after the blood sample was obtained. The lipid extracts (0.5 ml of serum in 10 ml chloroform–methanol, 2:1 v/v) were stored at a temperature of −20° and native sera at −2° until analysis had been performed. Silica Gel G (32 g in 60 ml water) chromatoplates 20 × 20 × 0.25 cm were prepared with a Desaga apparatus and activated for 1 h at 120°. An aliquot of the total lipid extract corresponding to 15 μl of serum was evaporated to dryness under a stream of N_2, redissolved in 10 μl of propanol–chloroform–methanol (3:2:1, v/v/v) and spotted in 1.5 cm long bands with a 5 μl micropipette. Ascending chromatography was performed in 9 × 22 × 20 cm jars without prior equilibration with a paper lining in *n*-heptane–toluene, 80:20 v/v. After evaporation of the solvent (under a light flow of air) the plate was rechromatographed in the same solvent. The running time was 50 min at room temperature, and the distance run 16 cm between the start and the solvent front. An ammonium sulphate reagent according to ZIMINSKI *et al.*[26] was used for detection of the separate bands. The chromatoplates were dried with hot air and heated for 45 min at 200°.

Quantitative densitometry on a Vitratron apparatus was performed under the same conditions as for the paper chromatograms, except that the speed of the recorder was raised from 8 to 9.

SAMPLES

Venous blood was drawn from subjects who had fasted overnight. Lipid extracts were separated by TLC, whole serum by PC, both types of sample in duplicate. Twenty samples of serum from two alcoholics, collected during a 14 day period of convalescence after a debauch, were compared. A hundred consecutive estimations by TLC, and 30 on different sheets of paper, were performed for the R_F values.

TABLE I

R_F VALUES OF DIFFERENT CHOLESTEROL ESTERS ON PC AND TLC
For PC $n = 30$; for TLC $n = 100$.

Cholesterol esters with	*PC R_F values*		*TLC R_F values*	
	Mean	*S.D.*	*Mean*	*S.D.*
Saturated FA	0.537	0.033	0.486	0.023
Monoenoic FA	0.415	0.034	0.438	0.022
Linoleic FA	0.305	0.036	0.386	0.018
Tri-tetraenoic FA	0.186	0.027	0.313	0.017
Penta-hexaenoic FA	0.115	0.026	0.267	0.015

RESULTS

Reproducibility of R_F values on paper and TLC

The R_F values on paper do not vary much, if the amount of material applied is not too large. Different papers may show variations in R_F values, due to variations in the process of activation before chromatography. The temperature of 115–120° must be individually checked and rigorously maintained. The variation of R_F values on different TLC plates is usually less than on PC. A hundred consecutive samples on 13 plates were calculated. The results are shown in Table I.

Quantitative densitometry and its precision

It was found, that the extinctions of the densitograms from PC conform to Lambert-Beer's law more closely than those from TLC. Densitometry of PC was performed within 24 hours, and TLC within 4 hours. The intensity of the carbonized spots decreased, but the colour did not fade quite proportionally in all the fractions. Loss of colour was prevented by covering the plate with another glass plate. It was

TABLE II

COMPOSITION OF SERUM CHOLESTEROL ESTERS OBTAINED BY PC AND TLC
Values expressed in per cent (mean ± S.D.). The standard deviation was calculated on the basis of the results for 20 different samples (from 2 persons), each result being a mean from duplicate analysis. d = Difference between duplicate analysis of the same sample using the same method. $n = 20$. A.E. = Analytical error (BOBERG, 1966), S = Fisher's test of duplicate differences; $S_{0.05} = 1.693$, $S_{0.01} = 2.114$.

Cholesterol esters with	*PC*				*TLC*				*t*	*S*
	Mean	*S.D.*	*A.E.*		*Mean*	*S.D.*	*A.E.*			
			$\sqrt{\frac{d^2}{2n}}$	*% of mean*			$\sqrt{\frac{d^2}{2n}}$	*% of mean*		
Saturated FA	13.3	2.6	1.135	8.5	11.8	1.3	0.974	8.3	0.765	1.358
Monoenoic FA	27.6	1.9	1.489	5.4	24.3	2.8	1.195	4.9	1.360	0.789
Linoleic FA	44.4	1.8	2.477	5.6	45.5	1.9	1.691	3.8	0.059	2.145
Tri-tetraenoic FA	10.1	1.4	1.688	15.8	12.8	1.2	1.109	8.6	0.217	2.316
Penta-hexaenoic FA	4.6	1.1	1.593	35.0	6.3	1.5	1.090	16.1	1.263	2.152

not possible to establish separate extinction curves of each fraction. This should be done in view of the higher extinction values of polyunsaturated FA cholesterides, but we did not have all the relevant standards. We prepared standards from a pool of serum (band 4 and 5), but the material was partially hydrolysed. Part of the standard remained on the starting point during chromatography. Similar results were obtained with a "pure" cholesterol-linoleate standard. The results from PC and TLC, respectively, are shown in Table II. On PC, the bands showed a wider variation in fractions with a low concentration, particularly in the last fraction of penta-hexaenoic FA cholesterides. The analytical error of TLC is definitely lower. By means of Fiscer's S-test it was shown that TLC is more precise in bands III, IV and V (polyenoic fatty acid cholesterides) than PC (the difference is statistically significant on the 0.01 level, see Table II).

The means and their S.D. and t- values are shown in Table II. The somewhat higher values in monoenoic cholesterides on PC and penta-hexaenoic FA cholesterides on TLC do not differ significantly ($t < t_{05}$).

DISCUSSION

Both methods can be used as routine methods for semiquantitative estimation of serum cholesterol esters. PC is less exact, but requires a minimum of equipment and is therefore preferable as a screening method. With the use of U.V. light, results can be obtained within 2 h after the blood has been taken and without any complicated extraction of lipids. TLC is suitable for preparative work and is more sensitive when used for detection of auto-oxidation products formed from cholesterol esters. Some unidentified additional bands between the start and the fraction of monoenoic FA cholesterides were obtained subsequent to exposure of the plate to daylight for several hours between two runs. Study of the plate under U.V. light can be recommended for immediate analyses. If a good chromatoplate with normal material is viewed under U.V. light after suitable spraying[7], several distinct bands can be observed in the first, second, fourth and fifth spots. These subfractions in the main bands apparently correspond to single cholesterol esters with FA's of different chain lengths. Data of separate bands obtained with GLG have been discussed by PORTMAN *et al.*[17]. Only the middle fraction, containing pure cholesterol-linoleate, is homogenous under U.V. The variations of R_F values on PC and TLC do not permit direct identification of individual fractions. For this purpose, standards prepared, say, from normal serum, are necessary for identification of individual spots.

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DISCUSSION

GÄNSHIRT: The reproducibility of the R_F value is worst for that component of the cholesterol ester mixture which exhibits the lowest R_F value and simultaneously represents the smallest proportion of the mixture. Is this poor reproducibility due to the low absolute R_F value or to the small absolute amount?

TICHÝ: The considerable variation of the quantitative values for the fractions for the penta- to hexaenoic fatty acids in cholesterol esters is given, on the one hand, by their absolute low concentration (approximately 0.2 to 0.5 μg per spot), on the other hand, by the difficulty in establishing the densitometric zero (baseline). For this fraction, TLC was more than twice as accurate as PC.