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MICROANALYTICAL QUANTITATION OF SERUM LIPIDS BY THIN-LAYER CHROMATOGRAPHY UTILIZING AN AUTOMATED APPLICATOR

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SUMMARY

A simple, accurate and convenient method for simultaneously quantitating triglycerides, free fatty acids, and free and esterified cholesterols on a micro-sample of human serum is presented. The method involves the utilization of an automated thin-layer plate applicator which also eliminates the necessity of a separate step for deproteinization.

INTRODUCTION

Thin-layer chromatography (TLC) is a particularly convenient method for separating and detecting small amounts of the major classes of serum lipids (phospholipids, free fatty acids, triglycerides and free and esterified cholesterol). Often less than five micrograms of each moiety are visualizable after suitable staining and/or oxidation. However, TLC has not been widely applicable to quantitation of serum lipids due to the laboriousness of application of sample to thin-layer plates, the necessity of prior lipid extraction and difficulties in quantification of the visualized spots.

Recently an automated device has been developed[1] with which lipid extraction and application of a serum sample to a thin-layer plate is automatically and expeditiously accomplished. An investigation was undertaken to indicate the efficiency and accuracy of quantifying serum lipid fractions by TLC using this device. A method was developed by which all classes of lipids with the exception of phospholipids can be conveniently and accurately quantitated by subsequent densitomometric scanning with the use of the automatic device.

MATERIALS AND METHODS

For each of the various steps described below, the automated thin-layer applicator was utilized. The operating features of the automatic applicator are elaborated in detail in a previous publication[1]. Consequently, only a brief description of the mode of utilization of this device will be given. Ten microliters of serum were added to the

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sample cups which had been filled with 100 μl of isopropanol. After a 2- to 5-min wait for deproteinization to occur the thin-layer plates (Silica Gel G) were spotted for 45 min; a pattern of 0.1 sec of pumping every 4 sec produced spots of less than 0.4 cm in diameter. Approximately 0.5 ml of effluate was applied during this period. The device incorporates proportionate refilling of the sample cups in which isopropanol is added to the sample cups as their contents are pumped out so that a constant volume was present in the cups at all times. The refilling pump was disabled during the last 10 min of the application to enable complete expression of the contents of the sample cups onto the plate.

Efficiency of extraction

Radiolabelled cholesterol, cholesterol palmitate, triolein, stearic acid and lecithin were obtained commercially. The activity of 10 μl of these substances was determined by counting in the Tricarb scintillation counter after placing the substance in a counting vial with 10 ml of BRAY's solution[2] and 100 μl of water. The efficiency of extraction was determined by comparing the activity of the effluent from the spotting needles with the known activity of the lipids. This experiment was performed with and without the addition of serum.

Quantitation of serum lipids

Ten microliters of serum were added to four of the eight sample containers. In three channels standards consisting of a mixture of lipids of known concentration in chloroform were added. In one channel a control pooled serum was present.

Hand determinations

The control sera were analyzed by hand reference methods for total and esterified cholesterol[4,5], triglycerides[6], and free fatty acids[7]. After separation of the lipids in petroleum ether (b.p. 30–60°)–diethyl ether–acetic acid (90:10:1) and drying, the plates were sprayed with 10% phosphomolybdic acid in 95% ethanol and heated at 70° for 10 min. The plates were then scanned with a Zeiss chromatogram spectrophotometer equipped with a recorder and integrator. Initially we used the instrument in the densitometric mode at 700 nm, expecting Beer's law to apply. However, we determined that near linearity was achieved in the more convenient reflectance scanning mode as has been reported[3] and the results described here are derived from this mode of quantitation.

Standard curves were calculated for each plate using both the least squares and point-to-point method for comparisons by a Fortran IV program created by Mr. U. ORMISTE for the IBM 360 computer. The values for the samples were interpolated automatically from each standard curve. Statistics were generated which indicated the reproducibility of the quantitation between identical samples applied on the same and on different plates. Phospholipids were not quantitated.

RESULTS

The efficiency of extraction of the lipids by the applicator system was determined for each of the various lipid classes studied. The efficiency was quantitated by comparing the known activity of the lipid placed in the extraction container to that of

the effluent delivered through the applicator needle over a 45-min period. These results are tabulated in Table I, and represent the means of sixteen trials. Stearate, triolein, and cholesterol were recovered with great efficiency (93–96%) reflecting their relatively high degree of solubility in isopropanol. Less soluble lipids, cholesterol palmitate and lecithin, were extracted with an efficiency of 87% and 90%, respectively. A small and statistically insignificant decrement in recovery was noted with the addition of serum to the radiolabeled lipids.

TABLE I

EFFICIENCY OF LIPID EXTRACTION BY AUTOMATED THIN-LAYER PLATE APPLICATOR
The results represent the means of sixteen trials.

Mode	*Counts per minute (± S.D.)*			
	Triolein	*Stearic acid*	*Lecithin*	*Cholesterol palmitate*
10 μl into counting vial	6,720 ± 410	12,640 ± 460	9,674 ± 590	10,936 ± 797
10 μl through system, no serum added	6,601 ± 304	12,391 ± 197	8,503 ± 401	10,348 ± 512
Recovery	0.98	0.98	0.87	0.94
10 μl through system with serum added	6,510 ± 612	12,071 ± 611	8,499 ± 584	9,929 ± 740
Recovery	0.96	0.94	0.87	0.90

TABLE II

COMPARISON OF VALUES OBTAINED ASSAYING TWO ALIQUOTS OF IDENTICAL SERUM ON THE SAME PLATE

	Free cholesterol	*Free fatty acids*	*Tri-glycerides*	*Cholesterol esters*
Number of pairs	16	14	16	16
r	0.99	0.96	0.99	0.99

TABLE III

COMPARISON OF VALUES OBTAINED ASSAYING IDENTICAL SERA ON DIFFERENT PLATES
N = number of samples.

	Free cholesterol	*Free fatty acids*	*Tri-glycerides*	*Cholesterol esters*
Value (mg %), N = 34	35.3	42.5	124.5	168.5
Standard deviation	3.88	3.82	9.96	11.79
Coefficient of variation	0.11	0.09	0.08	0.07
Hand reference methods, N = 15	30.0	38.4	120.0	168.0

A high degree of correlation ($r = 0.99$), was obtained among duplicate serum samples extracted simultaneously and quantitated on the same plate (Table II). This finding indicates that while lipid extraction may not be completely efficient, sera processed simultaneously are extracted with equal efficiency. It also provides additional support for the importance of including standards on each run to compensate for defects in extraction efficiency.

The precision of the quantitation of a serum on different runs is indicated in Table III. Values of lipid concentration were determined by constructing curves from the standards run concurrently with the test sera on each plate. Standard deviations and the coefficient of variation have been calculated for each of the lipid classes studied. It must be emphasized that the measures of error include all losses due to extraction, a factor which is often considered separately in the discussion of other methods. The coefficient of variation for the determination of triglycerides and cholesterol esters is quite good (0.08 and 0.07) and may be related to the fact that these moieties travel furthest during the development of the plate. Their spots are, consequently, larger and more uniformly distributed than those of slower migration. The increased coefficient of variation for free cholesterol and free fatty acids (0.11 and 0.09) may also be due to some trailing by the more rapidly traveling spots.

The values obtained by the thin-layer technique show good agreement with both the reference hand methods and the methodology used by the University of Chicago Clinical Laboratories and a commercial laboratory.

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REFERENCES

1 E. Fosslien and F. Musil, *J. Lipid Res.*, 11 (1970) 605.
2 G. Bray, *Anal. Biochem.*, 1 (1960) 279.
3 J. Lines, in A. Frazer (Editor), *Biochemical Problems of Lipids*, Elsevier, New York, 1963.
4 L. Abell, B. Levy, B. Brodie and F. Kendall, *J. Biol. Chem.*, 115 (1952) 357.
5 W. Sperry and M. Webb, *J. Biol. Chem.*, 187 (1950) 957.
6 L. Carlson and L. Wadström, *Clin. Chim. Acta*, 6 (1960) 590.
7 V. Dole, *J. Clin. Chem.*, 35 (1956) 150.